



III Symposium on Agricultural and Agroindustrial Waste Management
March 12-14, 2013-Sao Pedro, SP, Brazil

A SPE-LC-MS/MS METHOD FOR VETERINARY ANTIBIOTIC ANALYSIS IN SWINE MANURE

Vanessa Gressler^{1*}; Patrícia Bilotta²; Ricardo Luís Radis Steinmetz^{1,3}; Marcelo Bortoli³; Airton Kunz¹

¹Embrapa Swine and Poultry, Concórdia-SC, 89700-000, BR 153, Km 110, Brazil

²Environmental Management Post-Graduation Program, UP, Curitiba-PR, Brazil

³Chemical Engineering Post-Graduation Program, UFSC, Florianópolis-SC, Brasil
vanessa.gressler@embrapa.br

ABSTRACT: Veterinary antibiotics evaluation in environmental samples is a very important subject principally because of the development of antibacterial resistance. In this work two SPE-LC-MS/MS methods (acid and basic) were compared and adapted for amoxicillin, lincomycin and tiamulin antibiotics analysis in swine manure. The results showed that the basic method is more suitable for the simultaneous determination of the selected antibiotics with recoveries of $52.1 \pm 7.7\%$ for amoxicillin, $64.6 \pm 1.6\%$ for lincomycin and $74.7 \pm 9.5\%$ for tiamulin.

Keywords: residues, veterinary drugs, liquid chromatography-mass spectrometry, swine manure.

INTRODUCTION

Antibiotics are widely used in animal production as a growth promoter as well as prevent or treat microbial infection. Most of the veterinary antibiotics are not fully metabolized by animals being excreted in their original form or primary metabolites (Tylova et al., 2010).

Confined Animal Feeding Operations (CAFOs) produces a large amount of waste which contains a relatively high level of antibiotic residues. When this waste reaches the environment it can cause an impact principally because of the emergence and development of antibacterial resistance in wildlife and humans.

Some data indicate that these compounds are not efficiently eliminated during conventional sewage-treatment plants (Díaz-Cruz and Barceló, 2006; McArdeall et al., 2003), so, depending on the characteristics of antimicrobials and metabolites in the soil-water environment, they may threaten surface waters and groundwaters (Hernández et al., 2007).

In recent years, many analytical methods have been developed for the detection of antibiotics in different matrices. The most sensitive methods are commonly based in solid phase microextraction (SPE) and detection by mass spectrometry (MS) coupled with liquid chromatography (LC).

Amoxicillin, lincomycin and tiamulin are widely used in Brazilian swine production. However, the information about the contamination level of these antibiotics in swine manure is very limited. Therefore, the aim of this study is to develop a sensitive and selective method by SPE-LC-MS/MS for simultaneous determination of these three veterinary antibiotics in swine manure once they have different pKa (2.8 for amoxicillin, 7.8 for lincomycin and 7.6 for tiamulin) which is highly affected by the pH in each extraction step.

MATERIAL AND METHODS

Samples

For this method development, the reference swine wastewater samples were collected from solid-liquid separation unit effluent of the swine wastewater treatment plant from Embrapa Swine and Poultry. The swine wastewater physic-chemical characterization was done according to APHA, 2012.

Sample pre-concentration and clean-up evaluation



**III Symposium on Agricultural and Agroindustrial Waste Management
March 12-14, 2013-Sao Pedro, SP, Brazil**

According to the literature and the physic-chemical properties of the selected antibiotics, the extraction and clean-up method was verified in two pH ranges (2.5-3.0, the acid method, and 8.0-9.0, the basic method).

In addition, in this work, several sample pre-conditioning and clean-up parameters were tested to reach better recoveries of the antibiotics, they were: the addition or not of Na₂EDTA (to complex potentially interference metals), centrifugation rotations and time, SPE pre-conditioning (type and solvent volumes) and the use or not of a 5 mL HCl 0.5 mol/L step (in the acid method), flow rate, washing systems (to better remove the interferences), elute volumes and solvents which were tested individually or in combinations (dichloromethane/acetone 3:2 v/v, Ben et al., 2008, acetonitrile and methanol) and the use or not of basic elution methanol/NH₃ 30% 95:5 v/v, Beneti et al., 2006, in the basic method.

Liquid chromatography-mass spectrometry

The LC system was a Survayor Plus Chromatography (Thermo Scientific, USA). An ACE 5 C18 column (150 mm x 4.6 mm, 5 μm pore size) was used for separation of antibiotics. Various LC conditions were tested to achieve a good antibiotics separation and peak symmetry in the shortest time possible. Mobile phases consisted of water, methanol and acetonitrile together or not with the formic acid additive were also tested.

