Development of a multi-class method for the identification and quantification of residues of antibiotics, coccidiostats and corticosteroids in milk by liquid chromatography-tandem mass spectrometry

*C. Nebot, A. Iglesias, P. Regal, J. Miranda, A. Cepeda and C. Fente

Department of Analytical Chemistry, Nutrition and Bromatology, Faculty of Veterinary Medicine, University of Santiago de Compostela, 27002, Lugo, Spain. Tel.: +34 982 285900 Fax: +34 982 254592.

Even if coccidiostats are not generally employed in dairy cows, maximum levels for these substances in milk samples have been set up by the Commission Regulation 124/2009. Due to public health concern regarding the presence of residues of veterinary drugs in milk, the aim of the present work is to report a multi-class HPLC-MS/MS method for the simultaneous extraction, identification and quantification of seven coccidiostats, seven antimicrobial agents, three corticosteroids and an antifungal agent. Validation was conducted following the guidelines established in the European Commission Decision 657/2002 at the maximum concentration permitted in milk by the European Union for each drug. The method was successfully applied in 100 raw milk samples collected from ten local dairy farms and in fifteen milk samples bought in supermarkets. This method could be applied in routine analysis of milk samples by the dairy industry and replace the use of the current, non-differentiating, screening method.

Keywords: multi-class; residues; coccidiostats; veterinary drugs; milk; LC–MS/MS; 657/2002/EC.

*Corresponding author: Carolina.nebot@usc.es

Introduction

Veterinary drugs are generally used to treat disease and to protect animals' health. In dairy cattle, for instance, antibiotics are widely used for the treatment of diseases involving bacterial infections, especially mastitis (Shao et al., 2009). Application of a higher dose than the recommended, failure respecting proper withdrawal times or irresponsible use of the veterinary drugs can result in the occurrence of unwanted residues of drugs in food of animal origin (Shao et al., 2009). The presence of veterinary drugs in food and foodstuffs has been frequently reported (Bando, Oliveira, Ferreira & Machinski, 2009; Berendsen et al., 2010; Lopez, Pettis, Smith & Chu, 2008; Sheridan, Policastro, Thomas & Rice, 2008). Residues of drugs in food can endanger consumers' health. Short-term health effects include allergic and toxic reactions, and long-term exposure could result in chronic toxic effects or the development of antibiotic-resistant bacteria in humans (Gomes & Demoly, 2005; Raison-Peyron, Messaad, Bousquet & Demoly, 2001). Antimicrobial agents in food, regardless of their minute amounts, can be potentially carcinogenic (Koesukwiwat, Jayanta & Leepipatpiboon, 2007b).

Milk consumption has been promoted around the world as it is an inexpensive source of saturated fats, proteins and calcium. It provides the primary source of nutrition for young mammals before they are able to digest other types of food. Milk is known to be a nutritious, wholesome food that is consumed globally by humans. It is recommended for children and elderly woman. To protect milk consumers' health from the presence of residues of veterinary drugs, maximum residue levels (MRLs) of veterinary drugs in food have been set up in the Commission Regulation 37/2010 (EU, The European parliament and the Council of the European Union, 2010). The Commisssion includes MRL for substances which are normally employed to treat or prevent animal diseases. However, as a consequence of the unavoidable carry-over of coccidiostats in non-target feed, maximum levels (ML) for residues of these substances in food of animal origin have been set up in the Commission Regulation 124/2009 (EU, The European parliament and the Council of the European Union, 2009). It has to be pointed out that MLs have been established for food obtained from animals which are not normally treated with coccidiostats such as dairy animals. This decision was taken with the overall aim of protecting consumer's health within the European Union.

Due to the high number of veterinary drugs that need to be controlled, the food industry generally employs bioassay methods (Lamar & Petz, 2007). The screening methods employed are immunological techniques (ELISA test kits), radioimmunoassay (RIA), and biosensors (Toldrá & Reig, 2006). These methods permit the rapid detection of numerous analytes at low concentrations, but they have some disadvantages. These methods provide a semi-quantitative estimate of 'total' residues detected but they do not distinguish among members of a class of antibiotics (Stolker & Brinkman, 2005). Another disadvantage of the screening methods is that, before a sample is declared non-compliant, confirmation by sufficiently selective and sensitive instrumental methods such as LC–MS or GC–MS is required (EU, The European parliament and the Council of the European Union, 2002). Generally, milk industries reject milk which is positive by screening methods to avoid waiting for confirmation results. This action causes high economic lost to farmers and to the whole sector. The problem is that in many cases, positive samples obtained with screening methods are for concentrations below the established MRL and ML.

Antimicrobial are the compounds most frequently detected in milk (Ortelli, Cognard, Jan & Edder, 2009) and their control is highly important for the milk industry. Many methods for analysis of antimicrobial agents in food such as sulfonamides, tetracyclines, penicillins have been reported (Cui et al., 2006; Gamba et al., 2009; Hermo, Nemutlu, Kr, Barrón & Barbosa, 2008; Hoof et al., 2005; Koesukwiwat, Jayanta & Leepipatpiboon, 2007a; Koesukwiwat et al., 2007b; Li, Wu, Yang & Zhang, 2010; Marazuela & Moreno-Bondi, 2004; Ramírez et al., 2003; Volmer, 1996). The tendency nowadays is to develop multi-class methods which permit analysis for a variety of drugs with a single procedure. These methods maximize laboratory resources and sample throughput so their importance in residue control laboratories is increasing. Multi-class methods have been developed in matrices such as pig liver, kidney and muscle where 16 β-agonists could be detected (Shao et al., 2009), 12 coccidiostats were detected in chicken liver (Olejnik et al., 2009) and 42 antibiotics in honey (Hammel, Mohamed, Gremaud, LeBreton & Guy, 2008).

For milk analysis different multi-class methods have been reported. Some are screening methods, which permit the analysis of more than 100 veterinary drugs (Ortelli et al., 2009; Stolker et al., 2008), and other are confirmatory methods, which allow identification and quantification of antimicrobial agents such as sulfonamides,

93 tetracyclines, quinolones, penicillins (Bohm et al., 2009; Gamba et al., 2009;
94 Koesukwiwat et al., 2007b). Even with a wide range of multi-class methods for milk
95 analysis have been reported, to date, no method permits the simultaneous determination
96 and quantification of various coccidiostats in milk at the MLs established in the
97 Commission Regulation 124/2009. Considering the fact that coccidiostats are now
98 monitored in milk samples and that the development of HPLC-MS/MS multi-class
99 methods is the new trend in laboratory dedicated to residue analysis, the present article
100 focuses on the analysis of a group of coccidiostats, antimicrobial agents, corticosteroids
101 and an antifungal agent.

