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Determination of veterinary antibiotics in cow milk using rotating-disk sorptive extraction and liquid chromatography

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ABSTRACT

A novel application of rotating disk sorptive extraction (RDSE), using a styrene-divinylbenzene (S-DVB) sorbent for the extraction of antibiotic residues in milk was developed. The analytes studied were oxytetracycline and its 4-epimer, enrofloxacin, ciprofloxacin, sulfadoxine and trimethoprim. After RDSE, the analytes were determined by performing both high-performance liquid chromatography coupled to diode array detection (HPLC-DAD) and ultrahigh-performance liquid chromatography coupled to ultraspray-electrospray-time of flight-mass spectrometry (UPLC-TOF/MS).

By using HPLC-DAD, the absolute recoveries were between 85.5% and 106.4% with relative standard deviations between 3.7% and 9.9%. The obtained limits of quantification (LOQs) were lower than the respective maximum residue levels (MRLs) reported for each analyte, demonstrating that the methodology was applicable for residue depletion studies. UPLC-TOF/MS showed absolute recoveries from 88.5% to 114.1%, with RSDs between 4.3% and 15.4%. The LOQs obtained using UPLC-TOF/MS were also lower than the MRLs for each respective analyte.

Compared with other analytical methods previously reported for some of the analytes, the present method is simpler and less expensive and utilizes green chemistry, all while providing comparable figures of merit.

1. Introduction

Currently, the use of antibiotics in the veterinary industry is of the utmost importance for the control of diseases in animals that produce meat and milk in order to maintain a high level of productivity. However, there is great concern among consumers about the possible persistence of residual antibiotics in these foods [1].

Specific concerns have been raised for the presence of residual antibiotics in cow milk since the consumption of dairy products has increased and, at present, the dangers caused by long term exposure to these residual antibiotics have not been objectively determined. The potential for residual antibiotics in milk to create bacterial resistance or induce hypersensitivity to antibiotics are particularly worrisome. In addition, the heating or pasteurization of milk does not affect the content of an antibiotic, in many cases only the microbial activity is affected by pasteurization [2,3].

International organizations, such as the Food and Agriculture Organization of the United Nations (FAO) through the Codex Alimentarius and the European Medicine Agency (EMA), have established maximum acceptable levels for the concentrations of residual antibiotics in food, defining the maximum residue limit (MRL) as the maximum acceptable concentration of a residual drug in a food of animal origin that is intended for human consumption. In the case of milk, the MRLs are stricter than other tissues of animal origin intended for consumption, so it is necessary to develop analytical methods that allow for preconcentrate analytes and can reach sensitivities that enable accurate and precise determination of the concentrations of residual antibiotics. This will ensure that the regulations set by the FAO are met through residual depletion studies and milk quality controls [4,5]. The low MRLs established for milk imply that products have long withdrawal periods, which affects production considerably. Consequently, the development of new pharmaceutical formulations is the main method used to reduce withdrawal periods [6].

In this study, four antibiotics have been selected which are used in milk producing cows: oxytetracycline (OTC, ((4*S*,4a*R*,5*S*,5a*R*,6*S*,12a*S*)-4-(dimethylamino)-3,5,6,10,11,12a-hexahydroxy-6-methyl-1,12-dioxo-

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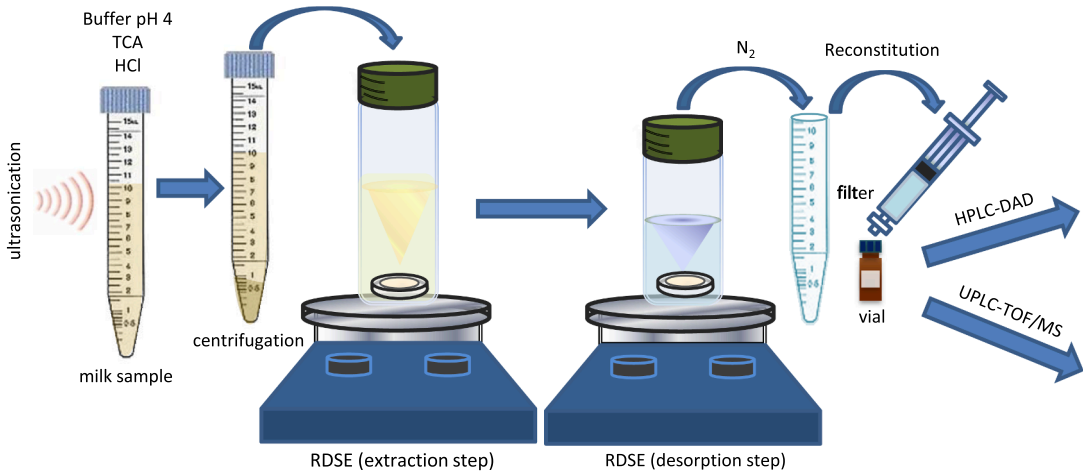

Fig. 1. Schematic procedure for the preparation of milk samples.

