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A new extraction procedure to abate the burden of non-extractable antibiotic residues in manure

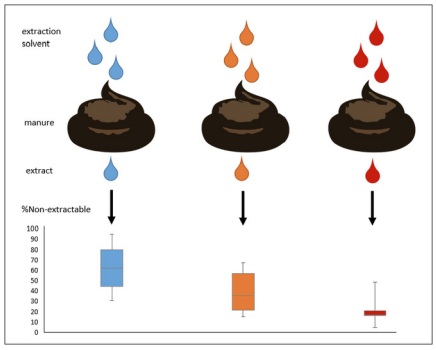
Larissa J.M. Jansen [a, *], Milou G.M. van de Schans [a], Diana de Boer [a], Irma E.A. Bongers [a], Heike Schmitt [b], Paul Hoeksma [c], Bjorn J.A. Berendsen [a]

[a] RIKILT Wageningen University & Research, Wageningen, The Netherlands Akkermaalsbos 2, 6708 WB, Wageningen, the Netherlands
[b] National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands
[c] Wageningen Livestock Research, Wageningen, the Netherlands

HIGHLIGHTS

- The amount of non-extractable residues of 10 antibiotics was determined in manure using 24 different extraction solvents.
- Results of a longitudinal study to further investigate the fate of non-extractable residues over time is reported.
- An optimized method, validated for the analysis of 48 antibiotics in manure is presented.
- This data contributes to the understanding of the processes during extraction of manure.

GRAPHICAL ABSTRACT



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ABSTRACT

Through agricultural soil fertilization using organic manure, antibiotic residues can accumulate in the environment. In order to assess the risks of environmental pollution by veterinary drugs, monitoring of manure for antibiotic residues is necessary. As manure is a complex matrix, extraction of antibiotics proved to be challenging. In this study, 24 extraction solvents were assessed for the extraction of residues from manure representing ten antibiotics from the antibiotic classes tetracyclines, quinolones, macrolides, lincosamides and sulfonamides. Especially for the tetracyclines and quinolones the extraction solvent selection is critical, due to high fractions of non-extractable residues especially when using aqueous solvents (62–77% and 90–95% respectively when using milli-Q water). In contrast, sulfonamides can effectively be extracted with aqueous solvents. Overall, 0.125% trifluoroacetic acid in acetonitrile in combination with McIlvain-EDTA buffer proved to be the most effective extraction solvent. A longitudinal study pointed out that most antibiotics bind to solid manure particles instantaneously after addition. Trimethoprim is an exception, but because by using the optimal extraction solvent, the optimum fraction of bound residues is desorbed, this does not hamper quantitative analysis when using spiked manure quality control samples. Based on these new insights, the current in-house multi-residue LC-MS/MS

Abbreviations: ACN, Acetonitrile; CTC, chlortetracycline; CIP, ciprofloxacin; CID, collision-induced dissociation; DMC, demeclocycline; DC, doxycycline; ENR, enrofloxacin; EA, ethyl acetate; EDTA, ethylenediaminetetraacetic acid; ES, extraction solvent; FLU, flumequine; FA, formic acid; LIN, lincomycin; MAR, marbofloxacin; MeOH, methanol; OTC, oxytetracycline; PP, polypropylene; RSD_r*, relative standard deviation of the repeatability including matrix influence; RF, response factor; SRM, selected reaction monitoring; S/N, signal-to-noise; SPE, solid phase extraction; SDZ, sulfadiazine; SDD, sulfadimidine; SMZ, sulfamethoxazole; TC, tetracycline; TIL, tilmicosin; TUL, tulathromycin; TFA, trifluoric acetic acid; TYL, tylosin.

* Corresponding author.
E-mail address: larissa.jansen@wur.nl (L.J.M. Jansen).

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method for manure analysis, containing 48 antibiotics, was revised, additionally validated and applied to 34 incurred manure samples.

1. Introduction

Antibiotics are commonly used in animal husbandry to treat bacterial infections. After intake, depending on the pharmacokinetics of the compounds, large fractions (30–90%) are excreted mainly as the parent compound via urine or faeces (Sarmah et al., 2006). The use of antibiotics in animal husbandry is also likely to cause selection of resistant bacteria in the gut, which (including resistant genes) might thereafter also be excreted (Wellington et al., 2013).

Bacterial resistance has become a major global issue, with an increasing number of bacterial pathogens showing multidrug resistance to antimicrobial agents (Knapp et al., 2010; Roca et al., 2015). An important factor in the selection of resistant bacteria is the increasing accumulation of antibiotic residues in the environment (Wellington et al., 2013), where low residue concentrations can already have an impact (Gullberg et al., 2011). In the Netherlands, the great majority of manure is stored for up to 6 months, depending on the local storage time and spreading regulations, and applied as a fertilizer on agricultural land. Only a small fraction (depending on the species) of manure is processed (Lahr, 2017). Therefore manure is known to be one of the major pathways though which antibiotic residues are introduced into the environment (Halling-Sørensen et al., 1998; Chee-Sanford et al., 2009).

The contribution of manure as a carrier of antimicrobial environmental contaminants becomes gradually more recognized in the world. Several studies analysed specific groups of antibiotics (Haller et al., 2002; Carballo et al., 2016), more compounds from multiple antibiotic groups (Jacobsen and Halling-Sørensen, 2006; Martínez-Carballo et al., 2007; Karcı and Balcıoğlu, 2009; Gorissen et al., 2015; Van den Meersche et al., 2016), or a wide variety of compounds from different antibiotic groups (Christian et al., 2003; Berendsen et al., 2015) in manure or faeces. These studies pointed out that many manure samples contain antibiotics, sometimes up to 1000 $\mu g\,kg^{-1}$ and some tetracyclines even up to 90000 $\mu g\,kg^{-1}$. Nevertheless, manure, the source of a significant part of the veterinary drug pollution in the environment, is currently not actively monitored. The lack of data on the amount of antibiotic residues in manure applied to agricultural land, with or without processing, hampers an adequate risk assessment of the environmental pollution by veterinary drugs.

However, surveillance data alone is insufficient to assess the risks connected with manure application. Research on the fate of antibiotic residues in manure is mandatory. Several studies investigated the fate of selected antibiotics in manure (Berendsen et al., 2018), or soil after applying antibiotic containing manure (Hamscher et al., 2002; Jacobsen et al., 2004; Burkhardt et al., 2005; Brambilla et al., 2007; Martínez-Carballo et al., 2007; Stoob et al., 2007; Sukul et al., 2008; Uslu et al., 2008; Domínguez et al., 2014; Spielmeyer et al., 2017). These studies pointed out that antibiotics can undergo a wide variety of interactions based on their physico-chemical properties, like binding to solid material or complex formation (Jacobsen and Halling-Sørensen, 2006), whereas other antibiotics quickly migrate through the solid material into the ground or surface water. Especially when compounds interact with matrix, it could result in non-extractable residues, influencing the estimation of the amount of veterinary drugs analysed and/or overestimating the bioavailable fraction of antibiotics in manure and manure amended soil.

