Analysis of veterinary antibiotics in dairy environments by liquid chromatography – mass spectrometry

Baena-Nogueras R.M.[1,*], Ortori C.[2], Barrett D.A.[2], Gomes R.L.[1]

[1] BECT – Bioprocess, Environmental & Chemical Technologies Research Group. Faculty of Engineering, University of Nottingham, NG7 2RD, United Kingdom.

[2] School of Pharmacy, Faculty of Science, University Park, University of Nottingham, NG7 2RD, United Kingdom.

*corresponding author:

e-mail: Rosa-maria.BaenaNogueras1@nottingham.ac.uk

Abstract

Veterinary antibiotics are widely used for disease treatment, protection and to promote animal's growth. The emerging concern regarding the diffusion of antibiotic resistant microorganisms (AMR) is directly linked to the use of antibiotics in animal husbandry as they act as co-selective drivers for AMR development and spread. Therefore, a new analytical method for the detection of pharmaceuticals at trace levels to be able to study their occurrence, behaviour and fate in complex environmental matrices (including milk, wastewater slurry and soil) has been developed. Several extraction techniques were tested in solid matrices for analytes under study such as ultrasound assisted extraction (UAE) and microwave assisted extraction (MAE), using different buffers and additives. Clean-up and pre-concentration of the extracts were performed by means of solid-phase extraction (SPE), using different cartridges (e.g. SAX-HLB) and methanol as eluting solvent. Antibiotic residues were also extracted from milk samples using acetonitrile previous to SPE purification or ultrafiltration. Recoveries were generally about 75% for most compounds, decreasing for those antibiotics having the highest instability (e.g. B-lactams). Identification and quantification of antibiotics were performed by liquid chromatography-mass spectrometry (LC-MS). Finally, the protocol proposed was validated by analysing the concentration of target compounds in samples collected from a dairy farm.

Keywords: Veterinary antibiotics, AMR, extraction methods, dairy environment, LC-MS

1. Introduction

As early as 2000, one-third of the antibiotics used in Europe were for veterinary purposes (Thiele-Bruhn, 2003). The exposure to constant concentrations of antibiotics can lead to the generation of Antimicrobial Resistance (AMR). AMR can be transferred by the soil amendment, crops and water affecting the therapeutic potential against human and animal pathogens and posing a high risk to public health. Antibiotics accumulation in soils has been found before at several ng g^{-1} as a result of manure amendment (Solliec *et al*., 2016) and the migration of these compounds through the soil column as well as the run-off from cropland. This has been demonstrated to pose a risk to subsoil, groundwater contamination and surface waters (Pan and Chu, 2017; Peru *et al*., 2006).

In this sense, concerns about development of AMR and its transference from animal to humans via the food chain, led to phasing out the antibiotics used as feed additives in the European Union since January 1, 2006. Maximum residue limits (MRLs) of veterinary antibiotics marketed in the European Union (EU) are assessing by the European Medicines Agency (EMA) for consumer safety and included in Commission Regulation (EU) No 37/2010. Rules for the validation of analytical methods used in the residue monitoring plan are described in Commission Decision 2002/657/EC and performance criteria and requirements for analytical methods are specifically drawn up in the framework of the Directive 96/23/EC. It is therefore necessary to understand antibiotic persistence in these environmental matrices by performing antibiotic analysis on the different sources from veterinary activities according to the framework.

Veterinary antibiotics include several structural classes with potentially diverse physico-chemical characteristics among them. In this regard, the development of extraction methodologies of the antibiotics under study in soil and slurry matrices is closely related to the p*Ka.* Therefore, while tetracyclines are stable in acidic conditions, macrolides are unstable in acid media. Briefly, the extraction methods are based on a liquid partitioning using different organic solvents combined with weakly acidic buffers followed by a solid phase extraction (SPE) clean-up step employing different cartridges and conditions. Several studies have employed a solid buffered extraction method by agitation (Pfeifer *et al*., 2002; Salvia *et al*., 2012: Berendsen *et al*., 2015), while other authors have evaluated different techniques such as sonication (Martínez-Carballo *et al*., 2007; Uslu *et al*., 2008; Solliec *et al*., 2006), MAE (Hu *et al*., 2010; Dorival García *et al*., 2013) or even a combination between shaking and ultrasound (Hu *et al*., 2011).