1,4,4a,5,5a,6,12,12a-octahydrotetracene-2carboxamide), enrofloxacin (EFX, 1-cyclopropyl-7-(4-ethylpiperazin-1-yl)-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid), sulfadoxine (SDX, 4-amino-*N*-(5,6-dimethoxy-4-pyrimidinyl)benzenesulfonamide) and trimethoprim (TMP, 5-(3,4,5-trimethoxybenzyl)pyrimidine-2,4-diamine). Each of these antibiotics are bacteriostatic and broad-spectrum bactericidal. In addition, the active metabolites ciprofloxacin (CFX, 1-cyclopropyl-6-fluoro-4-oxo-7-(piperazin-1-yl)-quinoline-3-carboxylic acid) and epimer (4E-OTC, 4-(dimethylamino)-1,5,6,10,11,12a-hexahydroxy-6-methyl-3,12-dioxo-3,4,4a,5,5a,6,12,12a-octahydrotetracene-2-carboxamide) were also included in this study (Fig. A1).

Dairy cows are commonly administered these types of antibiotics for the treatment of respiratory infections (laryngitis, bronchopneumonia, pneumonia and nonspecific), urogenital infections (pyelitis, cystitis and puerperal mastitis) and for general and local infections (septicemia, mastitis, wounds and joints) [7].

These analytes were previously detected using multiresidue approaches in bovine milk by liquid chromatography-tandem mass spectrometry using different sample preparation techniques, including: liquid–liquid extraction (LLE) [8–10], QuEChERs [11–13], solid phase extraction (SPE) using Strata-X [14] and Oasis® HLB cartridges [15–17], dispersive-solid phase extraction (d-SPE) [18] and magnetic-SPE [19].

In this study, a novel and simple application of rotating-disk sorptive extraction (RDSE) is demonstrated for the preparation of bovine milk samples for the determination of antibiotics. RDSE is an analytical strategy that has already been used for testing matrices of animal origin such as plasma or urine [20–22], however, this is the first time that the application of RDSE for the analysis of milk has been reported. The final determination of the analytes was performed by using liquid chromatography coupled alternatively to diode arrays (HPLC-DAD) or ultraelectrospray-time of flight-mass spectrometry detectors (UPLC-TOF/MS) (Fig. 1).

The use of RDSE for the extraction of antibiotics in bovine milk can result in faster, cheaper and more eco-efficient analysis with comparable figures of merit to the alternative methods that have been reported previously in the literature. The coupling of RDSE with liquid chromatography provides detection limits low enough to detect/quantify antibiotic analytes in real samples.

2. Material and methods

2.1. Chemicals

The standards OTC, 4E-OTC, EFX, CFX, TMP and SDX from Sigma-Aldrich (St. Louis, MO, USA) were used. Methanol, acetonitrile, hydrochloric acid, EDTA disodic salt, formic acid, citric acid, sodium monoacid sulfate, trifluoroacetic acid and trichloroacetic acid were provided by de Merck KGaA (Darmstadt, Germany). Styrene-divinylbenzene (S-DVB) was used as the sorptive phase and was purchased to United Chemical Technologies (Horsham, PA, USA). Other sorptive phases tested were: silica-octyl (C8), silica-octadecyl (C18), silica-cyanopropyl (CN-P), Florisil (FLO), Oasis HLB (HLB) and silica (Sil), also provided by United Chemical Technologies.

2.2. Sample preparation

2.2.1. HPLC-DAD

Samples of commercial milk were used for optimization and validation experiments. Samples were fortified with the six analytes at concentrations of 200 μg kg^{-1} for the optimization tests. For the validation experiments, the samples were fortified at concentrations of 50–300 μg kg^{-1} using a linear range.

An aliquot of 9 mL of McIlvaine buffer (pH 4) in the presence of EDTA (0.0475 mol L^{-1}) was added to 4.0 mL of each milk sample, followed by the addition of 0.5 mL of 50% (w/v) trichloroacetic acid and then acidification was performed using 60 μL of 6 mol L^{-1} hydrochloric acid. The milk samples were centrifuged at 4400 rpm for 5 min at 4 °C and the supernatants were diluted with 5 mL of water to be treated by RDSE.

2.2.2. UPLC-TOF/MS

A similar sample preparation procedure was used for the UPLC- TOF/MS measurements, except the sample volume was 8.0 mL. In this case, the samples were fortified in the range 5–50 μg kg^{-1} for TMP, CFX and EFX and in the range 50–500 μg kg^{-1} for SDX and OTC, due to the lower sensitivity for these analytes.

2.2.3. Rotating disk sorptive extraction procedure

The extraction device for RDSE was a Teflon disk 1.5 cm in diameter that contained an implanted magnetic bar (nickel-coated Micro Stir bar, Ningbo Xinghan Trading Co., LTD, China). The disk had a cavity on one face in which 50 mg of styrene–divinylbenzene sorbent was loaded. The cavity was covered with a fiberglass filter (1.4 cm diameter, 0.38 μm mean pore size) and then sealed with a Teflon ring. The sorptive phase of the disk was conditioned prior to each extraction by rotating the disk in 5 mL of methanol followed by 5 mL of deionized water for 5 min each. The methanol aliquot was reused for the conditioning of all the disks.