In the light of these findings, studying the extraction efficiency is of vital importance to allow accurate determination of the total antibiotic concentrations, especially for compounds that are expected to have large amounts of non-extractable residues. Ideally, information about the fate of the total fraction of antibiotics, including the irreversibly bound fraction as well as the part that is reversibly adsorbed and that is bioavailable under current soil conditions should be known, with the latter being the most important for ecotoxicological effects. This knowledge will aid a more accurate risk assessment (Aga et al., 2016; Larivière et al., 2017).

Some studies already investigated different extraction methods to extract antibiotics more effectively from manure (Janusch et al., 2014; Li et al., 2015; Wallace and Aga, 2016) or manure in combination with soil (Blackwell et al., 2004), achieving optimized methods with lower detection limits. However, additional to optimized methods, there is a need to clarify to what extent non-extractable residues influence the extraction process and outcomes for different compounds. This is especially important when using multi-residue methods, where compounds from different classes are extracted using a single extraction solvent.

In this study, many different extraction solvents were assessed with respect to the non-extractable fraction of residues in relation to the extraction recovery. For this purpose, ten different antibiotics (from six different classes) that are most often found in manure in The Netherlands (Berendsen et al., 2015) were extracted using 24 different extraction solvents. Additionally, a longitudinal study on the binding of residues was carried out. The impact of the outcomes on current monitoring procedures are discussed. Today, this study is, to our knowledge, the most in depth research published on the extraction procedure of antibiotics from manure, including the effectiveness of many different solvents and related extraction processes. Based on the new insights obtained, our in-house multi-residue LC-MS/MS method for manure analysis, containing 48 antibiotics, was revised, additionally validated and applied to 34 incurred samples to demonstrate its effectiveness.

2. Materials & method

2.1. Reference standards

Chlortetracycline (CTC, 90%), oxytetracycline (OTC, 99%), tetracycline (TC, 98%), ciprofloxacin (CIP, 99%), danofloxacin (99%), difloxacin (93%), enrofloxacin (ENR, 100%), flumequine (FLU, 99%), marbofloxacin (MAR, 98%), nalidixic acid (100%), norfloxacin (98%), oxolinic acid (98%), sarafloxacin (89%), erythromycin (89%), josamycin (98%), lincomycin (LIN, 96%), spiramycin (4674 IU mg^{-1}), tiamulin (99%), tylosin (TYL, 86%), valnemulin (95%), dapsone (99%), sulfacetamide (100%), sulfachlorpyridazine (99%), sulfadimethoxine (99%), sulfadimidine (SDD, 100%), sulfadoxine (98%), sulfamerazine (100%), sulfamethizole (100%), sulfamethoxazole (SMZ, 99%), sulfamethoxypyridazine (100%), sulfamoxole (98%), sulfaphenazole (99%), sulfapyridine (>99%), sulfaquinoxaline (93%), sulfathiazole (100%), sulfisoxazole (100%) and trimethoprim (100%) were

purchased at Sigma-Aldrich (St. Louis, MO, USA). Neospiramycin (96%), pirlimycin (96%), and natamycin (98%) were purchased at Toronto Research Chemicals (Toronto, ON, Canada). Doxycycline (DC, 97%) and sulfadiazine (SDZ, 100%) were purchased at Council of Europe (EDQM, Strasbourg, France). Gamithromycin (95%) and tulathromycin (TUL, 97%) were purchased at Santa Cruz Biotechnology (Dallas, TX, USA). Tilmicosin (TIL, 95%) was purchased at Dr. Ehrenstorfer GMBH (Augsburg, Germany), tylvalosin (94%) at ECO Animal Health (London, UK), tildipirosin (100%) at MSD Animal Health (Boxmeer, The Netherlands), and sulfamonomethoxine (94.5%) at TCI Europe (Zwijndrecht, Belgium).

The internal standards norfloxacin-d_5, CIP-d_8, ENR-d_5, sarafloxacin-d_8, difloxacin-d_3, oxolinic acid-d_5, nalidic acid-d_5, FLU-$^{13}C_3$, sulfathiazole-$^{13}C_6$, sulfapyridine-$^{13}C_6$, sulfamerazine-$^{13}C_6$, SDD-$^{13}C_6$, sulfamethizole-$^{13}C_6$, sulfachlorpyridazine-$^{13}C_6$, sulfadoxine-d_3, sulfisoxazole-$^{13}C_6$, sulfadimethoxine-d_6, and sulfaquinoxaline-$^{13}C_6$ were purchased at Witega (Berlin, Germany). Erythromycin-^{13}C-d_3, spiramycin-d_3, LIN-d_3, SDZ-d_4, and dapsone-d_8, MAR-d_8, tiamulin-d_{10}, valnemulin-d_6, TYL-d_3, pirlimycin-d_{12}, TIL-d_3, tylvalosin-d_9, TUL-d_7, sulfacetamide-d_4, sulfaphenazole-d_4 and trimethoprim-d_9 were purchased at Toronto Research Chemicals. TC-d_6 and gamithromycin-d_4 were purchased at Santa Cruz Biotechnology. Demeclocycline (DMC) was purchased at Sigma-Aldrich and tildipirosin-d_{10} at MSD Animal Health.

2.2. Reagents

Citric acid monohydrate, di-sodium hydrogen phosphate, ethylenediaminetetraacetic acid (EDTA), acetonitrile (ACN), ethyl acetate (EA), acetone, formic acid (FA), methanol (MeOH), ammonium acetate, ammonium (25%), were purchased at Witega (Darmstadt, Germany). Lead acetate trihydrate, trifluoric acetic acid (TFA) and ammonium formate were purchased at Sigma-Aldrich (St Louis, MO, USA).

McIlvain-EDTA buffer was prepared by adding 500 mL 0.1 M citric acid and 280 mL 0.2 M di-sodium hydrogen phosphate to 1 L water into a 2 L volumetric flask. The pH was adjusted to 4.0 using citric acid solution or di-sodium hydrogen phosphate solution and the solution was diluted with water up to the mark.

Stock solutions of reference standards and internal standards were prepared once at a concentration at 100 mg L^{-1} for the (fluoro)quinolones and at 1000 mg L^{-1} for the other compounds and stored by −80 °C (Berendsen et al., 2011). Tetracyclines, sulfonamides and the macrolides tildipyrosin and natamycin were dissolved in MeOH and (fluoro)quinolones in a solution of 2% 2 M ammonium hydroxide in MeOH. The lincosamides and the macrolides TYL, tiamulin and valnemulin were dissolved in water and the remainder of the macrolides and pleuromutilins in ACN. A mixed solution of reference standards and a solution of internal standards was made at a concentration of 10 mg L^{-1} in MeOH for the 10 compounds used in the extraction experiments. For the longitudinal experiment, a mixed solution of reference standards was made at a concentration of 20 mg L^{-1} and internal standards at a concentration of 5 mg L^{-1}, both in MeOH. A mixed solution containing all reference standards was prepared for the in-house validation in MeOH at a level of 0.25 mg L^{-1} for sulfonamides and 1 mg L^{-1} for the tetracyclines, quinolones and macrolides. A mixed solution containing all internal standards was made at a concentration of 5 mg L^{-1} in MeOH. Mix solutions were prepared fresh on the day of sample preparation.

