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Original Paper

Development and validation of an HPLC confirmatory method for the determination of seven tetracycline antibiotics residues in milk according to the European Union Decision 2002/657/EC

An HPLC method with diode-array detection, at 355 nm, was developed and validated for the determination of seven tetracyclines (TCs) in milk: minocycline (MNC), TC, oxytetracycline (OTC), methacycline (MTC), demeclocycline (DMC), chlortetracycline (CTC), and doxycycline (DC). Oxalate buffer (pH 4) was used with 20% TCA as a deproteinization agent for the extraction of analytes from milk followed by SPE. The separation was achieved on an Inertsil ODS-3, 5 μ m, 250 $\times$ 4 mm² analytical column at ambient temperature. The mobile phase, a mixture of A: 0.01 M oxalic acid and B: CH₃CN, was delivered using a gradient program. The procedure was validated according to the European Union decision 2002/657/EC determining selectivity, stability, decision limit, detection capability, accuracy, and precision. Mean recoveries of TCs from spiked milk samples (50, 100, and 200 ng/g) were 93.8–100.9% for MNC, 96.8–103.7% for OTC, 96.3–101.8% for TC, 99.4–107.2% for DMC, 99.4–102.9% for CTC, 96.3–102.7% for MTC, and 94.6–102.1% for DC. All RSD values were lower than 8.5%. The decision limits CC_a calculated by spiking 20 blank milk samples at MRL (100 μ g/kg) ranged from 101.25 to 105.84 μ g/kg, while detection capability CC_b from 103.94 to 108.88 μ g/kg.

Keywords: 2002/657/EC / HPLC / Milk / SPE / Tetracyclines / Validation

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1 Introduction

Tetracyclines (TCs) are antibiotics exhibiting activity against infections caused by both Gram-positive and Gram-negative bacteria as well as chlamydia, mycoplasmas, rickettsiae, and protozoan parasites. TCs have a fused, partially aromatic four-ring structure with various substituents. They are amphoteric compounds, soluble in acids, bases, and alcohols. Because of their polar character, TCs bind with proteins and chelate with divalent metal ions. Their chemical structures are given in Fig. 1. Due to their broad-spectrum activity TCs are widely used in animal husbandry not only for prevention and treatment but also to fraudulently promote growth. TC, chlortetracycline (CTC), and oxytetracycline (OTC) are

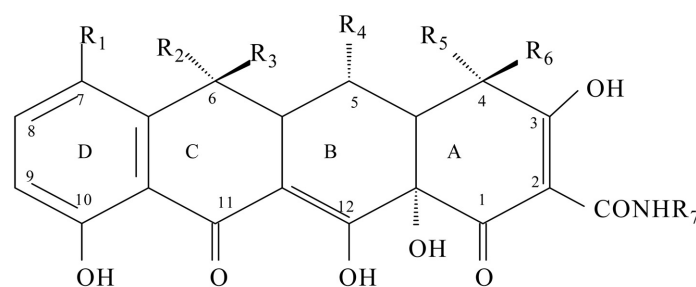
approved by the EU and FDA for therapeutic use in cattle, pigs, sheep, and poultries. Other TCs like minocycline (MNC), doxycycline (DC), demeclocycline (DMC), and methacycline (MTC) are commercially available as human drugs and can also be used for veterinary purposes [1–3].

In order to protect human health from the abundant use of TCs, which may result in the presence of residues in edible animal products, the European Union (EU) has enacted maximum residue levels (MRLs) for the presence of TCs in those products. The use of veterinary drugs in the EU is regulated by Council Regulation. Council Directive 96/23/EC describes the procedure for the establishment of MRLs for veterinary medicinal products in foodstuffs of animal origin. According to this directive TCs are classified in Group B1 (veterinary medicines and contaminants, with an MRL) of Annex I. Different MRL values have been set for TCs in different target tissues: 100 μ g/kg in milk and muscle, 200 μ g/kg in eggs, 300 μ g/kg in liver, and 600 μ g/kg in kidney [4, 5].