The supernatants obtained after centrifugation were diluted with 5 mL of water to be treated by RDSE. The conditioned disks were placed inside the sample vials and then rotated at 2000 rpm for 90 min in a multiposition magnetic stirrer (Heidolph Instruments, Germany), at room temperature. After each extraction, the disks were placed into new

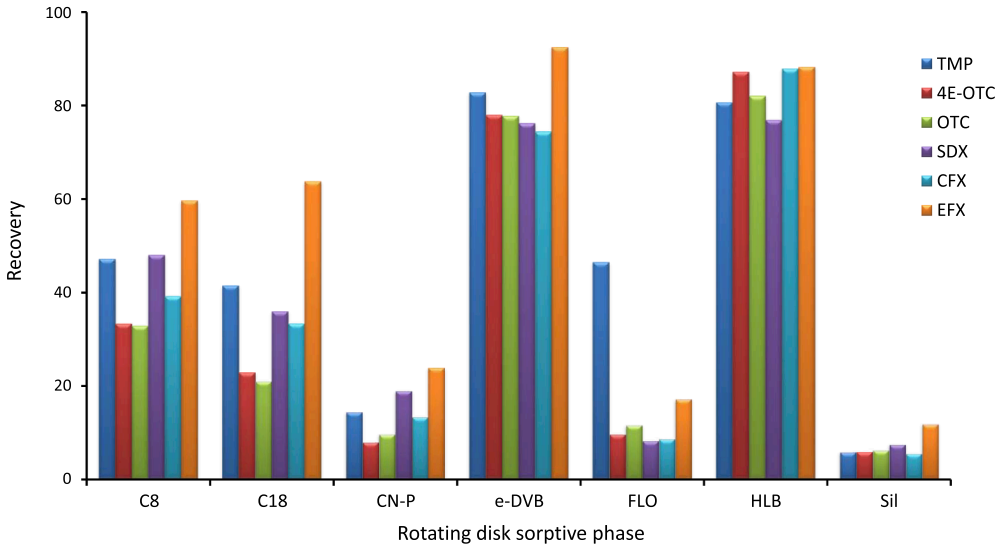

Fig. 2. Extraction efficiency of the different sorptive phases used in RDSE for the studied analytes (analyte concentration:100 μg kg^{-1}, extraction time 90 min).

vials containing 8 mL of methanol (desorbing agent) and then rotated for 30 min at 2000 rpm. The extract containing the analytes was then evaporated to dryness with a N_2 stream. The extract was redissolved in 1 mL of methanol and then mixed with either 1 mL of 0.2% (v/v) trifluoroacetic acid or 1 mL of 0.1 mol L^{-1} formic acid for HPLC-DAD and UPLC-TOF/MS, respectively. Finally, each solution was filtered with a 13 mm, 0.22 μm PVDF filter and then transferred to amber chromatographic vials.

2.3. Quantification of OTC, 4E-OTC, EFX, CFX, TMP and SDX

2.3.1. HPLC-DAD

Quantification was performed using a LaChrom Elite® HPLC System (Hitachi, Tokyo, Japan) equipped with a RP-C18 column 250 mm × 4.6 cm × 5 μm in size (Symmetry) and a L-2450 diode array detector (DAD) (Hitachi) (wavelength of 260–380 nm used for identification). 280 nm was the wavelength used for the simultaneous quantification of OTC, 4E-OTC, EFX, CFX, SDX and TMP over the concentration range of 50–300 μg kg^{-1} (this range contains the MRLs of each species analyzed). The mobile phase was 0.2% (v/v) trifluoroacetic acid/methanol/acetonitrile (20:3:2), the flow rate was 1.0 mLmin^{-1}, the injection volume was 100 μL, the oven temperature was 35 °C, and the analysis time was 24 min (Fig. A2).

2.3.2. UPLC-TOF/MS

UPLC-TOF/MS measurements were performed using a Flexar FX-15 ultrahigh-Performance Liquid Chromatography system coupled to an AxION 2 TOF-MS time of flight mass spectrometer system equipped with a dual-probe Ultraspray Electrospray Ionization Source (UESI) interface. The system had a binary pump system, a vacuum degasser, a cooling autosampler and a thermostat column compartment that was controlled by Chromera software and PerkinElmer TOF MS Driver software (PerkinElmer, MA, USA). Chromatographic separation was performed on a UPLC Brownlee HRes DB BiPh column that was 50 mm × 2.1 cm × 1.9 μm in size (Perkin Elmer®, USA). The column temperature was maintained at 25 °C and the injection volume was 10 μL. A 50:50 mobile phase (isocratic mode) of 1% formic acid (A) and acetonitrile (B) was used at a flow rate of 0.35 mL/min.