102 The antimicrobial agents selected for the study were sulfonamides and
103 quinolones. They are commonly employed in food production due to their relatively low
104 price (Clemente, Hermo, Barrón & Barbosa, 2006). The presence of sulfonamides and
105 quinolones, ciprofloxacin and enrofloxacin, in milk is permitted but only up to
106 concentration of 100 μg Kg^{-1}. Prolong exposure to residues of sulfonamides has resulted
107 in an increase of drug-resistant microbial strains (Koesukwiwat et al., 2007b) and
108 similar effects have been observed for quinolones (Rodriguez, Moreno-Bondi &
109 Marazuela, 2008).

110 The study also includes some corticosteroids which are anti-inflammatory drugs
111 sometimes illegally used in Europe as growth promoters. Fluoroquinolones exhibit
112 activity against Gram (+) and Gram (-) bacteria (Kowalski & Plenis, 2008) and they are
113 a very effective chemotherapeutic agent. Their use in lactating and breeding animals
114 may leave residues in the milk and tissue. They are hazardous for consumers, causing
115 allergic reactions and also leading to the emergence of drug resistance in bacteria (Tang,
116 Ho & Lai, 2006)

117 This paper presents a multi-class method for the analysis of 18 veterinary drugs
118 (Table 1) in milk samples. The veterinary drugs belong to different therapeutic classes
119 including antibiotics, coccidiostats, corticosteroids and an antifungal agent. The aim
120 was to develop and to validate a selective method for the identification and
121 quantification of veterinary drugs commonly analyzed in residue control laboratories,
122 by an LC-MS/MS and transfer it to routine laboratories for its implementation in milk
123 control plans.

124

2. Experimental

2.1. Chemicals, reagents and stock solutions

Griseofulvin, sulfadimethoxine, sulfamethoxazole, sulfamethoxypyridazine, sulfaquinoxaline, decoquinate, lasalocid, maduramicin, monensin, narasin, robenidine, salinomycin, ciprofloxacin, enrofloxacin, trimethoprim, flumethasone, cortisone, prednisolone (purity > 98%) and the internal standards (robenidine-d_8 and sulfadimethoxine-d_3) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile and methanol (HPLC grade) were obtained from Scharlau Chemie (Barcelona, Spain), and formic acid and ammonium acetate (purity > 99% for analysis) were from Acros Organics (Geel, Belgium). Purified water was prepared in-house with a Milli-Q water system from Millipore (Bedford, MA, USA). Nitrogen gas was generated using an in-house nitrogen generator from Peak Scientific Instruments, Ltd. (Chicago, IL, USA).

Drugs were accurately weighed to prepare stock solutions of individual compounds at a concentration of 0.6 mg mL^{-1} in 0.1% of formic acid in methanol. These stock solutions were mixed to obtain a stock solution mixture of all the selected drugs, concentrations of each analyte in this stock solution are summarized in Table 1. This stock solution was further diluted in 0.1% of formic acid in methanol to obtain a standard solution mixture of drugs at lower concentration. All standards were stored in the dark at −18°C and no longer than three months for low concentration (ng mL^{-1} and μg mL^{-1}) and no longer than one year for high concentrations (mg mL^{-1}).

2.2. Equipment.

The samples were analyzed on an LC–MS/MS system which consisted of an 1100 HPLC separation module bought from Agilent Technologies (Waldbronn, Germany) and a Qtrap 2000™ mass spectrometer bought from Applied Biosystems/MDS-Sciex (Toronto, Canada) equipped with a TurboIonSpray® source Software Analyst 1.4.1 from Applied Biosystems was used to control the whole system. Analyses were carried out by injecting 10 μL of sample into a Synergi 2.5 μm Polar-RP 100 Å (50 × 2.00 mm) HPLC column connected to a Polar-RP security-guard cartridge (4.0 × 2.0 mm), both from Phenomenex (Macclesfield, UK).

A H-103N series Kokusan centrifuge, a MS2 Minishaker vortex shaker from IKA (Staufen, Germany), a J.K. Ultrasonic Cleaner, a vacuum station Manifold (Phenomenex, UK) with Strata-X® SPE cartridges (60 mg, 3 mL) (Phenomenex, UK) and a Turbo Vap® II evaporator from Zyrmark (Hopkinton, MA, USA) were employed for the sample preparation and the extraction process.

2.3. HPLC-MS/MS conditions

Analytes were separated chromatographically using two solvents (A and B) mixed on a gradient mode. Solvent A contained 0.1% formic acid in water, and B 0.1% formic acid in acetonitrile. The flow rate was maintained at 0.2 mL min^{-1} during the whole run. The gradient program was as follows: 0–2 min, 98% A; 2–3 min, 85% A; 3–4 min, 75% A; 4–8 min, 55% A; 8–9 min, 50% A; 9–14 min, 30% A; 14–25 min, 7% A; 25–28 min, 0% A; 28–32 min, 75% A; 32–36 min, 100% A.

Mass-spectrometry measurements were performed using electrospray ion source (ESI) in positive mode. Selected veterinary drugs were identified by their retention times (t_R) and two multiple reaction monitoring (MRM). The MS conditions employed to monitor each transition are summarized in Table 2. Stock solutions of individual drugs at 100 ng mL^{-1} were analyzed to verify MRM transitions and t_R selected.

MS parameters set for the detection of each drug were: a source temperature of 400 ºC, a vacuum gauge of 2.2 atm, an ion spray voltage of 5.500 V and a curtain gas of 1.2 x 10^4 Pa, ion source 1 was set at 2.6 x 10^4 Pa and ion source 2 at 2.4 x 10^4 Pa. These parameters were set during the whole run

To monitor a specific transition between a precursor and a product ion the following MS parameters need to be set: declustering potential (DP), entrance potential (EP), collision cell entrance potential (CEP), collision energy (CE) and cell exit potential (CXP). These parameters were varied for each compound and changed automatically during the run. The dwell-time employed between transitions was 5 ms.

Repeatability of the t_R for each analyte was investigated by analyzing standard solutions that contained each drug at the following concentrations: 12.5, 25, 50, 75, 100, 150 and 250 ng mL^{-1}. Six injections form the same vial and from different vials were performed to investigate t_R repeatability.

187

188 ***2.4. Milk samples.***

189 The method was developed and validated with milk bought from local supermarkets;
190 this milk was enriched with known amounts of drugs. The applicability of the method
191 was investigated on raw milk samples obtained from ten local dairy farms; these
192 samples were collected during ten consecutive weeks. The applicability of the method
193 was also tested on whole, semi skimmed and skimmed milk bought from local
194 supermarkets. Samples, once in the laboratory, were frozen at -20 ºC until analysis.

