Elsevier Editorial System(tm) for Food Chemistry

Manuscript Draft

Manuscript Number:

Title: Antibiotic use in heavy pigs: comparison between urine and muscle samples from food chain animals analysed by HPLC-MS/MS

Article Type: Research Article (max 7,500 words)

Keywords: antibiotics; HPLC-MS/MS; muscle tissue; swine; urine.

Corresponding Author: Dr. Sara Panseri, Ph.D

Corresponding Author's Institution: Faculty of Veterinary Medicine

First Author: Luca M Chiesa, Associate Professor

Order of Authors: Luca M Chiesa, Associate Professor; Maria Nobile, PhD student; Sara Panseri, Ph.D; Francesco Arioli, Assistant professor

Abstract: The antibiotic overuse in zoothechnics, due to prophylactic and therapeutic treatments, or to their growth-promoting activity, is a major cause for the onset of widespread antibiotic resistance. Of particular relevance to this study, is the antibiotic abuse in pig breeding. Despite the comprehensive literature on residue controls in pig muscle, data on pig urine, a non-invasive, on-farm collectable matrix, are lacking. Therefore, we validated an HPLC-MS/MS method to detect 29 antimicrobials from eight classes and applied it to 43 anonymous pig urine and muscle paired samples and fulfilled the parameters in agreement with the Commission Decision 2002/657/UE. The analytical limits were moreover much lower than the maximum residue limits (MRLs) required by the Commission Regulation 37/2010/UE. In the samples, antibiotics were usually detected at higher frequencies and concentrations in urine than muscle. Urine proved a useful tool to detect antibiotic administration and their excessive use in pig farming is depicted

UNIVERSITÀ DEGLI STUDI DI MILANO
DIPARTIMENTO DI SCIENZE VETERINARIE
PER LA SALUTE, LA PRODUZIONE ANIMALE
E LA SICUREZZA ALIMENTARE

Dear Sirs,

The concern about misuse of antibiotics in zoothecnics is a major issue about food safety, broadly construed. The control on these drug residues must be therefore improved. Urine is not considered a classic matrix for control of residues in swine, perhaps due to the high frequency of empty bladders in stressed pigs. We wanted to demonstrate in this work that urine, despite the difficulties in its collection (reported by the official veterinarians, but not in literature and therefore not mentioned in the manuscript) is a better matrix than muscle, one of the most used tissues. A multiclass method for the detection of antimicrobials was therefore developed, validated and applied on a significant number of paired samples (to obtain the paired was a real difficulty due to the problem above mentioned). We think that the results show the usefulness of urine in controls of antimicrobial residues in pigs. Before the submission, the British English was checked and revised by Proof-reading.com.

Kind regards

Sara Panseri

Department of Health, Animal Science and Food Safety

University of Milan

Via Celoria, 10

20133 Milan

Italy

Highlights

Antibiotic overuse in pig breeding is of concern, due to antibiotic resistance onset.

Despite being invasive, muscle is usually the matrix used for the residue control.

Urine could allow improved monitoring, as on-farm sample collection is feasible.

A multiclass method for antibiotics detection in urine and muscle was validated.

Its application to 43 paired urine and muscle samples was effective.

Antibiotic use in heavy pigs: comparison between urine and muscle samples from food chain animals analysed by HPLC-MS/MS

Luca Maria Chiesa[a], Maria Nobile[a], Sara Panseri[a,*], Francesco Arioli[a]

[a]Department of Health, Animal Science and Food Safety, University of Milan, Via Celoria 10, 20133 Milan, Italy

*Corresponding author: Sara Panseri, Department of Health, Animal Science and Food Safety, University of Milan, Via Celoria 10, 20133 Milan, Italy. Phone: 0250317931; Fax: 00390250317941; E-mail: sara.panseri@unimi.it

Abstract

The antibiotic overuse in zoothechnics, due to prophylactic and therapeutic treatments, or to their growth-promoting activity, is a major cause for the onset of widespread antibiotic resistance. Of particular relevance to this study, is the antibiotic abuse in pig breeding. Despite the comprehensive literature on residue controls in pig muscle, data on pig urine, a non-invasive, on-farm collectable matrix, are lacking. Therefore, we validated an HPLC-MS/MS method to detect 29 antimicrobials from eight classes and applied it to 43 anonymous pig urine and muscle paired samples and fulfilled the parameters in agreement with the Commission Decision 2002/657/UE. The analytical limits were moreover much lower than the maximum residue limits (MRLs) required by the Commission Regulation 37/2010/UE. In the samples, antibiotics were usually detected at higher frequencies and concentrations in urine than muscle. Urine proved a useful tool to detect antibiotic administration and their excessive use in pig farming is depicted.