The MS measurement conditions were set as follows: drying gas temperature 300 °C; drying gas flow rate 10 L/min; nebulizer gas pressure 80 psi; capillary voltage 4000 V; nozzle voltage −125 V in positive ion mode; and the measured mass range was from $m/z = 100$ to 1000 Da.

The delta mass was 20 ppm for each compound. To improve the sensitivity of the analysis, the acquisition function was performed in trap mode, setting the parameters "IG Exit Low" and "Trap/Pulse Delay" at 10 and 33 μs, respectively.

3. Results and discussion

3.1. Selection of sorptive phase

A comparison of seven sorptive phases of diverse polarity (C8, C18, CN-P, S-DVB, FLO, HLB, Sil) was made using RDSE to perform 90 min of extraction for the six analytes from a solution of McIlvaine buffer (pH 4) in the absence of the milk matrix. As seen in Fig. 2, higher retention capability was achieved when HLB and S-DVB were used. According to these results the strongest intermolecular interactions between the sorptive phase and the analytes should be hydrophobic forces involving pi-stacking. The use of phases more hydrophilic or hydrophobic than either HLB or S-DVB resulted in lower retention of the studied analytes.

In the presence of the milk matrix, good performance was also obtained with S-DVB and HLB, however, S-DVB was selected because a better baseline and lower interference was observed in the chromatograms of S-DVB.

3.2. Experimental design for the optimization of the method

Preliminary assays applied to milk showed recoveries higher than 80% for TMP, CFX and EFX and lower than 70% for 4E-OTC, OTC and SDX. Considering that at the pH of the buffer that was used, 4E-OTC, OTC and SDX are in noncharged forms, and TMP, CFX and EFX are in ionic forms, it is possible that a loss of the noncharged species occurred due to affinity of the analytes with the proteins and/or milk fat that were centrifuged. However, it is not convenient to acidify the samples before adding EDTA since EDTA is stable at pH 4. For this reason, a new strategy where the pH 4 buffer (containing EDTA) was added to the milk samples, which were then acidified with 6 mol L^{-1} HCl, shaken, sonicated and then centrifuged for subsequent extraction by RDSE.

In parallel, the effects of the added volumes of both 6 mol L^{-1} HCl (to solubilize the analytes) and 50% w/V TCA (to precipitate the proteins) were studied by using a central compound design (2 K + 2 K + 2), that was centered on the faces, including the two centers. The following combinations were tested; 6 mol L^{-1} HCl (20, 40 and 60 μL) and 50% w/v TCA (0, 250 and 500 μL). The results of the 10 experiments showed there were significant effects for the six analytes (95% confidence level)

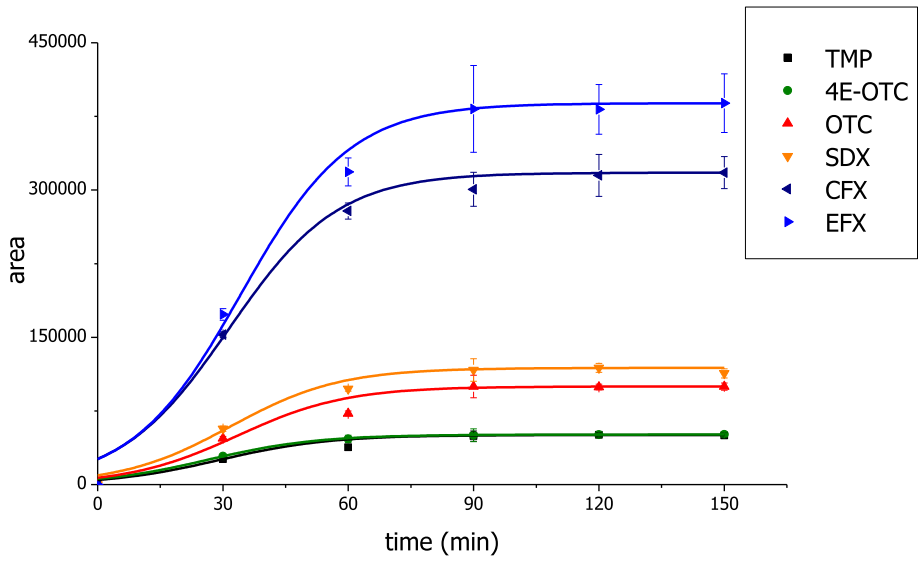

Fig. 3. Extraction profile for the studied analytes (concentration: 100 μg kg^{-1}).

Table 1
Validation parameters of the analytical method using two techniques.

