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Quantification of veterinary antibiotics (sulfonamides and trimethoprim) in animal manure by liquid chromatography–mass spectrometry

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Abstract

A fast and cost effective method was developed to extract and quantify residues of veterinary antimicrobial agents (antibiotics) in animal manure by liquid–liquid extraction and liquid chromatography–mass spectrometry. The compounds investigated include six sulfonamides, one metabolite, and trimethoprim. The method was performed without sample clean up. Recoveries from spiked manure slurry samples (spike level=1 mg/kg) were as follows: sulfaguanidine (52%), sulfadiazine (47%), sulfathiazole (64%), sulfamethazine (89%), its metabolite N^4-acetyl-sulfamethazine (88%), sulfamethoxazole (84%), sulfadimethoxine (51%), and trimethoprim (64%). Relative standard deviations of the recoveries were less than 5% within the same day and less than 20% between days. The limit of quantification was below 0.1 mg/kg liquid manure slurry for all compounds and calibration curves obtained from extracts of spiked samples were linear up to a level of 5 mg/kg liquid manure, except for trimethoprim (0.01–0.5 mg/kg). Analysis of six grab samples taken in Switzerland from manure pits on farms where medicinal feed had been applied revealed total sulfonamide concentrations of up to 20 mg/kg liquid manure. © 2002 Published by Elsevier Science B.V.

Keywords: Manure; Antibiotics; Sulfonamides; Trimethoprim

1. Introduction

The development and spread of antibiotic[1] resistant human pathogens is a major concern of global significance [1]. Increased frequencies of antibiotic resistance are a result of selective pressure exerted by the large amounts of antibiotics used. The estimated annual consumptions of antibiotics in the European Union and in the United States are both approximately 10 000 metric tons [2,3]. About half of the total antibiotic consumption in the European Union [2] and more than 80% of the consumption in the US [3] are used for livestock production. These figures include antibiotics added to animal feeds for disease prevention and treatment, and also for

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[1]For reasons of simplicity the term antibiotics is used in this paper for all antimicrobial agents.

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growth promotion. In Sweden, and in Switzerland, the use for growth promotion was banned in 1986 and in 1999, respectively, with the purpose to reduce selective pressure on antibiotic resistant pathogens. However, the legislation of the United States and the European Union only banned the use of certain antibiotics for growth promotion.

In some cases, the emergence and spread of antibiotic resistant human pathogens have been directly linked to the use of antibiotics in animal husbandry [4–6]. Generally, the most probable path for the infection of humans with antibiotic resistant bacteria from animal origin is considered to be the consumption of contaminated food products derived from treated animals. However, it should be noted that the quantity of antibiotics and antibiotic resistance genes excreted by the animals is far greater than the amount that ends up in food products, and that frequencies of antibiotic resistance are particularly high in bacteria isolated from animal manure [7–10]. With manure slurry being used as fertilizer, antibiotics, as well as antibiotic resistance genes, are distributed on fields and pastures on a large scale. Little is known about concentrations and fate of antibiotics in manure and soil. These parameters are of great importance when evaluating the role of contaminated manure in the spread of antibiotic agents and their corresponding resistance genes into the environment, and to assess the risk of water and food contamination through this pathway. Therefore, it is necessary to develop analytical methods for the quantification of the most important antibiotics in manure.

The most widely used groups of antibiotics in the European Union's animal husbandry are tetracyclines, macrolides, penicillins, aminoglycosides and sulfonamides/trimethoprim (trimethoprim is a potentiator often administered together with sulfonamides) [2]. Our focus was on substances that are likely to be transported into the aquatic environment. Tetracyclines are known to strongly sorb and are therefore expected to remain in the soil or to be transported into surface waters via particles. Penicillins, macrolides and aminoglycosides are expected to be fairly well degradable. However, sulfonamides appear to have a high potential to resist degradation and are hydrophilic enough to be transferred into the aquatic environment. For example, concentrations of the sulfonamide sulfamethazine (=sulfadimidine) measured by Alder et al. in a lake with intensive animal husbandry surroundings were higher than the concentrations in the effluents of waste water treatment plants in the same area [11]. This indicates that sulfamethazine in this case is not from human medicine, but from animal manure origin.