Keywords: antibiotics, HPLC-MS/MS, muscle tissue, swine, urine

Chemical compounds studied in this article

Amoxicillin (PubChem CID: 33613); benzylpenicillin (PubChem CID: 5904); nalidixic acid (PubChem CID: 4421); cefalexin (PubChem CID: 27447); chloramphenicol (PubChem CID: 5959); flumequine (PubChem CID: 3374); enrofloxacin (PubChem CID: 71188); tetracycline hydrochloride (PubChem CID: 54704426); lincomycin (PubChem CID: 3000540); sulphadimethoxine (PubChem CID: 5323)

1. Introduction

Over the last decade, the overuse of antimicrobial agents as growth promoters in food-producing animals have caused favourable conditions for the threat of bacterial resistance. It is well-established that multiantibiotic-resistant microorganisms results from chromosomal mutations or the exchange of mobile genetic elements, such as plasmids and transposons (Neu, 1992). The presence of antimicrobial residues in food, their environmental accumulation via the application of manure to soil as organic fertiliser or sludge storage, and the direct contamination of illicitly additivated water and feed, represents a threat to consumers.

It seems reasonable to hypothesise that an increase in the antibiotics concentrations in natural ecosystems may not only influence antibiotic resistance but also affect the broader microbial population dynamics in various natural environments (Martínez, 2008). The swine and poultry industries are the main users of antimicrobials. In an attempt to decrease their environmental and health risk, the use of antibiotics as animal growth promoters has been banned in EU countries and their monitoring is regulated in Council Directive 96/23/EC (European Union, 1996). In order to ensure food safety, the European Union (2010) has also set maximum residue limits (MRLs) for antibiotic residues in food of animal origin.

In the United States of America, in the year 2000, the Food and Drug Administration (FDA) approved 17 antimicrobial agents in swine feed (Cromwell, 2002). Some are permitted in combination like chlortetracycline plus penicillin plus sulphamethazine, or sulphathiazole, neomycin plus oxytetracycline. Most pigs receive antimicrobials in their feed. The chemical composition and mode of action of antibiotics are variable and heterogeneous, but all antimicrobials that are used in swine production should have one common goal. Namely, the capacity to inhibit or decrease the growth of systemic pathogens, even if these characteristics are less readily associated with the ability of a given antimicrobial agent to stimulate growth. The efficacy of antibiotics in improving the rate and efficiency of growth in pigs is well-documented in the scientific literature (Cromwell, 1991; Hays, 1981). Furthermore, antibiotics in breeding and during lactation provide reproductive benefits and improve lactational performance in sows. In this context, the economic benefits are several times greater than the cost of the antibiotic (Cromwell, 2002).

Despite researches that deal with the multiclass determination of antibiotics in soils and pig slurry (Blackwell, Lützhøft, Ma, Halling-Sørensen, Boxall, & Kay, 2004), swine wastewater (Tong, Li, Wang, & Zhu, 2009), in pig muscle and kidney (Granelli & Branzell, 2007) and nitrofurans in the retina of pigs (Cooper & Kennedy, 2005), the data on pig urine are lacking, except a work regarding only one antibiotic, chloramphenicol, in swine urine and muscle (Gantverg, Shishani, & Hoffman, 2003). To the best of our knowledge, no method has previously been reported for simultaneous screening of major antibiotics groups in swine urine. Urine analysis could be a useful alternative to tissues to improve the effectiveness of surveillance controls, as it offers several advantages compared to the analysis of other biological samples as well as muscle tissue. In particular, urine analysis is non-invasive, thus, it could permit the controls at farms and slaughterhouses. We previously reported the multi-residual screening of antibiotics in bovine urine by LC–MS/MS analyses that detected (Chiesa et al., 2015) 29 antimicrobial agents from eight different classes, as shown in Figure 1. In the current study, we used these detected antibiotics to develop a new method for the analysis of pig urine, enabling a direct comparison between the suitability of urine (not contemplated in most EU countries as a conventional matrix) and swine muscle tissue by analysing 43 paired urine and muscle samples. The multi-residual antibiotic strategies for the two matrices were developed and validated according to the Commission Decision 657/2002/CE (European Union, 2002), clarified by SANCO/2004/2726 revision 4. (European Union, 2008).

