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Multi-residue and multi-class method for the determination of antibiotics in bovine muscle by ultra-high-performance liquid chromatography-tandem mass spectrometry

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Abstract

A multi-residue quantitative screening method covering 41 antibiotics from 7 different families, by ultra-high-performance-liquid-chromatography tandem Mass Spectrometry (UHPLC-MS/MS), is described. Sulfonamides, trimethoprim, tetracyclines, macrolides, quinolones, penicillins and chloramphenicol are simultaneously detected after a simple sample preparation of bovine muscle optimized to achieve the best recovery for all compounds. A simple sample treatment was developed consisting in an extraction with a mixture of acetonitrile and ethylenediaminetetraacetic acid (EDTA), followed by a defatting step with n-hexane. The methodology was validated, in accordance with Decision 2002/657/EC by evaluating the required parameters: decision limit (CCα), detection capability (CCβ), specificity, repeatability and reproducibility. Precision in terms of relative standard deviation was under 20% for all compounds and the recoveries between 91% and 119%. CCα and CCβ were determined according the maximum residue limit (MRL) or the minimum required performance limit (MRPL), when required.

Keywords: Antibiotics, multi-class, multi-detection, UHPLC-MS/MS, muscle, validation.

1. Introduction

In food producing animals, antibiotics are widely used and administrated as feed additives and in drinking water to treat and prevent diseases but also to illegally stimulate animal growth (Wassenaar, 2005; Laxminarayan et al., 2013).

The continuous use of these drugs carries the risk of their presence in edible tissues which, for consumers, can be responsible for toxic effects and allergic reactions in hypersensitive individuals (Le Bizec, Pinel & Antignac, 2009). It can also result in the development of resistant strains of bacteria that might compromise the efficiency of antibiotics used for treatment of animals (Laxminarayan et al., 2013). When that occurs it became difficult to treat serious diseases, increasing the negative effects in animal welfare and consequently severe consequences for productivity and economy. Furthermore, the potential spread of resistant strains of bacteria from animals to humans can have the same effect when using antibiotics as human medicines (Doyle & Erickson, 2006). These concerns make the analysis of antibiotic residues in food producing animals an important field in food safety. To control abusive situations, and because food safety is a key police priority for the European Commission (Commission of the European Communities, 2000); several official documents were settled down to regulate the control of veterinary drugs in products of animal origin. The Council Directive 96/23/EC (European Commission, 1996) determines the measures to monitor certain substances and residues of veterinary medicines in living animals and in animal products. This directive foresees laboratorial control. For permitted veterinary drugs, tolerance levels were established as maximum residue limits (MRLs) in foodstuff of animal origin and listed in the EU Commission Regulation 37/2010 (European Commission, 2009 & European Commission, 2010). For non-authorized substances there are no tolerance levels but, for some compounds, to harmonize the analytical

performance of the methods, a minimum required performance limit (MRPL) had been set (European Commission, 2002; SANCO, 2007). The MRPL level is not a concentration obtained from toxicological data, but is only related with analytical performance. The European Decision 2002/657/EC (European Commission, 2002) describes the requirements for the performance and validation of the analytical methods employed in the official residues control. To fulfill such requirements it is important to have sensitive and specific analytical methodologies capable of monitoring the use or potential abuse of these drugs in the field of animal husbandry, ensuring that MRL levels are respected. The concern about having efficient screening methods is increasing and also about the improvement of cost-effectiveness of analytical procedures (Reig & Toldrá, 2008; Kaufmann, 2009; Martos et al., 2010). Typically the methods used in laboratory are multi-detection of related compounds, usually from the same family of antibiotics. That means that a single sample, to be analyzed for different groups of antibiotics, became part of a time consuming process that can last weeks. The delayed final result is associated with high cost and turns to be questionable in terms of usefulness of the result. This efficiency can be gathered in multi-class and multi-detection methods based on liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) being the tool of choice, providing the required degree of confidence for veterinary residues analysis in biological samples (Le Bizec, Pinel & Antignac, 2009; Kaufmann, 2009). Nowadays, the use of ultra-high performance liquid chromatography (UHPLC) provides numerous advantages in terms of resolution, sensitivity and also in minimizing time of analysis which is an important feature when running numerous samples in routine laboratories (De Brabander et al., 2009; Geis-Asteggiante et al., 2012, Lehotay et al., 2012 and Malik, Blasco, & Picó, 2010). Despite that, the simultaneous determination of antibiotics from different pharmacologic

families in complex biological matrices, such as bovine muscle, has several constrains mainly related with the differences in physicochemical properties of the compounds (De Brabander et al., 2009; Kinsella, O'Mahony, Cantwell, Furey & Danaher, 2009).