According to the European Agency for the Evaluation of Medicinal Products, a more intensive study of environmental safety of a veterinary medicinal product is necessary if any ingredient or metabolite is present in manure for spreading onto land in concentrations $\geq$0.1 mg/kg [13]. To our knowledge, methods for the simultaneous quantification of several sulfonamides and trimethoprim in manure slurry samples at this concentration level have not been published. However, Berger et al. presented a method to quantify sulfamethazine and chloroamphenicol [12] in manure slurry. The method consisted of liquid–liquid extraction with ethyl acetate, followed by liquid chromatography and ultraviolet detection (LC–UV). This method was used to measure concentrations in the mg/kg (wet manure) range. Limits of quantification (LOQs) were not reported. Hirsch et al. present a method to quantify sulfonamides in water samples [14], and a variety of methods are available to quantify residues of these antibiotics in food products [15–21]. The preferred technique for this task is liquid–liquid extraction followed by a sample clean up using solid-phase extraction and detection by liquid chromatography coupled with mass spectrometry (LC–MS) or tandem mass spectrometry (LC–MS–MS) [15–17]. Other methods include supercritical fluids for the extraction [18], gas chromatography (after derivatization) [19] or capillary electrophoresis [20] for separation, and UV [21] for the detection of these compounds.

The aim of this research was to develop a cost effective and precise method for the quantification of sulfonamides and trimethoprim in animal manure at the 0.1 to 10 mg/l level (structures and physico–chemical properties of the compounds investigated are given in Fig. 1 and Table 1). The methods used are liquid–liquid extraction followed by LC–MS. For this purpose, chromatography, mass spectrometry, and extraction parameters are optimized,