The high polarity of veterinary antibiotics with special emphasis on aminoglycosides, has led to experimenting

with chromatographic columns that differ from the conventional reverse phase ones, evolving towards the hydrophilic interaction liquid chromatography (HILIC) in combination with mass spectrometry (MS) (Chiaochan *et al*., 2010; Dasenaki *et al*., 2016). These columns allow polar compounds to be retained in a polar stationary phase from where the elution is achieved by increasing the portion of the aqueous mobile phase. In addition, this type of phase allows avoiding the use of ion pair reagents which significantly imped the MS response (Peru *et al*., 2006; Kahsay *et al*., 2014).

The aim of this work is to optimize an accurate, selective and robust analytical and extraction method for the simultaneously extraction of the most commonly used veterinary antibiotics belonging to different families in real dairy-farm environmental samples.

2. Materials and methods

2.1. Chemicals and standards

Antibiotics analytical standards used for quantification were obtained from Sigma Aldrich. Regarding deuterated compounds sulfadiazine-d_4 (benzene-d_4) was purchased from QMX Laboratories (Essex, UK), amoxicillin-d_4 was purchased from LGC Standards (Lancashire, UK), ceftiofur-d_3 and lincomycin-d_3 were purchased from Toronto Research Chemicals (Ontario, Canada) and enrofloxacin-d_5 hydrochloride and trimethoprim-d_9 were purchased from Sigma Aldrich (Dorset, UK). High-performance liquid chromatography (HPLC)-grade (or equivalent) methanol (MeOH), acetonitrile (ACN), Milli-Q water, formic acid (99%), citric acid (99.5%), ethylenediaminetetraacetic acid (Na_2EDTA, 99%), disodium salt dehydrate, sodium hydroxide (NaOH, 99%) were purchased from Fisher Scientific (Leicestershire, UK). 200 mL of McIlvaine extraction buffers at pH 3, 5 and 7 were prepared by adding a certain amount of 0.1M citric acid and 0.2M Na_2HPO_4 according to McIlvaine (1921). 0.1M EDTA solution was prepared by weighting 3.72 g in 100 mL. Finally, three different extraction solutions were prepared containing ACN, 0.1M EDTA solution and McIlvaine buffer solution at three different pH.

Individual stock solutions were prepared either in MeOH:Water (1:1) for cephalosporins, penicilins, novobiocin and florfenicol and MeOH for the rest of antibiotics at a concentration of 250 ng mL^{-1} and kept at -20ºC. An amount of 20 µl from 0.1M NaOH solution was added in those cases were it was necessary due to the low solubility (enrofloxacin and amoxicillin) according to previous studies (Ibáñez *et al*., 2009).

The Oasis HLB solid-phase extraction (SPE) cartridges used (500 mg, 6cc) were supplied by Waters Limited (Herts, UK).

2.2. Sample collection and storage

Wastewater slurry, soil and milk were collected from the University of Nottingham farm. This farm has 445 hectares of land, including a high performance working dairy unit and it operates both commercially and for research. All the samples were store at 4ºC and immediately processed after their transport to the lab.

2.3. Sample extraction and clean-up step

Dry weight was calculated for soil and total suspended solids (TSS) were calculated by filtering the wastewater slurry samples through 1 µm glass fibre filters (Fisher Scientific, Leicestershire, UK).

2 ml of liquid slurry or 2 g of fresh soil were measured and placed into 50 mL conical-bottom centrifuge tubes (Fisher, Leicestershire, UK), spiked with 2 ng mL^{-1} and 15 mL of extraction solution (ACN/EDTA/McIlvaine buffer pH3 or pH7, 25:25:50, v/v) were added. The samples were vortexed for 1 min and they were subsequently placed into a linear reciprocating shaker (Stuart, SSL2) during 60 min at 225 rpm. Twelve samples could be simultaneously extracted and they were then centrifuged at 6000 rpm for 10 min. The supernatant was collected and taken to 100 mL aliquots (solvent content <5%) with Milli-Q water prior SPE.