In the literature, only few methods, combining multi-detection and multi-class in a quantitative screening method for bovine muscle, are available. Martos et al. (2010) describes a LC-MS/MS method for the screening of 39 compounds from 7 families of antibiotics, although not validated. Granelli, Elgerud, Lundström, Ohlsson & Sjöberg (2009) presented an LC-MS/MS method for the determination of 19 compounds, from 5 classes. A group of the US Department of Agriculture (Geis-Asteggiante, et al., 2012 and Lehotay et al., 2012) described a qualitative screening method for the determination of more than 100 compounds in bovine muscle and/or in kidney, by UHPLC-MS/MS, including not only antibiotics, but several other drugs, such as anthelmintics, thyreostatics, beta-agonists, hormones, NSAIDS and tranquilizers. Although proved to be efficient for screening purposes, the validation presented is not based on European Commission requirements (European Commission, 2002). Recently, multi-detection methods for the analysis of veterinary drugs using liquid chromatography coupled with time-of-flight mass spectrometry (LC-ToF-MS) have been published (Peters, Bolck, Rutgers, Stolker & Nielen, 2009) and UHPLC-ToF-MS (Kaufmann, Butcher, Maden & Widmer, 2008). One of the main advantages is the possibility of analyzing an unlimited number of analytes in a single run, since the detection by ToF-MS is not limited by dwell time (Stolker, Zuidema & Nielen, 2007). Nevertheless, although it can be applied for screening and quantification purposes it cannot be used as confirmatory methods due to the requirements of legislation (European Commission, 2002) and always obliges the confirmation of positive findings using a MS/MS detector.

The present paper describes the development and validation of a simple and effective quantitative screening method by UHPLC-MS/MS for the simultaneous detection of 41 antibiotic compounds from sulfonamides, tetracyclines, penicillins, macrolides, quinolones, trimethoprim and chloramphenicol in bovine muscle. Validation procedure followed the requirements from the European Commission Decision 2002/657/EC (European Commission, 2002) in order to apply the method in routine analysis.

2. Material and Methods

2.1. Reagents, Solvents and Standard Solutions

All reagents and solvents used were of analytical grade with the exception of chemicals used for the mobile phase, which were of high-performance liquid chromatography grade. Methanol, acetonitrile and formic acid were supplied by Merck (Darmstadt, Germany). Ethylenediaminetetraacetic acid (EDTA) was purchased from Sigma-Aldrich (Madrid, Spain). All standards of sulfonamides, tetracyclines, penicillins, macrolides, quinolones, trimethoprim and chloramphenicol were supplied by Sigma-Aldrich (Madrid, Spain). The individual standards are listed in Table 1. Six internal standards were used: demethyltetracycline for tetracyclines, penicillin V for penicillins, lomefloxacin for quinolones, roxithromycin for macrolides, sulfameter for sulfonamides and for trimethoprim and chloramphenicol- d5 for chloramphenicol. All the internal standards were provided by Sigma-Aldrich. For all substances, stock solutions of 1mg mL^{-1} were prepared by weighing the appropriate amount of standard, diluted in methanol, and storing at -20ºC. Suitable dilutions were also prepared to have convenient spiking solutions for both the validation process and the routine analysis.

2.2. Instrumentation

For the sample preparation, the following equipment was used: Mettler Toledo PC200 and AE100 balances (Greifensee, Switzerland), Heidolph Reax 2 overhead mixer (Schwabach, Germany), Heraeus Megafuge 1.0 centrifuge (Hanau, Germany), Turbovap Zymark Evaporator (Hopkinton, MA, USA) and Whatman Mini-Uniprep PVDF 0.45 µm filters (Clifton, NJ, USA). Chromatographic separation and mass spectrometry detection was performed with a Xevo TQ MS – Acquity UHPLC system coupled to a triple quadrupole tandem mass spectrometer from Waters (Milford, MA, USA). The electrospray ion source in positive (ESI+) and negative (ESI-) mode was used with data acquisition in multiple reaction monitoring mode (MRM) and analysed using Masslynx 4.1 software (Waters). The MRM optimized conditions are presented in Table 1. The UHPLC system consisted of a vacuum degasser, an autosampler and a binary pump equipped with an analytical reverse-phase column Acquity HSS T3 2.1x100 mm with 1.8 µm particle size (Waters). The mobile phases used were: [A] formic acid 0.1% (v/v) in water and [B] acetonitrile. The gradient program used, at a flow rate of 0.45 mL min^{-1}, was: 0-5 min from 97% [A] to 40% [A]; 5-9 min from 40% to 0% [A]; 9-10 min from 0% back to 97% [A]; 11-12 min 97% [A]. The column was maintained at 40°C, the autosampler at 10°C and the injection volume was 20 µL.

