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Development and Validation of a Multi-class UHPLC-MS/MS Method for Determination of Antibiotic Residues in Dairy Products

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Abstract

In regard to the knowledge of provoking a worldwide resistance against antibiotics due to incorrect application and the resulting uptake of residues via the food chain, the European Union set maximum residue levels (MRLs) for these substances in animal derived products to protect the customers. In respect of these MRLs a multi-class UHPLC-MS/MS multiclass method has been developed for the simultaneous determination of 30 substances from different compound groups (quinolones, macrolides, lincosamides, β-lactams, sulphonamides, diamino-pyrimidine derivates and tetracyclines) in various kinds of dairy products. Since this method should be suitable for routine laboratory use, sample preparation requires an easy and fast approach to ensure high sample throughput. Therefore a sample preparation with C18EC dSPE bulk sorbent as a combination of the easy and quick sample preparation provided by QuEChERS and the clean-up principle of reversed phase SPE was applied. This method was validated for all compounds in all matrices in compliance with the requirements of Commission Decision 2002/657/EC.

Keywords Veterinary drug residues · UHPLC-MS/MS method · Milk and dairy products · C18EC bulk sorbent · Dispersive SPE

Introduction

Due to the increasing large-scale animal production, tons of veterinary drugs, especially antibiotic substances, are applied to treat animal diseases. Antibiotics are known as medical agents whose able to kill bacteria or inhibit their growth by different interfering mechanisms. The residues of these substances are remaining in the animal tissue, spreading via various routes into the food chain, and get finally taken up by human. The extensive consumption of animal-derived products like milk and dairy products results in a low-dose uptake of antibiotics over a long time period, increasing the risk to develop a worldwide antimicrobial resistance (e.g., MRSA—methicillin-resistant Staphylococcus aureus). Beside this main concerning risk, antibiotics are additionally able to provoke allergic reactions or toxicological symptoms like headache, nausea, diarrhea etc. (Austrian Agency for Health and Food Safety 2015; European Food Safety Authority 2017; Marazuela and Bogialli 2013; U.S. Department of Health and Human Services 2013) To protect the consumers from this additional antibiotic source, policies have become more stringent and the European Union set maximum residue levels (MRLs) for veterinary drug residues in raw animal food stuff in the commission regulation EU No. 37/2010 (The European Commission 2010).

For an efficient protection, a strict control of the goods is necessary, requiring powerful tools for determination of these pharmacologically active substances. LC-MS/MS technique is the method of choice for the confirmation of substances, because of its combination of analytical separation and structural information (Kennedy et al. 1998). Additionally, this technique fulfills the requirements demanded by Commission Decision 2002/657/EC (CRLs 2008).

A lot of work was done in the last decade in developing different methods for the determination of single or multiple antibiotic groups in different kinds of matrices, focused on raw materials (milk, meat, eggs, etc.) (Bohm et al. 2009; Chico et al. 2008; Dasenaki and Thomaidis 2015; Freitas et al. 2014; Frenich et al. 2010; Geis-Asteggiante et al. 2012; Granelli and Branzell 2007; Han et al. 2015; Hermo et al. 2008; Kaufmann et al. 2014; Martins-Júnior et al. 2007; Mastovska and Lightfield 2008; Ortelli et al. 2009; Stolker et al. 2008;

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Turnipseed et al. 2014; Wang et al. 2015) but there is a lack in the method development for processed dairy products, since antibiotics are not fully degraded during pasteurization process (Pérez et al. 2013).

Therefore, the aim of this work was the development and optimization of a qualitative multi-class residue method with UHPLC-MS/MS for the determination of selected antibiotics from seven different groups (quinolones, macrolides, lincosamides, ß-lactams, sulphonamides, diaminopyrimidine derivates, and tetracyclines) in raw bovine milk as well as in various types of dairy products (e.g., butter, curd, yogurt, cheese). The final method should be suitable in a routine laboratory work; therefore, sample extraction and clean-up procedures must be easy as well as less time and cost consuming.

Based on these requirements, a simple and fast UHPLC-MS/MS multi-class method for the determination of 30 antibiotic drugs in different kinds of dairy products was developed and validated in terms of linearity, trueness, precision, analytical limits (CCα, CCβ), and quantification (LOQs) according to Commission Decision 2002/657/EC (CRLs 2008).

Material and Methods

Chemicals and Reagents

Acetonitrile (ACN) (VWR Chemicals, Vienna, Austria) and methanol (MeOH) (Chem-Lab LV, Zedelgem, Belgium) were obtained in HPLC grade. Milli-Q water was prepared using a

Table 1 Antibiotic mix solutions with their corresponding internal standards (ISTD)

Compound		Concentration antibiotic mix standard (μg/l)	Corresponding internal standard compound	Concentration ISTD-mix solution (μg/L)
Ciprofloxacin	CH MIX	100	Ciprofloxacin-d8 hydrochloride hydrate	60
Danofloxacin	CH MIX	60	Danofloxacin-(methyl-d3)	100
Enrofloxacin	CH MIX	100	Enrofloxacin-d5 hydrochloride	100
Flumequine	CH MIX	100	Flumequine-1,2,carboxy-13C3	100
Marbofloxacin	CH MIX	150	Marbofloxacin-d8	150
Erythromycin A dihydrate	MALI MIX	80	Erythromycin-13C, d3	300
Lincomycin hydrochloride monohydrate	MALI MIX	300	Lincomycin-d3	400
Pirlimycin hydrochloride	MALI MIX	200		
Spiramycin	MALI MIX	400	Spiramycin I-d3	1000
Tilmicosin	MALI MIX	100		
Tylosin tartrate	MALI MIX	100		
Amoxicillin trihydrate	PEN MIX	80		
Ampicillin trihydrate	PEN MIX	80		
Ceftiofur	PEN MIX	200		
Cloxacillin sodium salt monohydrate	PEN MIX	60		
Penicillin G potassium salt	PEN MIX	40	Penicillin G-d7 N-ethylpiperidinium salt	400
Penicillin V potassium salt	PEN MIX	100		
Sulfadiazine	SAM MIX	20	Sulfadiazine-phenyl-13C6	20
Sulfadimethoxine	SAM MIX	20	Sulfadimethoxine-d6	20
Sulfadimidine = sulfamethazine	SAM MIX	20	Sulfamethazine-(phenyl-13C6)hemihydrate	20
Sulfadoxin	SAM MIX	20	Sulfadoxin-d3	20
Sulfamerazine	SAM MIX	20	Sulfamerazine-(phenyl-13C6)	20
Sulfamethoxazole	SAM MIX	20	Sulfamethoxazole-phenyl-13C6	20
Sulfamethoxypyridazine	SAM MIX	20	Sulfamethoxypyridazine-d3	20
Sulfanilamide	SAM MIX	20	Sulfanilamide-13C6	20
Sulfathiazole	SAM MIX	20	Sulfathiazole-(phenyl-13C6)	20
Trimethoprim	SAM MIX	100	Trimethoprim-d9	100
Chlortetracycline hydrochloride	TC MIX	200	Demeclocycline hydrochloride hydrate	200
Doxycycline hyclate	TC MIX	200	Doxycycline-d3 hyclate	200
Oxytetracycline hydrochloride	TC MIX	200	Demeclocycline hydrochloride hydrate	200
Tetracycline hydrochloride	TC MIX	200	Demeclocycline hydrochloride hydrate	200

Milli-Q Gradient Water System (Millipore, Billerica, MA, USA). Ammonium formate, citric acid monohydrate, disodium phosphate (Na_2HPO_4), and disodium ethylenediaminetetra acetate (Na_2EDTA) were purchased from Sigma-Aldrich (Vienna, Austria). Formic acid (HCOOH, purity 99–100%) was purchased from VWR Chemicals and C18 bulk sorbent from Agilent Technologies (Waldbronn, Germany).

The antibiotic substances ciprofloxacin, danofloxacin, enrofloxacin, flumequine, marbofloxacin, erythromycin A dehydrate, spiramycin, tilmicosin, tylosin tartrate, lincomycin hydrochloride monohydrate, amoxicillin trihydrate, ampicillin trihydrate, ceftiofur, cloxacillin sodium salt monohydrate, penicillin G potassium salt, penicillin V potassium salt, sulfadiazine, sulfadimethoxine, sulfadoxin, sulfamerazine, sulfamethazine, sulfamethoxazole, sulfamethoxypyridazine, sulfathiazole, trimethoprim, chlortetracycline hydrochloride, doxycycline hyclate, oxytetracycline hydrochloride, and tetracycline hydrochloride were obtained from Sigma-Aldrich (Vienna, Austria). Pirlimycin hydrochloride was purchased as Pirsue® sterile solution from Pfizer Ltd. (Tadworth, Surrey, UK).