2.3. Sample preparation

Of each manure sample 2 g was weighed in duplicate into 50 mL polypropylene (PP) tubes (Greiner Bio-One, Alphen aan de Rijn, The Netherlands). Standard solutions were added to one of the aliquots and internal standard solution to both. The aliquots were shaken for 5 s on a vortex mixer and then left at room temperature during 20 min in order to let the manure and antibiotics equilibrate. Hereafter 4 mL of a freshly prepared 0.125% TFA in ACN solution was added and samples were shaken thoroughly by hand. Subsequently 4 mL of McIlvain-EDTA buffer was added and samples were shaken head-over-head (Heidolph REAX-2, Schwabach, Germany) during 15 min. In order to further precipitate proteins and thus to prevent clogging of the Solid Phase Extraction (SPE) cartridge, 2 mL lead acetate solution (200 g L^{-1}) was added and samples were centrifuged (Biofuge Stratos centrifuge, Heraeus instruments, Germany) for 10 min at 3500 g. The supernatant was entirely decanted into a 12 mL glass tube. The ACN was evaporated at 40 °C under a gentle nitrogen flow (TurboVap LV Evaporator Zymark, Hopkinton, MA, USA), in order to remove the organic solvent to allow sufficient retention of even the most polar compounds on the SPE cartridge. The extracts were diluted by adding 13 mL of 0.2 M EDTA solution before SPE.

2.4. Sample clean-up

A reversed-phase polymeric SPE cartridge 200 mg, 6 mL (Strata-X, Phenomenex, Torrance, CA, USA) was subsequently conditioned with 5 mL of MeOH and 5 mL of McIlvain-EDTA buffer. The entire extract was transferred onto the cartridge, which was thereafter washed with 5 mL of water and dried by applying vacuum for 1 min. The residues were eluted into a 12 mL glass tube using 5 mL of MeOH which was then evaporated until dry (40 °C, N_2). Residues were reconstituted in 100 μL MeOH by vortex mixing and diluted with 400 μL of water. The final extracts were then filtered over 0.45 μm filters (REZIST, Schleicher & Schuell, München, Germany) into a 96-wells plate and analysed immediately or stored at −20 °C and analysed at a later point.

2.5. LC-MS method

Chromatographic separation in all cases was done using a Kinetex C18 2.1 × 100 mm 1.7 μm analytical column (Phenomenex), placed in a column oven operating at 40 °C. Analysis of incurred samples and method validation was carried out using an Acquity UPLC System, coupled to an AB Sciex Q-trap 6500 mass spectrometer. Both liquid chromatography and mass spectrometry settings, including ion transitions, were used as described by Berendsen et al. (2015). In brief; the mobile phases used were 2 mM ammonium formate and 0.16% FA in water (Solvent A) and 2 mM ammonium formate and 0.16% FA in MeOH (Solvent B). Operating at a flow rate of 0.3 mL min^{-1}, the used gradient was: 0–0.5 min, 1% B, 0.5–5.0 min, a linear increase to 100% B with a final hold of 1.0 min and an equilibration time of 3.5 min. The injection volume was 10 μL. Transitions of additional compounds are presented in Table 1. Data processing was done using MultiQuant 3.0.2 software.

Extraction experiments and the longitudinal experiment were analysed using a similar chromatographic system, however coupled to a Micromass Quattro Ultima Pt with ESI interface. Operating parameters for this system; capillary voltage 2.8 kV, cone voltage 65 V, source temperature 120 °C, desolvation temperature 450 °C, cone gas flow 120 L h^{-1} and desolvation gas 700 L h^{-1}. The system operated in positive mode. The detection mode used was selected reaction monitoring (SRM) with collision-induced dissociation (CID) using argon as the collision gas. Data processing was done using MassLynx 4.1 software (Waters).

Table 1
Additional transitions that were not included in a previous study (Berendsen et al., 2015).

Component	precursor ion (*m/z*)	product ion (*m/z*)	DP (eV)	CE (eV)	CXP (eV)
TMP	291	123	36	25	25
	291	230	36	25	25
TMP-d_9	300	123	36	25	25
neospiramycin	699	142	111	30	15
	699	174	111	27	14
marbofloxacin-d_8	371	79	66	27	10
tiamulin-d_{10}	504	202	71	29	18
valnemulin-d_6	571	269	10	25	24
TYL-d_3	919	177	11	49	16
pirlimycin-d_{12}	423	122	51	33	14
TIL-d_3	873	696	10	57	22
tylvalosin-d_9	1052	814	10	45	30
tulathromycin-d_7	407	577	41	21	22
sulfacetamide-d_4	219	160	36	15	14
sulfaphenazole-d_4	319	160	96	29	14

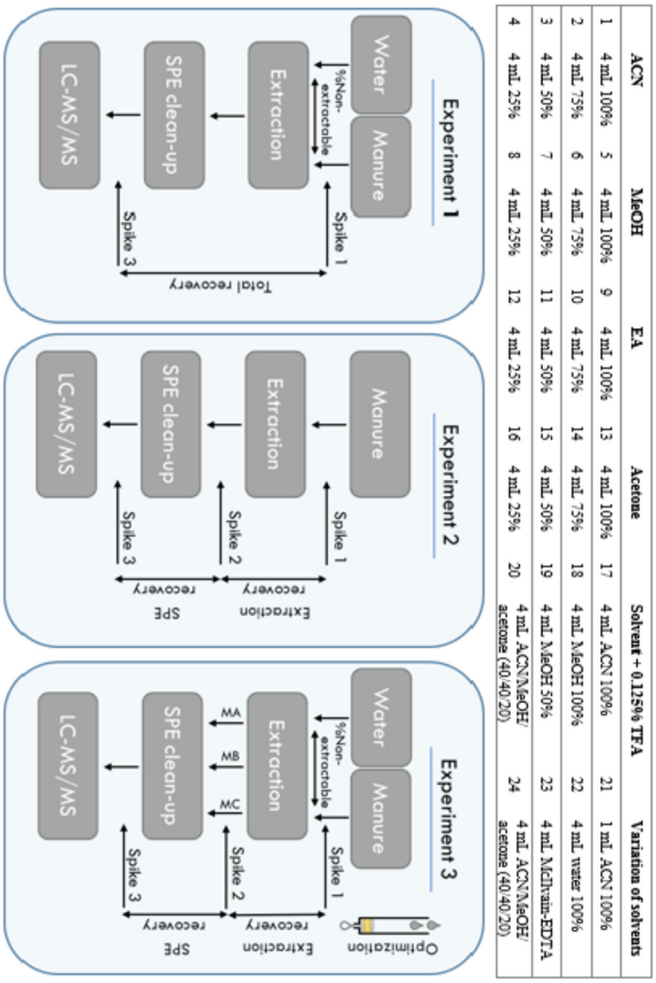
2.6. Method development

For all experiments, including the longitudinal experiment, semi-liquid bovine manure which did not contain any of the analysed compounds was used (this is referred to as 'blank manure'). The manure was obtained by scooping it from the slatted floors in the stable. It was then homogenized in the lab using an Ultra Turrax homogenizer (IKA, Staufen, Germany) and stored in the freezer until use.