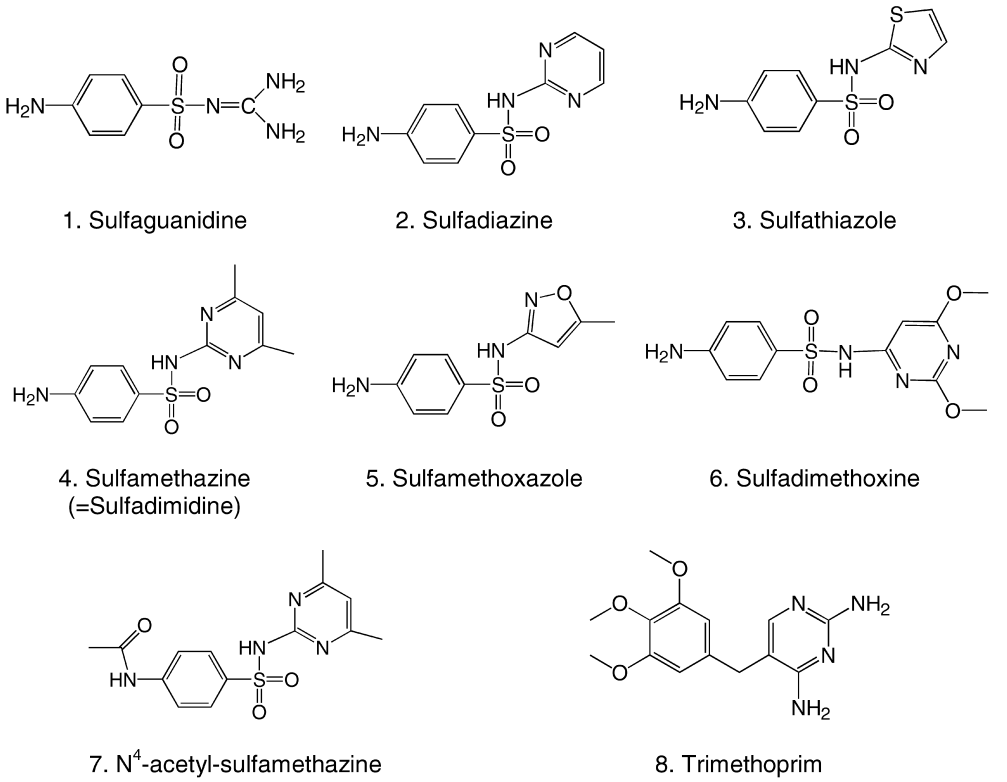

Fig. 1. Structures of the investigated compounds: six sulfonamides (1–6), one sulfonamide metabolite (7) and trimethoprim (8). Note that all sulfonamides investigated have at least two nitrogen functions. The amide attached to the sulfur is referred to as N^1 and is deprotonated at pH>5.5–7 (except for sulfaguanidine). The amine attached to the aromatic cycle is referred to as N^4 and is protonated at pH<2.5. For this reason, most sulfonamides are positively charged at acidic conditions, neutral between pH 2.5–6 (approximately) and negatively charged at alkaline conditions (see also Table 1).

Table 1
Physicochemical properties of the investigated compounds

	pK_a	t_R (min)	m/z* (V)	m/z (V)	m/z (V)	m/z (V)
Sulfaguanidine	pK_b=11.3[a]	4.4	215 (30)	156 (42)	92 (60)	–
Sulfadiazine	6.5[a]	7.3	251 (25)	156 (45)	92 (65)	65 (85)
Sulfathiazole	7.1[b]	7.8	256 (30)	156 (45)	92 (65)	65 (85)
N^4-Acetyl-sulfamethazine	7.1[c]	9.3	321 (40)	124 (70)	92 (75)	65 (90)
Trimethoprim	6.6[d]	9.7	291 (35)	261 (70)	230 (70)	–
Sulfamethazine	7.4[d]	10.9	279 (30)	156 (55)	92 (70)	65 (90)
Sulfamethazine-phenyl-$^{13}C_6$	–	10.9	285 (30)	284 (30)	114 (65)	98 (70)
Sulfamethoxazole	5.7[c]	18.6	−252 (30)	−156 (50)	−64 (75)	–
Sulfadimethoxine	5.9[c]	21.4	−309 (45)	−154 (75)	−66 (90)	–

Acidity and basicity constants are from (a) Ref. [24], (b) Ref. [25], (c) Ref. [26], (d) Ref. [27]. Retention times (t_R) are given in min. All mass per charge ratios (m/z) used for single ion monitoring are listed, followed by the optimal cone voltage setting (V) in parentheses. *Mass-to-charge ratio (m/z) used for quantification. All other mass to charge ratios were used for confirmation. Note that some compounds produced less than three fragments with sufficient intensity to be used as confirmation ions.

and then manure slurry grab samples from different farmyard manure pits are analyzed.

2. Experimental

2.1. Chemicals

Standards of sulfaguanidine, sulfadiazine, sulfamethazine (=sulfadimidine), N^4-acetyl-sulfamethazine, sulfamethoxazole, sulfadimethoxine and trimethoprim were purchased from Sigma–Aldrich (Seelze, Germany) and sulfathiazole from Fluka (Buchs, Switzerland). The isotope labeled internal standard sulfamethazine-phenyl-$^{13}C_6$ was obtained from Cambridge Isotope Labs. (Andover, MA, USA). All standards were dissolved in methanol (1 g/l) and kept at 4 °C. Ethyl acetate (EtOAc) was HPLC-grade and purchased from Fluka. Water and acetonitrile (ACN), used for mobile phases, as well as methanol (MeOH), used to dissolve standards, was HPLC-grade obtained from Scharlau (Barcelona, Spain). Ammonium-acetate (NH_4Ac), sodium chloride (NaCl), sodium sulfate (Na_2SO_4), potassium hydroxide (KOH), sodium hydrogen carbonate ($NaHCO_3$), sodium carbonate (Na_2CO_3), disodium hydrogenphosphate (Na_2HPO_4) and acetic acid glacial 100% (all analytical-reagent grade) were obtained from Merck (Darmstadt, Germany).