The SPE cartridges were conditioned with 10 mL of MeOH, 5 mL of deionized water and 5 mL of McIlvaine buffer. The samples were passed through the cartridges at approximately 2 mL min^{-1}, washed with 10 mL of Milli-Q water, and, finally, dried under vacuum for 30 min. The elution was carried out from the HLB cartridges with 10 mL of MeOH and the samples were then evaporated near to dryness using a Jouan vacuum centrifuge (DJB Labcare, Newport Pagnell, UK) and dried completely under a gentle stream of nitrogen at room temperature. The residues were dissolved in 1 mL of ACN:water 20:80 and filtered through 0.22 µm using syringe tip filters (Sigma Aldrich, Dorset, UK) for HPLC analysis. Antibiotic residues were also extracted from milk using acetonitrile to precipitate proteins and for SPE purification. Several parameters were optimized for all the matrices tested such as extraction solvent, extraction buffer, SPE cartridges or extraction technique.

2.4. Liquid chromatography-mass spectrometry

All analyses were performed by HPLC conducted with an Agilent 1100 HPLC coupled to an Applied Biosystems Q-Trap 5500 tandem mass spectrometer. The analytes were identified by multiple reactions monitoring (MRM) of a precursor ion and a single product ion using two transitions to confirm the compound identity (Table 1). LC-MS/MS instrument was operated in positive ion mode (ESI+) for all chemicals. The method for antibiotics and their isotope-labelled compounds were determined and optimized by infusion of individual standard solutions (1 mg L^{-1}) with the following instrumental analysis conditions: source temperature= 300ºC, cone gas flow= 30 psi, probe gas flow = 50 psi, nebulizer gas flow = 20 psi, collision gas pressure = 6.0 mTorr, and the ion spray voltage was 4500 V. An

Obelisc R 100 Å, 5 µm 2.1 x 100 mm analytical column and a 100 Å 2.1 x 10 mm guard column purchased from SIELC Technologies Inc. (Wheeling, Illinois, USA) was used and it was maintained at 40ºC. The mobile phases consist of ACN (A) and water with 1% formic acid (B). The HPLC gradient run for the veterinary antibiotics under study was increased from 5% to 95% A in 7 min and was held constant for 3 min. Finally, the mobile phase was brought back to initial conditions and held again for 4 min at a flow rate for 400 µl min^{-1}. Total run time was 15 min including re-equilibration. Standards solutions of analytes were prepared and analysed under the same conditions as the samples with a nine-point calibration curve from 0.1 to 200 ng mL^{-1} using a constant concentration of stable isotope-labelled internal standards (50 ng mL^{-1}) for the quantification method. Identification of compounds was performed basing on standards retention time and the constant ratio between the two MRM transitions employed for each compound.

Deuterated compounds were used to correct for losses during the extraction process and matrix effects during the injection. The method limits of detection (mLOD) and quantification (mLOQ) were determined from 100 mL spiked milk and 2 mL/2g spiked wastewater slurry/soil as the minimum detectable amount of analytes with a signal to noise ratio of 3 and 10 respectively. Instrument limits of detection (iLOD) were also calculated taking into account the amount of sample injected (10 µL). All the data were acquired and processed using MS Analyst 1.6.3 Software.

Table 1. MS/MS parameters for the analysis of target analytes and the corresponding isotopically labelled compounds by MRM positive ionization mode. Isotopically labelled compounds are indicated by numerated superscript for each compound: 1.Amnoxicillin-d_4, 2. Ceftiofur-d_3, 3.Enrofloxacin-d_5 hydrochloride, 4.Lincomycin-d_3, 5.Sulfadiazine-d_4, 6.Trimethoprim-d_9. [a]Values obtained from either Pubchem (https://pubchem.ncbi.nlm.nih.gov/) and/or Drugbank databases (http://www.drugbank.ca).

