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Determination of multi-class drugs in meat by liquid chromatography-mass spectrometry

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ABSTRACT

A simple liquid chromatographic-mass spectrometry (LC-MS/MS) method for the determination of different veterinary drugs, i.e. ß-lactams, quinolones, sulfonamides, tetracyclines, nitromidazoles, meloxicams and corticosteroids in meat is described. The sample was homogenized with extraction solution and centrifuged. The supernatant were left in the freezer (-20 °C) for 30 minutes and centrifuged. Thereafter 2.75 ml supernatant was evaporated to 0.4 mL. The sample was mixed, filtered, diluted and injected into the LC-MS/MS.

KEYWORDS

Multi-residue;
Multi-class antibiotic;
Multi Method;
Veterinary drugs;
Penicillins;
Sulfonamides;
Quinolones;
Tetracyclines;
Tiamulin;
Meloxicams;
Nitroimidazoles;
Corticosteroids;
Ketoprofen;
Naprofen;
Flunixin.

INTRODUCTION

In veterinary medicine drugs for the treatment of infections and other diseases in food-producing animals are widely used. The World Health Organization (WHO) has pointed out the risks associated with the use and misuse of drug treatments, both in human and veterinary medicine practices[1]. Drugs may be classified according to their chemical or therapeutic properties, but from an analytical point of view their chemical properties are the most important consideration. The presence of drug residues in food represents a potential health hazard to consumers. Residues of antibiotics can also lead to increasing numbers of antibiotic resistant bacteria[2].

To ensure food safety, the European Union has set maximum residue limits (MRLs) for veterinary drug residues in food of animal origin[3]. Routine laboratories have to analyze a large number of samples from different animal species and families of drugs to meet the requirements from the authorities. The cost-effectiveness of analytical procedures is important in drug residues analysis.

Bioassays are the most commonly used methods for monitoring residues of antibiotics in food. Microbial inhibition methods, such as the four-plate test[4], microbial receptor assays, such as the Charm test[5], immunoassays[6], Delvotest SP-NT[7], are sensitive for a limited number of antibiotics. The microbial inhibition tests rely on the inhibition of bacterial growth and are easy to

perform. However, they are non-specific and are not quantitative. These methods cannot be used for the detection of drugs belonging to the corticosteroid, meloxicams, nitroimidazole groups, etc.

From 2005 until today, only few papers describing multi class methods for screening and quantification of drugs in food are published[8-14]. These methods are, however, time-consuming, have poor sensitivity and difficult for use for routine analysis.

The intention of the present study was to develop a time saving, with a simple clean-up procedure and sensitive LC-MS/MS multi-class method for the determination and quantification of a broad range of veterinary drugs in meat. The sensitivity should at least meet the requirements of quantitative detection at the MRL level. The present method can also be used for analyzing egg, milk and plasma.

EXPERIMENTAL

Materials and reagents

Drug free meat from swine was used. These samples were used as control material and for spiking with the different drugs to conduct recovery experiments. The samples were stored frozen (-20 °C).

All chemicals and solvents were of analytical or HPLC grade. Ciprofloxacin, difloxacin, enrofloxacin and sarafloxacin were provided by Fluka Bio Chemica (Buchs, Switzerland). Marbofloxacin, norfloxacin and danofloxacin were supplied by Riedel de Häen (Germany). Amoxicillin, ampicillin, penicillin G, oxacillin, cloxacillin, dicloxacillin, tetracycline, oxytetracycline, chlortetracycline, doxycycline, sulfacetamide, sulfaguanidine, sulfadiazine, sulfathiazole, sulfapyridine, sulfamerazine, sulfamoxole, sulfameter, sulfamethazine, sulfamethizole, sulfamethoxypyradazine, sulfachloropyridazine, sulfamethoxazole, sulfamonomethoxine, sulfadoxine, sulfasoxazole, sulfadimetoxine, sulfaquinoxaline, prednisolone, prednisone, flumethasone, hydrocortisone-21-acetate, dexamethasone, bethamethasone, bethamethasone-17-valerate, meloxicam, tenoxicam, piroxicam, isoxicam, dimetridazole, metronidazole, metronidazole-OH, ronidazole, ketoprofen, naproxen and tiamulin were supplied by Sigma Co. (St. Louis, MO, USA). Flunixin and flunixin-OH was donated by Norbrook Laboratories Limited (Northern Ireland). Ipronidazole and Ipronidazole-OH were supplied by Witega Laboratorien Berlin (Germany). Sulfacloxine was supplied by Carbogen (Solution Pharmaceutical Services Division, Manchester, England).