2. Materials and methods

2.1. Chemicals and reagents

All HPLC or analytical grade solvents were from Fluka (Sigma–Aldrich, St. Louis, MO, USA). Formic (98–100%) and hydrochloric acid (37%) were from Riedel-de Haën (Sigma–Aldrich, St. Louis, MO, USA). Purified water was obtained through a Milli-Q system (Millipore, Merck KGaA, Darmstadt, Germany). Amoxicillin, ampicillin, cloxacillin, dicloxacillin, benzylpenicillin, oxolinic acid, nalidixic acid, cefquinome sulphate, cefalexin, florfenicol, florfenicol amine, chloramphenicol, flumequine, lomefloxacin hydrochloride, ciprofloxacin, enrofloxacin, marbofloxacin, tetracycline hydrochloride, doxycycline hyclate, chlortetracycline hydrochloride, oxytetracycline, lincomycin, sulphathiazole,

sulphadimidine, sulphadiazine, sulphadimethoxine, trimethoprim, erythromycin, tylosin and enrofloxacin d5 were used as the internal standards (IS) and purchased from Fluka.

For the preparation of EDTA-McIlvaine buffer solution (pH 4.0), 15 g of disodium hydrogen phosphate dihydrate,13 g of citric acid monohydrate and 3.72 g of EDTA were dissolved in water and diluted to 1 Trichloroacetic acid 20% (w/v) aqueous solution was also prepared. All these reagents were purchased from Fluka.

2.2. Sample collection

Paired urine and muscle samples from 27 male and 16 female heavy pigs (160–170 kg weight) derived from ten different farms, were collected from the food chain in different slaughterhouses of Lombardy, Italy. The samples were immediately frozen, taken to the laboratory and stored at -20°C until analysis.

2.3. Standard solutions

Stock solutions (1 mg mL^{-1}) for each standard were prepared in methanol and kept at -20°C. Working solutions containing each of the studied analytes at 10 and 100 ng mL^{-1} were prepared daily. Each working solution was maintained at 4°C during the method validation procedures.

2.4. Sample extraction

2.4.1. Urine

Each urine sample (5 mL) was centrifuged at 2500 x g at 4°C for 5 min, then spiked with the IS to give a final concentration of 2 ng mL^{-1}. The compounds of interest were extracted by using Oasis HLB cartridges (3 mL, 60 mg, Waters, Milford, MA, USA) under vacuum. The cartridges were preconditioned with 3 mL of methanol, 3 mL of 0.5 M HCl and 3 mL of Milli-Q water. The sample was loaded, and then the cartridges were washed with 3 mL of water and 3 mL of methanol:water (20:80, v/v). Finally, the analytes were eluted with 5 mL of methanol and collected in a 15-mL polypropylene

tube. The eluate was evaporated by rotary vacuum evaporation. The dried extract was reconstituted in 200 µL of methanol:water (10:90 v/v), then transferred to an HPLC vial.

2.4.2. Muscle tissue

Each minced muscle sample (1 g) was spiked with the IS to give a final concentration of 2 ng mL^{-1}. The analytes were then extracted by adding 5 mL of McIlvaine buffer (pH 4.0). Trichloroacetic acid (100 µl, 20% w/v) was added for protein precipitation and the sample then vortexed followed by sonication for 10 min. After centrifugation (2500 × g, 4°C, 10 min), the supernatant was transferred into a new polytetrafluoroethylene centrifuge tube and defatted with 2 × 3 mL of n-hexane. After each centrifugation (2500 × g, 5 min), the n-hexane layer was removed. The sample was further purified and extracted using Oasis HLB cartridges under vacuum. SPE cartridges were preconditioned with 3 mL of methanol and 3 mL of Milli-Q water. The sample was loaded, and then the cartridge was washed with 2 x 3 mL methanol:water (5:95 v/v). Finally, the compounds were eluted with 5 mL of methanol and were collected in a 15-mL polypropylene tube. The eluate was evaporated using a rotary vacuum evaporator. The dried extract was reconstituted in 200 µL of methanol:water (10:90 v/v) and then transferred to an HPLC vial.

2.5. HPLC-MS/MS analyses

The HPLC system (Thermo Fisher Scientific, San Jose, CA, USA) was equipped with a Surveyor MS quaternary pump with a degasser, a Surveyor AS auto-sampler with a column oven and a Rheodyne valve with a 20-µL loop. Analytical separation was carried out using a Synergi Hydro-RP reverse-phase HPLC column (150 x 2.0 mm, internal diameter 4 µm), with a C18 guard column (4 x 3.0 mm, Phenomenex, Torrance, CA, USA). The injection volume was 10 µL. The flow rate was 0.2 mL min^{-1}. The mobile phase consisted of a binary mixture of solvents A (0.1% aqueous formic acid) and B (MeOH). The elution started with 98% A, which was maintained for 5 min, followed by a linear gradient to 50% A at 22 min. Mobile phase B was then gradually increased to 95% at 24 min, which remained constant up to 29 min. The initial conditions were reached at 31 min, with an equilibration time that included the interval from 31–40 min. The mass spectrometer was a triple-quadrupole TSQ Quantum MS (Thermo Fisher) equipped with an electrospray interface (ESI) that was set both in both