195

196 ***2.5. Extraction procedure.***

197 Milk sample (2 mL) was transferred into a 10 mL glass centrifuge tube and 2 mL of
198 acetonitrile was added, together with 10 µL of the internal standards (IS) solution (1 µg
199 mL^{-1}). The mixture was vortexed (10 s), sonicated (10 min) and centrifuged at 1509 g
200 (10 min). The organic layer (the supernatant) was collected and transferred into a clean
201 graduated 40-mL falcon tube with 20 mL of Milli-Q water. The extraction procedure
202 was repeated with 1 mL of acetonitrile and the supernatant was diluted with 5 mL of
203 Milli-Q water.

204 The mixture (supernatant and Milli-Q water) was transferred into a Strata™-X
205 cartridge previously conditioned with 4 mL of methanol and 4 mL of Milli-Q water.
206 Once the whole sample had been loaded into the cartridge (flow rate of 1 mL min^{-1}), the
207 cartridge was dried with the vacuum pump for 20 min. Methanol (4 mL) was employed
208 to rinse the sample vessels and transferred to the SPE cartridge to elute the analytes.
209 First, the methanol impregnated the cartridge for 5 min and the eluent was collected into
210 a conical, glass Pyrex® tube. The elution step was completed with the addition of 2 x 3
211 mL of methanol. The whole eluent was evaporated to dryness under a gentle stream of
212 nitrogen in a water bath at 45 ºC. The residue was reconstituted in 200 µL of 0.1%
213 formic acid in methanol, vortexed, transferred into amber vials containing a 300 µL
214 micro-insert, and stored at -18 ºC. Extracts were analyzed within a month after the
215 extraction.

216 During analysis, the complete analytical procedure was applied to four quality
217 control samples: a blank sample (a sample negative for the drugs that are going to be

218 analyzed), a fortified sample (milk sample enriched with known amounts of analytes), a
219 reagent blank (reagents only, no milk) and fortified reagents (reagents spiked with
220 known amounts of analytes). These quality control samples were processed together
221 with samples to be confirmed fortified samples and reagents were prepared by adding
222 appropriate aliquots of the mixed standard, vortex, and allowing the sample to settle for
223 30 min.

224

225 ***2.6. Validation procedure***

226 The method presented was used to identify and quantify 18 drugs which belong to
227 different therapeutic classes, with established MRL and ML levels. Method validation
228 was conducted at the MRLs set by European legislation; drugs without MRL were
229 validated at the lowest detectable concentration. For simplification, the term validation
230 level (VL) was used instead MRL and ML. The validation process was conducted as
231 suggested by the 2002/657 Decision.

232 The Commission Decision specifies that, when no certified reference material is
233 available (CRMs), accuracies of the measurements should be assessed with recoveries
234 of known amounts of the analytes added to a blank matrix. The validation study was
235 conducted in order to determine recovery, precision, ruggedness, repeatability, decision
236 limit (CCα) and detection capability (CCβ) of the method. Design of the validation
237 study, as well as the calculations, were carried out with the software "ResVal 2.3"
238 obtained from the Community Reference Laboratory (CRL, Bilthoven, Netherlands).

239 Each IS was used to calculate correct recoveries, as described in the European
240 Commission Decision 2002/657. Robenidine-d_8 and sulfadimethoxine-d_3 were chosen
241 because: they are labeled forms of the analytes, particularly suitable for mass
242 spectrometric detection and stables isotopes. While sulfadoxine-d_3 was used to quantify
243 sulfonamides, penicillins, trimethoprim, enrofloxacin, and griseofulvin, robenidine-d_8
244 was employed to quantify decoquinate, lasalocid, maduramicin, monensin, narasin,
245 robenidine and salinomycin. A standard solution mixture of the two IS was always
246 added to the samples at the beginning of the extraction procedure to calculate
247 recoveries.

248 Validation was investigated by enriching blank milk samples with known
249 amounts of drugs. The samples were fortified as follows: a non-spiked milk sample

(blank), six samples spiked at 0.5 x VL, six spiked at .1.0 x VL, six spiked at 1.5 x VL, one spiked at 2 x VL and one spiked at 5 x VL. VL employed for each drug is summarized in Table 4. After fortification, samples were shaken vigorously for 30 min to homogenize them. The same procedure was performed on three different days (Day 1, 2 and 3). On each day, 21 samples were analyzed.

Linearity of the method was investigated with stock solutions and with blank milk samples enriched with drugs. The stock solutions employed contained the selected drugs at the following concentrations: 12.5, 25, 50, 75, 100, 150 and 250 ng mL^{-1} for griseofulvin (Table 1). The milk samples were those fortified at the following concentrations: 0, 0.5, 1, 1.5, 2, 5 x VL. Two types of calibration curves were generated; instrument calibration curves (ICCs), standard analyzed directly by the HPLC-MS/MS, and sample calibration curves (SCCs), milk samples enriched with a known amount of the analyte to be detected. The ICCs were generated by plotting peak areas against the concentrations of the analyte in the solution. The SCCs curves were constructed by plotting the ratio of the analyte/IS peak area versus the concentration of the sample. For each analyte and each day of the validation two calibration curves were obtained. Regression coefficients (r^2), slopes (b) and intercepts (a) were calculated for each curve.

According to European Commission Decision 2002/657/EC (EU, The European parliament and the Council of the European Union, 2002), CCα and CCβ can be defined for substances for which no permitted limit has been established, and for substances with established permitted limit. Depending on the case, different α error for CCα shall be applied (1 or 5 %). For this particular study, to simplify calculation the same α error was applied for all drug, the worst case scenario was considered, only an error of 1 % was admitted. Similarly, to calculate CCβ the same formula was applied to all analytes.

$$CC\alpha = [(Y_a + 2.33 \times \text{stdev } Y_a) - Y_a] / b \qquad \text{Equation 1}$$

$$CC\beta = [(Y_a + 2.33 \times \text{stdev } Y_a + 1.64 \text{ stdev } Y_a) - Ya] / b \qquad \text{Equation 2}$$

Where Y_a is the concentration corresponding to the *y*-intercept, stdev Y_a is the standard error at the intercept and b is the slope of the calibration curve.

On the fourth day of the validation, 20 different milk samples obtained from different farms were analyzed in order to evaluate the selectivity, and particularly, the

specificity of the method. Ten of the twenty samples were spiked with the drugs at the VL.