2.3. Sample preparation

A portion of 2.0 ± 0.05 g of minced and mixed bovine muscle sample was weighed into a 20 mL glass centrifuge tube. The internal standard solution was added, then vortexed for 30 ss and allowed to stand in the dark for at least 10 min.

Afterwards, twelve different extraction procedures were tested; the list of them and the main steps are presented in Table 2.

The liquid extraction was performed by shaking the sample with the solvent using a Reax shaker for 20 min followed by centrifugation for 15 min at 3100 *g*. The

supernatant was transferred into a new tube and, for extractions *ADry*, *MDry* and *EaDry* evaporated to dryness under a gentle stream of nitrogen, at 40 ºC. For the extract samples *A*, *M* and *Ea* the evaporation were just until 0.5 mL. Procedures *AHxDry*, *MHxDry*, *EaHxDry*, *AHx*, *MHx* and *EaHx* followed a defat step by adding 3 mL of n-hexane to the supernatant obtained after centrifugation. The extracts were vortexed for 30 s s and centrifuged for 15 min at 3100 *g*. The n-hexane layer were discarded and, for extractions *AHxDry*, *MHxDry* and *EaHxDry* evaporated to dryness under a gentle stream of nitrogen, at 40 ºC. For extract samples *AHx*, *MHx* and *EaHx* the evaporation were just until 0.5 mL. In all procedures, the residue was redissolved with mobile phase A (400 µL) or added to the 0.5mL of final extract, filtered through a 0.45 µm PVDF Mini-uniprep ^{TM}, transferred to vials and injected into the UHPLC-MS/MS under MRM optimized conditions for each compound (Table 1).

2.4. Validation procedure

The validation procedure followed the described by the EU Commission Decision 2002/657/EEC (European Commission, 2002). According to those requirements, specificity, recovery, repeatability, reproducibility, decision limit (CCα) and detection capability (CCβ) were determined.

The specificity was assessed by analyzing 20 bovine muscle samples from different origins to find possible peaks that could interfere with the detection of the analytes of interest. The same samples were spiked with all the compounds at the level of interest (VL) that, for most of them, corresponds to their MRL/MRPL level, in order to prove the identification capability of the method. Calibration curves were assembled with five concentration levels: 0.5xVL, 1.0xVL, 1.5xVL, 2.0xVL and 3.0xVL and carried out in three different days and with different operators. In each day six replicates of the 0.5xVL, 1.0xVL and 1.5xVL were executed in order to calculate repeatability,

reproducibility and recovery. Recovery determined in the validation process was estimated as a ratio between the determined concentration and the real concentration. CCα and CCβ were determined according to the following equations (European Commission, 2002):

$CC_\alpha = \mu_N + 2.33 \times \sigma_N$ (Equation 1, for compounds without MRLs)

$CC_\alpha = MRL + 1.64 \times \sigma_{MRL}$ (Equation 2, for compounds with established MRLs)

$CC_\beta = CC_\alpha + 1.64 \times \sigma_{VL}$ (Equation 3)

In which:

μ_N is the mean of noise amplitude of twenty blank samples; σ_N is the standard deviation of the noise amplitude of twenty blank samples at the retention time of the target antibiotic; σ_{MRL} or σ_{VL} is the standard deviation at the MRL or VL level in the twenty spiked blank samples at that level. For all the determinations, with the exception for the studies of absolute recoveries during sample preparation development, the peak areas of both the analytes and correspondent internal standard were measured, and the analyte/internal standard area ratios were determined. Internal standards were chosen in accordance with their similar physic-chemical behaviour with the antibiotics monitored and for that they were studied and selected before validation.

3. Results and Discussion

The principal limitation found while developing multi-detection and multi-class methods are related with the sample preparation, mainly due to the difficulty in achieve an efficient and generic procedure to extract simultaneously several compounds from diverse families with different physic-chemical properties. It is difficult to reach equally good recoveries in such methods and minimize the loss of all analytes during sample preparation. Multi-step and complex sample clean-up can result in total loss of some

target compounds and simplifying the procedure can be an improvement. Therefore and considering that the high selectivity of solid-phase-extraction (SPE) can be a problem in multi-class methods, a simple liquid extraction was tested and optimized. Twelve procedures were experienced and final results, in terms of individual absolute recovery, are presented in Table 3. The main purpose of these experiments was to evaluate the real impact/recovery that each procedure has in all compounds in order to select the best option possible. For that reason, absolute recoveries presented for each method did not take into account the presence of the internal standard, in opposition to the recovery obtained during validation.