2.2. Sampling and sample preparation

The sampling of slurry from large manure pits was done in accordance with established manure sampling techniques applied by the Swiss Federal Research Station for Agroecology and Agriculture [22]. Samples were collected from pig and cattle farms after stirring the manure slurry in the pits for a minimum of half an hour. Approximately 4 l was sampled three times and mixed well in a bucket. From this bucket 500 ml were sampled into a plastic container and transferred to the laboratory where they were ground in a kitchen blender before the samples were stored at −20 °C until extraction. Before the extraction, dry matter was determined after dehydrating a manure slurry subsample of 10 g at 60 °C until constant mass was reached.

2.3. Extraction procedure of the optimized method

The ground and frozen manure slurry samples were left to adjust to room temperature overnight. For the following subsampling procedure, the solution was stirred continuously to prevent sedimentation of particles, and a micro-pipette with an enlarged opening (the point cut off, leaving a hole of about 2 mm to let small particles pass) was used to sample. With this method, a subsample of approximately 20 ml was adjusted to pH 9 with KOH, and 3 g of this subsample was weighed into a 15 ml polypropylene tube (Cellstar, Greiner). The internal standard sulfamethazine-phenyl-$^{13}C_6$ was added (1 μg/g manure slurry), and the sample was vortexed (WhirliMixer from Fisons) and left to equilibrate for 10 min. After this, 1 g of NaCl was added, and the sample vortexed again. To extract the target compounds, 5 ml EtOAc was added, and then the sample was vortexed for 30 s, sonicated (Branson 3200) for 15 min and centrifuged (Ultrafuge Filtron, Heraeus) for 10 min at 5300 rpm. The organic phase was removed with a pasteur pipette and collected in a 20 ml borosilicate glass vial (Infochroma). The sample was extracted three times consecutively with EtOAc as described above and all three EtOAc extracts were collected in the same vial.

From the EtOAc extract, one third (5 ml) was filled into a reduction vial (Supelco) and reduced under nitrogen flow to almost dryness. 100 μl mobile phase A (see Section 2.4) was added and the supernatant EtOAc further reduced under nitrogen flow. Another 350 μl mobile phase A was added, the vial closed, and sonicated for 15 min. The resulting extract contained visible particles and was filtered with 0.45 μm regenerated cellulose filters (Spartan 13/0, 45 RC, Schleicher and Schuell). The filtrate was collected in a HPLC vial (11 mm amber, BGB Analytik). The reduction vial was washed with 600 μl mobile phase A and the washing liquid pressed through the same filter and collected in the same HPLC vial. The mass of the extract was adjusted to 1.00 g with mobile phase A.

2.4. HPLC–MS

The HPLC system consisted of the 1100 series from Hewlett-Packard with a binary pump (BinPump

G1312A, autosampler (G1313A), and a column compartment (ColComp G1316A). The separation of the antibiotics was performed on a reversed-phase C_{18} column with a pre-column for protection of the analytical column (125×3 mm I.D. Nucleosil 100-5 C_{18} HD, 5 μm particle size; and 8×3 mm I.D. Nucleosil 100-5 C_{18} HD, both from Macherey-Nagel). The injection volume was 50 μl, flow-rate 250 μl/min, and column temperature 25 °C. Mobile phase A was HPLC-water with NH_4Ac (1 m*M*), pH adjusted to 4.6 with acetic acid, filtered with cellulose nitrate 0.2 μm and combined with 10% (v/v) ACN. Mobile phase B was 100% ACN. All eluent changes were run linear. The eluent started at A–B (90:10). After injection, it was changed to A–B (85:15) within 6 min, where it was left constant for 4 min. Then, B was increased to 26% within 2 min and further to 65% within another 4 min. From there, B was further increased to 100% within 14 min where it was left for 5 min to flush the column before it was reduced to 10% within 5 min and left at this level to equilibrate for another 10 min.

