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Article

Simultaneous Determination of Sulfonamides, Penicillins and Coccidiostats in Pork by High-Performance Liquid Chromatography–Tandem Mass Spectrometry

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Veterinary drugs are widely and legally used to treat and prevent disease in livestock. However, drugs are also used illegally as growth-promoting agents. To protect the health of consumers, maximum residue limits (MRL) in food of animal origin have been established and are listed in Regulation 37/2010. According to this regulation, more than 300 drugs need to be controlled regularly in laboratories for residues of veterinary drugs. A cost-effective analytical method is very important and explains why the development of multi-residual methods is becoming popular in laboratories.

The aim of this work is to describe a simple, rapid and economical high-performance liquid chromatography–tandem mass spectrometry method for the simultaneous identification and quantification of 21 veterinary drugs in pork muscle samples. The sample clean-up procedure is performed with acidified dichloromethane and does not require solid phase extraction. The method is applicable to nine sulfonamides and seven coccidiostats identified within 36 min. Calculated relevant validation parameters such as recoveries (from 72.to 126 %), intra-precision and intermediate precision (relative standard deviation below 40 %) and decision limits (below 7 μg Kg^{-1}) were within acceptable range and in compliance with the requirements of Commission Decision 2002/657/EC.

Introduction

Veterinary drugs are widely and legally used to treat and prevent disease in livestock, but some are also used illegally as growth-promoting agents (1). Regardless of the use of these substances, they can remain as unwanted residues in food of animal origin, endangering the health of consumers. The European Parliament has set up maximum residue limits (MRLs) and maximum levels (ML) for residues of veterinary drugs in food of animal origin to protect consumer health. Evaluated drugs are described in Regulation 470/2009 (2) and their limits are listed in Table I of the Annex of Regulation 37/2010 (3). These regulations repeal Regulation 2377/90 and amend Directive 2001/82 and Regulation 726/2004. Food samples containing residues of veterinary drugs at concentrations below MRL are considered safe and designated as "compliant." In contrast, samples with concentrations of drugs above the MRL are considered non-safe and designated as "non-compliant."

Regulation 37/2010 sets MRL levels for more than 300 pharmacologically active substances in food matrices such as muscle, fat, liver, kidney and milk. To detect these residues, microbiological or bioassay techniques are widely used as screening methods. These methods either do not distinguish between members of a class of antibiotics or cannot quantify them.

Development of a new analytical technique such as atmospheric pressure ionization (API) permits high-performance liquid chromatography (HPLC) to be coupled with mass spectrometry (MS), which opened a new era in qualitative and quantitative analysis of veterinary drug residues. The combination of HPLC with MS or MS-MS techniques allows identification of veterinary drugs in classes that could not be chromatographically resolved with HPLC. In addition to the high level of specificity, these techniques are sensitive and sometimes require less than 1 pg of material. The specificity and sensitivity of these instruments enable the development of multi-residual methods for veterinary drugs at concentrations below MRLs.

The applications of HPLC–MS-MS to veterinary drug residue analysis and residue control have increased in the past five years. HPLC–MS-MS methods have been reported for the analysis of sulfonamides in salmon muscle (4), kidney (5, 6), egg (7) and milk (8). They have also been employed for the analysis of other veterinary drugs residues such as tetracycline (9), chloramphenicol (10), cyproheptadine (11) and coccidiostats (12, 13). Griseofulvine is widely used in veterinary medicine for the treatment of fungal infections, but it causes birth defects in laboratory animals (14) and its concentrations in food samples need to be controlled. Similarly, sulfonamide residues in food are of concern because of their potential carcinogenic nature and the possible development of antibiotic resistance in humans (15). Enrofloxacin, a fluoroquinolone, is a synthetic antibiotic with broad-spectrum antibacterial activity developed exclusively for use in animals (16). It is well known that continuous use of fluoroquinolons in poultry has led to the development of fluoroquinolone-resistant Campylobacter species in poultry, which is transferrable to humans. Anti-coccidial drugs (coccidiostats) are licensed to be used as feed additive and their presence in food samples is forbidden. However, because the unavoidable carryover of these substances in non-target feed and consequently in food of animal origin, MRLs for these substances were established in 2009 and regulated in the Commission Regulation (17). Toxicological studies indicate that some coccidiostats produce mutagens (18) and potential carcinogens. Poisoning in animals and humans caused by coccidiostats (particularly polyether antibiotics) are widely described in the literature (19, 20, 21, 22).

Each year sees an increase in the number of veterinary drug residues to be monitored; consequently, food laboratories are hastening the search for high-throughput techniques that may be more effective in tackling large amounts of samples in a limited time (23). New screening methods are still being

Table I
Formula, Maximum Residue Limit, Maximum Levels, Validation Level and SRM Parameters of the LC–MS-MS Analysis of the Selected Veterinary Drugs

	Formula	MRL (μg/kg)	VL (μg/Kg)	Rt	Precursor ion	Product ion 1	Product ion 2	DP (V)	EP (V)	CEP (V)	CE (V)	CXP (V)
Sulfachlorpyridazine	$C_{10}H_9ClN_4O_2S$	100	10	13.9	284	156	92	56	9	14	17	4
Sulfadimethoxine	$C_{12}H_{14}N_4O_4S$	100	10	14.9	310	156	92	66	10	14	25	4
Sulfamethazine	$C_{12}H_{14}N_4O_2S$	100	10	12.7	278	186	92	56	10	16	19	4
Sulfamethizole	$C_9H_{10}N_4O_2S_2$	100	10	13.0	270	156	92	56	9	14	17	4
Sulfamethoxazole	$C_{10}H_{11}N_3O_3S$	100	10	14.1	253	108	92	66	10	12	37	4
Sulfamethoxypyridazine	$C_{11}H_{12}N_4O_3S$	100	10	13.2	280	156	92	76	10	14	39	4
Sulfapyridine	$C_{11}H_{11}N_3O_2S$	100	10	13.1	249	92	108	61	10	12	35	4
Sulfaquinoxaline	$C_{14}H_{12}N_4O_2S$	100	10	15.1	300	156	92	81	10	16	19	4
Sulfathiazole	$C_9H_9N_3O_2S_2$	100	10	12.1	255	156	92	61	10	12	17	4
Penicilin G	$C_{16}H_{18}N_2O_4S$	25	10	14.8	335	217	160	46	10	24	17	4
Penicilin V	$C_{16}H_{18}N_2O_5S$	25	10	15.6	351	257	229	56	56	56	56	56
Trimethoprim	$C_{14}H_{18}N_4O_3$	50	15	12.2	291	230	123	50	10	13	10	3
Enrofloxacin	$C_{19}H_{22}FN_3O_3$	100	10	13.6	360	316	245	51	9	12	23	6
Griseofulvin	$C_{17}H_{17}ClO_6$	N/A	10	17.1	352	165	214	76	10	14	27	4
Decoquinate	$C_{24}H_{35}NO_5$	20	20	20.2	418	204	148	56	9	14	57	4
Lasalocid	$C_{34}H_{54}O_8$	5	3	21.8	613	377	559	71	10	24	35	6
Maduramicin	$C_{47}H_{83}NO_{17}$	2	1	22.1	939	877.5	859	46	12	24	41	34
Monensin	$C_{36}H_{62}O_{11}$	2	1	21.6	693	461.3	479	693	81	8	80	49
Narasin	$C_{43}H_{72}O_{11}$	5	3	22.4	787	430.9	279	126	11	32	69	4
Robenidine	25875-51-8	5	3	19.0	336	140.2	111	41	9	30	57	4
Salinomycin	$C_{42}H_{70}O_{11}$	2	1	21.8	773	431.3	531	151	8	18	55	20