2.6.1. Extraction experiments

Ten compounds were selected from six different antibiotic classes: the tetracyclines OTC and DC, the (fluoro)quinolones ENR and FLU, the macrolides TYL and TIL, the lincosamide LIN, the sulfonamides SDZ and SDD and additionally the pyrimidine inhibitor trimethoprim. These compounds were chosen since they were the most frequently found compounds in cattle manure in The Netherlands. The penicillin's (part of the β-lactam group), are another group which is frequently used to treat cattle, however these antibiotics are known to quickly degrade in manure and were therefore not included (Berendsen et al., 2018). A total of three different extraction experiments were carried out. A schematic overview of the experiments is given in Fig. 1. In experiment 1, the extraction capabilities of 24 different extraction solvents (ESs) were tested, determining the relative amount of non-extractable residues. This large range of extraction solvents was chosen based on the different (physico-)chemical properties the compounds. Different compositions of solvent mixtures were tested to do a thorough examination of the solvent effects. Additionally the total recovery of the method was determined. Based on the first experiment, some ESs were selected and further investigated in experiment 2, determining SPE recovery and extraction recovery. The most fit for purpose ES was chosen, resulting in the necessity to further optimize the SPE clean-up. In experiment 3 the SPE clean-up was optimized for the selected extraction solvent by comparing three different methods.

2.6.1.1. Experiment 1. For each ES tested (n = 24), 2 g of manure was weighed in fourfold and separately 2 g of water was weighed into 50 mL PP tubes, resulting in five different tubes per extraction solvent. The water sample and two of the manure samples were spiked at a concentration of 350 μg kg^{-1} (spike 1) for all ten antibiotics. The goal of this experiment was to visualize non-extractable residues. During all extraction experiments, internal standards were not added to the solid material but after extraction



	ACN	MeOH	EA	Acetone	Solvent + 0.125% TFA	Variation of solvents
1	4 mL 100%	5 4 mL 100%	9 4 mL 100%	13 4 mL 100%	17 4 mL ACN 100%	21 1 mL ACN 100%
2	4 mL 75%	6 4 mL 75%	10 4 mL 75%	14 4 mL 75%	18 4 mL MeOH 100%	22 4 mL water 100%
3	4 mL 50%	7 4 mL 50%	11 4 mL 50%	15 4 mL 50%	19 4 mL MeOH 50%	23 4 mL McIlvain-EDTA
4	4 mL 25%	8 4 mL 25%	12 4 mL 25%	16 4 mL 25%	20 4 mL ACN/MeOH/ acetone (40:40:20)	24 4 mL ACN/MeOH/ acetone (40:40:20)

Fig. 1. Schematic presentation of the three extraction experiments and the tested extraction solvents, where MA, MB and MC are method A, B and C as described in experiment 3.

in order to prevent them from interacting with the particles and correcting for the non-extractable part of residues. The internal standards were used for correction of minor recovery differences caused during sample clean-up and the instrumental analysis itself.

After spiking, the tubes stood for 20 min before adding extraction solvent. ES1 to ES24 were then added to their respective tubes and the method was proceeded according to the method described in sample preparation. Extraction of manure using head-over-head or ultrasonification were compared in previous research and both techniques worked sufficiently (Berendsen et al., 2015). However, after centrifuging, samples were diluted with 2 M EDTA. Internal standards were added at this point at a concentration of 350 μg kg^{-1} and extracts were subjected to SPE clean-up. The sample clean-up was carried out as described in the section sample clean-up. At the end, all spiked samples were reconstituted in 100 μL of MeOH, vortexed, and diluted with 900 μL of water instead of 400 μL. At this point spike 3, according to Fig. 1, is added to the third and fourth (not yet spiked) manure aliquots, which are used for the determination of the total recovery. They were spiked at a concentration of 350 μg kg^{-1} (70 μL of a 10 mg L^{-1} solution) and further reconstituted in 30 μL MeOH, vortexed and diluted with 900 μL water. All extracts were filtered, transferred into vials and analysed using LC-MS/MS. The total recovery and the amount of non-extractable residues were calculated according to formula 1

and 2 as described in the section "calculations" of Materials and method.

2.6.1.2. Experiment 2. For each of the extraction solvents selected from experiment 1, 2 g of manure was weighed in six fold into 50 mL PP tubes. The first two manure aliquots were spiked at a concentration of 350 μg kg^{-1} (spike 1) with all ten compounds. The same procedure as in experiment 1 was followed. After dilution with EDTA solution, the third and fourth manure aliquot were spiked (spike 2) with standard solution on a level of 350 μg kg^{-1}, which is used for the determination of the extraction recovery. Similar to experiment 1, internal standard solution was added to all aliquots at this stage. The clean-up procedure was carried out as described in sample clean-up. At the end spiked aliquots were reconstituted as in experiment 1. The final two manure aliquots were spiked at a concentration of 350 μg kg^{-1} (spike 3), which are used for the determination of the SPE recovery. Extraction recovery and SPE recovery were calculated according to formula 3 and 4.

2.6.1.3. Experiment 3. Three sets of seven aliquots, each consisting of six 2 g manure aliquots and one 2 g water aliquot, were prepared in 50 mL PP tubes. Antibiotic solutions were spiked according to experiment 1, where spike 1 was added to both the 2 g of water and the first duplicate of manure, spike 2 was added to the second manure duplicate after extraction and spike 3 was added to the last duplicate during reconstitution. Samples were extracted using 0.125% TFA in ACN. The first set of aliquots (n = 7) was then prepared using method A (MA), which is the procedure described in experiment 1. The second set was prepared using method B (MB), adding an evaporation step (40 °C, N_2) after centrifuging and pouring the supernatant into a glass tube in order to reduce the fraction of organic solvent to allow high recoveries during SPE. Hereafter, the concentrated extract was diluted with 13 mL 0.2 M EDTA and the procedure was followed as described for experiment 1 and 2. The third set of aliquots was prepared using Method C (MC). In this method, the extracts were centrifuged without adding lead acetate solution and immediately brought onto an OASIS HLB PRiME (3 cc 60 mg) cartridge (Waters, Milford, MA, USA), which is intended for high organic extracts not requiring concentration or salt removal. This specific cartridge was chosen because of the hypothesis that high organic solvents were the cause for low SPE recovery in experiment 1 and 2. For this experiment extraction recovery, SPE recovery and non-extractable residues were calculated using formula 2–4.

2.6.2. Longitudinal experiment

Based on the results of the extraction experiments, a longitudinal experiment was set up in order to gain more knowledge about how non-extractable residues behave in time, giving an indication of how representative spiked samples are for incurred material. The experimental set-up is shown in Fig. 2.