The MS system for ion analyses used was the triple quadrupole mass spectrometer TSQ Access Max (Thermo Scientific, USA). The MS parameters (spray voltage, vaporized temperature, capillary temperature, sheath gas pressure, auxiliary gas pressure and tube lens) which affect the ESI interface were optimized by flow injection analysis. The identification of individual antibiotics was based on the chromatographic retention time and two or more characteristics ions (Table 1). The protonated molecular ion [M+H]⁺ was used as the precursor ion for quantification of each antibiotic. Collision energies were also optimized to achieve higher amount of the quantifier ions selected.

RESULTS AND DISCUSSION

A good simultaneous extraction of the antibiotics was difficult to achieve because of the highly complex matrix (Table 2) and because of the different chemical properties between the antibiotics. The best antibiotics recoveries in the two different pH achieved were obtained as follow:

An aliquot of 10 mL of the reference swine wastewater spiked with the antibiotics (30 ng/mL each) and internal standard (IS, clindamycin, 5 ng/mL) was centrifuged at 3000 rpm for 10 min at room temperature. Then 3 mL of the centrifuged supernatant was adjusted to pH 2.5-3.0 with H₂SO₄ 40% for the acid method and to pH 8.0-9.0 with NH₄OH 1 mol/L for the basic method followed by vigorously mixing for 15 seconds. To each sample 100 μL of Na₂EDTA 5% (w/V) solution was added and then vigorously mixed again for 15 seconds, centrifuged (5000 rpm, 10 min) and submitted to a SPE extraction with an Oasis HLB cartridge (500 mg/6mL, Waters). The cartridge was pre-conditioned sequentially with 5 mL of methanol and 5 mL of Milli-Q water. The samples were then passed through the cartridge at a flow rate of 0.5 mL/min, followed by a cartridge wash step with 5 mL of Milli-Q water, (dehydration under vacuum for 3 min) and 5 mL of n-hexane (to remove the fat impurities and the remained water, dehydration under vacuum for 3 min) and then the analytes eluted with 10 mL of methanol followed by 3 min vacuum. The extracts were dried under nitrogen stream, re-dissolved in 1 mL of Milli-Q water/methanol 50:50 v/v and filtered in a 0.22 μm cellulose ester prior to LC-MS/MS analysis.

The HPLC mobile phase study showed that 0.1% formic acid in both, methanol and water phases were very important for the antibiotic peak shape. A good antibiotic separation was achieved with the following system: phase A, Milli-Q water with 0.1% of formic acid and phase B, acetonitrile with 0.1% of formic acid. Chromatographic separation was done isocratically with 50% A and 50% B, in a flow rate of 0.8 mL/min, 25 μL injection volume and 30 °C column temperature. After HPLC separation, the analyses were determined by



**III Symposium on Agricultural and Agroindustrial Waste Management
March 12-14, 2013-Sao Pedro, SP, Brazil**

electrospray-tandem mass spectrometry (ESI-MS/MS) in positive ion mode and selected reaction monitoring (SRM).

The MS spectrometer was operated under the following conditions: spray voltage, 3000 V; vaporized temperature, 380 °C; sheath gas pressure, 40 psi; auxiliary gas pressure, 20 psi; capillary temperature 220 °C; collision cell pressure, 1.6 mtorr; collision gas, argon. The ion characteristics and the optimal collision energy of the selected antibiotics are shown in Table 1.

For the antibiotics recovery calculation it was compared the results obtained between samples spiked after SPE extraction (as 100% reference) and samples spiked before the SPE extraction (Table 3).

Acid methods are more often described in the literature for sulfonamide, tetracycline and macrolide antibiotic analysis (Ben et al., 2008; Pan et al., 2011a and 2011b). In spite of no excellent recoveries, in this paper, the basic method showed to be the best choice, principally because of the recovery gain for amoxicillin ($52.1 \pm 7.7\%$).

CONCLUSIONS

Swine manure is a highly complex matrix which results in low extraction efficiency and increased ionization suppression. Amoxicillin showed to be the most difficult molecule to analyze and the extraction efficiency is related to pH. In spite of this, our results showed a selective and sensitive method for simultaneous determination of three veterinary antibiotics commonly used in swine production (amoxicillin, lincomycin and tiamulin) in swine manure samples. Recoveries ranged between $52.1 \pm 7.7\%$ to $74.7 \pm 9.5\%$ for the basic method, which was selected for the continued work. The method can be improved through the comprehensive study of the matrix effects and extraction conditions for each analyte. The results obtained in this work will be validated for future use in antibiotics monitoring in swine manure treatment systems studied by the group and for veterinary drugs occurrence tracking in the swine production system.