Through an electronically controlled valve (C2-0004EH from Valco) it was possible to either dispose of the outflow of the ultraviolet detector or to transfer it into the electrospray interface of an MS Platform LC (Micromass). To protect the MS system from contamination and clogging, the eluent was prevented from entering the MS system during the first 3.5 min and the last 25 min of each run. The MS was run in the positive and negative single ion modes (for cone voltages and mass to charge ratios refer to Table 1 and Results and discussion). The source temperature was 150 °C.

2.5. Quantification of grab samples

In some cases the amount of sulfonamides in extracts of grab samples was above the linear range of the calibration curve, and the extract was diluted to measure these compounds accurately. Thus, the quantity of internal standard in the diluted samples was too low to calculate a reliable ratio of the analyte peak area to the internal standard peak area. For this reason, the following procedure was applied for all grab sample quantifications: analyte concentrations, as well as the concentrations of the internal standard, were determined in the extracts of the grab samples with external calibration (and with standard addition where indicated). Then, the recovery of the internal standard was calculated individually for each sample. The concentrations of the analytes in the

Table 2
Absolute recoveries of spiked cow manure slurry[a] and deionized water extracted at different pH values[b]

	Cow manure slurry				Deionized water		
	pH 5.0, recovery (%), (RSD=2–8%)	pH 7.0, recovery (%), (RSD=2–6%)	pH 9.0, recovery (%), (RSD=1–4%)	pH 9.0[c], recovery (%), (n=1)	pH 5.0, recovery (%), (RSD=2–5%)	pH 7.0, recovery (%), (RSD=0–12%)	pH 9.0, recovery (%), (RSD=1–3%)
Sulfaguanidine	39	44	**52**	50	59	47	48
Sulfadiazine	68	80	**47**	34	88	84	48
Sulfathiazole	62	74	**64**	47	88	85	70
N^4-Acetyl-sulfamethazine	94	86	**88**	81	90	85	77
Trimethoprim	55	65	**64**	63	81	81	81
Sulfamethazine	45	59	**89**	66	85	87	80
Sulfamethoxazole	89	98	**84**	78	86	83	73
Sulfadimethoxine	31	47	**51**	54	89	90	84

Extract contents were determined with external calibration with the exception of the values in column 3 (see footnote b). Results in general are given as the average of n=3 extractions. Recoveries used for the final method are printed in bold.

[a] Spike level was 1 mg/kg wet manure slurry sample. Dry matter content was 2.9%. All parameters except pH were the same as in the ''optimized method''.

[b] The pH of manure samples was adjusted with KOH and acetic acid. The pH of deionized water was buffered with NH_4Ac–AcOH (pH 5.0), with Na_2HPO_4–AcOH (pH 7.0) and with Na_2CO_3–$NaHCO_3$ (pH 9.0).

[c] Values given in this column were determined with standard addition on one extract (four calibration points).

samples were calculated using the above calculated recovery of the internal standard and the relative recoveries to the internal standard known from spiked manure samples (see Table 2, pH 9). Extraction recoveries of sulfamethazine and the isotope labeled sulfamethazine used as internal standard were assumed to be equal.