Pharmaceutical group	Compound	p*Ka*[a]	t_R (min)	MRM $[M+H]^+$	Collision Energy	iLOD (pg)
Penicillins	Amoxicillin[1]	0.87	0.60	366.14>114.00	31	1.15
				366.14>208.00	19	
	Ampicillin[1]	2.5-7.3	0.68	350.15>106.10	25	8.04
				350.15>160.10	21	
	Cloxacillin[1]	2.78	1.70	436.06>178.10	47	7.50
				436.06>160.10	27	
	Penicillin-G[1]	2.74	1.80	335.16>217.00	23	4.41
				335.16>91.10	83	
Cephalosporins	Cephalexin[2]	5.2-7.3	0.67	348.13>158.10	15	1.56
				348.13>106.10	45	
	Cephalotin[2]	3.8	2.43	397.29>338.4	13	29.41
				397.29>88.10	29	
	Cefoperazone[2]	3.38	2.92	646.14>530.20	17	11.54
				646.14>143.10	49	
	Cefquinome[2]	2.43	2.10	529.19>134.00	27	6.25
				529.19>125.20	81	
	Ceftiofur[2]	2.83	2.30	524.03>125.00	81	0.83
				524.03>126.20	53	
Tetracyclines	Oxytetracycline[3]	3.27	0.61	461.18>426.00	29	2.72
				461.18>201.10	57	
Fluoroquinolones	Enrofloxacin[3]	5.94	0.70	360.31>316.10	33	1.54
				360.31>282.30	17	
Amphenicols	Florfenicol[3]	10.73	0.80	358.06>241.00	25	11.39
				358.06>103.20	91	
Macrolides	Tylosin[6]	7.73	0.56	916.46>174.20	55	0.18
				916.46>772.50	43	
Lincosamides	Lincomycin[4]	7.97-12.37	0.75	407.23>126.10	41	0.04
				407.23>359.20	27	
Sulfonamides	Sulfadiazine[5]	6.36	0.82	251.07>65.00	65	1.13
				251.07>92.20	43	
Aminocoumarin antibiotic	Novobiocin[3]	4.3	1.10	613.39>189.10	45	3.13
				613.39>332.30	25	
	Trimethoprim[6]	7.12	0.59	291.16>230.10	35	0.03
				291.16>123.10	45	

3.Results

3.1. Analysis optimization

The use of concentrated buffers is known to be necessary for the elution of polar compounds when reversed or even HILIC phases are used. However, a recent study found that Obelisc R stationary phase provide better sensitivity for highly hydrophilic compounds using only formic acid as additive (Díez *et al*., 2015).

Calibration curves from UPLC-QqQ-MS/MS had strong linearity ($r^2 > 0.9$) for all target analytes. The instrumental limits of detection (iLOD) provided by this method were lower than 30 pg of the injected amount in all the cases (Table 1) and the antibiotics considered in this study showed values between 0.02-2.94 ng mL^{-1} for both detection (mLOD) and quantification (mLOQ) limits in real samples, being lower than 1 μg L^{-1} for 60% of the target compounds. Detection and quantification limits showed similar or better results compared to previous studies (Peru *et al*., 2006; Solliec *et al*., 2016). In addition, those limits were lower than the MRLs established by the European Union.

3.2. Extraction and clean-up optimization

The most challenging matrix under study was the wastewater slurry with a TSS value of up to 7 g L^{-1} for settled slurry taken from bottom of tank. In terms of matrix effects, an average ratio of 0.1 was found between the matrix and the calibration curve signal of slurry samples. Among the extraction optimization of the wastewater slurry, the pH was revealed as one of the most influential parameters within the different antibiotics families tested. For instance, various studies have stated that the predominant cationic form of amphoteric quinolones such as enrofloxacin, may enhance the extraction efficiency at acidic conditions (Martínez-Carballo *et al*., 2007; Dorival-García *et al*., 2013). This is evident in the present study with an increase in the recovery of up to 42% when pH3 extraction buffer was used. On the other hand, an alkaline extraction was employed before for sulfonamides showing recoveries from 47 to 89% (Haller *et al*., 2002), showing an improvement for sulfadiazine (92.3±5%) when pH7 McIlvaine buffer was added. Other antibiotics such as tetracyclines are known to form chelate complexes with metal ions, so the addition of chelating agents (EDTA) is commonly accepted and the obtained recovery near 100% for oxytetracycline was in consonance with previous studies (Blackwell *et al*., 2004; Berendsen *et al*., 2015). Cephalosporins generally showed recoveries above 75% regardless of pH conditions, while penicillins were recovered better at acidic pH. Furthermore, the addition of acetonitrile has been proved as effective procedure to remove interferences derived from proteins content in manure samples (Ho *et al*., 2012).