Greiner tubes were covered with aluminium foil and 60 times 2 g of blank manure was weighed in. Of the 60 tubes, firstly 12 tubes were randomly selected to serve as a blank control for the longitudinal experiment. Secondly 24 tubes were randomly selected to form 12 duplicates and were spiked at a concentration of 500 μg kg^{-1} (spike 1) before incubation. The final 24 tubes were selected to form 12 duplicates and were reserved to be spiked after extraction (for determination of the extraction recovery).

Based on the results of the extraction experiments two different extraction solvents were compared during this experiment: water and TFA in ACN. As described in Fig. 2, from the above prepared manure samples, 1 blank control, 1 spiked duplicate and 1 reserved blank duplicate were randomly selected for each of the 2 extraction methods, adding up to 10 tubes in total. These 10 samples were

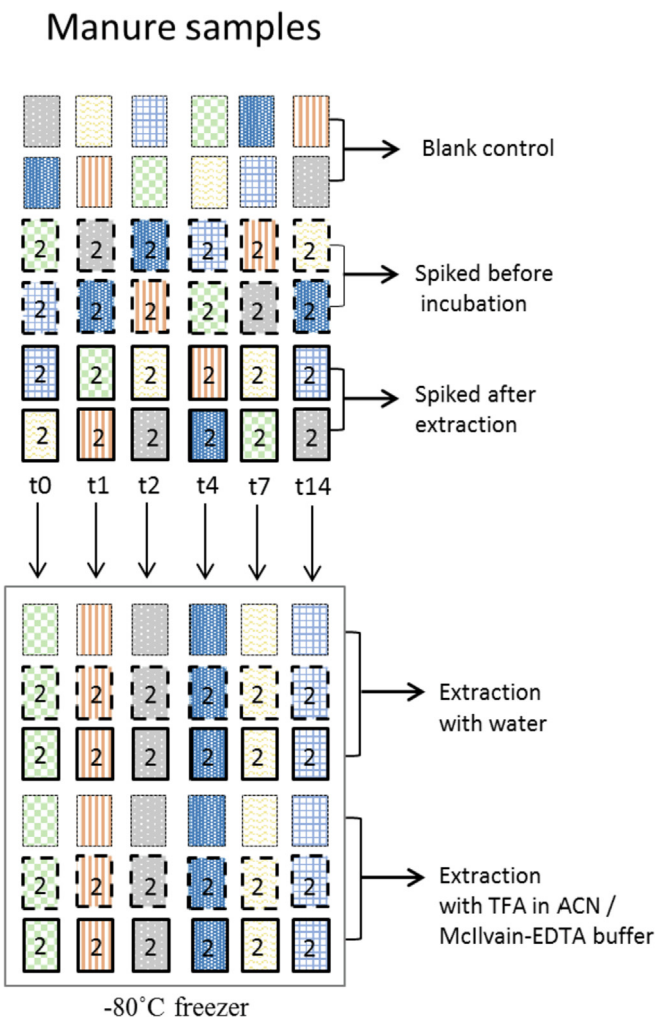


Fig. 2. Set-up of the longitudinal experiment. Blocks with a 2 inside indicate duplicates, blocks without a number are singular. For each sample preparation method a total of 5 manure samples were randomly selected per time point; 1 blank control, an incubated spiked sample in duplicate and a blank sample to spike after extraction in duplicate.

vortexed and stored immediately at −80 °C to serve time point 0 days (t = 0). The remaining samples were incubated at a temperature of 15 °C. After t = 1, 2, 4, 7 and 14 days, samples were randomly selected and stored at −80 °C according to above procedure. On day 14 all tubes were stored at −80 °C.

Before sample preparation, samples were defrosted. Samples for the water extraction were prepared by extracting with 8 mL of water and the other samples were extracted with 4 mL of a freshly prepared 0.125% TFA in ACN solution, followed by addition of 4 mL McIlvain-EDTA buffer. All aliquots were then treated according to the sample preparation procedure and after decanting the supernatant into a clean 50 mL tube, internal standard solution was added at a level of 100 μg kg^{-1} to all aliquots. At this phase, the blank duplicates were spiked as well, at a concentration of 500 μg kg^{-1} (spike 2). The rest of the procedure was followed as described in sample preparation, sample clean-up and LC-MS/MS analysis. Extraction recovery was calculated according to formula 3.

Additionally, for each time point, a tube with standard solution in water was also incubated to determine the stability of the antibiotics in the absence of manure matrix. These solutions were analysed without any sample preparation.

2.7. Calculations

Comparisons were made using response factors (RF), which were calculated by dividing the peak area of the most abundant ion of the individual antibiotics by the peak area of the corresponding internal standard. During the extraction experiments, RF were calculated using stable isotopically labelled internal standards for the compounds ENR, FLU, LIN, SDZ, SDX and TMP. DC and OTC were corrected using DMC. During extraction experiments, TIL was corrected using pirlimycin and TYL was corrected using tiamulin. During the longitudinal experiment, both TIL and TYL were corrected using gamithromycin-d_4.

Aiming for an optimal quantitative confirmatory monitoring method, additional internal standards were obtained. During the in-house one-day validation and the analysis of the incurred samples almost all compounds were corrected using labelled internal standards. These internal standards will from here on be used in practise as well. The following compounds are an exception because no labelled internal standard was available on the market and had therefore to be corrected using a similar compound; josamycin and natamycin were corrected using erythromycin-^{13}C-d_3, Neospiramycin using gamithromycin-d_4, sulfamonomethoxine using sulfisoxazole-^{13}C, sulfamoxole using sulfadimethoxine-d_6 and CTC, DC and OTC using DMC.

All losses during the sample preparation, including non-extractable residues, are expressed by the total recovery of the method (%Total recovery) and calculated according to formula 1. The spike numbers (e.g. spike 1, spike 2) correspond to the numbers given in Fig. 1. The fraction of non-extractable residues (Formula 2) is the amount of residues that could not be extracted from manure but could be extracted in absence of manure matrix (here the manure matrix was replaced by water). Note that losses or degradation (if any) during the entire sample preparation are taken into account by comparing water and manure. The extraction effectiveness is expressed as the extraction recovery (%Extraction recovery), calculated according to formula 3. The SPE recovery is calculated according to formula 4.

During the longitudinal experiment one duplicate of manure was spiked before incubation and one duplicate after extraction during sample preparation. Extraction recovery was determined according to formula 3.

Formula 1: %Total recovery = RF spike 1/RF spike 3 × 100%

Formula 2: %Non-extractable residues = 100 − (RF spike 1/RF spike 2) x 100%

Formula 3: %Extraction recovery = RF spike 1/RF spike 2 × 100%

Formula 4: %SPE recovery = RF spike 2/RF spike 3 × 100%

2.8. In-house validation and analysis of incurred samples

An in-house one-day validation was carried out to test whether performance criteria comply with 2002/657/EC (Commission, 2002) for the 48 compounds using the optimized method. For quality control, a total of 6 blank bovine manures (including veal calf and dairy cow) were collected. For each sample 2 g was weighed in triplicate and spiked at a level of 0, 5 and 10 μg kg^{-1} for sulfonamides and 0, 20 and 40 μg kg^{-1} for tetracyclines, quinolones, macrolides, lincosamides, pleuromutilins and trimethoprim. For the matrix calibration curve, six aliquots (2 g) of a blank manure batch were spiked at a level of 0, 2.5, 5, 10, 25 μg kg^{-1} for sulfonamides and 0, 10, 20, 40, 100 μg kg^{-1} for the other compounds. The samples were prepared according to the optimized sample preparation procedure, sample clean-up and LC-MS analysis.