Stock solutions (1 mg/mL) of quinolones was prepared in 0.3 M NaOH and working standards were diluted with 0.01 M HCl – acetonitrile (60 + 40) to appropriate concentration. Penicillins, tetracyclines and flunixin stock solutions and working standards were diluted with water. Meloxicams stock solutions were diluted in tetrahydrofuran. Stock solutions of flunixin-OH were diluted in methanol-acetonitrile (1+1). All other stock solutions were diluted in water-dimethyl sulfoxide (DMSO)-methanol-acetone (1+1+2+6).

The working standards of all analytes were mixed and diluted to the concentrations appropriate with methanol. For penicillins, tetracyclines and quinolones the working standards were maintained separately.

Ethylenedinitrilo tetraacetic acid, disodium salt, dihydrate (EDTA) and ammonium acetate were supplied by Merck, Darmstadt, Germany. Oxalic acid and DMSO were supplied by Sigma (St. Louis, USA). Spin-X centrifuge filter units (0.22 µm nylon type) from Costar (Corning, NY, USA), were used for filtration.

Solution A consisted of DMSO-methanol-acetone (1+3+6) and 0.15 % trichloroacetic (TCA). The TCA stock solution was prepared by dissolving 85 g TCA in 15 g water (85 % TCA in water). The stock solution was stored at + 4 °C. For drug extraction 150 µL TCA stock solutions were diluted with DMSO-methanol-acetone (1 + 3 + 6) to 100 mL.

Solution B consisted of 0.1 M EDTA in 0.5 M Na_2HPO_4 with pH 4.2. The pH of the buffer was adjusted to 4.2 with concentrate phosphoric acid and with 3 M phosphoric acid to pH 4.2.

Solution C consisted of 150 µL 85 % TCA stock solution diluted to 100 mL with acetone.

Chromatographic conditions

The LC-MS/MS instrumentation used for the present method consisted of a system with binary pump (Agilent 1100, Palo Alto, CA, USA) and a Sciex API 4000 QTrap triple stage quadrupole mass spectrometer (Applied Biosystems, Foster City, Ca, USA). Nitrogen was used for both nebulizer and collision gas. The MS was set to collect ion data in positive multiple

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reaction monitoring mode (MRM). The ion transitions are shown in TABLE 1. For all transitions, the dwell time was 75 ms, the source temperature was fixed at 400 °C and the ion spray voltage was 5000 V.