the positive (ESI+) and negative (ESI-) modes. Acquisition parameters were optimised in the ESI mode by direct continuous pump-syringe infusion of the standard analyte solutions at 1 μg mL^{-1}, a flow rate of 20 μL min^{-1} and an MS pump rate of 100 μL min^{-1}. The following conditions were used: capillary voltage 3.5 kV; ion transfer capillary temperature 340°C; nitrogen as sheath and auxiliary gas at 30 and 10 arbitrary units, respectively; argon as the collision gas at 1.5 mTorr; and peak resolution 0.70 Da at full-width half-maximum (FWHM). Three diagnostic product ions were chosen for each analyte and IS. The multiple reaction-monitoring (MRM) mode was used for all data acquisition. The selected diagnostic ions, one of which was chosen for the quantification, the collision energies and the relative intensities are reported in Table 1. The HPLC–MS/MS chromatograms for the antibiotics at the lowest concentration level of the validation and of the IS (2 ng mL^{-1}) are shown, together with the ion spectra, in Fig. 1. Acquisition data were recorded and elaborated using Xcalibur™ software from Thermo Fisher.

2.6. Methods validation

After the preliminary screening of a few samples of urine and muscle tissue, to identify “blank” samples, the validation was performed according to the criteria of the Commission Decision 2002/657/EC (European Union, 2002). For each analyte, the method performance was evaluated through its qualitative parameters, as well as molecular identification by retention time (RT) and transition ion ratios; through its quantitative parameters, such as linearity, recovery, accuracy in terms of trueness, and precision expressed as the intra- and inter-day repeatability; and through the analytical limits, i.e. decision limit (CCα) and detection capability (CCβ), as clarified in SANCO/2004/2726 revision 4. (European Union, 2008).

A total of 20 blank samples was analysed to evaluate the specificity and to check for any interference (signals, peaks, ion traces) in the region of interest where the target analyte was expected to elute. We also tested the selectivity by verifying a signal-to-noise ratio of >3 at the expected RT, and the ion abundance ratio relating to the different fragmentations. Validation was performed by spiking the samples with each of the analytes, resulting in three analytical series (matrix validation curves). Each series had six replicates for three concentration levels that were previously chosen according to the minimum concentration detectable with our instrumentation (C0), which was 0.5 ng

mL^{-1} for all analytes in urine and ranged from 1.0–10 ng mL^{-1} for the different analytes in the muscle samples.

The instrumental linearity was also evaluated by drawing six-point calibration curves in the solvent containing a fixed amount of the IS (2 ng mL^{-1}), with the initial analyte concentration corresponding to the minimum detectable concentration for each group up to 100 ng mL^{-1}.

The trueness was assessed through recovery and was evaluated using the data from the validation points of the three analytical series, expressed as a percentage of the measured concentration with respect to the spiked concentration. The precision in terms of intra- and inter-day repeatability was evaluated by calculating the relative standard deviation (SD) of the results obtained for six replicates of each analyte at three concentration levels of the three analytical series. We evaluated robustness using the fractional factorial Youden design (European Union, 2002).

The experiments to evaluate matrix effects corresponded to the strategy used by Matuszewski, Constanzer and Chavez-Eng (2003) that compares sample extracts plus the analyte of interest added post-extraction, with pure solutions prepared in the mobile phase containing equivalent amounts of the analyte of interest. The percentage ratio between the corresponding peak areas for standards spiked after extraction and the peak areas obtained in neat solution standards determines the extent of the matrix effect occurring for the analyte in question under chromatographic conditions.

3. Results and discussion

3.1 Validation performances

The mean recoveries for all analytes ranged between 90–107%, considering both matrices and all analytes. The 20 urine and muscle blank swine samples analysed to evaluate specificity, did not show any interference (signals, peaks, ion traces) in the region of interest, i.e. where the target analytes were expected to elute. The selectivity showed a good compliance with the relative RTs for each analyte, which was found to be within the 2.5% tolerance, with a signal-to-noise ratio >3 when compared with the standards. Moreover, the three chosen transitions showed an ion ratio within the recommended tolerances (European Union, 2002), when compared with the standards. The matrix validation curves constructed for each analyte demonstrated a good fit for all the analytes with a

correlation coefficient >0.99 in both matrices. The intra- and inter-day repeatability (Thompson, 2000), representing precision, were calculated using one-way analysis of variance (ANOVA) and expressed as coefficients of variation (CVs) For all analytes, the intra-day repeatability values were below 15 and 17% in the urine and muscle tissue samples, respectively, while the corresponding inter-day repeatability values were below 20 and 22%.