ACKNOWLEDGEMENT

This research was supported financially by CAPES-PNPD (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Programa Nacional de Pós-Doutorado) and FAPESC (Fundação de Amparo à Pesquisa e Inovação do Estado de Santa Catarina).

REFERENCES

- APHA; AWWA; WEF. Standard methods for the examination of water and wastewater. 21th Ed. Washington: APHA, 2012, 1325 p.
- Ben, W.; Quiang, Z.; Adams, C.; Zhahg, H.; Chen, L.; Simultaneous determination of sulfonamides, tetracyclines and tiamulin in swine wastewates by solid-phase extraction and liquid chromatography-mass spectrometry. *Journal of Chromatography A*, 1202, 2008, 173-180.
- Benetti, C.; Piro, R.; Binato, G.; Angeletti, R.; Biancotto, G. Simultaneous determination of lincomycin and five macrolide antibiotic residues in honey by liquid chromatography coupled to electrospray ionization mass spectrometry. *Food additives and Contaminants* 23(11), 2006, 1099-1108.
- Diaz-Cruz, S.M. and Barcelo, D. Determination of antibacterial residues and metabolites in the aquatic environment by liquid chromatography tandem mass spectrometry. *Analytical and Bioanalytical Chemistry*, 386, 2006, 973-985.
- Hernández, F.; Sancho, J.V.; Ibáñez, M.; Guerrero, C. Antibiotic residue determination in the environment Waters by LC-MS. *Trends in Analytical Chemistry*, 26(6), 2007, 466-485.
- McArdell, C.S.; Molnar, E.; Suter, M.J.-F.; Giger, W. Occurrence and fate of macrolide antibiotics in wastewater treatment plants and in the Glatt Valley Watershed, Switzerland. *Environmental Science Technology*, 37, 2003, 5479-5486.

Pan, X.; Quiang, Z.; Ben, W.; Chen, M. Simultaneous determination of three classes of antibiotics in the suspended solids of swine wastewater by ultrasonic extraction, solid-phase extraction and liquid chromatography-mass spectrometry. *Journal of Environmental Sciences*, 23(10), 2011, 1729-1737.

Pan, X.; Quiang, Z.; Ben, W.; Chen, M. Residual veterinary antibiotics in swine manure from concentrated animal feeding operations in Shandong Province, China. *Chemosphere*, 84, 2011, 695-700.

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

Tylova, T.; Olsovska, J.; Novak, P. Flieger, M. High-throughput analysis of tetracycline antibiotics and their epimers in liquid hog manure using ultra performance liquid chromatography with UV detection. *Chemosphere*, 78, 2010, 353-359.

Table 1. Quantification and identification transitions and optimal collision energy (eV) of the selected antibiotics in the positive ESI and SRM mode.

Antibiotics	Qualification transition	Quantification transition	Retention time (min)
Amoxicillin	366.2>208.1 (29)	136.6>114.1 (16)	2.05
Lincomycin	407.1>359.1 (17)	407.1>126.2 (28)	3.10
Tiamulin	-	494.3>192.1 (20)	5.15
Clindamycin (IS)	427.1>126.2 (27)	425.1>126.2 (29)	8.16

Table 2. Characteristics of swine wastewater used as the reference matrix.

Parameter	Value (mg/L)
TKN	1.108,0
NH ₃ -N	972,1
Alkalinity (CaCO ₃)	4.067,7
Total carbon	2.570,0
Inorganic carbon	951,7
Organic carbon	1.618,3
Total soluble carbon	1.407,0
Inorganic soluble carbon	654,9
Organic soluble carbon	752,1

TKN – Total Kjeldhal Nitrogen

Table 3. Recoveries of the reference matrix in acid and basic extraction methods.

Antibiotic	Spiked concentration (ng/mL)	Acid Recovery ± SD (%)	Basic Recovery ± SD (%)
		Swine wastewater	Swine wastewater
Amoxicillin	30	25.5 ± 4.3	52.1 ± 7.7
Lincomycin	30	106.9 ± 11.1	74.7 ± 9.5
Tiamulin	30	81.5 ± 7.0	64.6 ± 1.6