Reliability of the method was investigated with in-house blind samples enriched with known amounts of the drugs. A total of fifteen blind samples were analyzed. A mean RF was obtained from the analysis of the 73 milk samples employed for the validation procedure. Then the mean RF was employed to calculate the concentration of the analyte in the sample and the recovery of the analyte.

2.7. Matrix effect.

Matrix effects are alterations in the ionization efficiency due to the presence of analytes that coelute with the compounds of interest. These effects are unseen in the chromatogram but they have a deleterious impact on the accuracy and sensitivity of the method (Taylor, 2005). Matrix effects were, therefore, investigated by comparing the MS response (peak area) to the analytes at a given concentration in acidic methanol with the MS response to the same analytes present in a milk sample which was spiked before and after the extraction.

2.8. Screening method

Blind, raw and commercial milk samples were analyzed with the HPLC-MS/MS method presented and with a microbiological test called Milk Rapid Antibiotic (Mira) (Liofilchem, Italy) Test. MiRA Test is a microbiological test which permits the detection of residual antibiotics in milk such as sulfonamides. The kit uses spores of *Geobacillus stearothermophilus* and a culture medium designed for that purpose.

3. Results and discussion

3.1. Optimization of LC-MS/MS conditions

The method development started with the optimization of the MS parameters for the detection of the selected drugs. Parameters were optimized by automatic tuning, standard solution of individual drugs at 1000 ng mL^{-1} were infused directly into the MS. Precursor and product ions selected for identification of each drug were those which

311 gave the highest instrument signal response (Table 2). Drugs which required negative
312 ionization were not included in the study i.e. diclazuril. Positive ionization was already
313 reported for the selected drugs (Martins-Júnior, Kussumi, Wang & Lebre, 2007; Olejnik
314 et al., 2009).

315 The next step was to select an adequate analytical column. Based on previously
316 reported results (Turnipseed, et al., 2008) and those obtained for the analysis of
317 coccidiostats, the Synergi 2.5 µm Polar-RP 100A (50 × 2.00 mm) HPLC column was
318 considered the best option. To protect the analytical column from damaging by
319 chemical contaminants, it was connected to a security-guard cartridge filled with the
320 same material.

321 The combination of several mobile components, such as ammonium acetate,
322 formic acid, acetic acid, methanol, acetonitrile and water, were comprehensively
323 investigated to achieve an optimal chromatographic separation and high MS signal
324 response for the drugs selected.

325 Since ammonium acetate signal suppression has been shown to be lower than
326 other buffers (Heller, Nochetto, Rummel & Thomas, 2006; Ortelli et al., 2009) its
327 applicability in the method was investigated. The objective was to maintain mobile
328 phase pH. However, it produced a decrease of the signal intensity (5 times).
329 Consequently, its use in the mobile phase was discarded.

330 A multi-class HPLC-MS/MS method to determine different veterinary drugs
331 including quinolones, sulfonamides, macrolides and tetracyclines was reported by
332 Aguilera et al. (2008). The authors preferred the use of formic acid instead of acetic acid
333 because it improved ionization (Aguilera-Luiz, Vidal, Romero-González & Frenich,
334 2008). Even if some of the drugs selected for this study are different from those
335 employed by Aguilera et al (2008) similar ionization results were observed, and formic
336 acid was chose as ionization component.

337 The highest resolution among peaks, as well as symmetric peak shapes, was
338 achieved with the combination of formic acid, acetonitrile and water mixed on a
339 gradient mode. Formic acid helped with the prevention of peak tailing and provided
340 sufficient ionization.

341 To prevent incorrect identification of the analytes t_R variability was investigated
342 for each pharmaceutical. Standard deviation (STD) of the t_R was for all drugs < 0.4 min

343 (Table 3), the highest STD was observed for sulfamethoxazole. Similarly, the relative
344 standard deviation (RSD) of the t_R was < 0.4 % (Table 4). It was concluded that the
345 selected drugs could be satisfactory identified by their t_R and two SRM transitions as
346 their t_R did not vary more than 0.4 min.

347

348 ***3.2. Optimization of extraction procedure***

349 The optimization of the extraction procedure started with a simple liquid-liquid
350 extraction. The solvents tested were hexane and dichloromethane as they are immiscible
351 in milk. None of these two solvents could be employed; hexane could not extract all the
352 selected drugs because of its low polarity and dichloromethane extracts were very
353 greasy and not homogenous. The second approach was to mix acetonitrile or acidify
354 methanol with milk for protein precipitation (Stolker et al., 2008) and use this step to
355 extract the drugs. The resultant supernatants obtained with acetonitrile or acidify
356 methanol were evaporated (45 ºC), reconstituted in methanol and injected into the
357 HPLC-MS/MS. Nevertheless, recoveries were poor (below 40 %), certainly due to the
358 amount of fat present in the extract and matrix complexity.

359 Stolker *et al.* (2008) reported a screening method for drugs in milk where milk
360 proteins are precipitated with acetonitrile and the supernatant diluted in water. The
361 mixture is then loaded into a SPE cartridge. This procedure was reproduced in the
362 laboratory and all the selected drugs could be detected at concentrations below MRL
363 levels. However, recoveries were lower than expected and some improvements were
364 introduced. Acetonitrile extraction was performed twice instead of once. First and
365 second extractions were performed with 2 and 1 mL of acetonitrile, respectively. The
366 mixture of supernatants was diluted in 25 mL of Milli-Q water. Elution with methanol
367 was also performed twice, both with 3 mL of methanol. Recoveries improved,
368 approximately 5 %, when methanol was firstly poured into the samples vessel to dilute
369 the residual drugs and then poured into the cartridge. Methanol was allowed to soak the
370 cartridge for 5 min before elution of the analytes.

371 Matrix effects, certainly ion-suppression, were observed for the analysis of
372 ciprofloxacin, decoquinate, enrofloxacin, flumethasone, lasalocid, monensin, narasin,
373 sulfamethoxypyridazine, sulfaquinoxaline and trimethoprim. RSD of peak areas of these
374 drugs decreased approximately 30 % when they were spiked in milk extracts. Similar

RSDs of peak area were observed for the other investigated drugs. Therefore no matrix effects were observed for the other analytes. Recoveries of drugs in milk samples spiked before and after the extraction were compared. The results indicated that less than 20 % of cortisone, flumethasone, griseofulvin, maduramicin, monensin, narasin, salinomycin and sulfaquinoxaline were lost during the extraction procedure. Approximately 30 % of trimethoprim was also lost during the extraction.