reported (23, 24), and offered on the market. Screening methods permit rapid analysis of numerous drugs at μg/kg and even at pg/g. However, they also have numerous disadvantages: no specificity, unavailability for all active compounds, high cost and many false positives. It is well known that a suspect positive requires further analysis with a confirmatory method. However, it is actually more practical to directly analyze samples with a multi-class confirmation than to first use a screening method followed a confirmatory method (twice), than to use a screening method once and an HPLC–MS-MS twice. Nowadays, the tendency is to develop multi-class HPLC–MS-MS methods. These methods have been developed in matrices such as pig liver, kidney and muscle where 16 β-agonists have been detected (25); 12 coccidiostats have been detected in chicken liver (12) and 42 antibiotics have been detected in honey (26, 27), egg (28) and, more recently, in milk (29, 30, 31, 32). Multi-class methods are an effective way to monitor a wide variety of drugs in a single analysis, maximizing laboratory resources and sample throughput. However, only a few multi-class confirmatory methods for analysis of residues of veterinary drugs in muscle samples have been reported (23, 25).

HPLC–MS-MS methods, multi-class or not, for the analysis of residues of drugs in muscle involve long and tedious extraction protocols. Generally, two extractions are employed: one with water and a one with solid-phase extraction cartridges. The combination of these two extraction steps is sometimes time-consuming, and in many cases, low recoveries are attributable to losses during these steps.

This paper describes a rapid, straightforward, reliable and economical HPLC–MS-MS method for the identification and quantification of 21 drugs in pork muscle samples. The drugs belong to different therapeutic classes, including sulfonamides, penicillins, quinolones and coccidiostats, commonly analyzed by European laboratories dedicated to residue control. The extraction protocol is simple and fast and does not require solid-phase extraction. After evaporation and reconstitution of the extracts, the presence of 21 analytes was confirmed by HPLC–MS-MS within 36 min. The whole method has been validated according to the guidelines of Commission Decision 657/2002 (23) and the decision limits (CCα) achieved were <2 μg/kg for most coccidiostats, <4 μg/kg for sulfonamides, 4 μg/kg for enrofloxacin and 6 μg/kg for trimethoprim.

Experimental

Reagents and stock solutions

Sulfachloropyridazine, sulfadimethoxine, sulfamethazine, sulfathiazole, sulfamethoxazole, sulfamethoxypyridazine, sulfapyridine, sulfaquinoxaline, sulfamethizole, trimethoprim, penicillin G, penicillin V, decoquinate, lasalocid, maduramicin, monensin, narasin, robenidine, salinomycin, enrofloxacin, griseofulvin (purity higher than 98 %) and the two internal standards (IS), sulfadoxine-d3 and robenidine-d8, were obtained from Sigma-Aldrich (St. Louis, MO). Formic acid (purity higher than 99% for analysis) was purchased from Acros Organics (Geel, Belgium) and acetonitrile, methanol and dichloromethane were purchased from Scharlau Chemie (Barcelona, Spain). Ultrapure water was made in-house with a Milli-Q water system (Millipore, Bedford, MA) and the nitrogen was generated by an in-house nitrogen generator from Peak Scientific Instruments Ltd. (Chicago, IL)

Stock solutions of individual analytes were prepared by diluting 50 mg of drug with 50 mL of 0.1% formic acid in methanol. A standard solution mixture of drugs was prepared by appropriate dilution of the stock solution of individual analytes, and the final concentrations of drugs were: 5,000 ng/mL of decoquinate; 3,750 ng/mL of trimethoprim; 2,500 ng/mL of sulfachlorpyridazine, sulfadimethoxine, sulfamethazine, sulfathiazole, sulfamethoxazole, sulfamethoxypyridazine, sulfapyridine, sulfaquinoxaline, sulfamethizole, penicillin G, penicillin V, enrofloxacin and griseofulvin; 1,250 ng/mL of lasalocid, narasin and robenidine; 500/ng mL of maduramicin, monensin and salinomycin. This solution was diluted several times with 0.1% of formic acid in methanol to obtain a serial standard solution containing 5, 10, 15, 25, 37.5, 50, 125, 200, 500 and 750 ng/mL

Table II
Concentration (ng/mL) Drugs in the Standard Solutions Employed to Build Instrument Calibration Curves

Sulfachlorpyridazine	2,500	1,000	750	500	200	20	50	37.5	25	15	10	5
Sulfadimethoxine	2,500	1,000	750	500	200	20	50	37.5	25	15	10	5
Sulfamethazine	2,500	1,000	750	500	200	20	50	37.5	25	15	10	5
Sulfamethizole	2,500	1,000	750	500	200	20	50	37.5	25	15	10	5
Sulfamethoxazole	2,500	1,000	750	500	200	20	50	37.5	25	15	10	5
Sulfamethoxypyridazine	2,500	1,000	750	500	200	20	50	37.5	25	15	10	5
Sulfapyridine	2,500	1,000	750	500	200	20	50	37.5	25	15	10	5
Sulfaquinoxaline	2,500	1,000	750	500	200	20	50	37.5	25	15	10	5
Sulfathiazole	2,500	1,000	750	500	200	20	50	37.5	25	15	10	5
Penicilin G	2,500	1,000	750	500	200	20	50	37.5	25	15	10	5
Penicilin V	2,500	1,000	750	500	200	20	50	37.5	25	15	10	5
Trimethoprim	3,750	1,500	1,125	750	300	30	75	56.25	37.5	22.5	15	7.5
Enrofloxacin	2,500	1,000	750	500	200	20	50	37.5	25	15	10	5
Griseofulvin	2,500	1,000	750	500	200	20	50	37.5	25	15	10	5
Decoquinate	5,000	2,000	1,500	1000	400	40	100	75	50	30	20	10
Lasalocid	1,250	500	375	250	100	10	25	18.75	12.5	7.5	5	2.5
Maduramicin	500	200	150	100	40	4	10	7.5	5	3	2	1
Monensin	500	200	150	100	40	4	10	7.5	5	3	2	1
Narasin	1,250	500	375	250	100	10	25	18.75	12.5	7.5	5	2.5
Robenidine	1,250	500	375	250	100	10	25	18.75	12.5	7.5	5	2.5
Salinomycin	500	200	150	100	40	4	10	7.5	5	3	2	1

of sulfachlorpyridazine (Table II). These standard solutions were employed to build instrument calibration curves for each analyte.

Stock solutions containing individual IS were prepared at 1 mg/mL in methanol containing 0.1% of formic acid. This solution was then diluted to a final concentration of 1 μg/mL of each IS.