Decision 2002/657/EC [6] defines the performance criteria and the interpretation of results for analytical methods in the official control of residues in products of

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Abbreviations: CTC, chlortetracycline; DC, doxycycline; DMC, demeclocycline; MNC, minocycline; MRL, maximum residue level; MTC, methacycline; OTC, oxytetracycline; TC, tetracycline



Compound		Chemical name	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆	R ₇
TC	Tetracycline	2-naphthacenecarboxamide, 4-(dimethylamino)-1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-mono-hydrochloride	H	CH ₃	OH	H	N(CH ₃) ₂	H	H
OTC	Oxytetracycline	5-hydroxy-tetracycline	H	CH ₃	OH	OH	N(CH ₃) ₂	H	H
CTC	Chlortetracycline	7-chloro-tetracycline	Cl	CH ₃	OH	H	N(CH ₃) ₂	H	H
DC	Doxycycline	6-deoxy-5-hydroxy-tetracycline	H	CH ₃	H	OH	N(CH ₃) ₂	H	H
MNC	Minocycline	7-dimethylamino-6-demethyl-6-deoxy-tetracycline	N(CH ₃) ₂	H	H	H	N(CH ₃) ₂	H	H
MTC	Methacycline	6-methylene-5-hydroxy-tetracycline	H	= CH ₂		OH	N(CH ₃) ₂	H	H
DMC	Demeclocycline	6-demethyl-7-chloro-tetracycline	Cl	H	OH	H	N(CH ₃) ₂	H	H

Figure 1. Chemical structures of examined TCs.

animal origin. In this version, some new parameters are included, such as the limit of decision (CC_a) and detection capability (CC_b).

The development of reliable and multiresidue analytical methods to establish whether residues of TCs are present in various matrices has been the target of several analytical studies. Although most of these studies deal with the presence of TCs in edible animal tissues and milk, since an important amount of TCs used in veterinary medicine are consumed in lactating dairy cattle. Microbiological assays are used as screening methods to detect residues of TCs in milk [7–9]. However, these have important disadvantages, such as poor sensitivity and selectivity and they provide semiquantitative results. According to legislation, in order to confirm and quantify the presence of residual TCs in milk chromatographic techniques are required. Among them HPLC coupled with UV [10–13], MS [14–17], or electrochemical detector [18] is the most widely used technique for multi-

residue analysis of TCs in milk. TLC can also be used as a screening method for the analysis of TCs in milk [19, 20].

Isolation of TCs from milk involves precipitation of proteins, an extraction step with a suitable solvent system and a cleanup procedure. Difficulties in isolating these compounds from complex matrices like milk arise from their potency to form chelate complexes with divalent metal ions like calcium and to combine with sample matrix proteins. TCs chemical behavior also depends on pH, as they form various degradation products in very low or very high pH values [21]. Due to these characteristics, a deproteinizing agent and a mild acidic extraction buffer are demanded for the isolation of TCs from milk samples. McIlvaine buffer (citric acid with disodium hydrogen phosphate) [10, 12, 19], succinate [14, 13], and acetate [16] buffers are used as extracting solvents, while TCA [10] and HCl [18] as deproteinizing agents.

In order to reduce the interference and achieve analytes' concentration, the primary sample extract requires

a cleanup procedure prior to the chromatographic analysis. Isolation of TCs from milk is usually followed by an SPE cleanup procedure using RP materials and metal blocking agents [10, 11, 14–17, 19], while liquid–liquid extraction is rarely used [18], as it is time consuming and needs large amounts of organic solvents. Another technique used is metal chelate affinity chromatography (MCAC), based on the chelating ability of TCs. In most of the cases this technique shows poor recoveries, so that an extra SPE step is demanded [13, 16].