3. Results and discussion

3.1. LC and MS method development

In accordance with published LC–MS methods, ammonium acetate buffered water and acetonitrile were used to separate the sulfonamides on a reversed-phase chromatographic column [14,15]. With a buffer content of 1 m*M*, a very low salt concentration was chosen to reduce the loss of sensitivity of the MS during measurements. A comparison between pH 4.6 and pH 6.0 for the ammonium acetate buffer, as used by other authors [15], revealed more stable retention times and better peak shapes for some analytes at pH 4.6, due to the closeness of pH 6.0 to the pK_a of these analytes (see Table 1). At pH 4.6, baseline separation was achieved for all analytes with exception of the pairs trimethoprim/N^4-acetyl-sulfamethazine and sulfadiazine/sulfathiazole (see Fig. 2). The chosen gradient allows baseline separation for almost all compounds. This allows quantification not only with the $[M+H]^+$ ion trace, but also with fragment ion traces which are identical for all sulfonamides (see below). Further, the eluent bypasses the MS with a large fraction, reducing the contamination of the interface and therefore the loss of sensitivity.

Mass spectra of all antibiotics and of the internal standard were acquired in the full scan mode with cone voltages of 25 and 75 V, using positive and negative electrospray ionization. All compounds except sulfadimethoxine and sulfamethoxazole produced higher signal-to-noise ratios in the positive ion mode. Typical sulfonamide fragments in this mode were detected at m/z 156, 108, and 92 (all known from the literature [15]). A further typical fragment not found in literature was found at m/z 65. Because

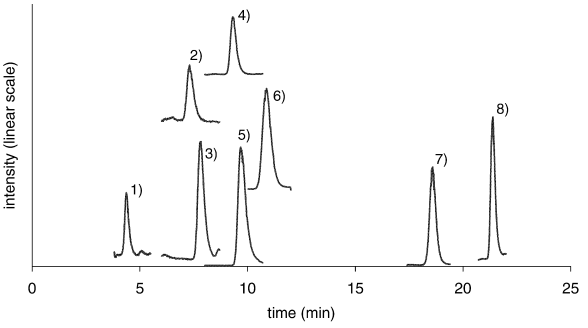

Fig. 2. SIM chromatograms of extracts obtained from manure spiked with 1 mg of each compound per kg liquid manure. Quantifying ion traces (see Table 1) of (1) sulfaguanidine; (2) sulfadiazine; (3) sulfathiazole; (4) N^4-acetyl-sulfamethazine (intensity reduced by factor 5); (5) trimethoprim (intensity reduced by factor 10); (6) sulfamethazine (intensity reduced by factor 5); (7) sulfamethoxazole; (8) sulfadimethoxine (intensity reduced by factor 5).

sulfamethazine-phenyl-$^{13}C_6$ produced an ion signal at m/z 70 instead of m/z 65, this fragment must be a $[C_5H_5]^+$ fragment of the aromatic cycle. Cone voltages were optimized for maximum signal intensity of typical ions during continuous injection of single compounds into the mass spectrometer (see Table 1). Either the $[M+H]^+$ or the $[M-H]^-$ ions were selected for quantification and two to three additional ions with the best signal-to-noise ratios were selected for confirmation.

3.2. Extraction method development

Because concentrations in manure slurry were considered to be high enough to be analyzed without sample enrichment, spiked manure slurry was diluted, filtered and injected directly onto the chromatographic column. However, this simple and direct approach did not yield satisfying results due to matrix interferences and unstable retention times. Therefore, a liquid–liquid extraction step with ethyl acetate as used in food analysis [16] was introduced. The extracts were reduced under nitrogen and taken up in mobile phase A without further sample clean up and then analyzed. Further, this method was

optimized by the control of extraction time, pH and salting out.