The internal standard (ISTD) substances ciprofloxacin-d8 hydrochloride hydrate, danofloxacin-(methyl-d3), demeclocycline hydrochloride hydrate, enrofloxacin-d5 hydrochloride, flumequine-1,2,carboxy-(13C3), penicillin G-d7-N-ethylpiperidinium salt, sulfadiazine-(phenyl-13C6), sulfadimethoxine-d6, sulfadoxin-d3, sulfamerazine-(phenyl-13C6), sulfamethazine-(phenyl-13C6)hemihydrates, sulfamethoxazole-phenyl-13C6, sulfamethoxypyridazine-d3, sulfathiazole-(phenyl-13C6), and trimethoprim-d9 were obtained from Sigma-Aldrich (Vienna, Austria). Doxycycline-d3 hyclate, erythromycin-13C-d3 and lincomycin-d3, and marbofloxacin-d8 and spiramycin I-d3 were bought from Toronto Research Chemicals (Toronto, ON, Canada).

Stock solutions were prepared at a concentration of 1000 mg/l of each compound by exactly weighing and dissolving in their individual solvent solution and stored at − 18 °C in the dark. The compounds erythromycin A, tylosin tartrate, tilmicosin, and spiramycin are soluble in ACN, the ß-lactams in H_2O:ACN (50:50), the sulphonamides, tetracyclines, trimethoprim and ciprofloxacin in MeOH, and the remaining quinolones in alkalized MeOH.

The working antibiotic mix standard solution was prepared by using five mixes to obtain concentrations as described in Table 1.

Individual stock solutions of the ISTD compounds were also prepared at a concentration of 1000 mg/l and stored at

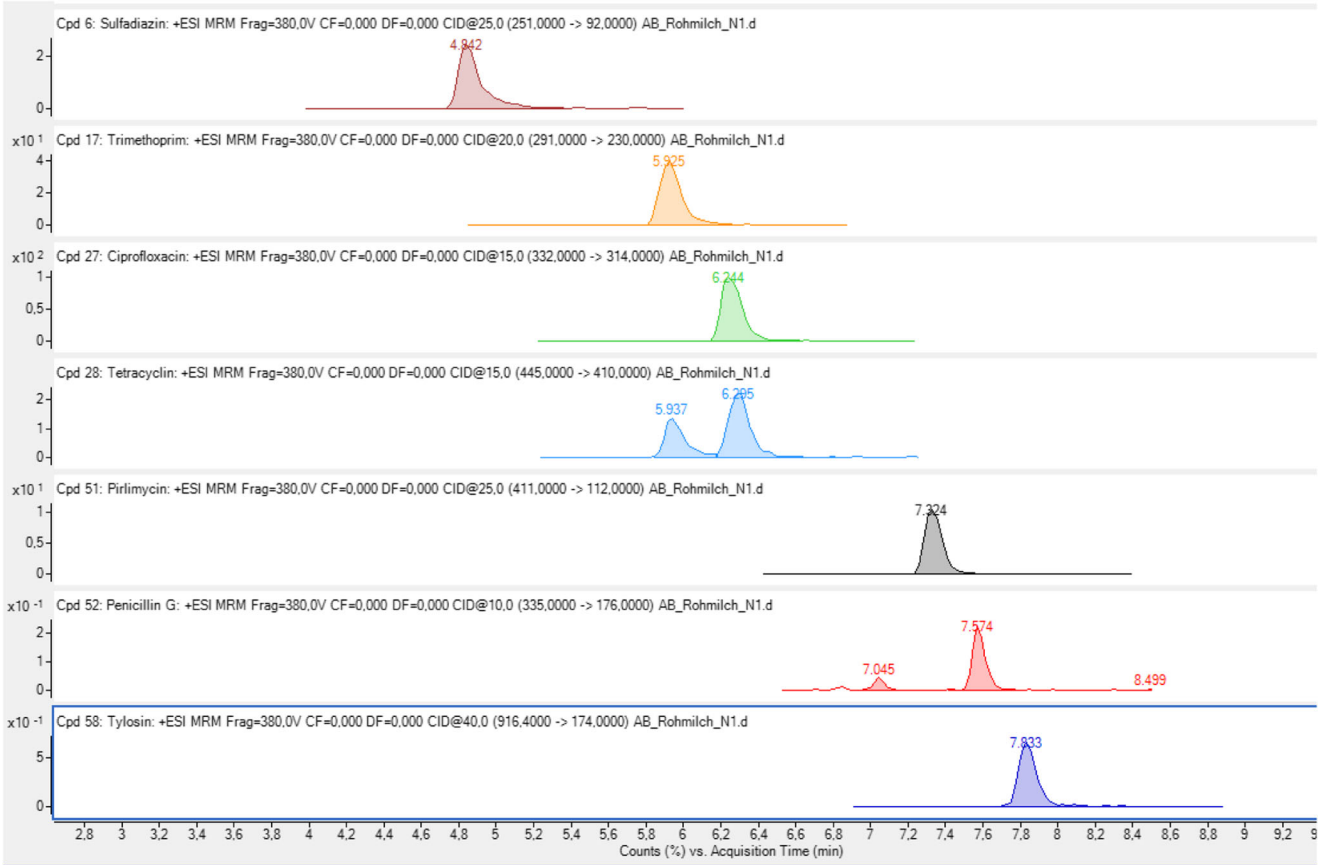

Fig. 1 Examples of compound chromatograms. UHPLC-MS/MS chromatograms from one representative compound of each substance group achieved from a spiked raw milk sample (0.1 of their MRLs)

Table 2 dMRM parameters of the antibiotics and their internal standards.

Component	Precursor ion (m/z)	Product ion (m/z)	tR (min)	CE (eV)	CAV (ev)
Quinolones					
Ciprofloxacin	332	314	6.3	15	1
		245		20	1
Ciprofloxacin-d_3	340	322	6.3	25	1
		235		45	1
Danofloxacin	358	340	6.3	20	1
		314		15	3
Danofloxacin-d_3	361	343	6.3	20	1
		317		15	3
Enrofloxacin	360	342	6.3	15	1
		245		20	1
Enrofloxacin-d_5	365	347	6.3	20	1
		245		30	1
Flumequine	262	244	7.8	25	3
		202		35	5
Flumequine-$^{13}C_3$	265	247	7.8	15	1
		205		35	1
Marbofloxacin	363	320	6	15	1
		345		10	3
Marbofloxacin-d_8	371	79	6	25	1
		353		20	1
Macrolides					
Erythromycin A	734	158	8	35	1
		576		20	1
Erythromycin A–^{13}C-d_3	739	580	8	15	1
		162		30	3
Spiramycin	844	174	6.9	40	1
		101		45	1
Tilmicosin	870	174	7.3	45	1
		696		45	5
Tylosin	916	174	7.9	40	3
		101		55	3
Lincosamides					
Lincomycin	407	126	5.9	30	1
		359		15	1
Lincomycin-d_3	410	129	5.9	30	3
		392		15	1
Pirlimycin	411	112	7.4	15	1
		56		45	3
ß-lactams					
Amoxicillin	366	208	3.3	10	3
		114		20	5
Ampicillin	350	192	6.3	15	5
		174		10	5
Ceftiofur	524	241	7.1	15	3
		126		35	5
Cloxacillin	436	277	8	15	1
		160		15	5

Table 2 (continued)

Component	Precursor ion (m/z)	Product ion (m/z)	tR (min)	CE (eV)	CAV (ev)
Penicillin G	335	176	7.6	10	1
		160		10	1
Penicillin G-d_7	342	183	7.6	5	5
		160		1	8
Penicillin V	351	160	7.9	10	3
		192		15	1
Sulphonamides					
Sulfadiazine	251	92	4.8	25	3
		156		10	1
Sulfadiazine-$^{13}C_6$	257	98	4.8	30	3
		162		15	3
Sulfadimethoxine	311	156	7	15	3
		108		30	3
Sulfadimethoxine-d_6	317	156	7	20	1
		108		25	3
Sulfamethazine	279	186	6.1	15	3
		156		20	1
Sulfamethazine-$^{13}C_6$	285	186	6.1	20	3
		162		20	1
Sulfadoxin	311	156	6.5	10	3
		108		25	5
Sulfadoxin-d_3	314	156	6.5	15	1
		92		30	1
Sulfamerazine	265	156	5.7	15	1
		92		25	3
Sulfamerazine-$^{13}C_6$	271	98	5.7	35	1
		172		15	5
Sulfamethoxazole	254	156	6.4	10	5
		92		25	3
Sulfamethoxazole-$^{13}C_6$	260	162	6.4	15	1
		114		20	1
Sulfamethoxypyridazine	281	156	6.2	10	1
		92		35	1
Sulfamethoxypyridazine-d_3	284	156	6.2	15	3
		108		30	5
Sulfathiazole	256	156	5.3	5	1
		92		15	3
Sulfathiazole-$^{13}C_6$	262	162	5.3	10	1
		114		20	5
Diamino-pyrimidine-derivate					
Trimethoprim	291	230	6	20	3
		261		25	3
Trimethoprim-d_9	300	234	6	25	1
		264		30	1
Tetracyclines					
Doxycycline and its 4-epimer	445	428	7.3	15	5
		154	6.3 (4-epi)	35	1
Doxycycline-d3	448	431	7.3	20	1
		413		25	3