The present work describes a simple, rapid, and reliable method for the simultaneous determination and confirmation of seven TCs (MNC, TC, OTC, DMC, CTC, MTC, and DC) in milk. The extraction of analytes from the matrix is performed using oxalate buffer (pH 4) with 20% TCA as a protein precipitation agent and is followed by a simple SPE procedure, using a C₁₈ sorbent. Determination of analytes is then performed by HPLC–diode-array detection. Validation of the method was based on the Commission Decision 2002/657/EC of the EU.

2 Experimental

2.1 Chemicals and reagents

TC and CTC were purchased from Fluka (Buchs, SG, Switzerland), while OTC, MNC, DMC, MTC, and DC from Sigma (St. Louis, MO, USA). Colchicine, used as the internal standard, was supplied by Fluka. HPLC grade methanol and ACN were obtained from Carlo Erba (Milano, Italy). Citric acid monohydrate and sodium citrate trihydrate of analytical grade were supplied by Riedel-de-Haen (Seelze, Germany), whereas oxalic acid, hydrogen sodium phosphate, and Na₂EDTA were purchased from Merck (Darmstadt, Germany). High-purity water obtained by a Milli-Q purification system (Millipore, Bedford, MA, USA) was used throughout the study. Milk samples were supplied from the local market.

Buffer solutions used for the extraction of TCs from milk samples were prepared as follows: citrate buffer (0.4 M, pH 4, 5, or 6) by mixing appropriate volumes of 0.4 M citric acid, 0.4 M sodium citrate and oxalate buffer (0.26 M, pH 4) by mixing 0.4 M oxalic acid and 0.1 M sodium hydroxide.

2.2 Instrumentation

A Shimadzu (Kyoto, Japan) quaternary low-pressure gradient system was used for the chromatographic determination of the examined TCs. The solvent lines were mixed in an FCV-10AL_{VP} mixer. An LC-10AD_{VP} pump was used to deliver the mobile phase to the analytical column, equipped with a Shimadzu SCL-10AL_{VP} System Controller, permitting fully automated operation, used to deliver the mobile phase to the analytical column. Sam-

ple injection was performed *via* a Rheodyne 7725i injection valve (Rheodyne, Cotati, California, USA) equipped with a 20 μ L loop.

Detection was achieved by an SPD-M10A_{VP} Photodiode Array Detector, in compliance with data acquisition software LabSolutions-LCSolutions by Shimadzu. Functions of the whole system were controlled by an SCL-10A_{VP} controller. Degassing of the mobile phase was achieved by a continuous helium sparging in the solvent reservoirs by a DGU-10B degassing unit.

The analytical column, an Inertsil ODS-3, 5 μ m, 250 $\times$ 4 mm², was purchased from MZ-Analysentechnik (Mainz, Germany).

A glass vacuum-filtration apparatus obtained from Alltech Associates was employed for the filtration of the buffer solution, using Whatman cellulose-nitrate 0.2 μ m membrane filters.

A Glasscol small vortexer (Terre Haute, IN, USA) and a Hermle centrifuge, model Z 230 (B. Hermle, Gosheim, Germany) were employed for the pretreatment of milk samples.

The SPE study was performed on a Vac-Elut vacuum manifold column processor, purchased from Analytichem International, Varian (Harbor City, CA, USA). Nexus cartridges (30 mg/cm³) were supplied from Varian, Discovery DSC-18 (500 mg/3 mL) from Supelco (Bellefonte, PA, USA), and Lichrolut (200 mg/3 mL) from Merck. All evaporations were performed with a Supelco 6-port Mini-Vap concentrator/evaporator.

2.3 Chromatography

The separation of the examined TCs was achieved on an Inertsil ODS-3, 5 μ m, 250 $\times$ 4 mm² analytical column, operated at ambient temperature, with observed back-pressure ranging from 240 to 270 kg/cm². The mobile phase, a mixture of 0.01 M oxalic acid as solvent A and CH₃CN as solvent B, was delivered to the analytical column according to a gradient program shown in Table 1. The flow rate was changing during the analysis under the gradient program shown in Table 1. An equilibration time of 3 min was required between runs. The monitoring of the examined TCs was performed at 355 nm.