Based on the methods described in SANCO/2004/2726 revision 4 (European Union, 2008), the CCα ranged from 0.54–0.86 and 0.95–10.09 ng mL^{-1} in the urine and muscle tissue, respectively, while the CCβ values ranged from 0.60–1.10 ng mL^{-1} in urine and 1.13–11.02 ng mL^{-1} in muscle tissue.

The method ruggedness, evaluated using the fractional factorial Youden design (European Union, 2002), was good in both matrices. Using the strategy of Matuszewski et al. (2003), a modest matrix effect was obtained, with values ranging from 89–104 and 82–109% for the different compounds in the urine and muscle tissue samples, respectively.

3.2 Application of the method

The developed and validated methods were applied to the analyses of 43 urine and muscle paired samples from male and female heavy pigs, collected from different slaughterhouses. The samples were completely anonymous and represented official controls for monitoring residues of antibiotics within the food chain. A comparison of the average concentrations ± SD and the medians for the analytes detected in the paired urine and muscle tissue samples (Table 2), revealed the suitability of urine samples for the majority of detected antibiotics and for the antibiotics present at higher concentration in the urine than muscle tissue.

These data provide evidence that supports urine as the preferential elimination route of most antibiotics in their unchanged form, as observed previously in bovine urine (Chiesa et al., 2015). The possibility to do on-farm controls using this matrix is controversial, considering that most illegal treatments could be detected in the instance of illicit drug administration or non-recorded administration of regulated drugs. Even if most of the antibiotics found are below the MRLs set by the European Regulation (European Union, 2010), the results reveal the contemporary presence of different classes of antibiotics in the urine of the analysed sample. Moreover, identical combinations of

antibiotics were found in the samples belonging to the same farm, but distinct from those obtained from different slaughterhouses. In particular, chloramphenicol, a strictly forbidden antibiotic, was detected both in urine and muscle tissue samples coming from the same farm (on four occasions in urine and five occasions in muscle). In this instance, their urine concentrations were more than four-fold higher than in muscle tissue.

Regarding the other antimicrobial agents studied, when they were found in muscle, the MRLs were never exceeded. For all the antimicrobials studied, the MRL value was 100 μg kg^{-1}, except for florfenicol, whose MRL is 200 μg kg^{-1} because it represents the sum of the antimicrobial and its metabolite, florfenicol amine (European Union, 2010).

Doxycycline was one of the antibiotics most frequently found in urine (in 37 samples, at a maximum of 339.45 μg L^{-1}). In the muscle tissues, doxycycline was found 15 times and the maximum recorded was 21.05 μg kg^{-1}. When it was present in the muscle, it was always detected in the urine but not vice versa. However, when the concentration in the urine was > 13.28 μg L^{-1} it was also detected in the muscle. When compared with muscle tissue, the urinary doxycycline concentration was 1.3–50 times higher. In the muscle tissue, its concentration never exceeded 100 μg L^{-1}. In contrast, this value was exceeded in six urine samples, four of which belonged to the same farm. However, all the samples were from anonymous animals. Consequently, we could neither discern if the animals had been illicitly treated, or if they had undergone a prophylactic or therapeutic treatment nor identify the route of administration, so it is not possible to evaluate pharmacokinetic considerations.

For the other antimicrobial agents evaluated, oxytetracycline was detected in some urine samples from four different farms, but only once in a muscle sample (1.89 μg kg^{-1}) with its paired urine sample showing the highest concentration, which was 52.14 μg L^{-1}. Tetracycline was found in all urine samples derived from one farm and sporadically in other instances, but was never detected in the muscle. Its highest concentration was 4.28 μg L^{-1}. Chlortetracycline was found in all urine samples from one farm, and in one another urine sample derived from a different farm (maximum value 1.27 μg L^{-1}), but was never detected in the muscle. Lincomycin was in 17 urine samples at a maximum of 120.69 μg L^{-1} and only three times in the muscle tissue. Its urine-to-muscle concentration ratio ranged from 0.2–12.88. Florfenicol and florfenicol amine, alone or combined, were detected in 17 urine samples at a maximum of 40.28 μg L^{-1}. Neither florfenicol nor its metabolite was found in the corresponding muscle samples. Tylosin was detected seven times, mostly in urine samples from two

different farms but never in the muscle samples. The highest concentration detected was 267.74 μg L^{-1}. Sulphadiazine was found at a maximum 180.42 μg L^{-1} in five samples from one farm and concomitantly with trimethoprim, but never in the muscle. Enrofloxacin was detected in almost all the urine samples originating from two farms and in one other sample from a different farm (3.72 μg L^{-1} maximum), but never in the muscle.