Table 2 (continued)

Component	Precursor ion (m/z)	Product ion (m/z)	tR (min)	CE (eV)	CAV (ev)
Chlortetracycline and its 4-epimer	479	444	7	20	5
		462	6.7 (4-epi)	15	5
Oxytetracycline and its 4-epimer	461	426	6.4	15	5
		443	6.2 (4-epi)	5	5
Tetracycline and its 4-epimer	445	410	6.3	15	5
		427	6 (4-epi)	10	5
Demeclocycline (ISTD for all three remaining TCs)	465	448	6.6	5	8
		430		15	8

tR retention time, *CE* collision energy, *CAV* cell accelerating voltage

− 18 °C in the dark. Penicillin G-d7-N-ethylpiperidinium salt was dissolved in H_2O:ACN (50:50), marbofloxacin-d8 and erythromycin-13C-d3 in chloroform, ciprofloxacin-d8 hydrochloride hydrate, and enrofloxacin-d5 hydrochloride in H_2O. The remaining substances were dissolved in MeOH. An ISTD mix solution was prepared reaching concentrations between 20 and 1000 μg/L (Table 1).

Apparatus

Samples were homogenized using the blender "Robot coupe blixer 3" (Robot Coupe Ltd., 153 Vincennes Cedex, France). For weighing the samples and the standard substances, analytical scales were used (Sartorius Ltd., Göttingen, Germany). Shaking process was fulfilled by using a Collomix device (Collomix Ltd., Gaimersheim, Germany) and vortexing was done with Reax Control (Heidolph Instruments Ltd. & Co. KG, Schwabach, Germany). For centrifugation of the samples, an Eppendorf centrifuge 5430 was used (Eppendorf Ltd., Vienna, Austria). Supernatant evaporation was conducted using a TurboVap® LV automated evaporation system (Biotage Ltd., Uppsala, Sweden). Chromatographic separation was made by using an UHPLC 1290 system connected to a triple quadrupole mass spectrometer 6490 from Agilent Technologies Ltd. (Waldbronn, Germany).

Samples

Different kinds of common organic dairy products were purchased at a local supermarket. Pasteurized milk, whipped cream, butter, curd, sour cream, yogurt, buttermilk, soft cheese covered with white mold and red cultures (contains 55% fat), and hard cheese (50% fat in dry matter) were investigated. Bovine raw milk was obtained from a local dairy.

Sample Preparation

Butter and cheese samples were homogenized together with dry ice using a blixer (Robot Coupe), all other samples were homogenized by shaking or stirring the sample with a spoon.

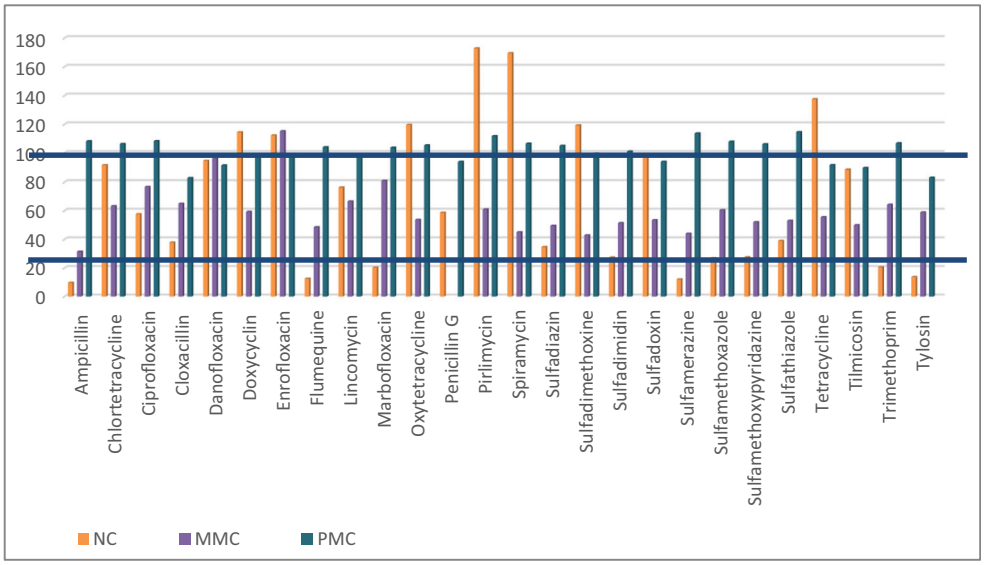

Fig. 2 Comparison of recovery rates (%) after quantification with different calibrations. Shown are the mean recovery rates (%) after sample preparation with C18 bulk sorbents quantified with normal calibration (NC), matrix-matched calibration (MMC), and procedure matched calibration (PMC). After quantifying with PMC for all substances recoveries between 70 and 120% could be achieved ($n = 3$)

2 ± 0.1 g of the homogenized samples were weighed into a 50 mL polypropylene centrifuge tube and 100 μl of ISTD mix solution (Table 1) was added. After adding 2 mL of Na_2EDTA-McIlvaine buffer solution (0.1 M, pH 4.0) and vortexing (Reax, Reidolph) for 30 s, 8 mL of acetonitrile for protein precipitation was added, vortexing again for 30 s, followed by shaking for 2 min (Collomix). Afterwards, the samples were centrifuged at 4220×g for 5 min and the supernatant was transferred to a 15 mL polypropylene centrifuge tube, containing 500 mg C18 bulk sorbent. For sample clean-up, the supernatant was vortexed with the bulk sorbent for 1 min and shaken for 2 min using the Collomix device, followed by centrifugation at 4220×g for 5 min. After centrifugation, 5 mL of the clean supernatant is transferred to a new 15 mL centrifuge tube followed by evaporation under nitrogen stream (45 °C, 17 psi). The residue was redissolved in 2 mL initial mobile phase solution and filtered (0.2 μm, PTFE) into a HPLC glass vial followed by chromatographic separation and measurement. Figure 1 shows a typical chromatogram of representative compounds of each group obtained from a fortified raw milk sample (1/10 of their MRLs) after extraction.

Validation

The previous described method (2.4) was validated for 10 different kinds of dairy matrices (bovine raw milk, pasteurized milk, butter, whipped cream, curd, sour cream, yogurt, buttermilk, soft cheese, and hard cheese) in accordance to Commission Decision 2002/657/EC (CRLs 2008), regarding

Table 3 Validation data from all compounds in all matrices. Repeatability is expressed as RSD. Matrix: bovine raw milk, 3.5% fat content

Compound		Spike concentration (μg/kg)		Recovery (%) and RSD (%)		CCα (μg/kg)	CCβ (μg/kg)	LOQ (μg/kg)
	MRL (μg/kg)	Low level	High level	Spike low concentration	Spike high concentration			
Amoxicillin	4	4	20	118 (4)	122 (5)	6.00	7.00	4.00
Ampicillin	4	4	20	71 (13)	74 (8)	6.00	9.00	4.00
Ceftiofur	100	10	50	111 (7)	94 (3)	103.00	105.00	10.00
Chlortetracycline	100	10	50	60 (4)	65 (10)	107.00	114.00	10.00
Ciprofloxacin	100	5	25	96 (18)	115 (9)	55.00	57.00	5.00
Cloxacillin	30	3	15	79 (16)	84 (5)	31.00	33.00	3.00
Danofloxacin	30	3	15	83 (7)	73 (15)	33.00	37.00	3.00
Doxycycline	no MRL	10	50	113 (7)	96 (4)	4.00	9.00	10.00
Enrofloxacin	100	5	25	88 (8)	105 (16)	56.00	62.00	5.00
Erythromycin A	40	4	20	182 (37)	179 (11)	43.00	47.00	20.00
Flumequine	50	5	25	78 (10)	153 (9)	53.00	57.00	5.00
Lincomycin	150	15	75	85 (12)	74 (12)	162.00	175.00	15.00
Marbofloxacin	75	7.5	37.5	100 (11)	107 (6)	78.00	82.00	7.50
Oxytetracycline	100	10	50	67 (15)	62 (19)	115.00	130.00	10.00
Penicillin G	4	2	10	157 (14)	169 (7)	5.00	6.00	2.00
Penicillin V	no MRL	5	25	75 (5)	83 (8)	4.00	8.00	5.00
Pirlimycin	100	10	50	85 (7)	82 (6)	105.00	110.00	10.00
Spiramycin	200	20	100	144 (52)	171 (19)	230.00	260.00	20.00
Sulfadiazine	100	1	5	79 (7)	83 (4)	100.00	101.00	1.00
Sulfadimethoxine		1	5	91 (8)	86 (16)	101.00	102.00	1.00
Sulfadimidine		1	5	96 (8)	94 (12)	101.00	102.00	1.00
Sulfadoxin		1	5	94 (10)	98 (9)	101.00	102.00	1.00
Sulfamerazine		1	5	91 (9)	92 (11)	101.00	102.00	1.00
Sulfamethoxazole		1	5	58 (13)	81 (13)	101.00	102.00	1.00
Sulfamethoxypyridazine		1	5	93 (11)	85 (7)	101.00	101.00	1.00
Sulfathiazole		1	5	84 (8)	75 (9)	101.00	102.00	1.00
Tetracycline	100	10	50	93 (11)	95 (7)	105.00	110.00	10.00
Tilmicosin	50	5	25	64 (22)	90 (14)	55.00	60.00	5.00
Trimethoprim	50	5	25	98 (4)	120 (4)	51.00	52.00	5.00
Tylosin	50	5	25	47 (20)	54 (14)	54.00	58.00	5.00

the terms of repeatability, reproducibility, precision, intermediate precision, trueness, linearity, selectivity, limit of detection (LOD), and quantification (LOQ).