2.4 Preparation of standards

Stock standard solutions of each TC were prepared in methanol at a concentration of 100 ng/ μ L. These solutions were found to be stable for 2 months, when stored, refrigerated, and wrapped in an aluminum foil. Working methanolic standards were prepared once a week from stocks by the appropriate dilution at 0.5, 0.8, 1, 2, 5, 8, 10, and 15 ng/ μ L. All working standards contained colchicine as the internal standard at a concentration of

Table 1. The gradient program followed for the elution of the examined TCs

<i>t</i> (min)	A: C ₂ H ₂ O ₄ (0.05 M)	B: CH ₃ CN	<i>t</i> (min)	Flow rate (mL/min)
0	88	12	0	1.65
2	80	20	3	1.65
4	73	27	5	1.2
10	70	30		

5 ng/μL. All solutions were protected from light during usage.

A 20-μL of aliquot was injected into the column and quantitative analysis was based on peak area measurements as ratios toward the peak area of the internal standard.

2.5 Method validation according to European Commission Decision 2002/657/EC

The proposed method was validated according to the European Commission Decision 2002/657/EC using spiked milk samples, since certified reference materials (CRM) for TCs in milk were not available. From the performance characteristics enacted by EU, linearity, accuracy, precision, sensitivity, and stability were examined using spiked milk samples at various concentrations.

The linearity response of TCs was first studied in standard solutions, using ten working standards injected three times, covering the entire working range of 0.5–15 ng/μL. In milk samples linearity response was examined by fortifying matrix samples with a series of mixed standards of the examined TCs, covering a broad range from 20 to 300 μg/kg. Calibration curves were constructed with these samples, injected three times, using analyte/internal standard peak area ratio. The LOD values were assessed as the minimum quantity of each compound (S/N of 3:1) that could be detected without interference from the baseline noise. The LOQ values were assessed as the minimum quantity of each compound (which is not less than three times the detection limit) that could be quantified.

Precision and accuracy expressed in terms of TCs recovery from milk samples were studied by analyzing spiked samples at three concentration levels (50, 100, and 200 μg/kg). Intra-assay precision was studied by five replicate measurements at these concentration levels, while inter-assay precision was conducted during routine operation of the system over the period of five consecutive days. Recovery was calculated as the percentage of the found mass of the analyte on the spiked sample toward the mass that was initially spiked and was tested after

the replicate analysis of spiked samples in various concentrations.

The decision limit, CC_a, was calculated as the mean measured concentration at the MRL (100 μg/kg tissue) plus 1.64 times the SD of within-day precision at this concentration. The detection capability, CC_b, was calculated as CC_a plus 1.64 times the SD of within-day repeatability at CC_a. Statistical analysis was performed at the 95% confidential level and the number of replicate analyses was 20.

Peak identification was performed by spectral information provided by diode array detector.

The selectivity of the procedure as interference from endogenous compounds was investigated by the analysis of ten different blank milk samples. For monitoring the stability of the examined TCs in standard solutions, stock solutions of the examined TCs and a mixture of a working solution were stored at +4 °C in the dark, after analyzing one fresh aliquot. Aliquots from the solutions were analyzed after 1, 2, 4, and 8 wk.

2.6 Sample preparation

2.6.1 Isolation from matrix

Aliquots of 1 mL from homogenized and pasteurized milk were placed into 10 mL centrifuge tubes. Fortified samples were prepared by adding 100 μL of TCs standard working solutions at different concentration levels (1, 2, 5, 8, 10, 15 ng/μL) containing the internal standard, to the milk samples that were subsequently homogenized in a vortexer for 2 min. After 15 min in calm, 0.5 mL of 20% TFA and 5 mL of 0.4 M oxalate buffer (pH = 4) were added. The tubes were vortexed for 5 min and left to stand for 15 min. The mixtures were then centrifuged at 4000 rpm for 15 min and the supernatants were removed.