Three organic solvents were tested for sample extraction: acetonitrile, methanol and ethyl acetate. The addition of a quelating agent was also performed, EDTA, especially to compete with antibiotics as tetracyclines and macrolides. It is known that these compounds can form complexes with the bi- and trivalent cations present in the sample extraction solution which can lead to significant losses of those compounds during the procedure. The presence of another compound, as EDTA, which has similar behavior, is responsible for the improvement of performance of these antibiotics avoiding drastically those losses.

In some of the experiments a defatting step of the organic layer was introduced, with n-hexane, to minimize the lipid content from the muscle and thus the potential interferences during analysis. Also, because some compounds have better affinity with aqueous phase, the same assays were performed without total dryness at the end of the extraction (until 0.5 mL).

Absolute recoveries were calculated for each compound and each methodology in order to understand the effects of all variants. The results are presented in Table 3 and, graphically compared in Figure 1, by the representation of the minimum and maximum

absolute recoveries obtained. In a first analysis of Table 3 and Figure 1 it can be seen that worse results were achieved when using ethyl acetate as extracting solvent, followed by methanol, being the acetonitrile the organic solvent of choice for the most compounds. Comparing the performance of the methods that involved evaporation until dryness or until 0.5 mL, it can be easily concluded that the second option gives better results. There are two reasons that can justify these data. First of all, the higher affinity of polar compounds with aqueous phase can be responsible for a significant amount of antibiotics concentrated in the aqueous content of the sample, turned miscible in the acetonitrile during homogenization. Also the well-known instability of antibiotics (Freitas, Leston, Barbosa, & Ramos, 2013) can be a problem during a longer evaporation process of the remaining aqueous layer. Being the acetonitrile the chosen organic solvent it remains the comparison between methods *A* and *AHx*, with or without a defatting step. It can be observed that the recovery is significantly higher when the lipid content is reduced from the matrix. The possibility of diminishing the interferences coming from the matrix can be responsible for reducing effects like ion suppression or enhancement of signal (Kaufmann, 2009; Kinsella, O'Mahony, Cantwell, Furey & Danaher, 2009), a common problem in the detection system when working with less specific methods such as multi-detection and multi-class and biological samples. Nonetheless a compromise had to be adopted selecting the most suitable method, although, for some compounds, the recoveries obtained are still significantly low, being the worse result the obtained for sulfanilamide with 22%. Briefly, the selected method listed with the code *AHx* above in the sample preparation, in the Table 2 and Figure 1, was determinate to be as follow: 2g of homogenized bovine muscle extracted with 10mL of acetonitrile with 1mL of 0.1M EDTA; after centrifugation the supernatant was defatted with n-hexane; centrifuged and evaporated until 0.5 mL of final extract.

For recovery correction and to control possible matrix effects, internal standards were selected for each group of compounds. The selection was based on their similarities with the target compounds, meaning that they should, as much as possible, be equally affected by the same fluctuations during extraction procedure, ionization efficiency, detection response and chromatographic behavior. Thereby, quantification by matrix based calibration curve using internal standards allows to monitor the efficiency of the extraction procedure and also to correct possible matrix effects.

Chromatographic and detection parameters were optimized: mobile phase, flow rate, gradient steps and ionization conditions. The conditions described above allow the determination of all 41 compounds in less than 10 min, one of the huge advantages of UHPLC and for that, chromatographic conditions were tested with the purpose of achieve the better efficiency in peak separation and peak shape along with a short run time.

In terms of detection, the ideal MRM conditions were obtained by direct infusion into the detector of each standard solution at the concentration of 10μg mL^{-1}. The use of an acidified mobile phase, 0.1% of formic acid, promotes the positive ionization, which improved the detection of almost all compounds since only chloramphenicol is ionized in negative mode. To fulfill the identification criteria demanded in the Decision 2002/657 (European Commission, 2002), two ion transitions were selected for each compound (Table 1). In Figure 2 a representative chromatogram of a spiked bovine muscle sample, at the corresponding validation level (VL) is presented. As an example, individual MRM of one compound per family of monitored antibiotic is also presented in Figure 2.

The method was validated in accordance with the European Commission Decision 2002/657 (European Commission, 2002) that establishes performance criteria for the methods and the procedures for their validation.