Method linearity was evaluated by performing calibration curves prepared in initial mobile phase solvent (95% mobile phase A and 5% mobile phase B), using ten concentration levels. The ranges were adapted in accordance to the MRLs of the compounds, starting at 1/500 × MRL rising up to 2 × MRL. Calibration curves were constructed by least-squares linear regression analysis of the peak area, corrected by the corresponding internal standard, versus the added concentration. Linearity was achieved when the coefficient of correlation was at least 0.990.

To assess the trueness of the method, six blank samples of each matrix were spiked with an antibiotic mix solution at two concentration levels in accordance to 0.1 × and 0.5 × of their MRLs. Only amoxicillin, ampicillin, and penicillin G were added to the samples in a higher concentration (see Tables 3, 4, 5, 6, 7, 8, 9, 10, 11, 12).

Repeatability (intraday precision) of the method was carried out on six different days, selecting two blank samples of each matrix per day and fortifying at the two concentration levels.

LODs and LOQs were established by fortified blank samples with antibiotics at two concentration levels (0.1 and 0.5 × MRLs) in order to determine the lowest amount of the analytes for which signal-to-noise ratios (S/N) were 3 and 10, respectively.

CCα and CCβ were calculated for all matrices by calibration curve procedure as described in the Commission

Table 4 Validation data from all compounds in all matrices. Repeatability is expressed as RSD. Matrix: pasteurized bovine milk, 3.5% fat content

Compound		Spike concentration (μg/kg)		Recovery (%) and RSD (%)		CCα (μg/kg)	CCβ (μg/kg)	LOQ (μg/kg)
		Low level	High level	Spike low concentration	Spike high concentration			
Amoxicillin	4	4	20	58 (16)	67 (20)	10.00	16.00	4.00
Ampicillin	4	4	20	104 (24)	88 (18)	10.00	16.00	4.00
Ceftiofur	100	10	50	93 (6)	84 (14)	110.00	120.00	10.00
Chlortetracycline	100	10	50	85 (11)	78 (16)	110.00	120.00	10.00
Ciprofloxacin	100	5	25	79 (21)	94 (16)	56.00	61.00	5.00
Cloxacillin	30	3	15	91 (19)	94 (18)	34.00	38.00	3.00
Danofloxacin	30	3	15	104 (7)	85 (16)	34.00	37.00	3.00
Doxycycline	no MRL	10	50	83 (19)	77 (21)	20.00	41.00	10.00
Enrofloxacin	100	5	25	106 (9)	116 (18)	57.00	64.00	5.00
Erythromycin A	40	4	20	58 (14)	68 (53)	53.00	67.00	4.00
Flumequine	50	5	25	89 (9)	118 (10)	53.00	58.00	5.00
Lincomycin	150	15	75	92 (7)	90 (17)	167.00	183.00	15.00
Marbofloxacin	75	7.5	37.5	77 (21)	97 (17)	84.00	93.00	7.50
Oxytetracycline	100	10	50	88 (19)	77 (21)	116.00	133.00	10.00
Penicillin G	4	2	10	130 (37)	164 (37)	9.00	14.00	2.00
Penicillin V	no MRL	5	25	93 (13)	93 (15)	8.00	16.00	5.00
Pirlimycin	100	10	50	85 (20)	98 (17)	112.00	123.00	10.00
Spiramycin	200	20	100	80 (22)	110 (21)	229.00	258.00	20.00
Sulfadiazine	100	1	5	98 (15)	109 (21)	102.00	103.00	1.00
Sulfadimethoxine		1	5	95 (7)	91 (14)	101.00	102.00	1.00
Sulfadimidine		1	5	94 (13)	77 (20)	102.00	103.00	1.00
Sulfadoxin		1	5	94 (15)	93 (22)	102.00	103.00	1.00
Sulfamerazine		1	5	87 (15)	95 (15)	101.00	102.00	1.00
Sulfamethoxazole		1	5	67 (19)	72 (19)	102.00	103.00	1.00
Sulfamethoxypyridazine		1	5	82 (20)	93 (15)	101.00	102.00	1.00
Sulfathiazole		1	5	109 (31)	108 (20)	102.00	103.00	1.00
Tetracycline	100	10	50	102 (13)	93 (19)	115.00	129.00	10.00
Tilmicosin	50	5	25	123 (53)	117 (33)	61.00	72.00	5.00
Trimethoprim	50	5	25	114 (16)	116 (12)	54.00	59.00	5.00
Tylosin	50	5	25	40 (21)	44 (16)	54.00	58.00	5.00

Decision 2002/657/EC. In order that MRLs of the compounds are only set for milk, we have taken these MRLs for all other matrices, too.

LC-MS/MS Conditions

Chromatographic analyses were performed using an Agilent UHPLC 1290 system connected to a 6490 mass spectrometer (Agilent Technologies, Waldbronn, Germany). Separation was achieved by using an UHPLC RRHD eclipse plus C18 column (100 mm × 2.1 mm × 1.8 μm particle size) from Agilent Technologies, placed in the column oven at a temperature of 40 °C and an operating flow rate of 0.25 mL/min. Injection volume was defined with 20 μl. The gradient started with 95% mobile phase A (H_2O, 5 mM ammonium formate, 0.1% HCOOH) holding for 2 min, decreasing to 83% within 1 min, followed by a further decrease to 30% within the next 3 min. This composition was hold for 2 min, followed by an increase to 100% mobile phase B (MeOH, 5 mM ammonium formate, 0.1% HCOOH) within 1 min, which was hold for 2 min. For column equilibration, initial mobile phase composition (95% solvent A) was reached within 4 min. According to this gradient, the whole separation was fulfilled within 15 min. Mass spectrometry measurement was achieved using an Agilent 6490 triple quadrupole mass spectrometer operating in positive electrospray ionization (ESI) mode with the following adjusted parameters: capillary voltage 3.5 kV, nozzle voltage 300 V, gas temperature 200 °C, gas flow 15 L/min, nebulizer gas 30 psi, sheath gas temperature 375 °C, and sheath gas flow 11 L/min. Nitrogen was used for collision-

Table 5 Validation data from all compounds in all matrices. Repeatability is expressed as RSD. Matrix: cream, 36% fat content