To optimize the extraction of TCs from milk samples, a variety of protein precipitation solvents such as TCA, TFA, acetone, CH₃CN, and various buffer solutions such as citrate buffer (0.4 M, pH = 4) and oxalate buffer (0.4 M, pH 4), in a range of combinations were tested. For the purification step, the supernatants were applied to pre-conditioned SPE cartridges after filtration through 0.2 μm Whatman filter papers. The optimized SPE protocol was subsequently applied to isolate TCs from the samples.

2.6.2 Cleanup procedure by SPE

SPE was used as a purification step after the extraction of TCs from milk. Three different sorbents were tested, Absolut Nexus by Varian, Discovery by Supelco, and Lichrolut by Merck. Cartridges were preconditioned by flushing 2 mL of methanol and 2 mL of water. Then 100 μL from the extract of TCs from milk were applied by

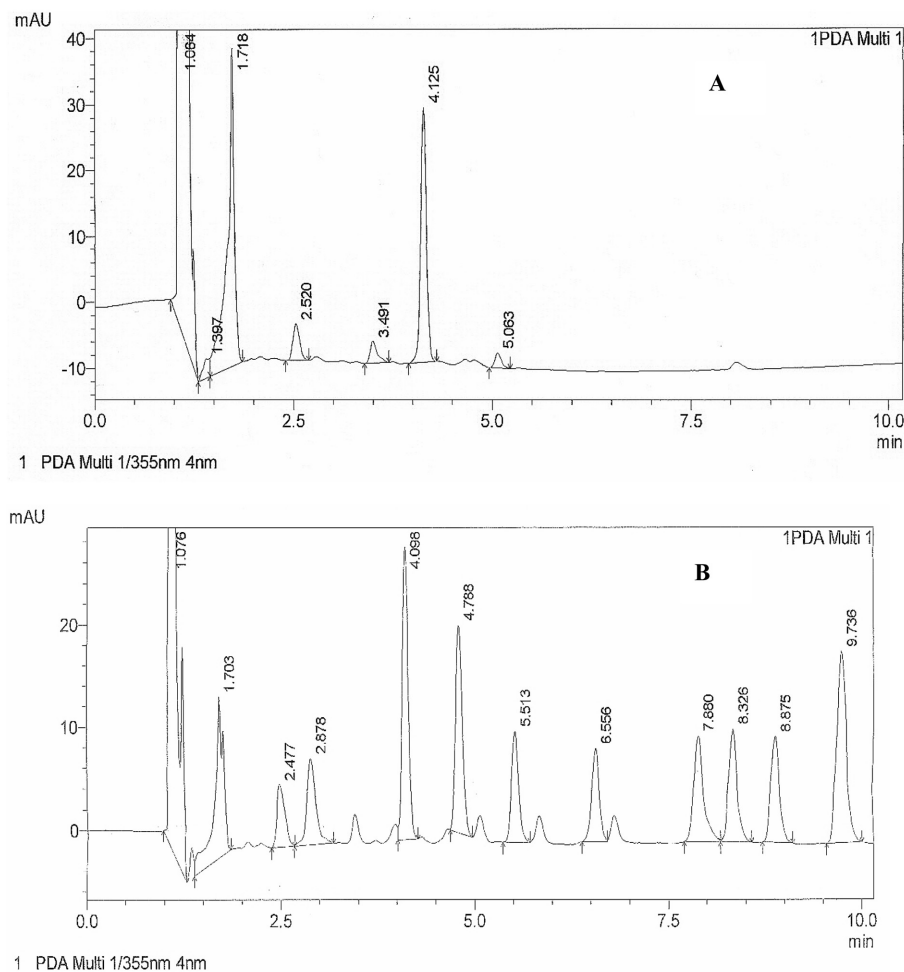


Figure 2. High-performance liquid chromatogram of (A) blank bovine milk and (B) spiked milk bovine milk at 5 ng/ μ L, after SPE using the conditions described in text. Peaks: (1) MNC 2.878 min, (2) OTC 4.788 min, (3) TC 5.513 min, (4) DMC 6.556 min, (5) CTC 7.880 min, (6) MTC 8.326 min, (7) DC 8.875 min, and colchicine (IS) 9.736 min.