Analyte	Technique* [Linear Range, μg kg^{-1}]	Recovery (%) n = 6		RDS (%) n = 6		Slope n = 5		R^2 n = 5		LOD (μg kg^{-1})	LOQ (μg kg^{-1})	MRL (μg kg^{-1})
		Low**	High***	Low**	High***	Standard	Fortified	Standard	Fortified	n = 6	n = 6	
TPM	1 [50–300]	102.8	103.6	9.9	5.3	297.8	293.8	0.997	0.996	11.0	33.4	50
	2 [5–50]	92.1	99.9	6.5	3.2	8125.1	5299.3	0.998	0.998	0.6	1.9	
SDX	1[50–300]	87.2	94.5	7.9	3.1	686.1	642.0	0.996	0.997	15.4	46.6	100
	2[50–500]	114.1	91.7	4.3	11.7	499.1	300.4	0.999	0.995	36.5	76.0	
CFX	1[50–300]	100.5	97.8	7.9	4.1	1916.0	1841.3	0.997	0.997	3.0	9.2	100
	2[5–50]	89.5	102.1	9.6	3.2	1302.4	767.0	0.996	0.999	2.4	7.6	
EFX	1[50–300]	106.4	101.4	3.7	4.8	2263.9	2468.7	0.997	0.997	3.5	10.7	
	2[5–50]	98.5	101.4	7.9	3.9	4232.5	3160.8	0.991	0.999	1.4	4.4	
OTC	1[50–300]	104.9	98.2	3.9	3.9	966.2	572,7	0.996	0.990	9.1	27.5	100
	2[50–500]	88.5	90.7	15.4	8.8	926.9	636.2	0.992	0.992	16.1	44.3	
4E-OTC	1[50–300]	85.5	100.6	9.8	7.1	324.8	295.2	0.998	0.994	10.9	33.3	
	2	–	–	–	–	–	–	–	–	–	–	

* 1 = HPLC-DAD; 2 = UPLC-TOF/MS.
** Low = lower concentration in the range.
*** High = higher concentration in the range.

(Fig. A3). A combined response surface was obtained for the six analytes through the designed experiment, where the optimal values for each of the variables were obtained by maximizing the desirability function. The optimized quantities were 60 μL of 6 mol L^{-1} HCl and 500 μL of 50% w/v TCA (Fig. A4).

The optimized method resulted in absolute recoveries higher than 80% for the six analytes. Under these conditions, recovery profiles were obtained for different extraction times to establish equilibrium times for the extraction process. Equilibrium was reached at approximately 90 min, as seen in Fig. 3.

3.3. Validation of the method

The analytical methodology was validated according to the criteria set out in the guide VICH GL 49, which establishes that analytical parameters must fulfill a methodology for residue depletion studies in matrices for human consumption [23]. The measurements were performed using HPLC-DAD and the methodology was validated for the concentration range 50–300 μg kg^{-1}. Determination coefficients (R^2) from 0.990 to 0.998 were obtained for the six analytes in milk, with average recoveries for the lowest concentration level (50 μg kg^{-1}) ranging from 85.5% to 106.4%. The relative standard deviation (RSD) with intermediate precision at the lowest concentration level ranged from 3.7% to 9.9% (Table 1). In addition, the limits of quantification (LOD) and detection (LOQ) obtained during the measurements were lower than the reported MRLs for each respective analyte. These results demonstrate that the methodology in this study can be utilized for residue depletion studies.

In parallel, some analytical features were also determined for UPLC-TOF/MS, which has improved the rapidity of testing compared to HPLC-DAD. The methodology was validated over the range of 5–500 μg kg^{-1}, with R^2 values from 0.990 to 0.999 (for five of the six analytes in milk), with an average recovery for the lowest concentration level ranging from 88.5% to 114.1% and RSDs for the lowest concentration ranging from between 4.3% and 15.4% (Table 1). However, the LOQ and LOD were lower than those obtained by HPLC-DAD for TMP, CFX and EFX because good ionization by UESI was observed for these analytes. Lower sensitivity was observed for SDX and OTC due to poorer ionization in the UESI source (Table 1).

Fig. 4 shows an example of the extracted ion chromatograms $[M-H]^+$ and mass spectra that were obtained in UPLC-TOF/MS by fortifying the milk matrix with a multistandard solution containing each of the analytes at a concentration of 100 μg kg^{-1}. The UPLC-TOF/MS measurements had significantly shorter chromatographic run time