Compound		Spike concentration (μg/kg)		Recovery (%) and RSD (%)		CCα (μg/kg)	CCβ (μg/kg)	LOQ (μg/kg)
	MRL (μg/kg)	Low level	High level	Spike low concentration	Spike high concentration			
Amoxicillin	4	4	20	43 (21)	60 (20)	10.00	16.00	4.00
Ampicillin	4	4	20	85 (28)	75 (20)	10.00	17.00	4.00
Ceftiofur	100	10	50	93 (8)	92 (14)	110.00	120.00	10.00
Chlortetracycline	100	10	50	86 (21)	80 (16)	113.00	125.00	10.00
Ciprofloxacin	100	5	25	81 (13)	100 (13)	54.00	59.00	5.00
Cloxacillin	30	3	15	90 (14)	88 (18)	34.00	38.00	3.00
Danofloxacin	30	3	15	101 (20)	101 (23)	35.00	39.00	3.00
Doxycycline	no MRL	10	50	91 (16)	88 (15)	15.00	31.00	10.00
Enrofloxacin	100	5	25	96 (14)	107 (17)	57.00	63.00	5.00
Erythromycin A	40	4	20	73 (19)	54 (21)	46.00	52.00	4.00
Flumequine	50	5	25	83 (11)	115 (11)	54.00	58.00	5.00
Lincomycin	150	15	75	92 (16)	94 (19)	169.00	187.00	15.00
Marbofloxacin	75	7.5	37.5	68 (18)	100 (19)	85.00	96.00	7.50
Oxytetracycline	100	10	50	81 (20)	70 (21)	118.00	136.00	10.00
Penicillin G	4	2	10	127 (31)	152 (23)	7.00	10.00	2.00
Penicillin V	no MRL	5	25	94 (13)	88 (18)	10.00	20.00	5.00
Pirlimycin	100	10	50	87 (19)	92 (15)	111.00	122.00	10.00
Spiramycin	200	20	100	130 (21)	114 (19)	233.00	267.00	20.00
Sulfadiazine	100	1	5	98 (8)	114 (18)	101.00	103.00	1.00
Sulfadimethoxine		1	5	94 (6)	91 (9)	101.00	101.00	1.00
Sulfadimidine		1	5	86 (10)	84 (18)	102.00	103.00	1.00
Sulfadoxin		1	5	80 (9)	87 (15)	101.00	102.00	1.00
Sulfamerazine		1	5	84 (13)	90 (14)	101.00	102.00	1.00
Sulfamethoxazole		1	5	79 (15)	74 (14)	101.00	102.00	1.00
Sulfamethoxypyridazine		1	5	80 (15)	98 (15)	101.00	102.00	1.00
Sulfathiazole		1	5	104 (18)	120 (16)	101.00	103.00	1.00
Tetracycline	100	10	50	109 (16)	83 (18)	114.00	127.00	10.00
Tilmicosin	50	5	25	118 (60)	113 (38)	63.00	77.00	5.00
Trimethoprim	50	5	25	107 (9)	108 (9)	53.00	56.00	5.00
Tylosin	50	5	25	56 (20)	51 (11)	53.00	57.00	5.00

induced fragmentation of the antibiotics under dynamic multiple reaction monitoring (dMRM) mode. The specific fragments and parameters for each substance are listed in Table 2. Data acquisition was processed using MassHunter software version B06.00 from Agilent Technologies, Inc.

Results and Discussion

Due to the lack of MRLs set for antibiotic residues in dairy products, the optimized methodology has been developed according to the set MRLs for bovine milk. The compounds were chosen according to the standard method from the EURL BVL 01.00 85 (Bundesamt für Verbraucherschutz und Lebensmittelsicherheit (BVL) 2011) complemented with the frequently used antibiotics from the group of the ß-lactams. Spiked concentrations were listed in Table 1. To that extend, 100 µl of the spiking solution was added to 2 g sample prior to extraction.

UHPLC-MS/MS Conditions

For separation of the compounds, a C18 reversed phase column was used in combination with an aqueous-methanol mobile phase including ammonium formate as buffer and 0.1% formic acid. To elute all analytes with their different chemical properties, a gradient program started with 95% aqueous phase rising up to 100% methanol phase

Table 6 Validation data from all compounds in all matrices. Repeatability is expressed as RSD. Matrix: butter, 80% fat content

Compound		Spike concentration (µg/kg)		Recovery (%)		CCα (µg/kg)	CCβ (µg/kg)	LOQ (µg/kg)
	MRL (µg/kg)	Low level	High level	Spike low concentration	Spike high concentration			
Amoxicillin	4	4	20	33 (31)	78 (58)	20.00	36.00	4.00
Ampicillin	4	4	20	49 (41)	50 (21)	11.00	17.00	4.00
Ceftiofur	100	10	50	78 (9)	75 (20)	114.00	128.00	10.00
Chlortetracycline	100	10	50	83 (16)	76 (13)	110.00	120.00	10.00
Ciprofloxacin	100	5	25	91 (12)	111 (18)	56.00	63.00	5.00
Cloxacillin	30	3	15	45 (16)	61 (17)	33.00	37.00	3.00
Danofloxacin	30	3	15	100 (8)	84 (20)	34.00	39.00	3.00
Doxycycline	no MRL	10	50	100 (24)	113 (19)	17.00	34.00	10.00
Enrofloxacin	100	5	25	93 (17)	104 (10)	54.00	58.00	5.00
Erythromycin A	40	4	20	80 (22)	72 (15)	44.00	49.00	4.00
Flumequine	50	5	25	84 (11)	105 (12)	55.00	60.00	5.00
Lincomycin	150	15	75	89 (20)	80 (19)	169.00	189.00	15.00
Marbofloxacin	75	7.5	37.5	87 (19)	98 (16)	84.00	92.00	7.50
Oxytetracycline	100	10	50	80 (20)	72 (19)	116.00	132.00	10.00
Penicillin G	4	2	10	128 (48)	139 (36)	9.00	14.00	2.00
Penicillin V	no MRL	5	25	63 (18)	65 (19)	10.00	21.00	5.00
Pirlimycin	100	10	50	80 (16)	87 (19)	114.00	128.00	10.00
Spiramycin	200	20	100	89 (19)	118 (22)	234.00	267.00	20.00
Sulfadiazine	100	1	5	89 (21)	90 (16)	101.00	102.00	1.00
Sulfadimethoxine		1	5	95 (10)	91 (15)	101.00	102.00	1.00
Sulfadimidine		1	5	87 (13)	85 (16)	101.00	102.00	1.00
Sulfadoxin		1	5	85 (8)	84 (12)	101.00	102.00	1.00
Sulfamerazine		1	5	80 (14)	85 (7)	101.00	102.00	1.00
Sulfamethoxazole		1	5	78 (8)	77 (13)	101.00	102.00	1.00
Sulfamethoxypyridazine		1	5	88 (16)	95 (15)	101.00	102.00	1.00
Sulfathiazole		1	5	78 (17)	83 (11)	101.00	102.00	1.00
Tetracycline	100	10	50	90 (20)	90 (21)	115.00	129.00	10.00
Tilmicosin	50	5	25	156 (46)	119 (34)	63.00	75.00	5.00
Trimethoprim	50	5	25	110 (9)	100 (8)	53.00	56.00	5.00
Tylosin	50	5	25	57 (21)	60 (33)	59.00	69.00	5.00

was used to ensure elution of all compounds. Experiments to shorten the gradient program after elution of the last compound (RT 8 min) were unsuccessful due to the needed time for the column to re-equilibrate.

The optimization of the MS parameters (collision energy (CE) and cell accelerating voltage (CAV)) was conducted by injecting a standard solution of 500 μg/L of each antibiotic compound diluted in initial mobile phase solvent. For determination of parent ion, a full-scan spectrum of each substance was collected in order to select the most abundant m/z value. For all substances protonated, $[MH]^+$ ions were detected with the highest abundance, using these precursor ions to obtain at least two typical fragments after exerting different CEs and CAVs. All MS/MS transitions with their CEs, CAVs, and RTs are shown in Table 2.

Extraction and Clean-up Procedure

Sample extraction and clean-up is always a compromise between purity of the sample and recovery rates of the compounds of interest, and is therefore the most critical step during method development. To define the best compromise, different sample preparation procedures were conducted as described in the following sections. Finally, we decided in accordance to the obtained recovery rates for a dSPE sample clean-up using C18 bulk sorbent as described in section 2.4.

Modified QuEChERS Approaches

Based on these requirements to ensure a quick and easy sample treatment, it was obvious to try modified QuEChERS

Table 7 Validation data from all compounds in all matrices. Repeatability is expressed as RSD. Matrix: buttermilk, 1% fat content

Compound		Spike concentration (μg/kg)		Recovery (%) and RSD (%)		CCα (μg/kg)	CCβ (μg/kg)	LOQ (μg/kg)
	MRL (μg/kg)	Low level	High level	Spike low concentration	Spike high concentration			
Amoxicillin	4	4	20	60 (17)	57 (21)	11.00	18.00	4.00
Ampicillin	4	4	20	40 (18)	30 (22)	12.00	20.00	20.00
Ceftiofur	100	10	50	34 (8)	34 (7)	105.00	109.00	10.00
Chlortetracycline	100	10	50	118 (11)	100 (18)	114.00	128.00	10.00
Ciprofloxacin	100	5	25	78 (16)	91 (19)	57.00	63.00	5.00
Cloxacillin	30	3	15	14 (32)	16 (18)	34.00	38.00	3.00
Danofloxacin	30	3	15	86 (19)	71 (7)	32.00	34.00	3.00
Doxycycline	no MRL	10	50	98 (20)	81 (20)	22.00	43.00	10.00
Enrofloxacin	100	5	25	125 (14)	118 (14)	56.00	62.00	5.00
Erythromycin A	40	4	20	n.d.	n.d.			
Flumequine	50	5	25	90 (25)	110 (8)	54.00	57.00	5.00
Lincomycin	150	15	75	80 (17)	89 (16)	166.00	181.00	15.00
Marbofloxacin	75	7.5	37.5	49 (21)	68 (15)	84.00	92.00	7.50
Oxytetracycline	100	10	50	94 (14)	62 (23)	119.00	138.00	10.00
Penicillin G	4	2	10	n.d.	n.d.			
Penicillin V	no MRL	5	25	n.d.	n.d.			
Pirlimycin	100	10	50	34 (22)	35 (12)	108.00	116.00	10.00
Spiramycin	200	20	100	94 (20)	120 (19)	227.00	255.00	20.00
Sulfadiazine	100	1	5	117 (18)	118 (22)	102.00	104.00	1.00
Sulfadimethoxine		1	5	94 (21)	86 (12)	101.00	102.00	1.00
Sulfadimidine		1	5	85 (18)	84 (17)	101.00	103.00	1.00
Sulfadoxin		1	5	74 (16)	80 (18)	101.00	103.00	1.00
Sulfamerazine		1	5	86 (12)	92 (19)	101.00	103.00	1.00
Sulfamethoxazole		1	5	70 (20)	72 (19)	102.00	103.00	1.00
Sulfamethoxypyridazine		1	5	93 (19)	102 (19)	101.00	103.00	1.00
Sulfathiazole		1	5	164 (62)	217 (35)	103.00	106.00	1.00
Tetracycline	100	10	50	125 (10)	98 (10)	107.00	115.00	10.00
Tilmicosin	50	5	25	83 (33)	82 (20)	57.00	65.00	5.00
Trimethoprim	50	5	25	103 (17)	105 (14)	55.00	60.00	5.00
Tylosin	50	5	25	7 (21)	7 (24)	56.00	63.00	5.00