allowing them to pass through the bed without suction. A mixture of $\text{CH}_3\text{OH}/\text{CH}_3\text{CN}/0.01$ M oxalic acid (30:30:40 v/v/v) was chosen as the eluting solvent according to our method described in ref. [22]. The samples were subsequently evaporated to dryness under a nitrogen stream in a water bath at 35°C and the residues were dissolved in 100 μL of methanol. Aliquots of 20 μL of the resulting sample were injected into the HPLC system.

3 Results and discussion

3.1 Chromatography

The multistep gradient elution yielded optimum separation of the seven studied TCs and the internal standard in less than 10 min. Retention times of the examined analytes were 2.9 min for MNC, 4.8 min for OTC, 5.5 min for TC, 6.6 min for DMC, 7.9 min for CTC, 8.3 min for MTC, 8.9 min for DC, and 9.7 min for colchicine (IS). A typical chromatogram is shown in Fig. 2. Under the conditions described above, the studied TCs and the internal standard were well resolved. Resolution factors (R_s) were calculated according to the formula: $R_s = 2(t_2 - t_1)/$

$(t_{w1} + t_{w2})$, where t_1 and t_2 are the retention times and t_{w1} and t_{w2} the baseline peak widths of successive peaks, and they were found to be 3.5 for MNC–OTC, 1.3 for OTC–TC, 2.3 for TC–DMC, 2.2 for DMC–CTC, 1.0 for CTC–MTC, 1.4 for MTC–DC, 1.7 for DC–IS, indicating a satisfactory separation. Moreover, endogenous compounds eluted at 2.5 and 4.1 min are well resolved from vicinal peaks: for MNC peaks 1.0 min (2.5 min) and 3.5 min (4.1 min). Typical high-performance liquid chromatograms of a blank milk sample and a spiked milk sample are shown in Figs. 2A and B, respectively.

Method robustness was studied with respect to slight modifications in temperature, flow rate, and pH value and no significant changes were observed. System suitability was checked by means of repeatability of peak area and retention time of analytes. Results are summarized in Table 2.

3.2 Sample preparation

In order to optimize extraction the of TCs from milk samples, two buffer solutions were examined regarding their extraction efficiency: oxalate buffer (0.4 M, pH 4) and cit-

Table 2. System suitability data ($n = 6$)

Analyte	Average time $\pm$ SD	RSD	Average area $\pm$ SD	RSD
MNC	2.87 $\pm$ 0.04	1.3	79 991 $\pm$ 1392	1.7
OTC	4.71 $\pm$ 0.02	0.5	128 694 $\pm$ 973	0.8
TC	5.43 $\pm$ 0.02	0.4	98 735 $\pm$ 1316	1.3
DMC	6.47 $\pm$ 0.03	0.4	85 102 $\pm$ 2639	3.1
CTC	7.77 $\pm$ 0.03	0.4	79 955 $\pm$ 1428	1.8
MTC	8.22 $\pm$ 0.03	0.4	89 885 $\pm$ 2195	2.4
DC	8.73 $\pm$ 0.12	1.4	87 403 $\pm$ 1978	2.3
IS	9.72 $\pm$ 0.02	0.2	182 324 $\pm$ 9834	5.4

rate buffer (0.4 M, pH 4). For the precipitation of milk proteins before adding the extraction buffer, HCl (1 M), acetone, CH₃CN, TCA, and TFA were examined. As already mentioned after the extraction of TCs, an extra purification step with SPE was necessary. According to our previous experience, optimum extraction includes CH₃OH/CH₃CN/0.01 M oxalic acid (30:30:40 v/v/v) as the eluting solvent. To avoid the presence of unknown peaks at chromatograms of blank milk samples, three different cartridges were studied: Nexus, Discovery, and Lichrolut.