TABLE 1

Compound	Recovery % Variation	S.D. % Variation	LOD ng/g	Parent ion $[M+H]^+$	Transition 1	Transition 2
Amoxicillin	91–92	1.4–1.6	3.0	366	349	114
Ampicillin	92 – 93	0.9 – 1.0	0.5	350	106	160
Cloxacillin	90 – 92	0.4 – 1.0	0.5	436	160	178
Dicloxacillin	84 – 88	0.5 – 1.1	0.5	470	160	311
Oxacillin	93 – 96	0.9 – 1.0	0.5	402	182	160
Penicillin G	91 – 94	1.3 – 2.0	0.5	335	160	176
Bethamethasone	97 – 99	0.8 – 0.9	0.25	393	355	373
Bethamethasone 17 – Valerate	95 – 96	0.5 – 1.6	0.25	477.3	355	279
Dexamethasone	89 – 90	1.0 – 1.2	1.0	393	147	237
Flumethasone	91 – 93	0.8 – 0.9	0.5	411	253	121
Hydrocortisone-21- acetate	89 – 91	1.3 – 1.8	0.5	405.5	309	327
Prednisolone	92 – 94	0.9 – 1.3	1.0	361	147	171
Prednisone	92 – 93	0.9 – 2.6	0.5	359	147	237
Flunixin	91 – 93	1.3 – 1.6	0.5	297	279	264
Flunixin – OH	84 – 85	1.1 – 2.1	0.5	313	295	280
Ciprofloxacin	95 – 99	0.2 – 1.8	0.5	332	288	245
Danofloxacin	94 – 95	1.5 – 1.8	1.0	358	340	255
Difloxacin	96 – 97	0.6 – 0.8	0.5	400	356	299
Enrofloxacin	92 – 93	0.8 – 1.4	0.5	360	316	245
Marbofloxacin	92 – 94	0.5 – 1.8	0.5	363	72	320
Norfloxacin	93 – 95	0.5 – 1.8	1.0	320	276	231
Sarafloxacin	95 – 96	0.9 – 0.9	1.0	386	342	299
Ketoprofen	86 – 91	0.4 – 0.8	0.5	255	209	105
Naproxen	90 – 95	1.1 – 1.2	1.0	231	185	170
Dimetridazole	93 – 94	0.5 – 0.8	5.0	142	122	96
Ipronidazole	94 – 95	1.0 – 1.3	0.5	170	109	123
Ipronidazole - OH	95 – 96	1.4 – 1.7	0.5	186	168	122
Metronidazole	96 –97	1.3 – 2.0	0.5	172	128	82
Metronidazole - OH	94 – 95	0.4 – 0.5	1.0	188	126	123
Ronidazole	91 – 94	1.5 – 1.6	0.5	201	140	55
Isoxicam	90 – 96	1.3 – 2.8	0.5	336	99	125
Meloxican	89 – 91	1.6 – 2.3	0.5	352	115	141
Piroxicam	88 – 96	2.1 – 2.2	0.5	332	95	121
Tenoxicam	96 – 97	0.5 – 0.7	0.5	338	121	95
Sulfacloxine	89 – 90	1.8 – 2.6	1.0	285	156	108
Sulfachloropyridazine	94 – 95	0.7 – 1.3	1.0	285.7	156	108
Sulfadiazine	95 – 97	1.4 – 1.5	1.0	251	156	92
Sulfadimethoxine	91 – 94	0.5 – 1.8	0.5	311	108	156
Sulfadoxine	97 – 98	0.3 – 1.0	0.5	311	108	156
Sulfaguanidine	91 – 97	1.5 – 2.3	0.5	215	92	156
Sulfamerazine	91 – 95	0.6 – 1.0	0.5	265	156	92
Sulfameter	94 – 97	0.9 – 2.4	0.5	281	156	92
Sulfamethazine	95 – 96	0.6 – 2.4	0.5	279	204	186
Sulfamethizole	95 – 95	0.8 – 2.8	0.5	271	156	108
Sulfamethoxazole	90 – 93	0.4 – 0.8	0.5	254	156	108
Sulfamethoxypyridazine	95 – 98	0.9 – 2.0	0.5	281	156	126
Sulfamonomethoxine	93 – 95	0.6 – 1.0	0.5	281	156	92
Sulfamoxole	94 – 98	0.7 – 1.5	0.5	268	156	92
Sulfapyridine	98 – 99	0.6 – 0.9	0.5	250	92	156
Sulfaquinoxaline	85 – 90	0.5 – 0.6	0.5	301	156	92
Sulfathiazole	95 – 97	0.8 – 1.0	0.5	256	156	92
Sulphisoxazole	98 – 99	0.7 – 1.4	0.5	268	156	113
Chlortetracycline	75 – 80	1.0 – 1.6	1.0	479	444	462
Doxycycline	79 – 82	1.2 – 1.4	2.0	445	154	98
Oxytetracycline	85 – 90	1.8 – 2.5	1.0	461	426	283
Tetracycline	81 – 83	1.2 – 1.4	2.0	445	427	241
Tiamulin	89 – 96	0.4 – 2.9	0.5	494	192	119

The column Hypersil Gold 1.9 µm 50 x 2.1 mm (Thermo Fisher Scientific Inc., Waltham, MA. USA) was operated at a constant temperature of 25 °C. The mobile phase A consisted of 0.1 % formic acid, 0.1 mM oxalic acid, 0.1 mM ammonium acetate and 0.5 % methanol in water. The mobile phase B was 0.1 % formic acid in methanol. The mobile phase A was prepared every 48 hour. The mobile phase operating conditions are shown in TABLE 2.