Pork muscle samples

The development of the extraction procedure and validation of the method were conducted with commercial meat. Pork muscle samples bought from local supermarkets were minced, stored in plastic bags and frozen. The applicability of the method was investigated in pork muscle samples obtained from different Spanish slaughterhouses that collaborated with the laboratory in a national control program of residue of veterinary drugs.

Sample preparation

Sample homogenization and extraction

Defrosted pork sample was homogenized with a hand food blender Mini Chopper (Moulinex Ind. Ltd, France). The homogenized sample was weighted (500 mg ± 10) in a 2-mL Eppendorf tube (Hamburg, Germany) and 1 mL of 0.1% of formic acid in dichloromethane, 10 μL of a mixture of IS and 200 μL of water were added to the tube. The mixture was vortex mixed (10 s), sonicated (10 min), vortex mixed again (10 s) and centrifuged at approximately 1,500 × *g* (15 min). The organic phase (lower layer) was transferred into a graduated 10-mL PYREX conical centrifuge tube and the entire extraction procedure was repeated with a subsequent 1 mL of 0.1% of formic acid in dichloromethane. The final mixture of extracts (approximately 2 mL of extract) was evaporated to dryness with a stream of nitrogen at 37 °C on a Turbo Vap II from Zyrmark (Hopkinton, MA).

The dried extract was reconstituted with 0.2 mL 0.1% of formic acid in methanol and transferred into an Ultrafree-MC centrifugal filter (Millipore, Bedford, MA). After filtration, the extract was transferred into an amber HPLC vial containing a 200 μL insert and the vial was kept at −18 °C until sample analysis by HPLC–MS-MS. The concentration of the analyte in the sample was calculated with the instrument calibration curve of the day and correction was applied for recoveries and the IS. The applied correction was the one calculated for the fortified sample.

Quality control samples

During the routine analysis, the complete analytical procedure was applied to four quality control samples. These quality control samples were processed together with the samples to be confirmed: a blank sample (a sample from which the analyte is absent, negative for the analytes that are going to be analyzed), a fortified sample (fortified pork muscle sample containing known amounts of analyte), a reagent blank (reagents only, no muscle) and fortified reagents (reagents spiked to a known concentration of analytes). The fortified sample and fortified reagents were prepared by adding appropriate aliquots of the mixed standard, vortexing and allowing the sample to settle for 30 min in the dark.

HPLC–MS-MS system

The HPLC system consists of an HPLC 1100 separation module, a vacuum degasser and an auto-sampler from Agilent Technologies (Waldbronn, Germany) and connected to a Qtrap 2000 from Applied Biosystems, MSD Sciex (Toronto, Canada) equipped with a TurboIonSpray source. The whole system was controlled with the software Analyst 1.4.1 from Applied Biosystems, MSD Sciex (Toronto, Canada).

Ultra-filtrated extract was injected (10 μL) into a Synergi 2.5 μm Polar-RP 100Å 50 × 2.00 mm column connected to a Polar-RP 4.0 × 2.0 mm security guard cartridge, both obtained from Phenomenex (Macclesfield, UK). The mobile phase consisted of a mixture of 0.1% formic acid in water and 0.1%

formic acid in acetonitrile. Elution of the analytes was performed on a gradient mode (Table III). The flow rate was held at 0.150 mL/min throughout the analysis.

The entire effluent from the HPLC column was directed into the electrospray source of the MS, which was working in positive ion-mode. The optimum signal for $[M + H]^+$ ions was obtained with the source temperature set to 400°C, vacuum gauge to 2.2 atm, ion spray to 5.500 V, curtain gas to 25 psi, ion source 1−55 psi and ion source 2−50 psi.

In this work, the MS was used in selected reaction monitoring (SRM) mode. This mode allows the transition between a particular precursor ion and its respective product ion to be monitored, which provides excellent selectivity. The transition can be written as parent $m/z >$ fragment (product ion) m/z. According to the Commission Decision 2002/657/EC (33), four identification points are required to identify a residue in food of animal product. These four identification points are earned by monitoring the transition from a precursor ion to two product ions. Therefore, for this particular research, two SRM transitions were employed to monitor each veterinary drug. Table I summarizes precursor and product ions and the declustering potential (DP), entrance potential (EP), collision cell entrance potential (CEP), collision energy (CE) and cell exit potential (CXP) employed for their detection. The transitions were monitored with a dwell time of 5 ms. Veterinary drugs were identified by their retention times (Rt) in two SRM.

In a single HPLC run, 42 SRM transitions were monitored. Figure 1 represents the overlaid 42 SRM chromatograms. Figures 2, 3 and 4 show the two SRM chromatograms, parent $m/z >$ product m/z monitored for each veterinary drug. The transition between parent > product ion 1 was employed for quantification.

The reproducibility of the Rt of the analytes was investigated by performing six injections from different vials and from the same vial employing standard solutions containing the same concentration of all analytes. Mean Rt, standard deviations (STD) and relative standard deviations (RSDs) of the Rt for injection from the same and different vials were calculated.

Table III
Percentage of 0.1% of Formic Acid in Milli-Q water in the Gradient Program

Time (min)	0.1% of formic acid in Milli-Q water
0	100
2	100
3	85
4	75
8	55
9	50
14	30
25	7
28	0
32	75
33	100
36	100

Validation

Selected veterinary drugs comprise several MRLs in addition to a substance without MRL (see Table I). The current method was validated on the basis of the Commission