4. Conclusion

The results shown in this study indicate that different classes of antibiotics are contemporary used for swine breeding and are generally found at a higher frequency and at higher concentrations in urine than in muscle tissue. We did not find a strict correlation between the two matrices, but usually, when the concentration in urine was very high, the analyte was detected in the paired muscle sample. The detection in urine depicts an overall framework of an excessive use of the antibiotics in pig farming. The use of urine as a control matrix may, therefore, be useful to ascertain any illicit treatment or to monitor the antibiotic concentrations after therapeutic uses. Consequently, withdrawal periods should be accurately scrutinised to ensure the safety of meat in the supply chain before it is presented to consumers. Moreover, it highlights the overuse of antibiotics associated with the increasingly urgent threat of antibiotic resistance.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Acknowledgement

Maria Nobile is the recipient of a Ph.D. fellowship in Veterinary and Animal Science in the Laboratory of Inspection of Food of Animal Origin at the University of Milan, Italy.

References

Blackwell, P. A., Lützhøft, H-C. H., Ma, H-P., Halling-Sørensen, B., Boxall, 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.

Chiesa, L., Nobile, M., Arioli, F., Britti, D., Trutic, N., Pavlovic, R., & Panseri, S. (2015). Determination of veterinary antibiotics in bovine urine by liquid chromatography–tandem mass spectrometry. *Food Chemistry*, *185,* 7–15.

Cooper, K. M., & Kennedy, D. G. (2005). Nitrofuran antibiotic metabolites detected at parts per million concentrations in retina of pigs—a new matrix for enhanced monitoring of nitrofuran abuse. *Analyst*, *130,* 466–468.

Cromwell, G. L. (1991). Antimicrobial agents. In E. R. Miller, D. E. Ullrey, & A. J. Lewis (Eds.), *Swine nutrition* (pp. 297–314). Stoneham, Massachusetts: Butterworth-Heinemann.

Cromwell, G. L. (2002). Why and how antibiotics are used in swine production. *Animal Biotechnology*, *13,* 7–27.

European Union. (1996). Council directive 96/23/EC on measures to monitor certain substances and residues thereof in live animals and animal products and repealing Directives 85/358/EEC and 86/469/EEC and Decisions 89/187/EEC and 91/664/EEC. *Official Journal of the European Union*, *125,* 10.

European Union. (2002). Commission Decision 2002/657/EC. Commission decision concerning the performance of analytical methods and the interpretation of results. *Official Journal of the European Union*, *221,* 8–36.

European Union. (2008). European Commission, Health & Consumer Protection, Directorate General, Directorate E, Safety of the Food Chain. Document SANCO/2004/2726-revision 4. Guidelines for the Implementation of Decision 2002/657/EC.

European Union (2010). Commission Regulation (EU) No. 37/2010 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin. *Official Journal of the European Union*, *18*(15), 1–72.

Gantverg, A., Shishani, I., & Hoffman, M. (2003). Determination of chloramphenicol in animal tissues and urine liquid chromatography–tandem mass spectrometry versus gas chromatography–mass spectrometry. *Analytica Chimica Acta*, *483,* 125–1350.

330 Granelli, K., & Branzell. C. (2007). Rapid multi-residue screening of antibiotics in muscle and kidney by
331 liquid chromatography-electrospray ionization–tandem mass spectrometry. *Analytica Chimica Acta*,
332 586, 289–295.
333 Hays, V. W. (1981). Effectiveness of feed additive usage of antibacterial agents in swine and poultry
334 production. Long Beach, CA: Rachelle Laboratories Inc.
335 Martínez, J. L. (2008). Antibiotics and antibiotic resistance genes in natural environments. *Science*,
336 *321*(5887), 365–367.
337 Matuszewski, B. K., Constanzer, M. L., & Chavez-Eng, C. M. (2003). Strategies for the assessment of
338 matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. *Analytical Chem*istry*, 75,*
339 3019–3030.
340 Neu, H. C. (1992). The crisis in antibiotic resistance. *Science, 257,* 1064–1073.
341 Thompson, M. (2000). Recent trends in inter-laboratory precision at ppb and sub-ppb concentrations
342 in relation to fitness for purpose criteria in proficiency testing. *Analyst*, *125,* 385–386.
343 Tong, L., Li, P., Wang, Y., & Zhu, K. (2009). Analysis of veterinary antibiotic residues in swine
344 wastewater and environmental water samples using optimized SPE-LC/MS/MS. *Chemosphere, 74,*
345 1090–1097.
346

Fig. 1. General structures of the nine classes of studied antimicrobial agents.

361 **Table 1** MS/MS conditions for the MRM acquisitions of investigated antibiotics. Ions for quantification
362 are in bold. The values in brackets represent the relative intensities (%). The collision energy (CE) is
363 subscripted and expressed in volts.