It was necessary to perform at least three consecutive liquid extraction steps. Although prolonging manure extraction times from 10 s to 3 min (stirring with the vortex) did not improve recoveries, as a margin of safety 30 s was chosen for the optimized method. The failure of prolonged extraction times to increase recoveries indicates that the extraction (of spiked samples) is controlled by distribution coefficients rather than the kinetics of desorption processes. The salting out by different amounts of NaCl or Na_2SO_4 added to the samples prior to extraction was studied and 6 *M* NaCl was determined to yield the best recoveries. To investigate the influence of the extraction pH, spiked samples of purified water were buffered at pH 5.0, 7.0 and 9.0 and extracted with EtOAc (for buffers used see Table 2). As expected, due to the negatively charged nature of most of the investigated compounds above pH 7, water samples showed decreasing recoveries for these compounds with an increasing extraction pH. At pH 5.0, all recoveries from water samples were above 80% except for sulfaguanidine (59%). When performing the same experiment with manure slurry samples recoveries were generally considerably lower compared to the water samples. Unexpectedly, recoveries of sulfamethazine, sulfadimethoxine and sulfaguanidine from wet manure slurry increased with an increasing extraction pH (see Table 2). This could be due to different particle sorption processes at different pH values. For the optimized method, pH 9.0 was chosen because it extracts sulfamethazine (and therefore also the isotope labeled sulfamethazine used as internal standard) more efficiently. Furthermore, chromatographic noise is lower compared to the pH 5.0 extracts.

3.3. Quality control

Recoveries of the optimized method were >50% for all compounds except sulfadiazine (47%) (see Table 2). Relative standard deviations (RSDs) of recoveries determined with three parallel extractions were below 5% and signals were without interferences when extracts of manure slurry spiked at the 1 mg/kg level were measured (Fig. 2). Between-day RSDs were below 20% and results obtained when measuring recoveries with standard addition differed by less than 30% from the ones mentioned above. Signal-to-noise ratios (S/N) of the quantifying ion chromatograms obtained from extracts of manure slurry samples spiked at the 0.1 mg/kg level varied greatly among different analytes. Sulfamethazine, sulfamethoxazole and trimethoprim had an $S/N>100$, whereas N^4-acetyl-sulfamethazine, sulfaguanidine and sulfadimethoxine had an S/N between 10 and 100 at this concentration level. S/N values for sulfadiazine and sulfathiazole were >100 at the 1 mg/kg level. For these two compounds, interferences were observed at concentrations of 0.1 mg/kg in some of the samples. If only one of the two compounds is present, fragment ions can be used for quantification. If both are present, the fragment ions of these two compounds interfere with each other because they are not baseline separated. In this case, LC–MS–MS may be employed.

Calibration curves acquired from extracts of manure slurry spiked with different concentrations of sulfonamides were linear over one order of magnitude and up to 5 mg/kg wet manure slurry. Trimethoprim, due to its excellent ion yield was only linear up to 0.5 mg/kg. Blanks did not contain interfering signals with the exception of the $[M+H]^+$ ion traces of sulfadiazine and sulfathiazole mentioned previously. A compound was considered to be identified only if the quantification ion signal could be confirmed by at least two confirmation ion signals with ratios that did not deviate by more than 50% from the ratio obtained from standard samples.

To validate the accuracy of unknown concentrations quantified with external calibration, the antibiotics in two of the analyzed grab samples were also quantified using the method of standard addition to the extracts (see Table 3, samples A and E). Differences between the results of the two methods were not significant (<12% for values above 0.02 mg/kg). Recoveries of the internal standard sulfamethazine-phenyl-$^{13}C_6$ were calculated with an external calibration and compared to dry matter of the samples. It was observed that these two parameters correlated negatively [slope=−9.9 (% recovery/% dry matter), $R^2=0.62$, $n=9$]. Therefore, samples

Table 3
Sulfonamide and trimethoprim residues in manure grab samples (A–F, $n=1$)

Compound	Mother pigs with farrows			Fattening pigs			Fattening calves,
	A	B	C	D	E		F
Sulfamethazine	8.7 (8.9)	5.5	3.3	0.23	0.13 (0.11)		3.2
N^4-Acetyl-sulfamethazine	2.6 (2.7)	0.59	0.15	nd	det		det
Sulfathiazole	12.4 (12.4)	det	nd	0.10	0.17 (0.17)		nd
Trimethoprim	det	nd	nd	nd	nd		nd
Dried mass content (%, w/w)	3.3	3.4	1.8	3.7	3.2		1.1

Results in mg compound per kg wet sample determined by external calibration (and determined by standard addition in parenthesis for samples A and E).
det=Detected, but below 0.1 mg/kg and therefore not quantified.
nd=Not detected; Sulfaguanidine, sulfadiazine, sulfamethoxazole and sulfadimethoxine were not detected in any of the samples.

with more than 3.0% (w/w) dry matter should be diluted to this value prior to extraction to achieve constant recoveries.