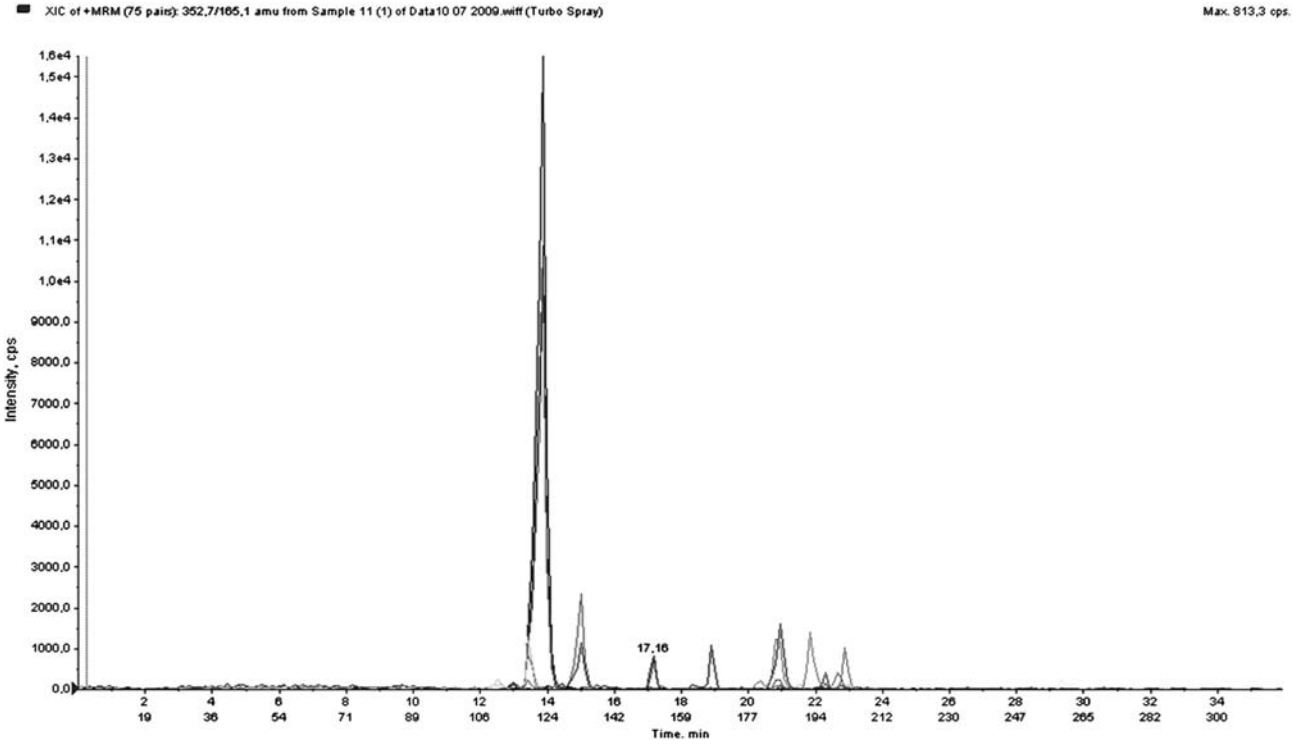

Figure 1. Overlaid SRM chromatogram of the two transitions of each of the 21 veterinary drugs obtained in a pork muscle sample spiked with the drugs at the validation level (see Table II).

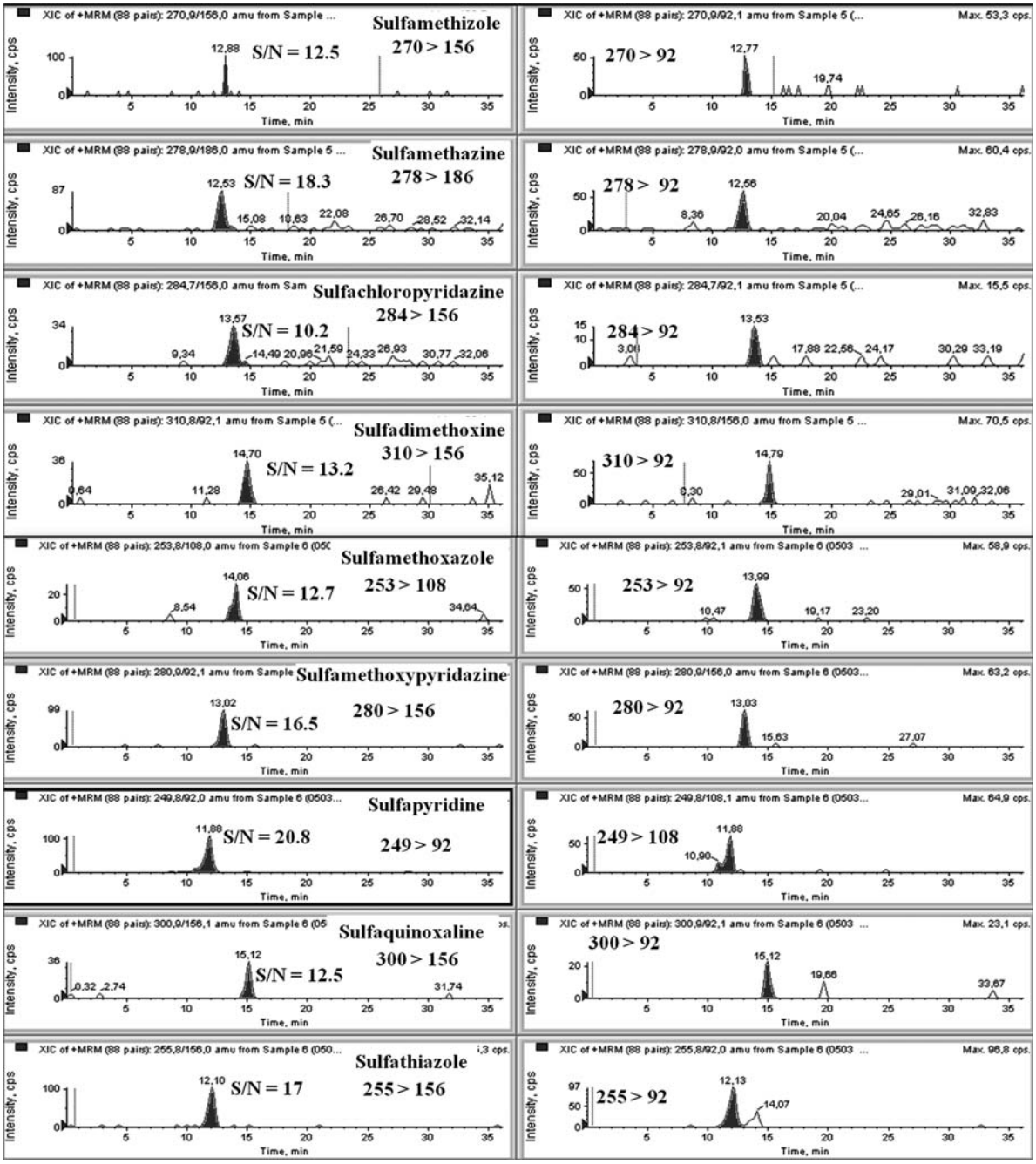

Figure 2. SRM chromatograms, parent ion $m/z >$ (product ion) m/z, monitored to identify each sulfonamide in a pork muscle sample containing 21 veterinary drugs at the validation level (see Table II).

Decision 2002/657/EC (33), this regulation requires a validation around the MRL for authorized drugs and at concentrations as low as possible for substances without MRL. The validation of coccidiostats was accomplished at the ML set up in the Commission Regulation 124/2009/EC. Although sulfonamides have MRL of 100 μg/Kg in muscle, they were validated at 10 μg/Kg because the Commission Regulation 37/2010 states that "the combined total residues of all substances within the sulfonamide group should not exceed 100 μg/Kg." Similarly, trimethoprim, enrofloxacin, the penicillins and griseofulvin were validated at 15, 10, 25 and 10 μg/Kg, respectively. Following recommendations of the Commission Decision 2002/657/EC (33), the validation process was conducted with six levels of concentrations: 0, 0.5, 1, 1.5, 2 and 5 × MRL or validation level (VL).

Intra-day and inter-day repeatability, within-laboratory reproducibility, sensitivity and specificity of the method were investigated. All calculation was performed on the program ResVal (version 2.2) from the Community Reference Laboratory (CRL) for hormones (RIVM, Bilthoven, Netherlands).