Two IS were employed for recovery control, robenidine-d_8 and sulfadimethoxine-d_8, other substances such as norfloxacin-d_5 and sulfadoxine-d_3 could have also been employed but they were not included due to their high prices. The number of IS employed for residue analysis in food is variable; two IS were used for the analysis of sixteen β-agonist in pig liver, kidney and muscle (Shao et al., 2009), three for the determination of 12 coccidiostats in chicken liver (Olejnik et al., 2009), and none for the determination of 47 antimicrobial agents (Malone, Dowling, Elliott, Kennedy & Regan, 2009).

3.3. Method validation

As there was no reference material, the method was validated using milk samples enriched with drugs at different concentrations. The developed method was validated in terms of sensitivity, accuracy, intraday and inter-day precision, as well as linearity.

ICC and SCC were built to assess the method linearity. Similar linear regression coefficients (r^2) were achieved during the four days of the validation, indicating the repeatability of the method. For the investigated drugs, r^2 of ICC were higher than 0.9800 and those of SCC were higher than 0.9600 (Table 4), r^2 of ICC and of SCC indicated good linearity of the method.

Data to estimate the precision of the method were generated during three days and for concentration levels but always within the requirement of the Commission Decision 657/2010. Recoveries of the selected drugs were higher than 80 %, except for the recoveries of cortisone, flumethasone, narasin and robenidine drugs, which had recoveries of 70, 71, 74 and 76 %, respectively. Intra-day sulfonamides recoveries were between 73 and 119 %, and inter-day recoveries between 80 and 118. These values were similar than those reported by other authors (Bohm et al., 2009; Huang, Yuan & Huang,

2007; Koesukwiwat etal., 2007b) but 10 % higher than those reported by Gamba *et al.* (2009) who reported an HPLC-DAD method for the analysis of sulfonamides alone. The lowest recoveries were obtained for the corticosteroids values which were similar to those reported previously (Cui et al., 2006; Stolker et al., 2008). However, recoveries of monensin and salinomycin achieved in milk samples by Stolker et al (2008) were 10 and 22 % higher than those achieved in this research. The advantage of the protocol presented is the posibility of identifying and quantifying seven conccidiostats in milk together with sulfonamides, quinolones and corticosteroids.

Repeatability, intra and inter-day, of the method was investigated at three concentrations (0.5, 1 and 1.5 x VL). The intra-day repeatability (RSDr) and inter-day repeatability (RSD_R) ranged from 1 to 19 % and from RSD below 20 %, respectively. RSD values were within the requirement of the Commission Decision 2002/657/EC (EU, The European parliament and the Council of the European Union, 2002).

Method specificity and selectivity were investigated on Day 4. Ten blank milk samples and ten enriched milk samples were analyzed at Day 4. No false positive or false negative results were obtained with any of the milk sample analyzed on Day 4. Analysis of the enriched milk samples resulted with accurate identification and quantification of the drugs. These results proved the selectivity and specificity of the method.

CCα and CCβ were determined in accordance with Section 3.1.2.4 and 3.1.2.6 of the Commission Decision 2002/657/EC by fortifying six milk samples at the VL on three different days. These two statistical limits permit critical evaluation of the method at various concentrations. Above these limits the method could reliably identify and quantify the selected drugs. CCα and CCβ also take into account method variability and statistical risk of making a wrong decision (Rambla-Alegre, Collado-Sánchez, Esteve-Romero & Carda-Broch, 2010). CCα and CCβ achieved are summarized in Table 4. CCα of sulfonamides ranged from 5.8 to 18.7 ng mL^{-1}, and CCβ from 9.1 to 31 ng mL^{-1}. The method presented could detect sulfonamides at concentrations more than 5-times lower than with other reported methods (Gamba et al., 2009; Stolker et al., 2008).

Multi-class methods to detect residues of veterinary drugs in milk have been reported previously. Stolker *et al.* (2008) reported an ultra-performance liquid

chromatography method combined with time-of-flight mass spectrometer (UPLC-Tof-MS) for the screening and quantification of more than 100 veterinary drugs. CCβ values achieved by Stolker *et al.* (2008) for enrofloxacin, flumethasone and the sulfonamides were higher than those presented in this article probably due to the characteristics of the mass spectrometer employed (Stolker et al., 2008). Another UPLC-Tof-MS method for the screening of 150 veterinary drugs was reported more recently but for this method no CCβ were published (Ortelli et al., 2009). In addition to the method presented in this article, other HPLC-MS/MS multi-class confirmatory methods for the detection of residues of veterinary drugs have been reported. The presented method is the only multi-class method able to identify and quantify different sulfonamides, quinolones, corticosteroids and coccidiostats in milk by HPLC-MS/MS.

3.4. Method application

The applicability of the method was investigated in milk samples collected from farms of the North-West of Spain. One hundred raw milk samples collected from local dairy farms and fifteen milk samples bought from local supermarkets were analyzed with the described method to determine the presence of the selected drugs. All sample resulted to be compliant according to the MRL and ML established in the different EU legislations (EU, The European parliament and the Council of the European Union, 2009; EU, The European parliament and the Council of the European Union, 2010). However, one of the raw milk samples resulted to be positive for decoquinate, concentration of the coccidiostat was 1 ng mL^{-1}. The samples were also analyzed with screening methods for comparison. None of the samples resulted to be non-compliant with the screening method either. However, the samples which resulted to be positive for decoquinate with the HPLC-MS/MS resulted to be compliant with the confirmatory method.

4. Conclusions

This work presents a suitable HPLC-ESI-MS/MS method for the simultaneous extraction, identification and quantification of residues of eighteen veterinary drugs (seven coccidiostats, seven antimicrobial agents, three corticosteroids and an antifungal agent) in milk samples. The authors would like to emphasize that the novelty of this

471 analytical methodology lays in its capacity to determine seven coccidiostats at the ML
472 in milk together with other groups of veterinary drugs (antimicrobial agents and
473 corticosteroids), something not reported previously for HPLC-MS/MS methods, on the
474 best of authors' knowledge.

475 Validation of the method, based on the factor-comprehensive in-house validation
476 concept and always in accordance with Commission Decision 2002/657/EC, showed its
477 applicability and reliability for the analysis of selected veterinary drugs in milk.

478 Additionally, The method was successfully applied in raw milk samples
479 collected from dairy farms and in fifteen milk samples bought in supermarkets. These
480 results indicated that the method could be successfully implemented for routine analysis
481 of milk samples in the dairy industry or a laboratory dedicated to residue control. The
482 present method combined with other reported methods could certainly replace the use of
483 the current, non-differentiating, screening methods.