The stability of the antibiotics, in solutions as well as final extracts, were reported in previous studies (Berendsen et al., 2011, 2015). Trueness and repeatability including matrix variation (RSDr*) were calculated and tested against the performance criteria (Commission, 2002). In case of trueness, because of the large matrix variation, the calculated percentage was accepted when between 80 and 120% for all compounds. The RSDr* is accepted when, according to the Horwitz equation (Horwitz et al., 1980), the result is below 23.4% or 21.1% on a level of 5 μg kg^{-1} and 10 μg kg^{-1} respectively for the sulfonamides and 19.0% or 17.2% on a level of 20 μg kg^{-1} and 40 μg kg^{-1} respectively for the other compounds. It has been found this formula is not applicable to lower concentrations (<120 μg kg^{-1}) (Thompson, 2000). However, manure is considered to be a more complex matrix compared to most products of animal origin. Therefore, the criteria as established in 2002/657/EC were adopted, instead of the more strict criteria set by Thompson (2000). The linearity was considered to be acceptable if the coefficient of correlation (r) was at least 0.990 based on the matrix calibration curve.

Selectivity is mainly acquired by the ion transitions when using a triple quadrupole (Berendsen et al., 2013). Here, selectivity was assessed by verifying the blank manure samples, without addition, for interfering signals at the retention time corresponding with the compounds of interest.

Confirmation of the identity in every spiked sample was evaluated by calculating the relative retention time and relative ion ratio (deviation of the relative abundance of two product ions) based on the matrix calibration standards. The retention time of the compound should not deviate more than 2.5% from the relative retention time, and ion ratio should comply with the reported ranges in 2002/657/EC (Commission, 2002).

The limit of detection (LOD) and limit of quantification (LOQ) are the concentrations where respectively the most and the least abundant product ion showed a signal-to-noise ratio of at least 6. For LOQ, additionally the antibiotic detected should comply with the conformation criteria in 2002/657/EC (Commission, 2002).

A total of 34 different veal calve manure samples were collected from different farms. These samples were analysed using the optimized method as described in the sample preparation, sample clean-up and LC-MS method sections.

3. Results & discussion

3.1. Extraction experiments

In order to assess the effects of different extraction solvents on different compounds in manure, the total recovery and the fraction of non-extractable residues were determined for 24 different extraction solvents (ES). In Fig. 3 the results of extraction experiment 1 are presented. Boxplots are shown for each ES tested, taking into account the calculated total recovery of 10 compounds in duplicate. TYL and TIL could not be analysed in the water samples during this experiment and therefore the fraction of non-extractable residues was not calculated for these compounds. Moreover, the fraction of non-extractable residues could also not be calculated for extraction solvents yielding 0% total recovery for specific compounds. In these results, only peaks with a signal-to-noise ratio (S/N) equal or higher than 6 were considered. Compounds having zero recovery are left out in the visualization of non-extractable residues in Fig. 3 because otherwise the boxplot will not give a correct representation of the reality.

Focussing on the fraction of non-extractable residues, it is observed that, overall, high percentages of organic solvents are more effective as is clear from the high fraction of non-extractable residues when using water as ES (ES22) in comparison with the fraction of non-extractable residues in the ACN (ES1 - 4) and MeOH (ES5 - 8) analogues. Also 1 mL of ACN seems insufficient (comparison of ES21 with ES1).

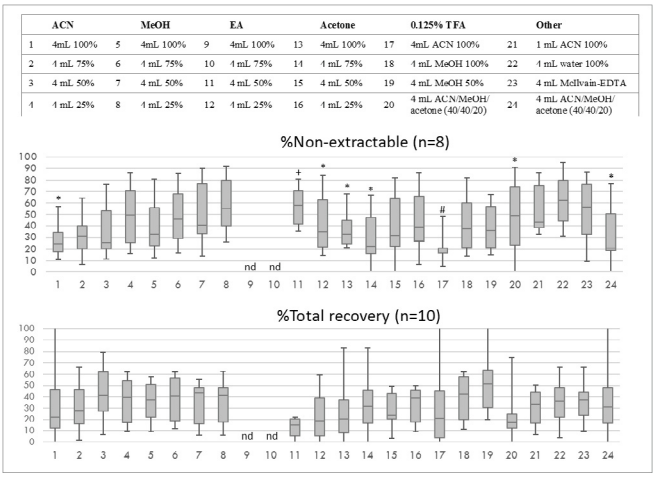


Fig. 3. Boxplots showing the percentage non-extractable residues and the total recovery for each extraction solvent. The compound with the highest and lowest percentage are indicated with the top and bottom error bars. The middle line in the boxes indicates the median. Compounds that were not detected are indicated as nd. For % Non-extractable residues n = 8 compounds, n = 7 compounds (*), n = 6 compounds (+) or n = 5 compounds (#).

A total recovery of 0% was found for all compounds in extracts containing 100% and 50% ethyl acetate (EA) (ES9, 10). EA extracts in general were unmanageable using the current procedure, because they caused major (100% EA) to minor (25% EA) clotting of the SPE cartridges. Therefore, the use of EA was regarded impractical and was therefore eliminated in further experiments.

LIN in ES 1, 11–14, 20, 24 and LIN, OTC and trimethoprim in ES17 also yielded an insufficient total recovery. All these extraction solvents contain a percentage of ACN or acetone of over 50% and yielded low method recoveries compared to MeOH, water and McIlvain-EDTA buffer extracts. As it was observed that the total recovery increased with a decrease of the percentage of ACN and acetone, it was hypothesised to be caused by low SPE recoveries and not to be related to the solvent extraction itself.

From the results of experiment 1, nine candidate ESs were selected for experiment 2, based on either high recovery or the low fraction of non-extractable residues. Additionally, water as ES was included as a reference.

Results of experiment 2, in which also the recovery of the SPE was determined, are shown in Fig. 4 for the individual compounds. As a comparison, the fraction of non-extractable residues determined in experiment 1 is also presented. The results of experiment 2 confirm that SPE is the main cause for the low total recoveries using ESs containing >50% ACN or acetone. In case the total recovery can be improved by optimising the SPE procedure, TFA in ACN (ES17) is considered as the most optimal extraction solvent, with the lowest fraction of non-extractable residues.