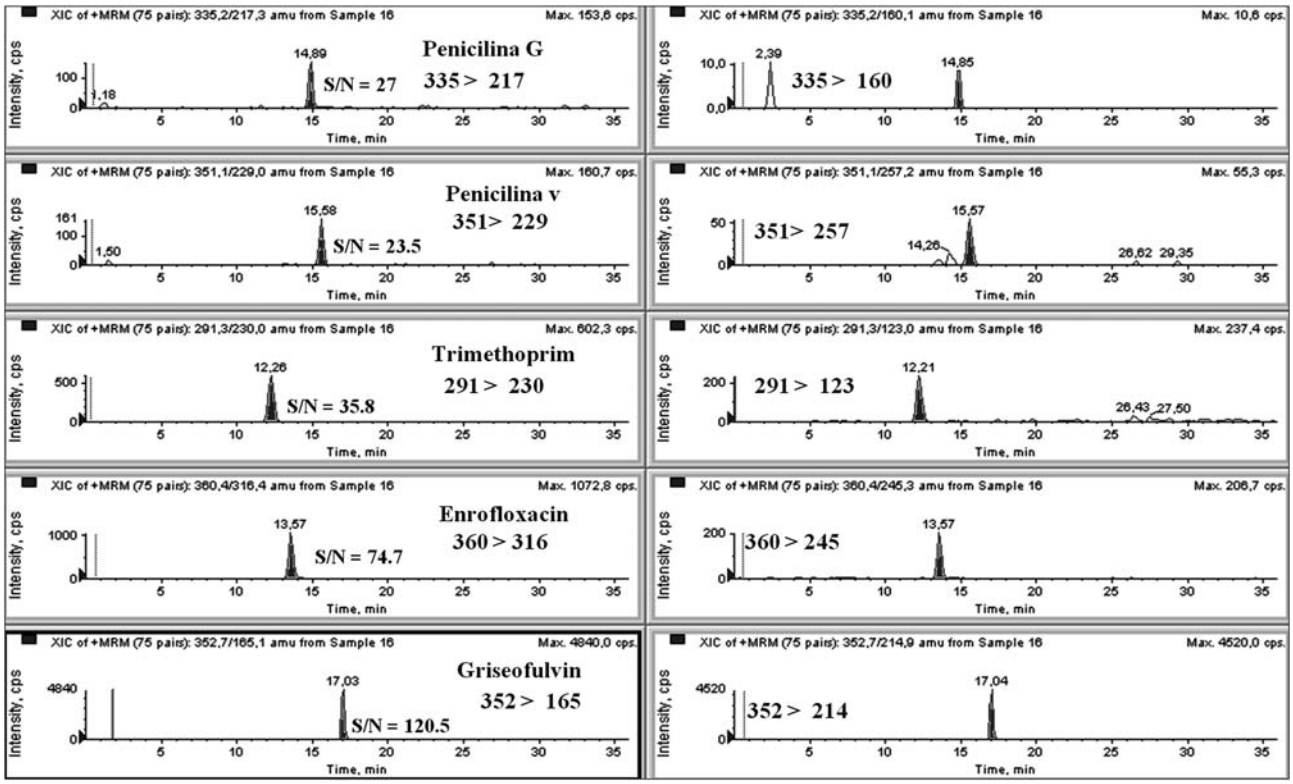

Figure 3. SRM chromatograms, parent ion $m/z >$ (product ion) m/z, monitored to identify each penicillin, trimethoprim, enrofloxacin and griseofulvin in a pork muscle sample containing 21 veterinary drugs at the validation level (see Table II).

Validation process

Four experiments (Exps. 1, 2, 3 and 4), conducted on four different days, were carried out for validation of the method. Each day, different concentrations, (15, 25, 37.5, 50 and 125, 200, 500 and 750 ng/mL of Sulfachloropyridazine) of standard solutions containing a mixture of drugs spiked with the ISs were analyzed to obtain instrument calibration curves (ICC). ICCs were built by representing the ratio of the area of the selected ion of the analyte and the IS against concentration of the coccidiostats in the standard.

To conduct Exps. 1, 2 and 3, a homogeneous muscle sample was divided into 63 sub-samples. Twenty-one samples were fortified and analyzed each day of the validation. The samples were fortified as follows: one sample was not spiked with drugs (blank), six samples were spiked at 0.5 × VL, six spiked at 1 × VL, six spiked 1.5 × VL, one spiked at 2 × VL and one spiked at 5 × VL. These samples were used to construct the sample calibration curves (SCCs) for each analyte on each day. SCCs were constructed by calculating the area of the selected ion of the analyte and the IS and their ratio was used as the response variable. ICC was constructed by linear curve fitting using linear regression. ICC were described by the equation $y = bx + a$, where b is the slope and a is the intercept.

Exp. 4 consisted of 20 muscle samples collected from different slaughterhouses. Ten were not spiked with drugs and 10 were spiked to a concentration of 1 × VL, and ten were only spiked with the IS. The samples were shaken vigorously for 30 min after being spiked with the coccidiostats to homogenize.

Exps. 1, 2 and 3 allowed precision, accuracy, decision limit (CCα) and detection capability (CCβ) to be estimated. According to European Commission Decision 2002/657/EC (33), 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 errors for CCα shall be applied (1 or 5%). During separation for this particular study, to simplify calculation, the same α error, 1%, was applied for all drugs. Similarly, to calculate CCα and CCβ, the same formula was applied to all analytes:

$$CC\alpha = \frac{(Ya + 2.33 STDEV\ Ya) - Ya}{b} \quad (1)$$

$$CC\beta = \frac{(Ya + 2.33 STDEV\ Ya + 1.64 STDEVYa) - Ya)}{b} \quad (2)$$

where Ya = concentration corresponding to the Y-intercept; STDEVYa = standard error at the intercept; b = slope of the calibration curve.

The IS was used to calculate a correction factor (RF) for each analyte. The calculation of an RF for each coccidiostats was as follows:

$$RF - (Ax\ X\ Cs)/(As\ X\ Cx)$$

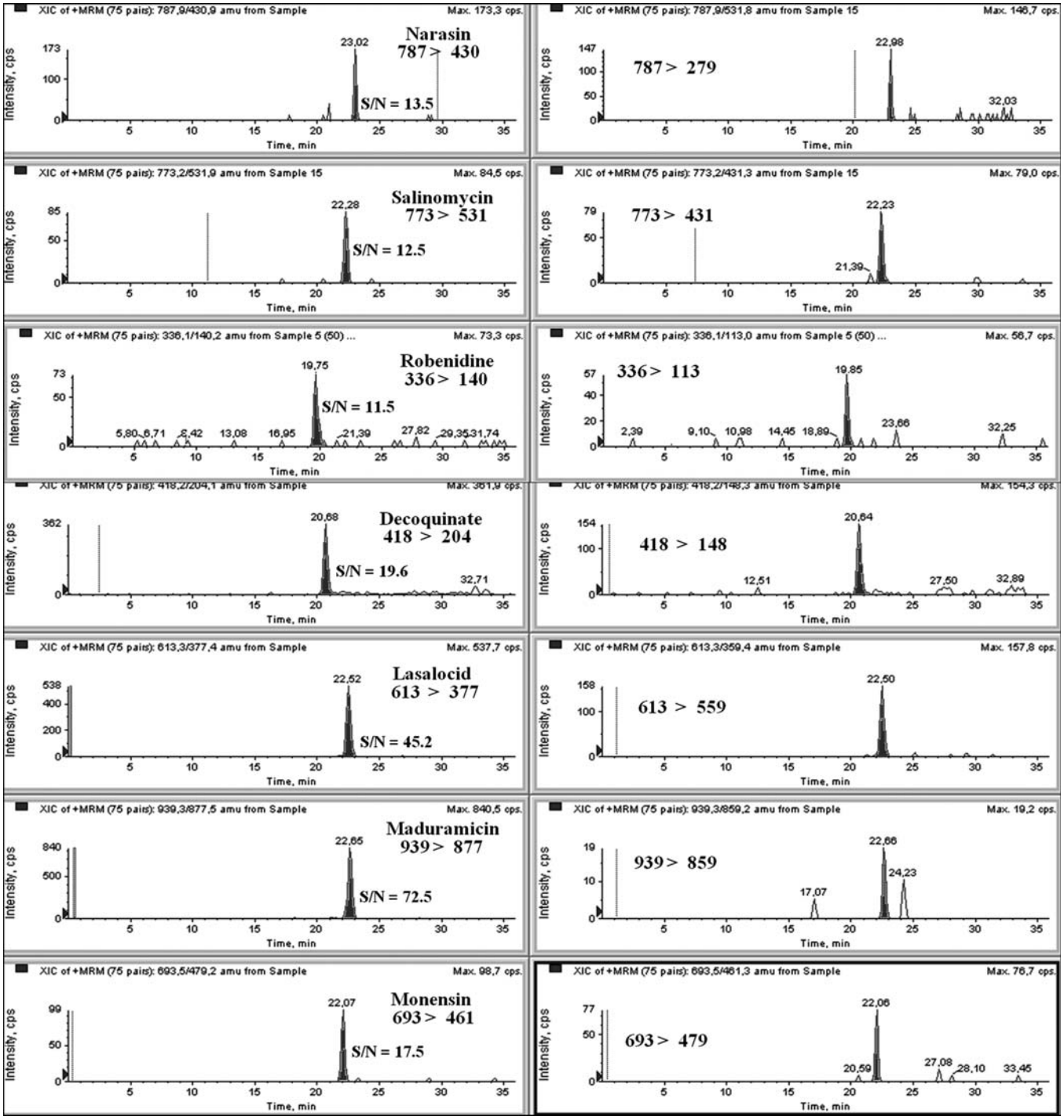

Figure 4. SRM chromatograms, parent ion $m/z >$ (product ion) m/z, monitored to identify each coccidiostat in a pork muscle sample containing 21 veterinary drugs at the validation level (see Table II).

where Ax = area of the target analyte to be measured; As = area of the appropriate IS; Cs= concentration of the IS in the extract; Cx = concentration of the analyte (coccidiostat) in the extract.

A mean RF was obtained from the analysis of the 73 pork muscle samples employed for the validation procedure. The mean RF was then employed to calculate the concentration of the analyte in the sample and the recovery of the analyte.

While sulfadoxine-d3 was used as IS to quantify sulfonamides, penicillins, trimethoprim, enrofloxacin and griseofulvin; robenidine-d8 was employed to quantify decoquinate, lasalocid, maduramicin, monensin, narasin, robenidine and salinomycin.

Result and Discussion

Method development

To identify adequate precursor and product ions for each analyte, standard solutions containing individual drugs at 1.0 μg/mL (0.1% formic acid in methanol) were infused directly into the electrospray ion source at 20 μL/min. The MS system employed in this study could alternate between positive and negative ionization mode, but the use of this switching mode results in a decrease in sensitivity and lifetime of the MS capillary (34). Both ionization modes were tested, and only drugs that ionize with positive electrospray ionization were selected. The same ionization mode was reported for coccidiostats (35, 36), sulfonamides (37) and penicillins (24). Selected precursor and product ions were those that gave a higher signal response; proton adducts $[M + H]^+$ were employed in most cases.

After selecting precursor and product ion and MS parameters for detection, 10 μL of a stock solution mixture of drugs at 100 ng/mL was injected directly into the mobile phase (50:50 of phase A:B). The mixture was introduced into the MS capillary at a flow rate of 0.15 mL/min. Under these conditions, other MS parameters were optimized to obtain the best signal response for all analytes; tested conditions include: source temperature (350, 400 and 450 °C), ion spray (4,500, 5,000 and 5,500 V) and curtain gas (20, 25 and 30 psi).

Based on supplier recommendations and satisfactory results obtained for sulfonamide analysis, an ether-linked phenyl column was selected as a suitable HPLC column. To obtain good resolution of the analyte, different combinations of solvents were tested, always on a gradient mode: a mixture of methanol–water containing 0.1% of formic acid, acetonitrile–water containing 0.1% formic acid and the same conditions with another buffer (1% of ammonium acetate). Because ammonium acetate signal suppression has been shown to be lower than other buffers (28, 38) it was included on the mobile phase components to maintain mobile phase pH. However, the use of ammonium acetate produced a decrease of the signal intensity (5 times) and was discarded.

The use of methanol as mobile phase components for analysis of coccidiostats has been reported previously (39), as has the use of acetonitrile (40). Methanol has also been used in sulfonamide analysis (41), but most published methods implement acetonitrile in the mobile phase (42). No significant differences were observed on peak shape, signal intensity or resolution; however, peak shapes were more symmetric with acetonitrile than with methanol. Therefore, acetonitrile was preferred.

The primary problem encountered during drug identification was the formation of ions by the sulfonamides; three product ions are generated in sulfonamide fragmentation: $[M\text{-}RNH2]^+$ 196 (m/z 156), $[M\text{-}RNH2\text{-}SO]^+$ (m/z 108) and $[M\text{-}RNH2\text{-}SO2]^+$ 197 (m/z 92). The first attempt was to elute the sulfonamides as much as possible to avoid signal contamination. This signal contamination could occur with high concentrations of a particular ion monitored in two SRM transitions. However, no signal contaminations were observed for sulfonamides or any other drugs (Figures 2, 3 and 4) with the gradient selected, although some sulfonamides coeluted. The HPLC method resulted in reproducible Rt for all selected drugs (Table IV) and RSDs for replicated injections from the same vial and different vials resulted below 0.5% for all concentrations tested. RSDs of mean Rt were below 1 for all drugs and at all concentrations. The developed method allows the selected drugs to be identified by their Rt and two SRM (Table I).