484

485 **6. References**

486

487

488 Aguilera-Luiz M. M., Vidal J. L. M., Romero-González R., Frenich A. G. 2008. Multi-
489 residue determination of veterinary drugs in milk by ultra-high-pressure liquid
490 chromatography–tandem mass spectrometry. *Journal of Chromatography A, 1205*,
491 10-16.

492 Bando E., Oliveira R. C., Ferreira G. M., Machinski M. 2009. Occurrence of
493 antimicrobial residues in pasteurized milk commercialized in the state of Parana,
494 Brazil. *Journal of Food Protection, 72*, 911-914.

495 Berendsen B., Stolker L., de Jong J., Nielen M., Tserendorj E., Sodnomdarjaa R.,
496 Cannavan A., Elliott C. 2010. Evidence of natural occurrence of the banned

497 antibiotic chloramphenicol in herbs and grass. *Analytical and Bioanalytical*
498 *Chemistry, 397*, 1955-1963.

499 Bohm D. A., Stachel C. S., Gowik P. 2009. Multi-method for the determination of
500 antibiotics of different substance groups in milk and validation in accordance with
501 Commission Decision 2002/657/EC. *Journal of Chromatography.A, 1216*, 8217-
502 8223.

503 Clemente M., Hermo M. P., Barrón D., Barbosa J. 2006. Confirmatory and quantitative
504 analysis using experimental design for the extraction and liquid chromatography–
505 UV, liquid chromatography–mass spectrometry and liquid chromatography–mass
506 spectrometry/mass spectrometry determination of quinolones in turkey muscle.
507 *Journal of Chromatography A, 1135*, 170-178.

508 Cui X., Shao B., Zhao R., Yang Y., Hu J., Tu X. 2006. Simultaneous determination of
509 seventeen glucocorticoids residues in milk and eggs by ultra-performance liquid
510 chromatography/electrospray tandem mass spectrometry. *Rapid Communications*
511 *in Mass Spectrometry, 20*, 2355-2364.

512 EU, The European parliament and the Council of the European Union. 2010.
513 Commission Regulation (EU) No 37/2010 of 22 December 2009 on
514 pharmacologically active substances and their classification regarding maximum
515 residue limits in foodstuffs of animal origin. *L 15*.

516 EU, The European parliament and the Council of the European Union. 2009.
517 Commission Regulation Nº 124/2009 of 10 February 2009 setting maximum levels
518 for the presence of coccidiostats or histomonostats in food resulting from the
519 unavoidable carry-over of these substances in non-target feed. *L 40*.

520 EU, The European parliament and the Council of the European Union. 2002.
521 Commisssion Decission of 12 August 2002 implementing Council Directive
522 96/23/EC concerning the performance of analytical methods and the interpretation
523 of results., *L 221*.

524 Gamba V., Terzano C., Fioroni L., Moretti S., Dusi G., Galarini R. 2009. Development
525 and validation of a confirmatory method for the determination of sulphonamides in
526 milk by liquid chromatography with diode array detection. *Analytica Chimica Acta,*
527 *637*, 18-23.

528 Gomes E. R., Demoly P. 2005. Epidemiology of hypersensitivity drug reactions.
529 *Current Opinion in Allergy and Clinical Immunology, 5*, 309-316.

530 Hammel Y., Mohamed R., Gremaud E., LeBreton M., Guy P. A. 2008. Multi-screening
531 approach to monitor and quantify 42 antibiotic residues in honey by liquid
532 chromatography–tandem mass spectrometry. *Journal of Chromatography A, 1177*,
533 58-76.

534 Heller D. N., Nochetto C. B., Rummel N. G., Thomas M. H. 2006. Development of
535 multiclass methods for drug residues in eggs: hydrophilic solid-phase extraction
536 cleanup and liquid chromatography/tandem mass spectrometry analysis of
537 tetracycline, fluoroquinolone, sulfonamide, and beta-lactam residues. *Journal of*
538 *Agricultural and Food Chemistry, 54*, 5267-5278.

539 Hermo M. P., Nemutlu E., Kır S., Barrón D., Barbosa J. 2008. Improved determination
540 of quinolones in milk at their MRL levels using LC–UV, LC–FD, LC–MS and LC–
541 MS/MS and validation in line with regulation 2002/657/EC. *Analytica Chimica*
542 *Acta, 613*, 98-107.

543 Hoof N. V., Wasch K. D., Okerman L., Reybroeck W., Poelmans S., Noppe H.,
544 Brabander H. D. 2005. Validation of a liquid chromatography–tandem mass
545 spectrometric method for the quantification of eight quinolones in bovine muscle,
546 milk and aquacultured products. *Analytica Chimica Acta, 529*, 265-272.

547 Huang X., Yuan D., Huang B. 2007. Simple and rapid determination of sulfonamides in
548 milk using Ether-type column liquid chromatography. *Talanta, 72*, 1298-1301.

549 Koesukwiwat U., Jayanta S., Leepipatpiboon N. 2007a. Solid-phase extraction for
550 multiresidue determination of sulfonamides, tetracyclines, and pyrimethamine in
551 Bovine's milk. *Journal of Chromatography A, 1149*, 102-111.

552 Koesukwiwat U., Jayanta S., Leepipatpiboon N. 2007b. Validation of a liquid
553 chromatography–mass spectrometry multi-residue method for the simultaneous
554 determination of sulfonamides, tetracyclines, and pyrimethamine in milk. *Journal*
555 *of Chromatography A, 1140*, 147-156.

556 Kowalski P., Plenis A. 2008. Simultaneous determination of six quinolone antibiotics in
557 poultry and porcine samples by capillary electrophoresis. *Bulletin of the Veterinary*
558 *Institute in Pulawy, 52*, 81-85.

559 Lamar J., Petz M. 2007. Development of a receptor-based microplate assay for the
560 detection of beta-lactam antibiotics in different food matrices. *Analytica Chimica*
561 *Acta, 586*, 296-303.

562 Li C., Wu Y., Yang T., Zhang Y. 2010. Rapid simultaneous determination of
563 dexamethasone and betamethasone in milk by liquid chromatography tandem mass
564 spectrometry with isotope dilution. *Journal of Chromatography A, 1217*, 411-414.

565 Lopez M. I., Pettis J. S., Smith I. B., Chu P. 2008. Multiclass Determination and
566 Confirmation of Antibiotic Residues in Honey Using LC-MS/MS. *Journal of*
567 *Agricultural and Food Chemistry, 56*, 1553-1559.

568 Malone E. M., Dowling G., Elliott C. T., Kennedy D. G., Regan L. 2009. Development
569 of a rapid, multi-class method for the confirmatory analysis of anti-inflammatory
570 drugs in bovine milk using liquid chromatography tandem mass spectrometry.
571 *Journal of Chromatography A, 1216*, 8132-8140.