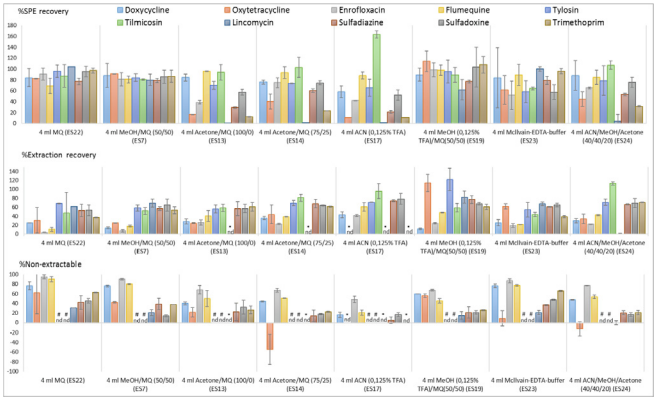


Fig. 4. SPE and extraction recovery for 10 extraction solvents, in comparison with the non-extractable residues. In some cases compounds were not detected (nd) because of low SPE recovery (*) or because they could not be analysed in water (#).

In experiment 3, the SPE recovery was optimized for TFA in ACN (ES 17), testing three different methods (method A, B and C). Method C caused major clotting of the PRiME cartridges, yielding insufficient results. Therefore this option was discarded. In Fig. 5 the SPE recovery (n = 2), extraction recovery (n = 1) and the fraction of non-extractable residues (n = 1) are shown for method A and method B. Once again, TYL and TIL could not be analysed in water.

Furthermore, OTC results did not meet the quality criteria. Therefore the results of OTC are considered to be unreliable. This experiment shows that, after evaporation of the organic fraction from the extraction solvent (method B), the SPE recovery is significantly improved for ENR, LIN, SDZ and TMP. These compounds in particular are relatively polar and therefore they are most prone to break through the SPE cartridge. As a result of the SPE optimization, these compounds yield a sufficient total recovery and allow the assessment of the extraction recovery and fraction of non-extractable residues.

After optimization of the SPE procedure, the non-extractable fraction of all residues, except TYL and TIL was determined using the described method. Clearly, even when using 0.125% TFA in ACN as the ES, for some antibiotics not all residues are extractable. Mainly the tetracyclines and (fluoro)quinolones show a relative high fraction of non-extractable residues. In this experiment as well as the longitudinal experiment no significant formation of the epi-tetracyclines was observed. Residues of tetracyclines and (fluoro) quinolones might be bound to solid particles or occur as stable complexes. In earlier research, it was found that these groups of antibiotics have a high persistence in manure (Loke et al., 2002; Martínez-Carballo et al., 2007; Conkle et al., 2010; Berendsen et al., 2018). Furthermore, tetracyclines are known to form complexes with doubly charged cations (Vartanian et al., 1998).

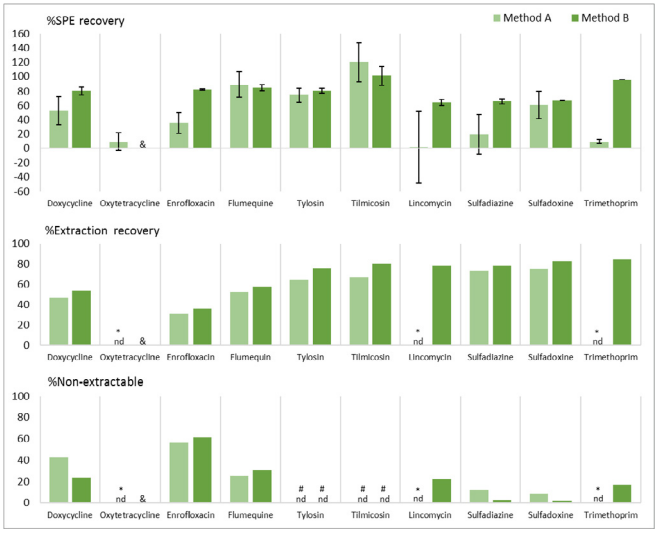


Fig. 5. SPE recovery, extraction recovery and fraction non-extractable residues for method A and B, the methods without and with evaporation step before SPE respectively. &: analysis did not meet the quality criteria. *: Not determined (nd) because of low SPE recovery. #: Not detected (nd) because they could not be analysed in water.

3.2. Longitudinal experiment

Losses caused by bound residues can be corrected for when adding an (isotopically) labelled internal standard which reacts in the same way as the analyte of interest present in the manure sample. However, in case the fraction of non-extractable residues changes over time, e.g. by adsorption or desorption, addition of internal standard at the beginning of the sample preparation will not accurately correct for this effect. Therefore, the longitudinal effects were studied, aiming to determine whether binding is an instantaneous process or a process occurring over time. This was done by plotting the extraction recovery of the water extraction and the TFA in ACN extraction (the extraction solvents yielding respectively the most and the least non-extractable residues) over time and comparing them.

Firstly, the antibiotics remained stable in aqueous solution during the 14 days of the experiment, which validates the results of the longitudinal study in manure. The results for all 10 compounds are presented in Fig. 6.

The data was evaluated statistically using linear regression analysis (Excel 2016, Microsoft, Redmond, WA, USA). Based on residual plots, it was determined whether a linear regression model was appropriate for data analysis. In case it was not, a natural logarithm transformation was applied and residual plots were assessed again. This pointed out that all compounds except TIL and sulfadoxine required natural logarithm transformation before comparison.

In order to compare the extraction recovery over time for the two ESs, two different aspects were tested using linear regression. First, whether a compound significantly decreases over time ($P = 0.05$) and second whether a compound that was extracted with water shows a significantly different trend compared to extraction with TFA in ACN. In order to test the latter hypothesis, the calculated 95% confidence interval of the slopes of both regression lines were used. Instantaneous binding is indicated by parallel lines for both ESs in Fig. 6 (no significant difference between the slopes) regardless whether there is a significant decrease in time or not. In case there is a significant difference between the slopes, the regression lines in Fig. 6 would clearly diverge or converge, indicating an increase respectively decrease of non-extractable residues over time.

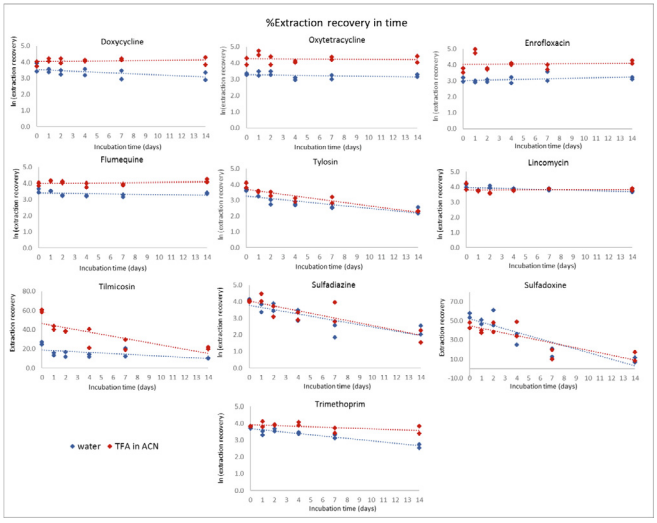


Fig. 6. Extraction recovery of ten different compounds in manure and sterile manure incubated during 14 days and extracted with water or TFA in ACN. Expressed as natural logarithm, 100% recovery equals 4.6.