The absence of interfering peaks, in the 20 blank bovine muscle analysed samples, above a signal-to-noise ratio of 3, was confirmed in all blank samples. Furthermore, after spiking the same blank samples, the identification of all compounds was effective without any false negative result. The results for precision, in terms of repeatability and reproducibility as relative standard deviation (RSD %), recovery, CCα and CCβ are summarized in Table 4. Values presented for precision and recovery were calculated for the VL that, for most of the compounds are the MRL. To prove the robustness of the method, precision is an important parameter that must be analyzed during validation since it measures the variability during the analytical process. In terms of repeatability, the higher value obtained was for sulfanilamide, with 17%. All the other compounds were under that RSD. Regarding reproducibility it was also for sulfanilamide the worse value, 22%, while the remaining compounds were below 20%. All these values are in accordance with the acceptance criteria, according to the Decision 657/2002 (European Commission, 2002). The calculated RSD cannot exceed the level calculated by the Horwitz equation that depends on the concentration level. The recovery determined during validation was calculated as a ratio between the determined concentration and the real concentration. The range values obtained were between 86 and 109% falling into the accepted range (European Commission, 2002). It is important to note that such values are different from the ones obtained during the development of sample preparation. In these cases the recoveries were calculated as absolute values, without having the correction of the internal standard addition, and for that reason values

presented in Table 3, for method *AHx*, are different from the ones calculated during validation and described in Table 4.

CCα and CCβ were calculated according to the equations described above (equation s 1, 2 and 3) depending if the MRL is established or not. As can be seen in Table 4, compounds without tolerance level have lower CCα and CCβ, closer to the limit of detection of the method although in the other cases these concentrations are always above MRL.

The results of the validation clearly demonstrated the suitability of this method for the detection and identification of all tested antibiotics.

4. Conclusions

A reliable multi-detection and multi-class method for the determination of 41 antibiotics from 7 different classes in bovine muscle was developed. The sample preparation has the main advantage of being inexpensive and low time consuming. Also the use of UHPLC-MS/MS provided the possibility of analyzing a wide number of samples in short period of time. By replacing the methods currently applied in the laboratory (one screening method for each class of compounds) the total time from sampling to the final result will be reduced in a very significant period of time.

The method developed was completely validated in order to be used in routine analysis of official control for quantitative screening purposes with the possibility of extending the method for confirmation. For a laboratory involved in food safety control with a large number of antibiotic residues and samples to analyze, the present method is a huge improvement.

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Figure Captions

Figure 1: Minimum and maximum absolute recoveries obtained for the twelve extraction procedures for all the antibiotics tested at the concentration of the VL (see Table 4 for the respective values).

Figure 2: Chromatogram of individual MRM of one compound per class of antibiotic for a spiked bovine muscle sample at the corresponding validation level (VL)