Analyte	Precursor ion (m/z)	Product ions CE (m/z)	ESI
Amoxicillin	366	**114(80)$_{20}$**, 134(21)$_{31}$, 349(100)$_{7}$	(+)
Ampicillin	350	**106(100)$_{18}$**, 114(14)$_{29}$**,** 160(14)$_{14}$	(+)
Cloxacillin	436	160(48)$_{13}$, 178(35)$_{33}$, **277(100)$_{14}$**	(-)
Dicloxacillin	468	291(100)$_{21}$, **327(63)$_{16}$**, 424(32)$_{12}$	(-)
Benzylpenicillin	335	114(61)$_{32}$, 160(92)$_{12}$, **176(100)$_{14}$**	(+)
Oxolinic acid	262	160(5)$_{35}$, **216(10)$_{29}$,** 244(100)$_{18}$	(+)
Nalidixic acid	233	159(22)$_{33}$, 187(69)$_{26}$, **215(100)$_{16}$**	(+)
Cefalexin	348	158(63)$_{5}$, **174(100)$_{15}$,** 191(23)$_{6}$	(+)
Cefquinome	529	**134(100)$_{15}$**, 324(43)$_{15}$, 396(44)$_{10}$	(+)
Ciprofloxacin	332	268(16)$_{22}$, **288(100)$_{17}$**, 314(94)$_{21}$	(+)
Enrofloxacin	360	245(49)$_{26}$, **316(100)$_{18}$**, 342(29)$_{21}$	(+)
lomefloxacin	352	**265(100)$_{23}$**, 288(16)$_{19}$, 308(63)$_{16}$	(+)
Marbofloxacin	363	72(83)$_{23}$, **320(100)$_{15}$**, 345(18)$_{21}$	(+)
Florfenicol	356	169(1)$_{39}$, 185(35)$_{21}$, **336(100)$_{12}$**	(-)
Florfenicol amine	248	130(24)$_{23}$, 134(8)$_{28}$, **230(100)$_{11}$**	(+)
Chloramphenicol	321	152(65)$_{20}$, 194(35)$_{16}$, **257(100)$_{14}$**	(-)
Flumequine	262	174(13)$_{39}$, **202(54)$_{32}$,** 244(100)$_{19}$	(+)
Chlortetracycline	479	154(39)$_{27}$, **444(100)$_{21}$**, 462(69)$_{16}$	(+)
Doxycycline	445	321(10)$_{31}$, 410(8)$_{24}$, **428(100)$_{19}$**	(+)
Oxytetracycline	461	337(26)$_{29}$, **426(100)$_{19}$**, 443(52)$_{12}$	(+)
Tetracycline	445	154(38)$_{30}$, **410(100)$_{19}$**, 427(43)$_{14}$	(+)
Lyncomicin	407	**126(100)$_{16}$**, 359(10)$_{18}$, 389(5)$_{28}$	(+)
Sulphathiazole	256	92(50)$_{27}$, 108(45)$_{25}$, **156(100)$_{15}$**	(+)
Sulphadimidine	279	108(32)$_{26}$, 124(39)$_{265}$, **186(100)$_{18}$**	(+)
Sulphadiazine	251	92(58)$_{27}$, **108(62)$_{23}$**, 156(100)$_{16}$	(+)

Sulphadimethoxine	311	$92(30)_{31}$, $108(34)_{28}$, **$156(100)_{20}$**	(+)
Trimethoprim	291	$230(100)_{22}$, **$261(75)_{24}$**, $275(47)_{21}$	(+)
Erythromycin	735	$116(32)_{36}$, **$158(100)_{30}$**, $576(37)_{19}$	(+)
Tylosin	817	$156(12)_{42}$, **$174(100)_{37}$**, $772(38)_{29}$	(+)
Enrofloxacin-d5 (IS)	365	$245(49)_{32}$, **$321(100)_{27}$**, $347(46)_{19}$	(+)

364

365 **Table 2** Average concentration ±SD, number and percentage of positives and median of the analytes

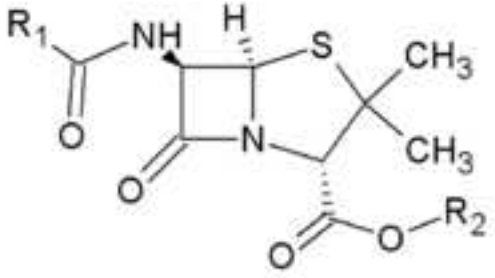
366 detected in urine and muscle samples.