(quick, easy, cheap, effective, rugged, and safe) approaches, originally developed as a powerful sample preparation to analyze hundreds of pesticides in fruits and vegetable samples (Anastassiades et al. 2003).

Based on already published work (Aguilera-Luiz et al. 2008; Stubbings and Bigwood 2009) for initial experiments, blank bovine milk samples were fortified with an antibiotic multi-standard mix according to 1/10 of their MRLs. For protein and fat precipitation acidified, ACN (1% formic acid) was used to extract the analytes from the samples. To prevent agglutination of the buffered extraction salts ($MgSO_4$ or Na_2SO_4), the addition of an aliquot of water to obtain a ACN:H_2O ratio of 1:1 is necessary. Instead of water, we added a 0.1 M Na_2EDTA solution to enhance the recovery rates of tetracyclines by avoiding their typical chelate formation (Berendsen and Nielen 2013). The obtained results showed that only 19 of 30 substances could be detected and most of them showed unsatisfying recovery rates below 70%, indicating possible matrix interferences diminishing the ionization of the compounds, which is typical for ESI. Based on these initial results, further clean-up steps were necessary to remove possible matrix interferences in order to achieve better peak shapes and recovery rates for all substances. Therefore, the effects of the addition of various amounts of primary and secondary amine exchange material (PSA) for removing sugars and fatty acids and C18 sorbent (for elimination of nonpolar interferences) were evaluated. The obtained results demonstrated no improvement of the recovery rates. The group of tetracyclines was no more

Table 8 Validation data from all compounds in all matrices. Repeatability is expressed as RSD. Matrix: sour cream, 15% fat content

Compound		Spike concentration (μg/kg)		Recovery (%) and RSD (%)		CCα (μg/kg)	CCβ (μg/kg)	LOQ (μg/kg)
	MRL (μg/kg)	Low level	High level	Spike low concentration	Spike high concentration			
Amoxicillin	4	4	20	56 (12)	59 (14)	9.00	13.00	4.00
Ampicillin	4	4	20	58 (16)	56 (21)	9.00	15.00	4.00
Ceftiofur	100	10	50	36 (17)	35 (19)	113.00	126.00	10.00
Chlortetracycline	100	10	50	100 (12)	82 (21)	118.00	135.00	10.00
Ciprofloxacin	100	5	25	69 (21)	103 (21)	56.00	63.00	5.00
Cloxacillin	30	3	15	11 (20)	9 (16)	34.00	38.00	3.00
Danofloxacin	30	3	15	103 (15)	97 (17)	34.00	38.00	3.00
Doxycycline	no MRL	10	50	94 (21)	92 (14)	14.00	27.00	10.00
Enrofloxacin	100	5	25	119 (13)	119 (8)	53.00	57.00	5.00
Erythromycin A	40	4	20	n.d.	n.d.			
Flumequine	50	5	25	88 (17)	112 (18)	57.00	64.00	5.00
Lincomycin	150	15	75	83 (17)	86 (19)	168.00	186.00	15.00
Marbofloxacin	75	7.5	37.5	63 (19)	84 (15)	83.00	92.00	7.50
Oxytetracycline	100	10	50	79 (19)	65 (16)	113.00	126.00	10.00
Penicillin G	4	2	10	n.d.	n.d.			
Penicillin V	no MRL	5	25	n.d.	n.d.			
Pirlimycin	100	10	50	39 (15)	34 (21)	115.00	131.00	10.00
Spiramycin	200	20	100	110 (32)	132 (20)	231.00	263.00	20.00
Sulfadiazine	100	1	5	106 (14)	118 (21)	102.00	103.00	1.00
Sulfadimethoxine		1	5	98 (17)	89 (16)	101.00	102.00	1.00
Sulfadimidine		1	5	85 (14)	84 (10)	101.00	102.00	1.00
Sulfadoxin		1	5	70 (21)	82 (20)	101.00	103.00	1.00
Sulfamerazine		1	5	86 (13)	83 (17)	101.00	103.00	1.00
Sulfamethoxazole		1	5	42 (17)	60 (13)	101.00	102.00	1.00
Sulfamethoxypyridazine		1	5	96 (11)	96 (16)	101.00	102.00	1.00
Sulfathiazole		1	5	131 (44)	183 (32)	103.00	106.00	1.00
Tetracycline	100	10	50	125 (14)	107 (15)	111.00	122.00	10.00
Tilmicosin	50	5	25	76 (38)	85 (19)	56.00	63.00	5.00
Trimethoprim	50	5	25	113 (20)	101 (10)	54.00	58.00	5.00
Tylosin	50	5	25	5 (14)	7 (20)	55.00	61.00	5.00

detectable and the quinolones danofloxacin and enrofloxacin showed recovery rates of nearly 200%, assuming possible various interactions with co-extracted compounds from the matrix. To assess the signal-influencing matrix effects, the samples were quantified by matrix-matched calibration (MMC). Matrix-matched calibration was carried out by fortifying blank extracts with various amounts of antibiotic mix standard automatically using MassHunter software injection program to achieve five different calibration levels in accordance to 0.01, 0.02, 0.04, 0.1, and 0.25 of the MRLs. Results demonstrated in a slight increase of the recovery rates as well as in removing the signal-enhancing effects on quinolones. However, the recovery values were not improved to a satisfying rate assuming that the analytes get lost during extraction and clean-up procedure. Another explanation for the low recovery rates could be different polarities of the compounds, assuming that the analytes partially migrated into the aqueous phase after separation.

Solid-Phase Extraction

To improve ionization of the analytes due to better matrix removal solid-phase extraction (SPE) was tested for sample clean-up. Based on already published studies (Bundesamt für Verbraucherschutz und Lebensmittelsicherheit (BVL) 2011; Galarini et al. 2015; Heller et al. 2006; Stolker et al. 2008), typical SPE cartridges in reversed phase mode (C18 Strata-X, Phenomenex Ltd., Aschaffenburg, Germany) were investigated. Acetonitrile was used as extraction solvent in order

Table 9 Validation data from all compounds in all matrices. Repeatability is expressed as RSD. Matrix: yogurt, 3.5% fat content