3.3. Validation in dairy farm samples

This method has been validated according to the European Commission 2002/657/EC and applied to real dairy samples such as wastewater slurry where the concentrations found were in accordance with levels from other studies in animal faeces (Berendsen *et al*., 2015; Hu *et al*., 2010).

Table 2. Concentration (μg L^{-1}) of detected target veterinary antibiotics in slurry samples.

Compound	Concentration (ng L^{-1}) ± SD
Amoxycillin	77900±16370
Ampicillin	25300±251
Cephalexin	6310±770
Enrofloxacin	1830±240
Novobiocin	7990±1190
Oxytetracycline	10150±1390
Sulphadiazine	8710±20
Trimethoprim	100±50

4. Conclusions

This study presents a viable method for simultaneously analysis of several classes of veterinary antibiotics in complex matrices from diary-farm environments. Particularly, the directly application of slurry onto fields as fertilizer may act not only as a non-point source of antimicrobial residues, but also as an impeller of the AMR generation, which makes this study the first step towards the evaluation of the impact of the antibiotics presence and fate on the AMR development.

References

Commission Regulation (EU) No 37/2010 of 22 December 2009 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin, Official Journal of the European Community L15 (2010) 1.

European Commission, Council Directive (EEC) 96/23/EC, Official Journal of the European Community. L125 (1996) 10.

Aguado J., Arsuaga J.M., Arencibia A., Lindo M. and Gascón V. (2009), Aqueous heavy metals removal by adsorption on amine-functionalized mesoporous silica, *Journal of Hazardous Materials*, **163**, 213-221.

Berendsen, B.J.A., Wegh, R.S., Memelink, J., Zuidema, T. (2015), The analysis of animal faeces as a tool to monitor antibiotic usage, *Talanta*, **132**, 258-268.

Blackwell , P.A., Lützhøft, H.C.H., Ma, H.P., Halling-Sørensen, B., Boxal, A.B.A., Kay, P. (2004), Ultrasonic extraction of veterinary antibiotics from soils and pig slurry with SPE clean-up and LC-UV and fluorescence detection, *Talanta*, **64**, 1058-1064.

Chiaochan, C., Koesukwiwat, U., Yudthavorasit, S., Leepipatpiboon, N. (2010) Efficient hydrophilic interaction liquid chromatography-tandem mass spectrometry for the multiclass analysis of veterinary drugs in chicken muscle, *Analytica Chimica Acta*, **682**, 117-129.

Dasenaki, M.E., Michali, C.S., Thomaidis, N.S. (2016), Analysis of 76 veterinary pharmaceuticals from 13 classess including aminoglycosides in bovine muscle by hydrophilic interaction liquid chromatography-tandem mass spectrometry, *Journal of Chromatography A*, **1452**, 67-80.

Díez, C., Guillarme, D., Spörri, A.S., Cognard, E., Ortelli, D., Edder, P., Rudaz, S. (2015), Aminoglycoside analysis in food animal origin with a zwitterionic stationary phase and liquid chromatography-tandem mass spectrometry, *Analytica Chimica Acta*, **882**, 127-139.

Dorival-García, N., Zafra-Gómez, A., Camino-Sánchez, A., Navalón, A., Vílchez, J.L. (2013), Analysis of quinolone antibiotic derivatives in sewage sludge samples by liquid chromatography-tandem mass spectrometry: Comparison of the efficiency of three extraction techniques, *Talanta*, **106**, 104-118.