572 Marazuela M. D., Moreno-Bondi M. C. 2004. Multiresidue determination of
573 fluoroquinolones in milk by column liquid chromatography with fluorescence and
574 ultraviolet absorbance detection. *Journal of Chromatography A, 1034*, 25-32.

575 Martins-Júnior H. A., Kussumi T. A., Wang T. A., Lebre D. T. 2007.
576 A Rapid Method to Determine Antibiotic Residues in Milk using Liquid
577 Chromatography Coupled to Electrospray Tandem Mass Spectrometry. *Journal of*
578 *the Brazilian Chemical Society, 18*, 397-405.

579 Olejnik M., Szprengier-Juszkiewicz T., Jedziniak P. 2009. Multi-residue confirmatory
580 method for the determination of twelve coccidiostats in chicken liver using liquid
581 chromatography tandem mass spectrometry. *Journal of Chromatography A, 1216*,
582 8141-8148.

583 Ortelli D., Cognard E., Jan P., Edder P. 2009. Comprehensive fast multiresidue
584 screening of 150 veterinary drugs in milk by ultra-performance liquid
585 chromatography coupled to time of flight mass spectrometry. *Journal of*
586 *Chromatography B, 877*, 2363-2374.

587 Raison-Peyron N., Messaad D., Bousquet J., Demoly P. 2001. Anaphylaxis to beef in
588 penicillin-allergic patient. *Allergy, 56*, 796-797.

589 Rambla-Alegre M., Collado-Sánchez M. A., Esteve-Romero J., Carda-Broch S. 2010.
590 Quinolones control in milk and eggs samples by liquid chromatography using a
591 surfactant-mediated mobile phase. *Analytical and Bioanalytical Chemistry*, - 1-11.

592 Ramírez A., Gutiérrez R., Díaz G., González C., Pérez N., Vega S., Noa M. 2003. High-
593 performance thin-layer chromatography–bioautography for multiple antibiotic
594 residues in cow's milk. *Journal of Chromatography B, 784*, 315-322.

595 Rodriguez E., Moreno-Bondi M. C., Marazuela M. D. 2008. Development and
596 validation of a solid-phase extraction method coupled to liquid chromatography
597 with fluorescence detection for the determination of fluoroquinolone residues in
598 powdered infant formulae: Application to the analysis of samples from the Spanish
599 and Latin American market. *Journal of Chromatography , 1209*, 136-144.

600 Shao B., Jia X., Zhang J., Meng J., Wu Y., Duan H., Tu X. 2009. Multi-residual
601 analysis of 16 β-agonists in pig liver, kidney and muscle by ultra performance
602 liquid chromatography tandem mass spectrometry. *Food Chemistry, 114*, 1115-
603 1121.

604 Sheridan R., Policastro B., Thomas S., Rice D. 2008. Analysis and Occurrence of 14
605 Sulfonamide Antibacterials and Chloramphenicol in Honey by Solid-Phase
606 Extraction Followed by LC/MS/MS Analysis. *Journal of Agricultural and Food*
607 *Chemistr* , 3509-3516.

608 Stolker A., Rutgers P., Oosterink E., Lasaroms J., Peters R., van Rhijn J., Nielen M.
609 2008. Comprehensive screening and quantification of veterinary drugs in milk
610 using UPLC–ToF-MS. *Analytical and Bioanalytical Chemistr , 391*, 2309-2322.

611 Stolker A. A. M., Brinkman U. A. T. 2005. Analytical strategies for residue analysis of
612 veterinary drugs and growth-promoting agents in food-producing animals-a review.
613 *Journal of Chromatography A, 1067*, 15-53.

614 Tang H. P., Ho C., Lai S. S. 2006. High-throughput screening for multi-class veterinary
615 drug residues in animal muscle using liquid chromatography/tandem mass
616 spectrometry with on-line solid-phase extraction. *Rapid Communications in Mass*
617 *Spectrometry, 20*, 2565-2572.

618 Taylor P. J. 2005. Matrix effects: the Achilles heel of quantitative high-performance
619 liquid chromatography–electrospray–tandem mass spectrometry. *Clinical*
620 *Biochemistry, 38*, 328-334.

621 Toldrá F., Reig M. 2006. Methods for rapid detection of chemical and veterinary drug
622 residues in animal foods. *Trends in Food Science & Technology, 17*, 482-489.

623 Volmer D. A. 1996. Multiresidue Determination of Sulfonamide Antibiotics in Milk by
624 Short-column Liquid Chromatography Coupled with Electrospray Ionization
625 Tandem Mass Spectrometry. *Rapid Communications in Mass Spectrometry, 10*,
626 1615-1620.

TABLES

Table 1. Therapeutic class, MRL, ML and chemical properties of the selected veterinary drugs

Analyte	Therapeutic	MRL[a] or ML[b] (ng mL^{-1})	CAS N^{o} [c]	MW	Formula	Concentration[d] [ng mL^{-1}]
Ciprofloxacin	Antimicrobial	100	85721-33-1	331	$C_{17}H_{18}FN_3O_3$	5002
Cortisone	Corticosteroid	N.P.[d]	53-06-5	360	$C_{21}H_{28}O_5$	500
Decoquinate	Coccidiostat	5	18507-89-6	418	$C_{24}H_{35}NO_5$	504
Enrofloxacin	Antimicrobial	100	93106-60-6	359	$C_{19}H_{22}FN_3O_3$	5016
Flumethasone	Corticosteroid	N.P.[d]	2135-17-3	410	$C_{22}H_{28}F_2O_5$	504
Gliseofulvin	Antifungal	No limit	126-07-8	353	$C_{17}H_{17}ClO_6$	504
Lasalocid	Coccidiostat	1	25999-31-9	591	$C_{34}H_{54}O_8$	100
Maduramicin	Coccidiostat	2	84878-61-5	934	$C_{47}H_{83}NO_{17}$	200
Monensin	Coccidiostat	2	17090-79-8	671	$C_{36}H_{62}O_{11}$	200
Narasin	Coccidiostat	1	555134-13-9	765	$C_{43}H_{72}O_{11}$	100
Prednisolone	Corticosteroid	6	50-24-8	360	$C_{21}H_{28}O_{25}$	600
Robenidine	Coccidiostat	5	25875-51-8	334	$C_{15}H_{13}Cl_2N_5$	504
Salinomycin	Coccidiostat	2	53003-10-4	751	$C_{42}H_{70}O_{11}$	200
Sulfadimethoxine	Antibiotic	100	122-11-2	310	$C_{12}H_{14}N_40_4S$	10008
Sulfamethoxazole	Antibiotic	100	723-46-6	253	$C_{16}H_{11}N_30_3S$	10008
Sulfametoxipyridazine	Antibiotic	100	80-35-3	280	$C_{11}H_{12}N_40_3S$	10008
Sulfaquinoxaline	Antibiotic	100	59-40-5	300	$C_{14}H_{12}N_4O_2S$	10008
Trimethoprim	Antibiotic	50	738-70-5	290	$C_{14}H^{18}N_4O_3$	5016