Figure 1

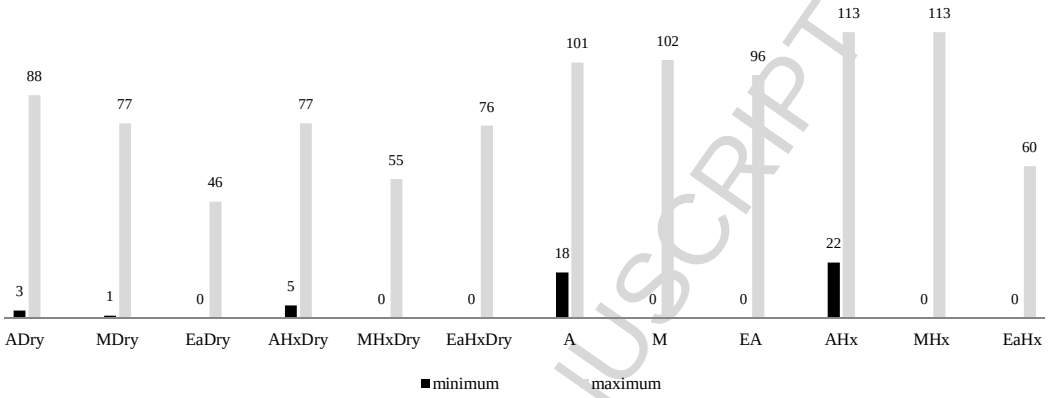

Figure 2

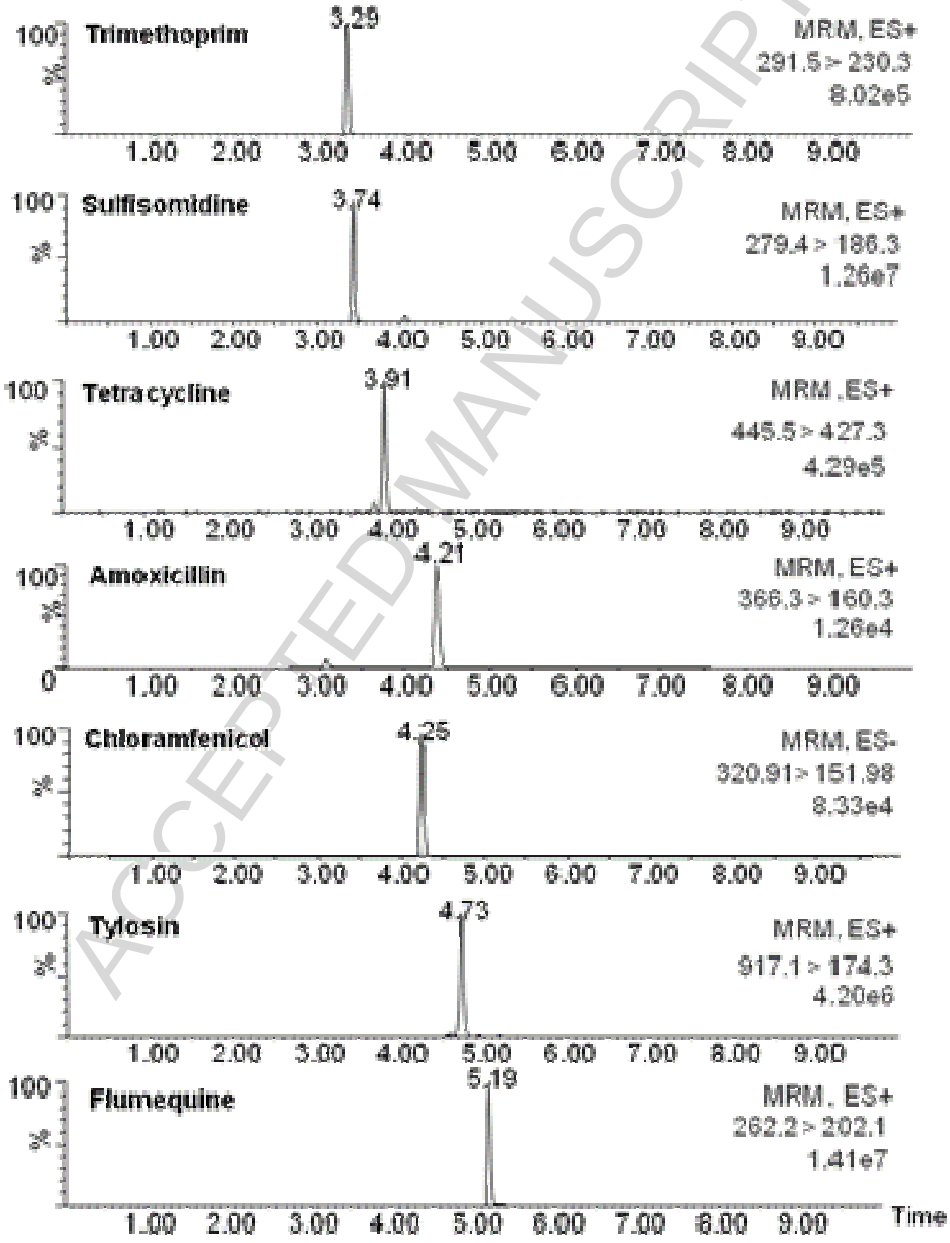

Table 1: Multiple reaction monitoring (MRM) acquisition conditions for each antibiotic and for the internal standards (IS) used.