Haller, M.Y., Müller, S.R., McArdell, C.S., Alder, A.C., Suter, M.J.F. (2002), Quantification of veterinary antibiotics (sulfonamides and trimethoprim) in animal manure by liquid chromatography- mass spectrometry, *Journal of chromatography A*, **952**, 111-120.

Hu, X., Zhou, Q., Luo, Y. (2010), Occurrence and source analysis of typical veterinary antibiotics in manure, soil, vegetables and groundwater from organic vegetable bases, northern China, *Environmental Pollution*, **158**, 2992-2998.

Hu, W., Ma, L., Guo, C., Sha, J., Zhu, X., Wang, Y. (2011), Simultaneous extraction and determination of fluoroquinolones, tetracyclines and sulfonamides antibiotics in soils using optimised solid phase extraction chromatography-tandem mass spectrometry, *International Journal of Environmental and Analytical Chemistry*, 1-16.

Ho, Y.B., Zakaria, M.P., Latif, P.A., Saari, N. (2012), Simultaneous determination of veterinary antibiotics and hormone in broiler manure, soil and manure compost by liquid chromatography-tandem mass spectrometry, *Journal of Chromatography A*, **1262**, 160-168.

Ibáñez, M., Guerrero, C., Sancho, J., Hernández, F. (2009), Screening of antibiotics in surface and wastewater samples by ultra–high-pressure liquid chromatography coupled to hybrid quadrupole time-of-flight mass spectrometry, *Journal of Chromatography A*, **1216** (12), 2529–2539.

Kahsay, G., Song, H., Chepdael, A.V., Cabooter, D., Adams, E. (2014), Hydrophilic interaction chromatography (HILIC) in the analysis of antibiotics, *Journal of Pharmaceutical and Biomedical Analysis*, **87**, 142-154.

Salvia, M.V., Vulliet, E., Wiest, L., Baudot, R., Cren-Olivé, C. (2012), Development of a multi-residue method using acetonitrile-based extraction followed by liquid chromatography–tandem mass spectrometry for the analysis of steroids and veterinary and human drugs at trace levels in soil, *Journal of Chromatography A*, **1245**, 122-133.

Solliec M., Roy-Lachapelle A., Gasser M.O., Coté C., Généreux M., Sauvé S. (2016), Fractionation and analysis of veterinary antibiotics and their related degradation products in agricultural soils and drainage waters following swine manure amendment, *Science of the Total Environment*, **543**, 524-535.

Thiele-Bruhn, S. (2003), Pharmaceuticals antibiotic compounds in soils – a review, *Journal of Plant Nutrition and Soil Science*, **166**, 145-167.

Uslu, M.O., Yediler, A., Balcıoğlu, I.A., Schulte-Hostede, S. (2008), Analysis and sorption behavior of fluoroquinolones in solid matrices, *Water Air & Soil Pollution*, **190** (55), 55-63.

Martínez-Carballo, E., González-Barreiro, C., Scharf, S., Gans, O. (2007), Environmental monitoring study of selected veterinary antibiotics in animal manure and soils in Austria, *Environmental Pollution*, **148**, 570-579.

McIlvaine, T. (1921), A buffer solution for colorimetric comparison, *Journal of Biological Chemistry*, **49**, 183-186.

Pan, M., Chu, L.M. (2007), Leaching behavior of veterinary antibiotics in animal manure-applied soils, *Science of the Total Environment*, **579**, 466-473.

Peru, K.M., Kuchta, S.L., Headley, J.V., Cessna, A.J. (2006), Development of a hydrophilic interaction chromatography-mass spectrometry for spectinomycin and lincomycin in liquid hog manure supernatant and run-off from cropland, *Journal of Chromatography A*, **1107**, 152-158.

Pfeifer, T., Tuerk, J., Bester, K., Spiteller, M. (2002), Determination of selected sulfonamide antibiotics and trimethoprim in manure by electrospray and atmospheric pressure chemical ionization tandem mass spectrometry, *Rapid Comunications in Mass Spectrometry*, **16**, 663-669.