DC, OTC, ENR, FLU and LIN show straight lines with no significant decrease over time for both ESs. Furthermore, no significant differences between the slopes of the two lines is observed. Therefore it is concluded that these compounds bind instantaneously. Note that since the variation between duplicates is very small for LIN, small decreases automatically lead to a significant p-value. The calculated p-value was considered and compared to the visual data, concluding no decrease occurs.

TYL, SDZ and SDX show a significant decrease in time for both of the ESs and TIL and TMP show a significant decrease in time for only one ES. Previous research showed that the decrease of TYL and TIL is due to degradation by micro-organisms, however for SDZ and SDX, a decrease due to micro-organisms is ruled out (Berendsen et al., 2018). Binding of sulfonamides is however very unlikely, since these compounds are known to have a high solubility and high mobility (Boxall et al., 2004; Burkhardt et al., 2005; Karcı and Balcıoğlu, 2009). Therefore we conclude that the negative slope is most likely the result of abiotic degradation.

TYL, TIL, SDZ and SDX show no significant difference between the two slopes. For TYL, SDZ and SDX it is concluded that binding occurs instantaneous. For TIL however, diverging lines are observed in Fig. 6, as only the TFA in ACN decreases significantly. The reason why no significant difference is indicated by statistical analysis is because of the differences between duplicates, which causes large uncertainties. Therefore, no clear conclusions for TIL can be drawn.

TMP shows a significant decrease in time for water only and the slopes of the two ESs is significantly different. Based on this data it is concluded that the fraction of non-extractable residues increases in time, but by using TFA in ACN as the ES, the maximum fraction of residues are released and extracted.

3.3. In-house validation and analysis of incurred samples

The bovine samples selected for validation were analysed and results were assessed against previously set criteria. Trueness complied for 45 out of the 48 compounds, exceptions were josamycin, sulfamoxole and OTC. The RSD_r* complied for 43 out of 48 of the compounds, exceptions were neospiramicin, TIL, sarafloxacin, OTC and sulfaquinoxaline. Most of the compounds that could not be quantified based on the outcomes of this validation, did not have isotopically labelled internal standards. It is expected that these compounds will comply to set criteria in case an isotopically labelled internal standard becomes available. Based on the validation results, using the developed method, a quantitative analysis can be carried out for all prioritized compounds (most often found in manure), except for TIL and OTC. Note that for OTC, isotopically labelled standards are commercially available, but have not been applied in the validation due to its extremely high costs. For TIL, it stood out that one of the six validation samples showed extremely deviating results. The other five were all within the acceptable ranges of the performance criteria.

The method was used to analyse a total of 34 veal calve manure samples from different origin. The validation was carried out on a broad scope of cattle manure, including veal calf manure, therefore the analysis of veal calf matches the scope of our validation. Quantification of all samples was done based on a calibration line prepared in semi-solid bovine manure which was blank for all analysed antibiotics. Every sample was analysed with and without addition of the analytes of interest for quality control purposes. The samples containing antibiotics are shown in Table 2. LODs for tetracyclines ranged from 2 to 25 $\mu g\,kg^{-1}$, for quinolones from 1 to 25 $\mu g\,kg^{-1}$, for macrolides, lincosamides and pleuromutilins from 1 to 50 $\mu g\,kg^{-1}$ and for sulfonamides, dapsone and TMP from 1 to 15 $\mu g\,kg^{-1}$. Samples were considered positive if the antibiotic

Table 2
Positive results (μg kg^{-1}) of the 34 manure samples using the in-house validated method.

	CTC	OTC [b]	TC	DC	MAR	CIP	ENR	FLU	TYL	TUL	TIL [b]	LIN	SDZ	SDD	SMZ
1		1000[a]	12[c]	3000 [a]	19			2100 [a]			24		34	3600 [a]	
2	61	11[c]	7	1900 [a]				1							
3		600 [a]		2300 [a]				190			71		60	1100 [a]	7
4		1200 [a]	20	2600 [a]		2		260			119				
5		1600 [a]	31	1800 [a]		9	4	120			26		1	22	
6		1800 [a]	34	3400 [a]		2		16			300		2	9	
7		480 [a]	8	1400 [a]				2			41		3		
8		1800 [a]	33	6900 [a]		5	3	22			6				
9		770 [a]	10	540 [a]		1		4000 [a]			8		65		
10		1800 [a]	24	1600 [a]		4	3	41			12		3	23	
11		71		7000 [a]				1200 [a]			21		35	12	
12		2000 [a]	34	5100 [a]				700 [a]			150				
13		1600 [a]	36	1800 [a]		4		7			25		1		
14		2200 [a]	31[c]	1600 [a]		1		171			29		17	2	
15		3100 [a]	24	3400 [a]				11	5	21	2000 [a]		2	5100 [a]	
16		200	8	1100 [a]		2 [c]		1700 [a]			76		60		3
17		86	7	530 [a]		2 [c]		19			8		3	2	
18		75	5	690 [a]				97			8		8	200 [a]	
19			12	2100 [a]		5 [c]		68			32		14	420 [a]	
20		2700 [a]	47	3400 [a]					4	18	300			1700 [a]	1 [c]
21		48		50								2			
22		42		63				15					3		
23		28		13				6							
24		8													
25		21	9	52				11							
26		10											9		
27		18													
28						9									
29			8												
30		3	11										7		
31		75	7												
32		5	7					4					3		
33						4		5				2	26		

[a] Quantitative result was extrapolated.
[b] Compound could not be quantified based on the validation, therefore the reported values should be considered estimations.
[c] Compound was detected and quantified, but the identity was not confirmed according to validation regulations (Commission, 2002).

detected complied with the conformation criteria in 2002/657/EC (Commission, 2002), and in case the least abundant product ion showed a signal-to-noise ratio of at least 6.

Out of a total of 34 samples, 33 were tested positive (97%). A single manure sample contained up to 8 different antibiotics. The antibiotics most frequently found with the highest concentrations are OTC, DC, FLU, TIL and SDD.

4. Conclusion

This research demonstrates that especially for the tetracyclines and quinolones the extraction solvent selection is critical due to a high fraction of non-extractable residues, whereas sulfonamides can effectively be extracted with aqueous solvents. Overall, 0.125% trifluoroacetic acid in acetonitrile in combination with McIlvain-EDTA buffer proved to be the most effective extraction solvent. Furthermore, for most antibiotics, spiked manure is considered representative for incurred manure samples in case extraction is done using TFA in ACN in combination with McIlvain-EDTA buffer. Based on these new insights, the current in-house multi-residue LC-MS/MS method for manure analysis was revised and additionally validated. The data presented in this research contributes to the understanding of the processes during extraction of manure and thereby presents valuable data that can be used when optimising methods to analyse multiple veterinary drugs in manure. Furthermore, it is a step closer to an adequate risk assessment of the environmental impact of contamination by veterinary drugs.

Declarations of interest

None.

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