		ESI	Precursor ion (*m/z*)	Product ion (*m/z*)	Cone voltage (V)	Collision energy (eV)	Retention time (min)
Sulfonamides	*sulfapyridine*	+	250.3	156.3	30	15	3.27
	sulfadiazine	+	251.2	156.2	30	15	3.24
	sulfamethoxazole	+	254.4	156.4	30	20	4.26
	sulfathiazole	+	256.4	156.3	25	15	3.35
	sulfisoxazole	+	268.3	156.2	25	15	4.37
	sulfamethiazole	+	271.0	156.2	25	15	3.86
	sulfisomidine	+	279.4	186.3	30	16	3.74
	sulfamethazine	+	279.4	156.3	30	15	3.77
	sulfamethoxypyridazine	+	281.2	156.2	30	15	3.84
	sulfachloropyridazine	+	285.3	92.3	30	28	4.15
	sulfadoxine	+	311.4	156.4	30	18	4.25
	sulfadimethoxine	+	311.4	156.4	30	20	4.65
	sulfanilamide	+	173.2	92.1	30	25	1.07
	sulfaquinoxaline	+	301.3	92.2	30	30	4.70
	sulfameter (IS)	+	281.3	92.2	25	30	3.86
	trimethoprim	+	291.5	230.3	25	23	3.29
Tetracyclines	*tetracycline*	+	445.5	410.3	25	20	3.91
	doxycycline	+	445.5	428.2	25	18	3.96
	oxytetracycline	+	461.5	426.3	25	20	3.46
	chlorotetracycline	+	479.3	444.2	25	20	3.86
	demethyltetracycline (IS)	+	465.2	448.3	25	17	3.69
Macrolides	*erythromycin*	+	734.5	158.2	25	30	4.22
	spyriamicin	+	843.5	174.0	35	35	3.71
	tilmicosin	+	869.3	174.2	35	45	3.94
	tylosin	+	917.1	174.3	35	35	4.73
	roxithromycin (IS)	+	837.7	679.5	30	30	5.43
Quinolones	*nalidixic acid*	+	233.2	215.1	40	14	3.81
	flumequine	+	262.2	202.1	30	32	5.19
	oxolinic acid	+	262.2	216.1	30	25	4.44
	cinoxacin	+	263.2	217.1	30	23	4.25
	norfloxacin	+	320.3	276.2	20	17	3.45
	enoxacin	+	321.2	303.2	35	18	3.40
	ciprofloxacin	+	332.2	288.2	35	17	3.48
	danofloxacin	+	358.3	96.1	33	21	3.52
	enrofloxacin	+	360.3	316.3	31	19	3.58
	ofloxacin	+	362.1	261.3	34	26	3.44
	marbofloxacin	+	363.3	72.1	30	20	3.36
	lomefloxacin (IS)	+	352.2	265.3	31	22	3.54
Penicillins	*penicillin G*	+	335.1	176.0	30	25	3.81
	ampicillin	+	350.4	106.3	25	20	3.34
	amoxicillin	+	366.3	160.3	25	20	4.21
	oxacillin	+	402.0	243.0	30	20	5.24
	nafcillin	+	415.0	199.0	30	25	5.47
	dicloxacillin	+	470.0	311.0	30	25	5.65
	penicillin V (IS)	+	351.0	160.2	25	25	5.07
Amphenicol	*chloramphenicol*	-	320.9	151.9	30	25	4.25
	chloramphenicol_d5 (IS)	-	326.0	157.0	30	25	4.24

Table 2: Schematic description of the twelve extraction procedures tested.

Procedure	Solvent extraction (10 mL) with 1 mL 0.1M EDTA	Deffating (2 mL)	Concentration
ADry	acetonitrile		evaporate until dryness
MDry	methanol		
EaDry	ethyl acetate		
AHxDry	acetonitrile	n-hexane	
MHxDry	methanol		
EaHxDry	ethyl acetate		
A	acetonitrile		evaporate until 0.5 mL
M	methanol		
Ea	ethyl acetate		
AHx	acetonitrile	n-hexane	
MHx	methanol		
EaHx	ethyl acetate		

Table 3: Absolute recoveries (expressed as %) of the target antibiotics for the twelve extractions procedures tested*

Method / *Antibiotics*	ADry	MDry	EaDry	AHxDry	MHxDry	EaHxDry	A	M	EA	AHx	MHx	EaHx
sulfapyridine	88	72	38	76	16	18	99	16	81	99	61	**9**
sulfadiazine	46	33	19	38	**11**	18	95	17	48	104	29	**13**
sulfamethoxazole	36	28	**2**	23	19	16	57	41	**6**	46	47	**14**
sulfathiazole	50	26	**5**	46	**6**	**8**	91	**12**	18	109	15	**6**
sulfisoxazole	36	27	**0**	**13**	**12**	**3**	53	**10**	**2**	45	42	**5**
sulfamethiazole	43	25	**6**	35	**6**	**14**	72	19	15	80	20	**11**
sulfisomidine	42	38	17	37	21	15	72	18	43	90	54	**13**
sulfamethazine	72	62	31	65	41	27	94	37	96	108	96	23
sulfamethoxypyridazine	28	22	**9**	24	15	**11**	60	**11**	24	64	42	**10**
sulfachloropyridazine	66	50	**10**	50	27	32	83	18	18	102	63	19
sulfadoxine	54	41	**7**	46	28	19	80	53	**14**	104	67	16
sulfadimethoxine	46	43	**12**	36	22	**14**	76	52	31	106	69	17
sulfanilamide	**3**	**1**	**3**	**5**	**0**	**10**	18	**1**	**3**	22	**1**	**9**
sulfaquinoxaline	27	30	**5**	23	18	**8**	35	36	**14**	56	47	**9**
trimethoprim	54	36	16	35	15	**2**	74	19	23	57	47	**4**
tetracycline	62	**11**	17	53	**8**	23	99	**10**	36	101	17	19
doxycycline	57	22	21	53	16	50	92	38	44	106	40	26
oxytetracycline	35	**4**	**9**	26	**5**	20	54	**7**	15	72	**5**	**14**
chlorotetracycline	35	**9**	15	37	**8**	49	85	**11**	42	90	15	46
erythromycin	64	59	**9**	45	42	**5**	93	61	17	98	62	16
spyriamicin	48	50	**5**	54	35	**0**	94	58	**14**	111	77	**0**
tilmicosin	27	30	**5**	25	19	**0**	69	40	25	81	56	**0**
tylosin	49	75	**3**	40	55	**0**	74	102	**6**	98	113	**0**
nalidixic acid	81	67	38	72	46	73	92	46	48	105	66	46
flumequine	46	42	37	42	29	59	75	50	62	107	69	50
oxolinic acid	62	48	46	56	34	66	87	47	58	106	65	48
cinoxacin	59	43	21	54	**7**	76	95	18	34	102	50	60
norfloxacin	67	45	**13**	60	27	**5**	92	40	35	95	56	**3**
enoxacin	57	35	**14**	40	18	**6**	96	19	33	100	47	**6**
ciprofloxacin	60	39	15	52	24	**6**	67	35	28	100	43	**3**
danofloxacin	58	37	**14**	43	23	**0**	97	41	30	98	52	**1**
enrofloxacin	51	37	16	37	22	**8**	83	33	36	84	47	**13**
ofloxacin	49	27	**9**	36	18	**1**	76	31	21	78	39	**2**
marbofloxacin	77	53	26	62	29	**1**	72	23	42	98	67	**2**
penicillin G	86	62	**12**	77	27	**0**	94	31	34	100	84	**0**
ampicillin	50	28	**2**	21	**11**	**0**	87	57	**0**	65	48	**0**
amoxicillin	45	33	22	34	18	**0**	51	**0**	**0**	52	**0**	**0**
oxacillin	39	32	**7**	39	27	**11**	101	50	24	101	87	**10**
nafcillin	34	23	**12**	44	17	17	60	36	30	85	40	**11**
dicloxacillin	18	22	**2**	31	16	**3**	46	31	**7**	57	33	**3**
chloramphenicol	57	77	**9**	24	30	**12**	56	**6**	**10**	113	**9**	50