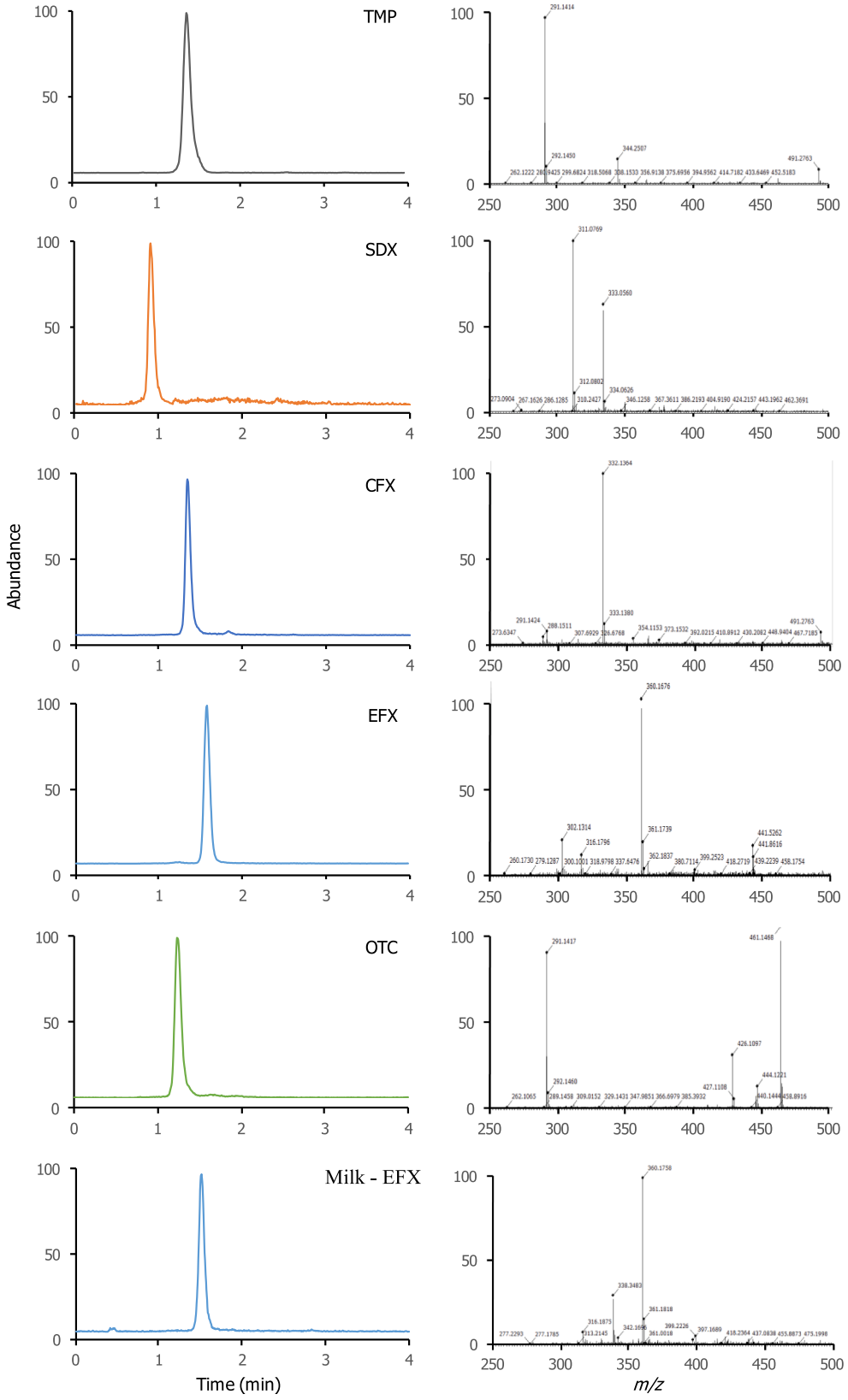

Fig. 4. Left: Extracted ion chromatograms $[M - H]^+$ obtained by UPLC-TOF/MS of each analyte at 100 μg kg^{-1} in a spiked milk sample. Right: Corresponding mass spectrum obtained in TOF mode, $m/z = 291.1414$ (TMP), $m/z = 311.0769$ (SDX), $m/z = 332.1364$ (CFX), $m/z = 360.1676$ (EFX), $m/z = 461.1468$ (OTC). The last chromatogram was obtained from a commercial whole milk sample showing the presence of EFX ($m/z = 360.1758$).

Table 2
Analytical features of different analytical methods reported for the determination of the analytes in milk.