3.4. Concentrations in manure grab samples

Grab samples were taken from six different pits containing manure slurry from cattle and pig farms that had been using medicinal feed including one or several of the investigated compounds. In the pits, manure slurry had been collected over time. Therefore, contaminated material had already been diluted with material from medication free time periods. Extracts revealing more than 5 mg of a single antibiotic per kg liquid manure slurry were diluted and measured again, as a precaution against measurements in the non-linear range. Parallel to these measurements, several blank manure slurry samples from different farms were analyzed and no (false) positive results were observed.

Sulfamethazine was detected in all six samples (see Table 3). Five samples contained its metabolite N^4-acetyl-sulfamethazine in concentrations 2 to 50 times lower than the concentrations of the parent compound. Although this metabolite itself is not antimicrobial, Berger et al. showed that it is transformed into its parent compound in manure [12]. Sulfathiazole was found in four samples and trimethoprim could be detected in only one sample. Maximum amounts of sulfamethazine, N^4-acetyl-sulfamethazine, sulfathiazole and trimethoprim were detected in sample A. This sample was taken during an ongoing treatment of the animals with a mixture of trimethoprim, sulfamethazine and sulfathiazole in a ratio of 2:5:5. Interestingly, despite its low detection limit, trimethoprim could only be detected in this sample in very low concentrations. It is not clear whether this is due to a fast degradation, or to an irreversible sorption process, which could have taken place in the gut of the animals, thereby hindering the extraction.

3.5. Application and environmental significance

The method developed allows the quantification of six sulfonamides, one sulfonamide metabolite and trimethoprim from below 0.1 mg/kg liquid manure slurry up to >10 mg/kg (by dilution of the extract). It is an advantage that no extract clean up (for example with solid-phase extraction) is necessary as this would increase cost and time for the analysis. Another advantage is that the method does not require tandem mass spectrometry, since this instrument is not available to a lot of laboratories and would further increase costs of the method. The analysis of grab samples showed that the method is capable of detecting the investigated pharmaceuticals in natural manure slurry as it is stored in pits on farms. Thus, this tool can be used to screen for the occurrence of these pharmaceuticals in manure

slurry, to determine half-lives of antibiotics in manure slurry, and to establish mass balances from antibiotic contents in medicinal feed to quantities spread on fields with manure slurry being used as fertilizer. Additionally, the method could be used as a tool for regulatory compliance control in cases of suspected illegal use of pharmaceutical substances.

Particle interference, however, can considerably affect extraction efficiency such that experiences from water or food samples (for example optimal extraction pH) cannot be transferred directly onto manure samples. The alkaline extraction pH used by this method contrasts neutral extraction pH published for the analysis of food samples [16].

Results of grab sample analysis showed that total active sulfonamide concentrations in natural manure slurry can be as high as 20 mg/kg. Minimum inhibition concentrations of sulfonamides (e.g., for *Escherichia coli*) are typically below 1 mg/kg [23]. It is therefore possible that a selection of antibiotic resistant bacteria does not only occur in the intestinal tract of treated farm animals, but also at concentrations encountered in manure slurry pits. With manure slurry being applied onto fields as fertilizer with a maximum of 50 m^3/ha [13], sulfonamide residues spread on fields could reach up to 1 kg/ha, a value comparable to application doses of modern pesticides. These calculations demonstrate that the use of antibiotics in animal husbandry could be a major source of antibiotic residues in the environment and that it could play an important role in the rise of resistance gene pools. The method presented in this paper can be used to elucidate aspects of these important issues.

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