Sample pretreatment

Meat: A volume of 400 µL water-methanol (1 + 1), 100 µL solution B and 4000 µL solution A were added to 1 g meat. The meat mixture was homogenized for ~15 sec. with an Ultra-Turrax S 25N - 10G dispersing tool (Ika – Warke, Staufen, Germany). After centrifugation for 5 min (2500 rpm) the supernatant was transferred to a graduate centrifuge tube and kept at -20 °C

for 30 min. After centrifugation for 3 min, 2750 µL supernatant was transferred to a graduate glass-stoppered tube and evaporated to 400 µL under a stream of air at 37 °C, mixed with a whirl mixer, and then filtered through a Spin - X centrifuge filter. To 75 µL of the filtered liquid 25 µL water was added and blended. For drugs with high MRL (> 50-100 ng/g) the sample could be diluted with higher proportion water. Aliquots of 6 µL were injected into the LC-MS/MS at intervals of 16 min. In the spiking experiments, the corresponding volumes of working standard solutions were diluted to 400 µL with water-methanol (1+1).

TABLE 2 : Mobile phase operating conditions.

Total time	Flow rate (min)	Solution A (µL/min)(%)	Solution B (%)
0.1	300	100	
1.0	300	100	
1.1	300	77	23
2.0	300	77	23
2.1	300	66	34
3.0	300	66	34
3.1	300	49	51
4.0	300	49	51
4.1	300	32	68
5.5	300	32	68
5.6	300	8	92
7.5	300	8	92
7.6	350	100	
15	350	100	

Other matrixes: For egg, milk and plasma the initial homogenization step was replaced by vigorously shaking for ~15 sec.

For corticosteroids in milk a volume of 400 µL water-methanol (1+1) and 4000 µL solution C was added to 2000 µL raw milk. The sample was shaken vigorously for 10 sec and centrifuged for 5 min at 2500 rpm. The supernatant was transferred to another centrifuge tube and 4000 µL chloroform was added. The sample was shaken vigorously for 10 sec and centrifuged for 4 min at 2500 rpm. The upper phase (water) was discarded and the organic phase was transferred to a new clean tube to avoid water residues. Thereafter the organic phase was evaporated to dryness under a stream of air at 45 °C. After the tube achieved room temperature the extract was dissolved in 100 µL methanol. Thereafter water (400 µL) was added and whirl-mixed for 3 sec. The sample was kept at -20°C for 6 min and centrifuged for 2 min (2500 rpm) and then filtered through a Spin – X centrifuge filter. Aliquots of 30 µL were injected into the LC-MS at intervals of 16 min.

Calibration curves and recovery studies

The precision, recovery, and linearity were determined by spiking drug-free meat samples with mixed solutions of working standards to yield 0, 0.25, 0.5, 1, 2, 5, 10, 15 and 20 ng/g. Duplicate samples were used. The recovery was determined by comparing the results from spiked meat samples with those of standard solutions prepared by diluting the corresponding standard with drug-free extract from meat. The linearity of the standard curves was calculated using peak area measurements.

RESULTS AND DISCUSSION

The standard curves for all drugs were linear in the investigated areas from their limits of quantification (LOQ) to 20 ng/g. The linear coefficient for all drugs varied from r=0.9989 to 0.9997. The recovery and standard deviations were calculated from samples (n=5) spiked with 5 and 10 ng/g (TABLE 1). The recovery was calculated directly, without correction for an internal standard. The detection limits were calculated from transition 1. Transition 1 was used for screening and transition 2 was used to confirm the drug identity. Chromatograms obtained from drug-free chicken meat and from swine meat spiked with 57 different drugs are shown in Figure 1.

The present multi class residue method describes the screening of 57 different drugs in meat with one transition. After a possible drug is identified, the extract with the suspect signal is re-injected into the LC-MS/MS to identify two or more transitions that could be present. We have used the present method for many years in routine analyzes for one or few specific drug family groups. No significant differences regarding recovery, standard deviation or repeatability have been observed when analyzing meat from different animal species, egg, milk or plasma.