Table IV
Mean Rt, Standard Deviation and RSD (%) Obtained from Replicated Injections (n = 6) from the Same Vial and Different Vials

	Replicate injections (1 vial)			Replicate injections (6 vials)		
	Mean	STD	RSD	Mean	STD	RSD
Sulfachlorpyridazine	14.1	0.1	0.4	13.9	0.1	0.5
Sulfadimethoxine	15.1	0.0	0.1	15.0	0.0	0.3
Sulfamethazine	12.7	0.0	0.4	12.7	0.0	0.3
Sulfamethizole	12.9	0.7	0.1	13.1	0.1	0.4
Sulfamethoxazole	14.3	0.1	0.4	14.1	0.1	0.5
Sulfamethoxypyridazine	12.9	0.7	0.3	12.8	0.7	0.4
Sulfapyridine	12.0	0.0	0.4	11.8	0.1	0.8
Sulfaquinoxaline	13.9	0.4	0.6	15.1	0.1	0.4
Sulfathiazole	12.3	0.0	0.3	12.1	0.1	0.5
Penicillin G	14.9	0.0	0.2	14.5	0.8	6.3
Penicillin V	15.4	0.0	0.2	15.6	0.0	0.1
Trimethoprim	12.3	0.0	0.2	12.2	0.0	0.3
Enrofloxacin	13.2	0	0.1	13.1	0.0	0.3
Griseofulvin	17.1	0.0	0.0	17.0	0.1	0.3
Decoquinate	20.3	0.0	0.1	20.2	0.0	0.1
Lasalocid	21.8	0.0	0.0	21.8	0.0	0.2
Maduramicin	22.1	0.0	0.1	22.1	0.0	0.1
Monensin	21.6	0.0	0.0	21.6	0.1	0.2
Narasin	22.4	0.0	0.0	22.4	0.0	0.2
Robenidine	19.2	0.0	0.2	19.0	0.0	0.1
Salinomycin	21.7	0.0	0.1	21.8	0.0	0.2

The aim of this work was to develop a simple, rapid, reliable multi-residue method applicable in pork muscle for the simultaneous extraction, detection and confirmation of the most frequently controlled veterinary drugs in laboratories dedicated to residue analysis. During the method development, different extraction solvents were tested to improve the extraction of the drugs and to decrease interferences (data not shown). Drug recoveries obtained with methanol, acetonitrile and dichloromethane acidified with 0.1% of formic acid and non-acidified were compared. A higher number of drugs and higher recoveries were achieved with 0.1% of formic acid in dichloromethane, probably because of dichloromethane polarity.

To avoid contamination, the complete extraction procedure was conducted in disposable plastic materials and the glassware was rinsed with acetone before and after its use. Matrix effects were reduced by reconstitution of the extract in 0.2 mL instead of lower volumes and applying ultra-filtration to the reconstituted extract.

Method performances and validation results

Specificity/selectivity

The specificity and selectivity of the method were evaluated on Day 4. Twenty pork muscle samples collected from different animals were divided into two sets of samples; one set was spiked with the drugs at the VL and the other set was not spiked. The method was considered selective and sensitive due to the absence of any interference peaks around the Rt of the analytes in their SRM chromatograms. The method was able to detect the drugs spiked in the samples without obtaining any

Table V
Validation levels, Regression Coefficients of Instrument Calibration Curves and Sample Calibration Curves, Decision Limits, Detection Capabilities and Relative Standard Deviation Achieved in the Validation Study

				Intra-day		Inter-day			
	VL	R^2 of ICC	R^2 of SCC	Recovery	RSD	Recovery	RSD	CCα	CCβ
Sulfachlorpyridazine	10	0.993	0.993	80	7	84	8	1.1	3.1
Sulfadimethoxine	10	0.971	0.991	95	15	110	16	2.3	4.3
Sulfamethazine	10	0.982	0.992	85	5	72	23	1.7	3.7
Sulfamethizole	10	0.987	0.976	83	9	79	10	3	5
Sulfamethoxazole	10	0.999	0.983	79	8	76	39	3	6
Sulfamethoxypyridazine	10	0.994	0.986	80	3	82	6	1	3
Sulfapyridine	10	0.989	0.968	85	12	82	39	3	5
Sulfaquinoxaline	10	0.993	0.978	78	15	88	19	4	7
Sulfathiazole	10	0.996	0.995	81	31	81	31	2.3	4.1
Penicilin G	25	0.993	0.973	89	10	84	14	3.4	6.1
Penicilin V	25	0.988	0.976	70	20	72	32	2	4
Trimethoprim	15	0.996	0.974	84	7	80	10	6.9	12
Enrofloxacin	10	0.993	0.974	111	9	114	7	4.3	7.3
Griseofulvin	10	0.992	0.980	120	12	126	14	2.9	4.8
Decoquinate	20	0.991	0.975	80	4	82	5	4	7
Lasalocid	5	0.993	0.983	91	9	91	12	1.9	4.3
Maduramicin	2	0.992	0.959	73	13	61	17	1.2	1.8
Monensin	2	0.988	0.975	75	13	73	16	0.9	2.1
Narasin	5	0.991	0.973	79	10	75	25	3	3.8
Robenidine	5	0.985	0.970	72	20	70	29	2	3
Salinomycin	2	0.987	0.963	74	10	71	31	1.2	2

false negatives. In addition, the method analyzed non-spiked milk samples without false positives, demonstrating its easy applicability in raw milk samples.

Linearity

For each veterinary drug, a quadratic regression mode was fitted to ICC and SCC. The regression coefficients (R^2) of ICC were higher than 0.98 during the four days of validation for all the analytes over the whole concentration range from 15 to 500 ng/mL (Table V). The linearity of the method was also observed in the SCC (Table V). R^2 of SCCs were slightly lower than those achieved for ICC, probably due to matrix effects. Overall, R^2 of the SCCs were higher than 95 over the whole concentration range (0.5, 1. 1.5, 2 and 5 × VL), as expected for the case of salinomycin (0.95).

Repeatability, reproducibility and recoveries

Inter-day and intra-day assays were performed to evaluate precision (RSD) and accuracy (% deviation). Inter-day precision and accuracy were determined by analyzing nine batches of six muscle samples spiked at three concentrations, 0.5, 1 and 1.5 × VL, and run within three days (Exps. 1, 2 and 3). Intra-day precision and accuracy were obtained by analyzing one batch of 10 muscle samples (Exp. 4) spiked at the VL. Accuracy represents the closeness of agreement between a test result and the acceptable value. Accuracy has to be estimated by the determination of trueness and precision. However, trueness can only be established by analyzing certificated reference materials (CRM). When no CRMs are available, recovery was calculated instead of trueness (33). Recoveries of the analytes were determined as the percentage of the drugs recovered during the analytical procedure, employing the correction factor. Inter-day recovery values obtained at the three levels were acceptable, at mean recoveries within 70 and 126% (Table IV). Intra-day recovery values were also acceptable, ranging from 70 to 120% (Table IV), but they were approximately 15% lower than those achieved for the intra-day study.

Repeatability, expressed as RSD of the recoveries, was between 3 and 20, with the exception of sulfathiazole (31%). This is an acceptable range, because the Commission Decision 657/2002/EC states that RSD of the mean shall not exceed 20% for concentrations between 10 and 100 μg/Kg. Inter-day reproducibility, expressed as RSD of the recoveries obtained in three different days, ranged between 5 and 39%, with the exception of robenidine (59%). The Commission Decision 657/2002/EC states that mass fractions lower than 100 μg/Kg should be as low as possible, and for mass fraction of 100, the RSD should be lower than 23. Therefore, taking into account the fact that the VLs employed in the validation were below 21 μg/Kg, RSD below 39% could be considered acceptable.