Stages of sample preparation	Technique	Run time (min)	Analyte	Recovery (%)	RDS (%)	LOD (μg kg^{-1})	Ref.
Thawed, protein precipitation, cooling, centrifugation, solvent evaporation, and reconstitution	HPLC-MS/MS	60*	TMP	108	11.2	2.5	[10]
			SDX	99	13.4	2.5	
			CFX	80	8.4	0.5	
			EFX	77	5.5	5.0	
			OTC	95	10.9	5.0	
Thawed, protein precipitation, centrifugation (x2), defatting (x2), solvent evaporation and reconstitution	LC–MS/MS	10	TMP	–	–	–	[9]
			SDX	–	–	–	
			CFX	114.2	9.2	0.2	
			EFX	117.7	9.4	0.1	
			OTC	–	–	–	
Thawed, protein precipitation, centrifugation, and microfiltration	UPLC-TOF-MS/MS	2.5	TMP	–	–	–	[8]
			SDX	–	–	–	
			CFX	–	–	–	
			EFX	–	–	–	
			OTC	72	12.7	0.1	
Protein precipitation, QuEChERS, centrifugation, solvent evaporation, and reconstitution	UPLC-TOF-MS/MS	12	TMP	88.7	7.6	–	[13]
			SDX	81.4	10.5	–	
			CFX	3.2	21	–	
			EFX	23	10.1	–	
			OTC	–	–	–	
Thawed, protein precipitation, centrifugation, QuEChERS, solvent evaporation and reconstitution	UPLC-TOF-MS/MS	10	TMP	–	–	–	[12]
			SDX	–	–	–	
			CFX	98	2.1	0.4	
			EFX	99.6	4.5	0.3	
			OTC	–	–	–	
Protein precipitation, QuEChERS, centrifugation, dispersive-SPE, and microfiltration	UPLC-TOF-MS/MS	10	TMP	88.7	7.6	–	[11]
			SDX	81.4	10.5	–	
			CFX	3.2	21	–	
			EFX	23	10.1	–	
			OTC	–	–	–	
Thawed, solvent extraction (MeCN x2), centrifugation, SPE (Strata™-X), solvent evaporation and reconstitution	HPLC-MS/MS	36	TMP	99	6	–	[14]
			SDX	–	–	–	
			CFX	83	1	–	
			EFX	84	8	–	
			OTC	–	–	–	
Protein precipitation, centrifugation, SPE (Oasis® HLB), solvent evaporation and microfiltration	LC–MS/MS	9	TMP	–	–	–	[17]
			SDX	118.2	11.6	0.01	
			CFX	–	–	–	
			EFX	–	–	–	
			OTC	–	–	–	
Protein precipitation, cooling, centrifugation, SPE (Oasis® HLB), solvent evaporation and reconstitution	HPLC-MS/MS	39	TMP	–	–	–	[15]
			SDX	–	–	–	
			CFX	–	–	–	
			EFX	–	–	–	
			OTC	93.5	6	25.0	
Thawed, protein precipitation (x2), centrifugation, SPE (Oasis® HLB), solvent evaporation and reconstitution	HPLC-MS/MS	31	TMP	–	–	–	[16]
			SDX	–	–	–	
			CFX	–	–	–	
			EFX	–	–	–	
			OTC	88.6	5.6	3.7	
Protein precipitation, centrifugation, filtration, SPE (Oasis® HLB), solvent evaporation and reconstitution	HPLC-MS/MS	25	TMP	100	4.4	–	[24]
			SDX	103	3.7	–	
			CFX	102	6.8	–	
			EFX	98	5.8	–	
			OTC	97	7.2	–	
Protein precipitation, centrifugation (x3), solvent extraction (ammonium formate buffer) and microfiltration	*c*LC-DAD-MS	15	TMP	–	–	–	[25]
			SDX	–	–	–	
			CFX	77	5.3	3.0	
			EFX	96	5	6	
			OTC	–	–	–	
Agitation, heating (90 °C with sand), solid phase dispersion (with hot water) and microfiltration	HPLC-MS/MS	–	TMP	–	–	–	[26]
			SDX	–	–	–	
			CFX	82	11	–	
			EFX	85	9	–	
			OTC	–	–	–	
Protein precipitation, centrifugation, SPE (Strata X), solvent evaporation and reconstitution	UPLC-MS/MS	3.64	TMP	–	–	–	[31]
			SDX	–	–	–	
			CFX	95	8.5	0.1	
			EFX	79	11.5	0.3	
			OTC	–	–	–	
Thawed, protein precipitation, centrifugation, SPE (Oasis® HLB), solvent evaporation, reconstitution and microcentrifugation	HPLC-MS/MS	21	TMP	–	–	–	[27]
			SDX	–	–	–	

(*continued on next page*)

Table 2 (*continued*)

Stages of sample preparation	Technique	Run time (min)	Analyte	Recovery (%)	RDS (%)	LOD (μg kg^{-1})	Ref.
			CFX	–	–	–	
			EFX	70	19	–	
			OTC	60	–	–	
Protein precipitation, centrifugation, solvent evaporation, fat removal (*n*-hexane), SPE (C_{18}), solvent evaporation, reconstitution and microcentrifugation	HPLC-MS/MS	15	TMP	–	–	–	[28]
			SDX	–	–	–	
			CFX	93.9	4	0.2	
			EFX	91.7	3.8	0.2	
			OTC	–	–	–	
Thawed, protein precipitation, centrifugation, microcentrifugation, solvent evaporation and reconstitution	UPLC-TOF/MS	9	TMP	–	–	–	[29]
			SDX	–	–	–	
			CFX	489	7	0.5	
			EFX	353	7	0.5	
			OTC	229	12	10	
Protein precipitation, centrifugation, SPE (Strata X, ENV + Isolute and Oasis MAX), solvent evaporation and reconstitution	HPLC-MS/MS	12	TMP	–	–	–	[30]
			SDX	–	–	–	
			CFX	87	2	1	
			EFX	91	5	0.5	
			OTC	–	–	–	
Thawed, protein precipitation, shaking, centrifugation, dispersive-SPE, solvent evaporation and reconstitution	LC-MS/MS	17	TMP	–	–	12.5	[18]
			SDX	–	–	25.0	
			CFX	–	–	12.5	
			EFX	–	–	12.5	
			OTC	–	–	25.0	
Protein precipitation, centrifugation (x2), cooling, magnetic-SPE, solvent evaporation and reconstitution	HPLC-DAD	10	TMP	96.8	3.8	8.0	[19]
			SDX	–	–	–	
			CFX	–	–	–	
			EFX	–	–	–	
			OTC	89.5	1.2	8.0	
Protein precipitation, centrifugation, RDSE and evaporation	HPLC-DAD	24	TMP	103.6	5.3	11.0	This work
			SDX	94.5	3.1	15.4	
			CFX	97.8	4.1	3.0	
			EFX	101.4	4.8	3.5	
			OTC	98.2	3.9	9.1	
Protein precipitation, centrifugation, RDSE and evaporation	UPLC-TOF/MS	4	TMP	99.9	3.2	0.6	This work
			SDX	91.7	11.7	0.8	
			CFX	102.1	3.2	2.4	
			EFX	101.4	3.9	1.4	
			OTC	90.7	8.8	4.8	