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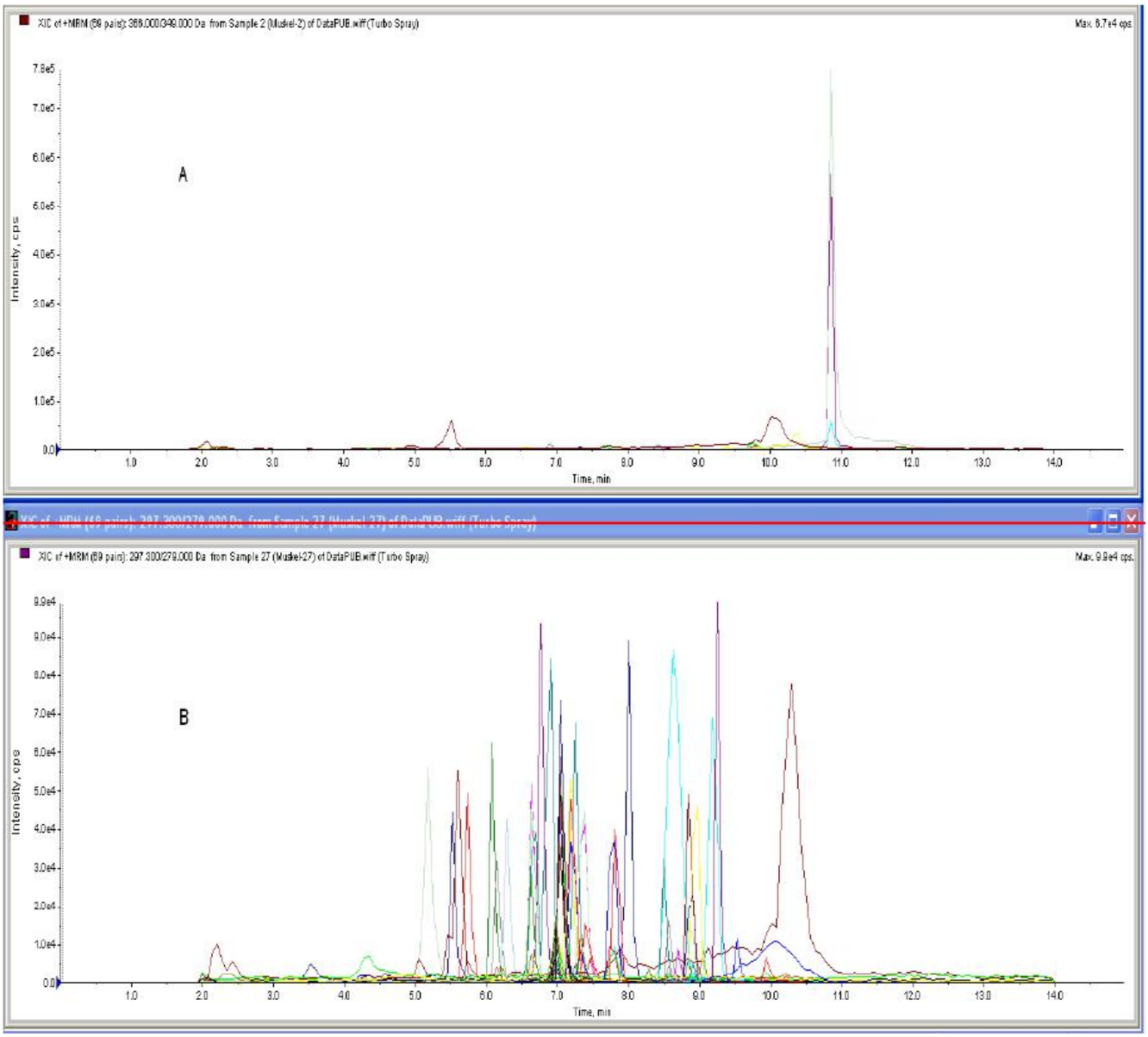

Figure 1 : Overlay of total ion chromatograms from drug-free egg, cow plasma, swine meat and milk (A). Total ion chromatograms from swine meat spiked with 10 ng/g of 57 different drugs (B).