Compound		Spike concentration (μg/kg)		Recovery (%) and RSD (%)		CCα (μg/kg)	CCβ (μg/kg)	LOQ (μg/kg)
	MRL (μg/kg)	Low level	High level	Spike low concentration	Spike high concentration			
Amoxicillin	4	4	20	61 (14)	65 (16)	9.00	14.00	4.00
Ampicillin	4	4	20	58 (31)	61 (17)	9.00	15.00	4.00
Ceftiofur	100	10	50	69 (12)	70 (12)	109.00	117.00	10.00
Chlortetracycline	100	10	50	88 (22)	86 (11)	109.00	118.00	10.00
Ciprofloxacin	100	5	25	73 (9)	89 (18)	57.00	64.00	5.00
Cloxacillin	30	3	15	55 (17)	56 (19)	34.00	39.00	3.00
Danofloxacin	30	3	15	105 (7)	94 (13)	33.00	35.00	3.00
Doxycycline	no MRL	10	50	84 (20)	82 (15)	14.00	28.00	10.00
Enrofloxacin	100	5	25	113 (18)	114 (10)	54.00	58.00	5.00
Erythromycin A	40	4	20	n.d.	n.d.			
Flumequine	50	5	25	86 (11)	110 (8)	53.00	57.00	5.00
Lincomycin	150	15	75	86 (20)	89 (17)	167.00	184.00	15.00
Marbofloxacin	75	7.5	37.5	66 (22)	93 (21)	86.00	98.00	7.50
Oxytetracycline	100	10	50	107 (19)	80 (16)	115.00	130.00	10.00
Penicillin G	4	2	10	125 (42)	198 (86)	13.00	23.00	2.00
Penicillin V	no MRL	5	25	63 (22)	64 (11)	6.00	12.00	5.00
Pirlimycin	100	10	50	78 (18)	82 (21)	116.00	132.00	10.00
Spiramycin	200	20	100	107 (38)	105 (25)	239.00	279.00	20.00
Sulfadiazine	100	1	5	102 (18)	113 (20)	102.00	103.00	1.00
Sulfadimethoxine		1	5	93 (12)	94 (15)	101.00	102.00	1.00
Sulfadimidine		1	5	86 (18)	84 (9)	101.00	102.00	1.00
Sulfadoxin		1	5	73 (13)	75 (16)	101.00	102.00	1.00
Sulfamerazine		1	5	87 (13)	91 (13)	101.00	102.00	1.00
Sulfamethoxazole		1	5	63 (10)	66 (16)	101.00	103.00	1.00
Sulfamethoxypyridazine		1	5	95 (14)	93 (20)	101.00	103.00	1.00
Sulfathiazole		1	5	149 (44)	153 (37)	103.00	106.00	1.00
Tetracycline	100	10	50	118 (11)	91 (12)	109.00	118.00	10.00
Tilmicosin	50	5	25	79 (42)	86 (17)	56.00	62.00	5.00
Trimethoprim	50	5	25	103 (12)	97 (7)	53.00	55.00	5.00
Tylosin	50	5	25	20 (10)	23 (19)	55.00	60.00	5.00

to get a clean supernatant preventing clogging of the cartridges. To ensure retention of the analytes on the C18 material of the cartridge, the supernatant was diluted ten times with water. After conditioning the cartridge with methanol and water, the diluted sample extract was applied to the SPE column, followed by washing the cartridge with water and elution of the analytes with methanol. However, only 21 out of 30 substances could be extracted and only nine of them showed recovery rates between 70 and 120%. To improve the recoveries especially for tetracyclines, the effect of a Na_2EDTA in the extraction solution was assessed. With this step, recovery rates for tetracyclines were three times higher than with acetonitrile, but unfortunately, the number of detected analytes did not increase. For a better explanation of the low recovery rates, the samples were quantified with matrix-matched calibration resulting in better recoveries for nearly all substances, except in case of tetracyclines and macrolides. In addition, possible losses of analytes during washing step were assessed and the elution step was conducted twice to evaluate if the volume of elution solvent was sufficient. Therefore, the washing solution was analyzed and it was noticed that sulfadiazine was partially getting lost during washing step. Tetracyclines were also found after second elution indicating that tetracyclines retained in the stationary phase maybe explaining the low recoveries. Due to the fact that not all substances were detectable and that SPE might be more time consuming, another sample preparation was examined.

Table 10 Validation data from all compounds in all matrices. Repeatability is expressed as RSD. Matrix: curd, 20% fat in dry matter

Compound		Spike concentration (μg/kg)		Recovery (%) and RSD (%)		CCα (μg/kg)	CCβ (μg/kg)	LOQ (μg/kg)
	MRL (μg/kg)	Low level	High level	Spike low concentration	Spike high concentration			
Amoxicillin	4	4	20	47 (10)	53 (9)	7.00	9.00	4.00
Ampicillin	4	4	20	72 (16)	56 (15)	9.00	14.00	4.00
Ceftiofur	100	10	50	63 (8)	55 (18)	113.00	126.00	10.00
Chlortetracycline	100	10	50	96 (16)	93 (9)	107.00	114.00	10.00
Ciprofloxacin	100	5	25	69 (17)	87 (19)	57.00	63.00	5.00
Cloxacillin	30	3	15	5 (42)	5 (17)	34.00	38.00	3.00
Danofloxacin	30	3	15	101 (10)	77 (17)	34.00	38.00	3.00
Doxycycline	no MRL	10	50	87 (21)	81 (15)	15.00	31.00	10.00
Enrofloxacin	100	5	25	111 (21)	117 (18)	57.00	64.00	5.00
Erythromycin A	40	4	20	n.d.	n.d.			
Flumequine	50	5	25	86 (11)	116 (9)	53.00	57.00	5.00
Lincomycin	150	15	75	81 (11)	83 (16)	165.00	181.00	15.00
Marbofloxacin	75	7.5	37.5	76 (20)	82 (11)	81.00	88.00	7.50
Oxytetracycline	100	10	50	84 (12)	72 (13)	111.00	122.00	10.00
Penicillin G	4	2	10	27 (162)	56 (145)	22.00	40.00	2.00
Penicillin V	no MRL	5	25	14 (32)	13 (38)	21.00	42.00	5.00
Pirlimycin	100	10	50	45 (16)	50 (12)	108.00	116.00	10.00
Spiramycin	200	20	100	84 (23)	114 (17)	225.00	249.00	20.00
Sulfadiazine	100	1	5	97 (18)	116 (21)	102.00	103.00	1.00
Sulfadimethoxine		1	5	91 (14)	81 (8)	101.00	101.00	1.00
Sulfadimidine		1	5	82 (16)	87 (18)	102.00	103.00	1.00
Sulfadoxin		1	5	82 (14)	76 (15)	101.00	102.00	1.00
Sulfamerazine		1	5	89 (21)	83 (17)	101.00	103.00	1.00
Sulfamethoxazole		1	5	52 (18)	72 (20)	102.00	103.00	1.00
Sulfamethoxypyridazine		1	5	87 (19)	96 (13)	101.00	102.00	1.00
Sulfathiazole		1	5	134 (47)	144 (29)	103.00	105.00	1.00
Tetracycline	100	10	50	117 (7)	103 (16)	112.00	124.00	10.00
Tilmicosin	50	5	25	73 (44)	86 (18)	56.00	62.00	5.00
Trimethoprim	50	5	25	98 (13)	105 (11)	54.00	58.00	5.00
Tylosin	50	5	25	8 (18)	9 (16)	54.00	59.00	5.00

Dispersive Solid-Phase Extraction

Dispersive solid-phase extraction (dSPE) is a combination of the easy and fast sample preparation provided by QuEChERS and the SPE principle to bind matrix co-extractives onto sorbents while the compounds of interest are remaining in the extract. In this work, C18 bulk sorbent alone as well as in combination with PSA or zirconia (ZSep) sorbents was examined. Based on already published studies (Lehotay et al. 2012; Schneider et al. 2015) sample preparation with C18 bulk sorbent was initially investigating in bovine milk samples. First results demonstrated that nearly all substances could be detected, except penicillin V, amoxicillin, and ceftiofur assuming that these substances are getting rapidly degraded. Unfortunately, only 12 substances obtained good recovery rates between 70 and 120%. Therefore, matrix effects were evaluated by quantifying with matrix-matched calibration fortifying a blank milk samples at five different concentration levels (0.01, 0.02, 0.04, 0.1, and 0.25 of the MRLs). The recovery rates demonstrated increasing as well as decreasing adjustments which let us to assume that the matrix is not the most important influence factor, subsequently performing quantification by procedure matched calibration (PMC) to eliminate possible influences from the matrix and sample preparation. For this, five blank milk samples were fortified with antibiotic standard mix at different concentrations (0.01, 0.02, 0.04, 0.1, and 0.25 of the MRLs) before sample preparation. In accordance with this quantification, acceptable recovery rates were obtained for all substances (Fig. 2). However, the application of procedure matched calibration

Table 11 Validation data from all compounds in all matrices. Repeatability is expressed as RSD. Matrix: soft cheese, 55% fat in dry matter