CCα and CCβ

The calculated mean CCα and CCβ are shown in Table IV. CCα and CCβ were calculated with Exps. 1, 2 and 3. Table IV summarizes the mean CCα and CCβ of three days. CCα values were below the VL selected for each analyte: sulfonamides (1–2.3 μg/Kg), coccidiostats (0.9–4 μg/Kg), penicillins (2–3.4 μg/Kg), trimethoprim (6.9 μg/Kg), enrofloxacin (4.3 μg/Kg) and griseofulvin (2.9 μg/Kg). Likewise, CCβ values were below the VL (Table IV).

Not many HPLC–MS-MS methods have reported CCα for the selected analytes in pork muscle samples, so comparison was difficult. Sergi *et al.* (2007) presented an LC–electrospray ionization tandem MS confirmatory procedure for monitoring 13 SAs in animal tissues with CCα between 104.9–108.2 μg/Kg (43), which are lower than those presented. Dubois *et al.* (2004) reported a method for simultaneously determining nine coccidiostats in muscle from chicken at very low levels (≤ 1 μg/Kg), lower than those reported for the same coccidiostats in liver (12). The fact that Dubois's method (35) is applicable only to coccidiostats could explain lower CCα, because

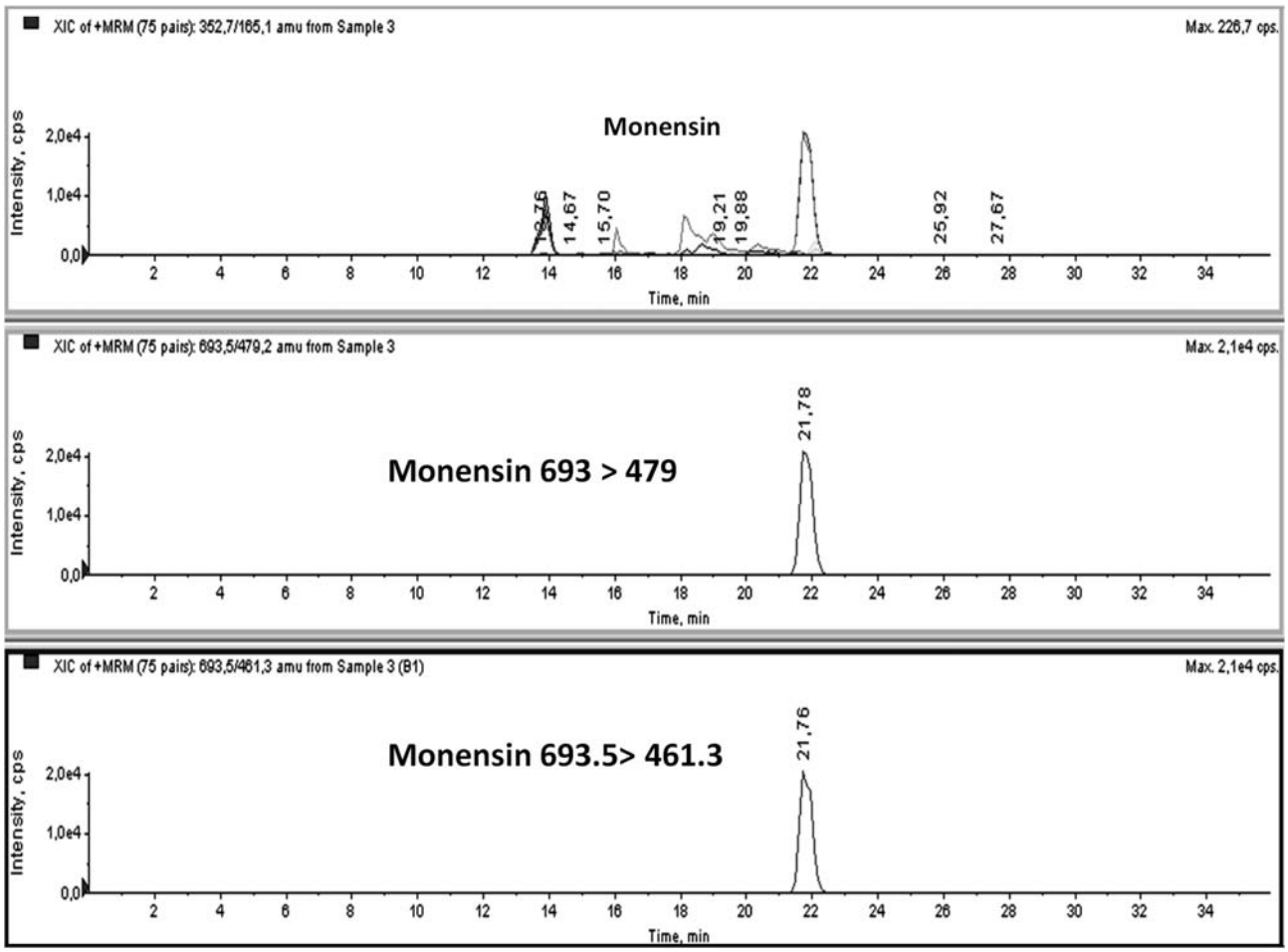

Figure 5. SRM chromatogram of pork muscle samples positive for monensin (525 μg/Kg).

the method is more specific than the method presented. Granelli *et al.* (2009) reported a multi-class method for enrofloxacin and sulfonamides, in addition to other antibiotics, in muscle with CCα for the same antibiotic of 100 ng/g. CCα of enrofloxacin achieved in this research was 25 times lower than those reported previously (44, 45, 46). A multi-class method that employs different extraction protocols, based on QuEChERS, reported higher CCα for maduramicin (9.31 μg/Kg), lasalocid (22.6 μg/Kg), narasin (8.04 μg/Kg) and salinomycin (6.45 μg/Kg) than those achieved with a simple extraction. Therefore, the presented method is able to detect the selected analyte with some of the lowest CCα reported.

Applicability of the method

The applicability of the presented method was demonstrated in real pork-muscle samples obtained from a quality control program in which 25 slaughterhouses participated. A total of 100 pork muscle samples were analyzed within four days for the presence of the selected drugs. All samples were found to be compliant with the exception of one, which was non-compliant for monensin (525 μg/Kg, Figure 5). After revising the results, 20 compliant and the non-compliant samples were re-analyzed with other procedures, set up in the laboratory. These procedures involved solid-phase extraction and HPLC–diode array detection analysis; these procedures could analyze sulfonamides, penicillins and monensin with limits of detection of approximately 50 μg/Kg. The results were the same than those obtained with the HPLC–MS-MS method; compliant samples were equally compliant and the non-compliant samples also resulted positive to monensin at the same range of concentration. These results demonstrate the practicability of the method.

Conclusion

There is an increasing interest in multi-residual methods, especially for the analysis of residues of veterinary drugs in food samples. The method presented in this article is able to extract and analyze 21 veterinary drugs using a single extraction procedure. The CCα and CCβ were found to be sufficiently low to determine the residues of the drugs in muscle below the MRL and VL set up by European legislations. The method saves cost and time and was validated in conformity with the primary revised EU requirements for detecting residues of veterinary drug substances in animal products; the method also demonstrated good linearity, accuracy and precision. Therefore, the method could be easily implemented in residue control laboratories in Europe where these drugs are constantly monitored.

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