When analyzing one or few drug family groups it is possible to change the mobile phase for a more optimal baseline separation. The method presented in this paper is selective, robust, sensitive, and accurate. The limits of detection were calculated as three times the peak-to-peak baseline noise (S/N = 3) from drug-free samples, and ranged from 0.25 ng/g to 5 ng/g for all drugs. The LOQ were the double of the limits of detection and varied from 0.5 to10 ng/g for all drugs. No interference was observed during analysis, neither for the calibration curves, or when performing the recovery studies. Some sulfonamides have identical parent ions and fragment ions. Therefore a good baseline separation is achieved.

For dimetridazole the method did not achieve the detection limit described in the authorities' regulations. However, it can be possible to meet the regulations exigency by other analytic columns and mobile phase composition.

The present method includes plasma as matrix. Based on this method, blood samples from living animals can be analyzed for drug misuse.

Previous works describe that tetracyclines could be successfully extracted from food matrices using

EDTA[15,16]. The recovery was increased by the addition of EDTA to the samples prior to extraction. EDTA zwitterionic form predominates at pH below 3.0 and the double charged anionic form predominates between pH 3.0 and 6.5. Accordingly, it might be deduced that interaction of the zwitterionic forms of EDTA and tetracyclines could by important under mildly acidic conditions[16]. The use of pH 4.2 buffer with EDTA is optimal for tetracycline's extraction[15]. Simultaneously, a lower pH leads to loss of acid-labile penicillin, while a higher pH-value will result in lower overall recovery. A balance must therefore be achieved between good extraction efficiency and loss of the substance cause by acid degradation[17].

The pH value, which influences the extraction of penicillins and tetracyclines, had to be optimized by adding a small amount of TCA to acetone[18]. In addition, TCA increase the denaturizing property of acetone to proteins. The addition of small amount of buffer (pH 4.2) keeps a more stable pH during extraction and gives a satisfactory extraction for tetracyclines, penicillin's and many other drugs.

The tetracyclines forms chelate complexes with metal ions and absorb on the silanol group[16,19]. The chelate complexes show tailing in a reversed-phase column. To avoid the formation of these complexes, mobile phases containing oxalic acid were used[20].

Acetone is a favorable solvent for extraction of drugs, because it is effective to denature proteins and is simple to evaporate. To ease the extraction a homogenizing step with an Ultra-Turrax is absolutely necessary. In this way, the contact surface between the extraction solution and the matrix sample is increased and the release of protein-bound drugs from natural samples is enhanced.

In many laboratories, a stream of nitrogen is used to evaporate sample extracts. In this study, air produced from a central air compressor was used for evaporation. For analytes that are not easily oxidized, the use of air is a practical and economically favorable alternative compared with nitrogen.

The presence of DMSO in the extraction solution increases the solubility of a wide range of drugs. In addition, DMSO reduces the binding of drugs to fat during the evaporation step. The high boiling point (189 °C) of DMSO is advantageous for the volume reduction to 400 µL during evaporation. In this way the degradation of specific drugs is avoided. If necessary, the volume can be adjusted with water to 400 µL after evaporation.

During sample preparation, low temperature (- 20 °C) and centrifugation were used to remove protein residues and fat.

The graduation of glass-stoppered tubes is not always accurate. To achieve a more precise volume-indication we pipetted 400 µL water and marked with a marker the 400 µL levels. This water was discarded before pipetting the extracts.

Working standards of penicillins and tetracyclines were made and stored separately because they are unstable and should be prepared weekly.

Quinolone standards are very stable, but they were stored separately because the low pH can affect other drugs.

European and Norwegian authorities have established very low MRL levels for a few corticosteroids in milk. To meet these regulations we have made a modification of the present method. The limits of detection for dexametasone were 0.1 ng/mL and the limit of quantification was 0.2 ng/mL. These levels are in accordance with the authorities' requirements.

CONCLUSIONS

The applications of the method presented here provide good evidence that a simple sample preparation in combination with LC-MS/MS can offer a number of significant advantages for the detection and quantification of selected classes and numbers of drugs in different matrixes compared with another published methods. LC-MS/MS methods generally require only a simple clean-up step or only a dilution procedure without derivatization. The validation data showed that the method performance is good and can be used for routine analysis.

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