[a]MRL: Maximum residue limit

[b]ML: Maximum level

[c]CAS N^{o}: Chemical Abstracts Service Registry Number

[d]Concentration of each drug in the stock solution

Table 2. Precursor and product ions and MS parameters employed to identify the selected drugs

Analyte	Precursor [m/z]	Product 1 [m/z]	Product 2 [m/z]	Precursor > Product ion 1				
				DP[a]	EP[b]	CEP[c]	CE[d]	CXP[e]
Ciprofloxacin	332	314	231	46	7	14	19	4
Cortisone	361	163	121	36	8	14	27	4
Decoquinate	418	373	204	51	9	18	21	4
Enrofloxacin	360	316	342	31	7	14	19	4
Flumethasone	411	253	121	16	6	14	19	4
Griseofulvin	353	69	165	31	6	14	41	4
Lasalocid	613	377	595	56	9	22	33	4
Maduramicin	934	629	393	21	8	38	35	6
Monensin	693	675	461	66	9	24	31	6
Narasin	787	431	531	86	7	26	47	4
Prednisolone	359	147	237	21	6	14	29	4
Robenidine	336	140	111	31	9	34	27	4
Salinomycin	773	431	531	81	8	26	47	4
Sulfadimethoxine	311	156	92	31	7	14	21	4
Sulfamethoxazole	254	92	156	21	6	14	35	4
Sulfamethoxypyridazine	281	156	92	26	7	14	17	4
Sulfaquinoxaline	301	166	92	26	7	14	17	4
Trimethoprim	291	230	123	41	7	14	21	4

[a]Declustering potential
[b]EP: Entrance potential
[c]CEP: collision cell entrance potential
[d]CE: collision energy
[e]CXP: cell exit potential

Table 3. Retention time (t_R) of each drugs obtained (min) with different standard solutions.

	12.5 (ng mL^{-1})	25.0 (ng mL^{-1})	50.0 (ng mL^{-1})	75.0 (ng mL^{-1})	100.0 (ng mL^{-1})	150.0 (ng mL^{-1})	Mean t_R	RSD[a] (%)
Ciprofloxacin	0.5	0.5	0.3	0.3	0.3	0.0	13.1	0.1
Cortisone	0.0	0.0	0.0	0.0	0.0	0.0	14.8	0.2
Decoquinate	0.2	0.0	0.0	0.2	0.0	0.3	20.2	0.1
Enrofloxacin	0.3	0.1	0.1	0.1	0.1	0.0	13.4	0.1
Flumethasone	0.0	0.0	0.0	0.0	0.0	0.0	15.5	0.2
Griseofulvin		0.0	0.0	0.0	0.0	0.0	17.0	0.2
Lasalocid	0.0	0.0	0.0	0.0	0.0	0.0	21.8	0.1
Maduramicin	0.2	0.2	0.0	0.2	0.2	0.0	22.1	0.1
Monensin	0.2	0.0	0.0	0.0	0.0	0.0	21.6	0.1
Narasin		0.0	0.0	0.0	0.0	0.0	22.4	0.0
Prednisolone	0.0	0.0	0.3	0.0	0.0	0.0	14.6	0.2
Robenidine	0.2	0.2	0.0	0.3	0.0	0.0	19.0	0.2
Salinomycin	0.2	0.0	0.0	0.2	0.0	0.2	21.8	0.1
Sulfadimethoxine	0.3	0.0	0.0	0.0	0.0	0.0	15.0	0.3
Sulfamethoxazole	0.5	0.5	0.3	0.3	0.3	0.4	14.1	0.4
Sulfamethoxypyridazine	0.4	0.6	0.5	0.3	0.3	0.0	12.8	6.2
Sulfaquinoxaline	1.0	1.0	0.6	0.5	0.4	0.3	15.1	0.3
Trimethoprim	0.4	0.3	0.4	0.0	0.0	0.0	12.2	0.1

[a]Relative Standard Deviation of the t_R

Table 4. Validation level (VL), regression coefficient (R^2), decision limit (CCα), detection capability (CCβ), recoveries and relative standard deviation (RSD) of the veterinary drugs at the validation level

Analyte	VL (ng mL^{-1})	ICC[a] R^2	SCC[b] R^2	Intra-day		Inter-day		CCα (ng mL^{-1})	CCβ (ng mL^{-1})
				Mean Recovery (%)	RSD (%)	Mean Recovery (%)	RSD (%)		
Ciprofloxacin	50	0.980	0.966	83	1	83	10	7.9	13.3
Cortisone	5	0.995	0.968	73	7	71	22	0.8	1.5
Decoquinate	5	0.990	0.981	80	9	80	15	0.5	0.9
Enrofloxacin	50	0.991	0.977	84	8	81	10	6.3	10.7
Flumethasone	5	0.990	0.979	76	13	71	17	0.6	1.0
Griseofulvin	5	0.990	0.976	110	7	105	11	0.6	1.0
Lasalocid	1	0.981	0.969	101	13	100	17	0.2	0.3
Maduramicin	2	0.986	0.960	104	11	105	16	0.4	0.6
Monensin	2	0.990	0.982	85	11	101	10	0.2	0.4
Narasin	1	0.991	0.983	74	13	72	14	0.1	0.2
Prednisolone	6	0.991	0.963	83	4	81	12	0.9	1.6
Robenidine	5	0.994	0.970	71	6	65	15	0.7	1.2
Salinomycin	2	0.980	0.981	76	9	76	19	0.2	0.4
Sulfadimethoxine	100	0.994	0.973	73	8	82	17	13.4	22.8
Sulfamethoxazole	100	0.995	0.976	75	13	80	10	14.7	25.0
Sulfamethoxypyridazine	100	0.998	0.984	119	6	119	9	9.3	15.8
Sulfaquinoxaline	100	0.997	0.956	78	19	81	18	18.7	31.8
Trimethoprim	50	0.998	0.980	99	6	100	15	5.8	9.9

[a]Instrument Calibration curves
[b]Samples calibration curves