	Urine samples				muscle samples			
Analyte	Average conc.±SD ($ng\ mL^{-1}$)	Positives	% Positives	Median	Average conc.±SD ($ng\ g^{-1}$)	Positives	% Positives	Median
Chloramphenicol	7.83±2.07	4	9	0	1.42±0.77	5	12	0
Florfenicol	7.27±9.92	5	12	0	0	0	0	0
Florfenicol amine	7.68±5.60	17	40	0	0	0	0	0
Doxycycline	46.59±74.89	34	79	4.55	6.12±6.53	15	35	0
Tetracycline	1.71±1.27	10	23	0	0	0	0	0
Oxytetracycline	17.06±16.55	12	28	0	1.89	1	2	0
Chlortetracyclin	0.86±0.38	7	16	0	0	0	0	0
Lyncomicin	0.86±0.38	17	40	0	2.66±1.34	3	7	0
Tylosin	110.27±112.09	8	19	0	0	0	0	0
Sulphadiazine	86.47±66.66	5	12	0	0	0	0	0
Trimethoprim	14.59±5.81	5	12	0	0	0	0	0
Enrofloxacin	2.36±1.20	6	14	0	0	0	0	0

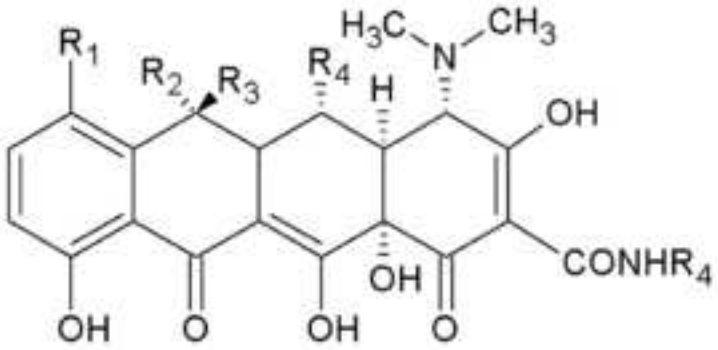
367

Figure(s)

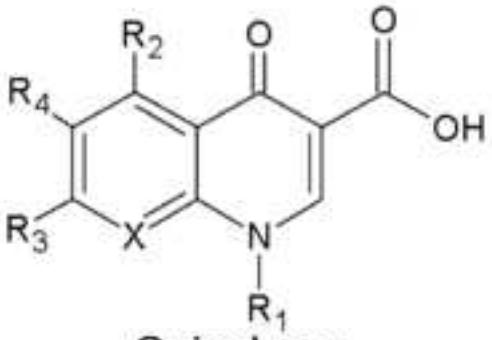
Click here to download high resolution image



Penicillins

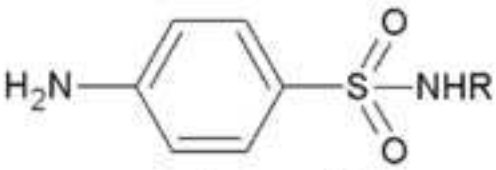


Tetracyclines

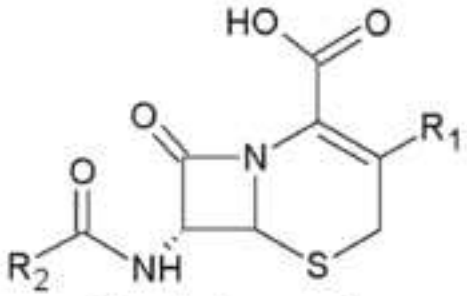


Quinolones

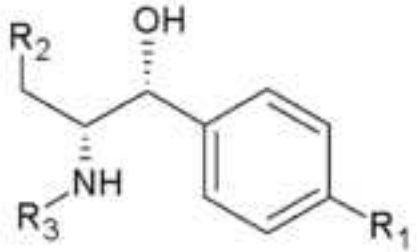
R_4=F Fluoroquinolones



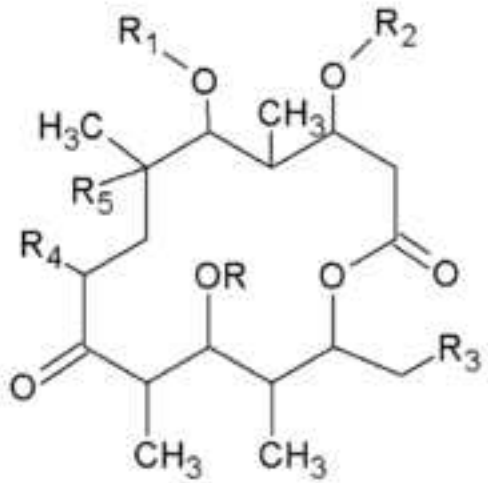
Sulphonamides



Cephalosporins



Amphenicols



Macrolides

Lincosamides