Compound		Spike concentration (μg/kg)		Recovery (%)		CCα	CCβ	LOQ
	MRL (μg/kg)	Low level	High level	Spike low concentration	Spike high concentration	(μg/kg)	(μg/kg)	(μg/kg)
Amoxicillin	4	4	20	n.d.	n.d.			
Ampicillin	4	4	20	n.d.	n.d.			
Ceftiofur	100	10	50	n.d.	n.d.			
Chlortetracycline	100	10	50	80 (20)	81 (10)	108.00	117.00	10.00
Ciprofloxacin	100	5	25	75 (21)	80 (16)	57.00	63.00	5.00
Cloxacillin	30	3	15	n.d.	n.d.			
Danofloxacin	30	3	15	93 (17)	71 (17)	34.00	37.00	3.00
Doxycycline	no MRL	10	50	123 (22)	92 (18)	21.00	42.00	10.00
Enrofloxacin	100	5	25	173 (28)	123 (13)	57.00	63.00	5.00
Erythromycin A	40	4	20	n.d.	n.d.			
Flumequine	50	5	25	89 (17)	113 (14)	55.00	60.00	5.00
Lincomycin	150	15	75	87 (16)	93 (17)	166.00	182.00	15.00
Marbofloxacin	75	7.5	37.5	56 (22)	80 (15)	83.00	90.00	7.50
Oxytetracycline	100	10	50	74 (20)	83 (18)	113.00	126.00	10.00
Penicillin G	4	2	10	n.d.	n.d.			
Penicillin V	no MRL	5	25	n.d.	n.d.			
Pirlimycin	100	10	50	11 (15)	18 (20)	113.00	127.00	10.00
Spiramycin	200	20	100	107 (36)	59 (11)	232.00	264.00	20.00
Sulfadiazine	100	1	5	103 (16)	117 (19)	101.00	103.00	1.00
Sulfadimethoxine		1	5	89 (21)	76 (18)	101.00	103.00	1.00
Sulfadimidine		1	5	87 (22)	76 (15)	101.00	103.00	1.00
Sulfadoxin		1	5	87 (22)	77 (14)	101.00	102.00	1.00
Sulfamerazine		1	5	89 (16)	94 (15)	101.00	102.00	1.00
Sulfamethoxazole		1	5	72 (17)	74 (19)	101.00	103.00	1.00
Sulfamethoxypyridazine		1	5	83 (20)	94 (20)	101.00	103.00	1.00
Sulfathiazole		1	5	173 (47)	214 (34)	103.00	106.00	1.00
Tetracycline	100	10	50	99 (18)	95 (15)	111.00	121.00	10.00
Tilmicosin	50	5	25	69 (54)	87 (21)	57.00	64.00	5.00
Trimethoprim	50	5	25	94 (15)	108 (13)	55.00	59.00	5.00
Tylosin	50	5	25	5 (12)	5 (32)	60.00	69.00	5.00

for various kinds of matrices is not suitable in a routine laboratory, indicating that the use of internal standard substances is essential. In order to confirm these findings, this sample preparation technique was additionally tested in further dairy products using ISTD mix solution to correct procedure and matrix effects. For optimization of the extraction solution, the already described positive effect of Na_2EDTA on tetracyclines was tested, receiving better results for all tetracyclines compared to the extraction only with water. With reference to the main components of dairy products, experiments were additionally carried out with C18/ZSep (usually used for fatty samples), and C18/PSA (for removing proteins) in expectation to reduce matrix interferences more effectively. Sample clean-up with the combination C18/ZSep showed recovery rates near 100% for sulphonamides and quinolones, but low recovery rates for macrolides/lincosamides and tetracyclines. Furthermore, the quality of the peak shapes for quinolones and tetracyclines was decreased. The combination C18/PSA indicated mean recovery rates of nearly 100% for all compounds in all matrices. Nevertheless, quinolones and tetracyclines showed a decrease in their peak shapes and it was not possible to analyze doxycycline.

It could be observed that in the matrices buttermilk and curd, the compounds of the group of β-lactams showed low recovery rates, whereas in the cheesy matrices, ß-lactams were not detectable anymore. This could be explained by the interaction of the antibiotics and the still present bacteria in the matrix, regarding that different dSPE salts for fat or protein removal could not increase the recoveries.

Table 12 Validation data from all compounds in all matrices. Repeatability is expressed as RSD. Matrix: hard cheese, 55% fat in dry matter

Compound		Spike concentration (μg/kg)		Recovery (%) and RSD (%)		CCα	CCβ	LOQ
	MRL (μg/kg)	Low level	High level	Spike low concentration	Spike high concentration	(μg/kg)	(μg/kg)	(μg/kg)
Amoxicillin	4	4	20	n.d.	n.d.			
Ampicillin	4	4	20	n.d.	n.d.			
Ceftiofur	100	10	50	n.d.	n.d.			
Chlortetracycline	100	10	50	83 (9)	95 (21)	115.00	131.00	10.00
Ciprofloxacin	100	5	25	58 (21)	78 (21)	57.00	64.00	5.00
Cloxacillin	30	3	15	n.d.	n.d.			
Danofloxacin	30	3	15	93 (21)	67 (10)	33.00	35.00	3.00
Doxycycline	no MRL	10	50	94 (17)	95 (18)	17.00	33.00	10.00
Enrofloxacin	100	5	25	107 (10)	118 (5)	52.00	54.00	5.00
Erythromycin A	40	4	20	n.d.	n.d.			
Flumequine	50	5	25	85 (9)	113 (9)	53.00	57.00	5.00
Lincomycin	150	15	75	94 (12)	99 (17)	167.00	184.00	15.00
Marbofloxacin	75	7.5	37.5	69 (20)	79 (16)	85.00	96.00	7.50
Oxytetracycline	100	10	50	100 (28)	88 (22)	118.00	137.00	10.00
Penicillin G	4	2	10	n.d.	n.d.			
Penicillin V	no MRL	5	25	n.d.	n.d.			
Pirlimycin	100	10	50	38 (13)	56 (25)	125.00	150.00	10.00
Spiramycin	200	20	100	100 (23)	73 (37)	228.00	256.00	20.00
Sulfadiazine	100	1	5	95 (21)	110 (16)	102.00	104.00	1.00
Sulfadimethoxine		1	5	112 (15)	102 (25)	102.00	103.00	1.00
Sulfadimidine		1	5	77 (17)	82 (19)	101.00	103.00	1.00
Sulfadoxin		1	5	78 (15)	73 (15)	101.00	103.00	1.00
Sulfamerazine		1	5	90 (18)	89 (18)	102.00	103.00	1.00
Sulfamethoxazole		1	5	99 (14)	77 (19)	101.00	103.00	1.00
Sulfamethoxypyridazine		1	5	95 (20)	92 (15)	101.00	101.00	1.00
Sulfathiazole		1	5	218 (57)	207 (9)	104.00	107.00	1.00
Tetracycline	100	10	50	100 (23)	102 (138)	114.00	129.00	10.00
Tilmicosin	50	5	25	107 (38)	85 (44)	58.00	66.00	5.00
Trimethoprim	50	5	25	107 (18)	101 (21)	55.00	60.00	5.00
Tylosin	50	5	25	10 (15)	11 (20)	53.00	57.00	5.00

Quantification of the Final Method

Quantification was performed by using a solvent standard calibration curve including the same concentration of internal standard as the samples were fortified described in section 2.4. For compounds without corresponding ISTD, a factor of 0.5 must be recognized.

Validation

Validation data (Tables 3, 4, 5, 6, 7, 8, 9, 10, 11, 12) provided the best results for trueness (recovery rates between 70 and 120%) and repeatability (RSD < 20%) for nearly all substances in the matrices raw milk, pasteurized milk, cream, butter, and yogurt. The most problematic substances with high recovery rates as well as high standard deviations were erythromycin A and penicillin G. In yogurt and curd, it was not possible to analyze erythromycin A. The compounds sulfathiazole and tilmicosin were providing recovery rate up to 200% and high standard deviations (up to 60%) in nearly all low spiked sample matrices.

The matrices sour cream and buttermilk showed decreased recovery rates for the substances of the group of β-lactams, whereas penicillin G and penicillin V, as well as erythromycin A could not be detected anymore.

Cheese demonstrated to be the most problematic matrix since the whole compound group of β-lactams was not detectable.

Sample preparation and measurement were controlled by analyzing a certified reference material (CRM) (from Progetto Trieste: code: MI1321-1/CM bovine raw milk incurred with oxytetracycline, code: MI1414_1/CM bovine raw milk incurred with doxycycline) simultaneously with each working batch. Results were always within satisfactory range, assuming that the sample preparation and LC/MS-MS measurement were valid.

Conclusions

In this work, a multi-class UHPLC-MS/MS method was presented to determine 30 veterinary drug residues from various antibiotic groups in different kinds of dairy products. The requirement to establish a sample preparation which is suitable in routine laboratory work enforces an easy and fast sample preparation technique. Therefore, several techniques were investigated (modified QuEChERS, SPE, dSPE) and compared concerning their easiness of handling and recovery rates of the compounds. The best combination in regard of the above mentioned requirements was achieved by sample preparation using C18 bulk sorbent for sample clean-up. With this developed sample preparation procedure, a sample throughput of about 20 samples in 4 h could be achieved.

The investigated dairy matrices were raw bovine milk, pasteurized bovine milk, cream, butter, buttermilk, sour cream, curd, yogurt, soft cheese, and hard cheese. Validation of the proposed method was fulfilled for all matrices in accordance to the European Commission EG 2002/657. The applicability of the method was confirmed by measuring naturally incurred certified reference material successfully. Validation data showed good recovery rates and repeatability for nearly all compounds in all matrices demonstrating a successful validated method to carry out routine analysis of veterinary drug residues in dairy products in accordance to their set MRLs.

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Compliance with Ethical Standards

Conflict of Interest Barbara Schwaiger declares that she has no conflict of interest. Céline Lesueur declares that she has no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed Consent Not applicable.

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