*Absolute recoveries below 15% are in bold and underlined.

Table 4: MRLs and MPRL set by EU for bovine muscle, validation level (VL) and validation parameters: decision limit (ccα), detection capability (ccβ), repeatability, reproducibility and recovery.

	MRL *MRPL (µg/kg)	VL (µg/kg)	CCα (µg/kg)	CCβ (µg/kg)	Repeatability (%RSD)	Reproducibility (%RSD)	Recovery (%)
sulfapyridine	100	100	132	164	8	12	109
sulfadiazine	100	100	113	125	5	8	93
sulfamethoxazole	100	100	108	117	7	10	108
sulfathiazole	100	100	107	115	6	8	105
sulfisoxazole	100	100	111	121	6	9	104
sulfamethiazole	100	100	110	120	3	5	101
sulfisomidine	100	100	104	108	3	4	93
sulfamethazine	100	100	105	110	6	9	100
sulfamethoxypyridazine	100	100	108	116	2	4	91
sulfachloropyridazine	100	100	104	108	7	11	103
sulfadoxine	100	100	110	121	3	5	91
sulfadimethoxine	100	100	107	114	4	5	93
sulfanilamide	100	100	105	111	17	22	102
sulfaquinoxaline	100	100	106	112	5	7	102
trimethoprim	100	100	108	116	5	7	98
tetracycline	100	100	125	149	13	20	109
doxycycline	100	100	123	147	13	20	103
oxytetracycline	100	100	124	148	13	19	102
chlorotetracycline	100	100	121	143	12	17	100
erythromycin	100	100	116	131	9	14	101
spyriamicin	200	200	226	252	15	20	101
tilmicosin	50	50	60	71	7	10	93
tylosin	100	100	116	133	9	14	116
nalidixic acid	-	100	0.01	0.02	8	13	102
flumequine	200	200	214	229	8	12	104
oxolinic acid	100	100	114	127	8	12	105
cinoxacin	-	100	0.02	0.04	10	14	108
norfloxacin	-	100	0.02	0.04	9	13	86
enoxacin	-	100	0.04	0.06	10	15	98
ciprofloxacin	-	100	0.09	0.12	9	14	95
danofloxacin	200	200	229	258	15	20	106
enrofloxacin	100	100	121	142	12	17	105
ofloxacin	-	100	0.01	0.02	10	15	105
marbofloxacin	-	100	163	176	7	11	100
penicillin G	50	50	69	87	11	17	94
ampicillin	50	50	61	73	7	10	97
amoxicillin	50	50	65	79	8	12	106
oxacillin	300	300	315	330.	9	13	101
nafcillin	300	300	307	315	4	6	103
dicloxacillin	300	300	310	319	6	9	96
chloramphenicol	0.3*	0.3	0.07	0.10	13	19	105