* Three runs of 20 min for each antibiotic family.

compared with the HPLC-DAD measurements (4 min vs 24 min). However, UPLC-TOF/MS was unable to separate the epimer of OTC under UPLC conditions, resulting in the epimer coeluting with OTC.

A comparison of the slopes of the calibration curves obtained in the absence (standards) and presence of the milk matrix indicates that a negative matrix effect between 42% and 25% was obtained for UPLC-TOF/MS.

Although the total time of the RDSE process was of 120 min, the use of two multiposition magnetic stirrers allowed the simultaneous processing of 20 samples in a space of approximately 1 m^2.

3.4. Real sample analysis

The analytical method developed in this study was utilized to determine the analytes in real samples of 10 commercial milk brands in Santiago, Chile. The samples were prepared in triplicate and each sample was injected twice into the UPLC-TOF/MS system. Considering the matrix effect observed for UPLC method, quantification based on matrix-matched calibration was performed.

Fig. 4 shows an example of the extracted ion chromatograms $[M-H]^+$ and mass spectra obtained in TOF mode for the analyzed samples, and only the EFX analyte ($m/z = 360.1758$) was quantified in one of the milk brands. The concentration was found to be 8.5 ± 0.5 μg kg^{-1}, with a mass (m/z) error lower than 10 ppm.

3.5. Comparison with other analytical methods

Table 2 shows a critical comparison of the analytical features of the sample preparation method used in this study with respect to other recently published studies which have involved the determination of antibiotics for veterinary use [8–19,24–30]. The number of steps in the sample preparation in most reported methodologies is between 5 and 6. The methodology in our study has reduced this number down to just few simple steps. According to Table 2, most of the methods reported involve the use of SPE or QuEChERS in the preparation of milk samples, which achieved very good analytical features, however the costs are considerably higher than in this study. On the contrary, some methodologies which do not use an extraction step, after deproteinization, have been reported, achieving also similar figures of merit than the obtained in this study. It should be stressed that almost all the methods reported used liquid chromatography coupled with triple quadrupole mass spectrometry as the determinative technique. Sample preparation through RDSE not only achieved good figures of merit when mass spectrometry is used as chromatographic detector, but also allows the use of a DAD detector, similar to the case of the extraction based on magnetic-SPE [19].

When considering multianalyte determination, the total time of the chromatographic run using UPLC-TOF/MS is much shorter (4 min) with respect to others methods, except for the determination of different antibiotics performed using UPLC-MS/MS [8,31]. However, the method in our study provides recovery, precision and LOD values similar to previous studies. Finally, the present sample preparation method uses an affordable device (rotating disk) that is easily reused, as opposed to the high costs of the cartridges used for SPE or the expensive high purity organic solvents used in solvent extraction. According to the analytical eco-scale proposed by Gałuszka et al. [32], the proposed method using RDSE is excellent as green methodology. Consequently, RDSE is an eco-

efficient methodology with comparable quality and rigor to other analytical methods.

4. Conclusions

A rapid, sensitive, and selective analytical method was developed which can be applied to carry out antibiotic depletion studies in milk to determine the storage time of formulations containing any of the studied analytes. In addition to being a useful tool to perform quality control trials of milk intended for human consumption, the processing time, economy, eco-efficiency and autonomy of the process make RDSE an interesting alternative for routine laboratory use. The combination of RDSE using styrene divinylbenzene with UPLC- TOF/MS made it possible to unequivocally identify and quantify the six analytes investigated in this study to levels below 1 μg kg^{-1}.

CRediT authorship contribution statement

Alver Castillo-Aguirre: Conceptualization, Formal analysis, Validation, Investigation, Writing - original draft. **Alejandro Cañas:** Conceptualization, Formal analysis, Validation, Investigation, Writing - original draft. **Luis Honda:** Supervision, Conceptualization, Validation, Investigation. **Pablo Richter:** Project administration, Funding acquisition, Conceptualization, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.microc.2020.105851.

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