As shown in the results summarized in Table 3, optimum results are obtained by using TCA or TFA as the precipitation agent, while oxalate and citrate buffers offer similar extraction efficiencies. Recoveries using Nexus are also comparable with those using Discovery or Lichrolut. Moreover, the same unknown peaks at the same retention times appear at all cases. In order to choose the optimum extraction and SPE protocol, recovery results were statistically treated using one factor ANOVA. Data are presented in Table 4. As it can be seen from the ANOVA results for the extraction medium, oxa-

late and citrate do not provide significantly different recovery rates. This holds for the use of TFA or TCA as deproteinization media. With regards to SPE sorbent, Lichrolut and Discovery provide similar results, while there is statistically significant difference when using NEXUS. Taking into consideration recovery and ANOVA results the combination of TFA as deproteinization agent, oxalate buffer as extraction solvent, and Discovery cartridges for SPE provided optimal conditions.

Moreover, extraction efficiency was studied using three different oxalate buffer concentration levels, TFA concentration levels, and three pH values. Results are shown in Figs. 3A–C. Optimum recovery rates were obtained with 20% TFA/0.4 M oxalate buffer at pH = 4.

3.3 Method validation

The described method was fully validated according to 2002/657/EC guidelines. The results of figures of merit that were investigated are described in the following paragraphs.

3.3.1 Linearity and sensitivity

Calibration curves constructed both for standard solutions and for milk samples after appropriate pretreatment, were obtained by least-squares linear regression analysis of the peak area ratio of analyte to the internal standard *versus* analyte injected amount. The method was linear up to 15 ng/ μ L for all TCs in both standard solutions and milk samples. All calibration data as well as LOD and LOQ are presented in Table 5. Regression equations revealed good correlation coefficients ranging between 0.994 and 0.999 over the examined range.

Table 3. Recoveries of the examined TCs after SPE in extracts using various extraction protocols (0.4 M, pH 4)

SPE sorbent	Deproteinization – extraction media	Recovery (%)						
		MNC	OTC	TC	DMC	CTC	MTC	DC
Nexus	TCA – oxalate	97.7	99.2	93.2	116.5	114.9	71.9	90.5
	TCA – citrate	92.0	93.7	88.1	91.5	98.2	65.4	71.1
	HCl – oxalate	137.8	29.0	16.5	100.1	52.4	44.0	65.0
	HCl – citrate	102.3	44.4	22.1	92.8	36.1	28.2	74.9
	TFA – oxalate	96.0	84.2	80.0	95.8	87.4	65.7	70.5
	TFA – citrate	104.5	83.5	82.6	89.9	90.9	72.0	72.4
	CH ₃ CN – oxalate	74.7	64.3	47.0	66.2	74.5	56.5	61.6
	Acetone – oxalate	43.3	23.6	18.5	62.1	54.7	38.1	44.6
Lichrolut	TCA – oxalate	76.9	95.9	50.2	127.6	113.6	87.0	94.8
	TCA – citrate	80.8	110.5	94.1	116.9	107.3	87.6	97.2
	TFA – oxalate	95.1	105.5	76.3	113.5	118.6	91.5	98.2
	TFA – citrate	111.6	105.2	94.9	117.6	104.2	84.2	97.0
Discovery	TCA – oxalate	111.7	101.0	68.2	102.6	104.8	92.1	99.4
	TCA – citrate	118.2	108.8	94.1	118.1	121.1	77.0	84.8
	TFA – oxalate	106.0	101.9	101.2	114.0	97.6	88.8	93.2
	TFA – citrate	110.1	109.3	101.7	113.2	106.1	86.0	92.3

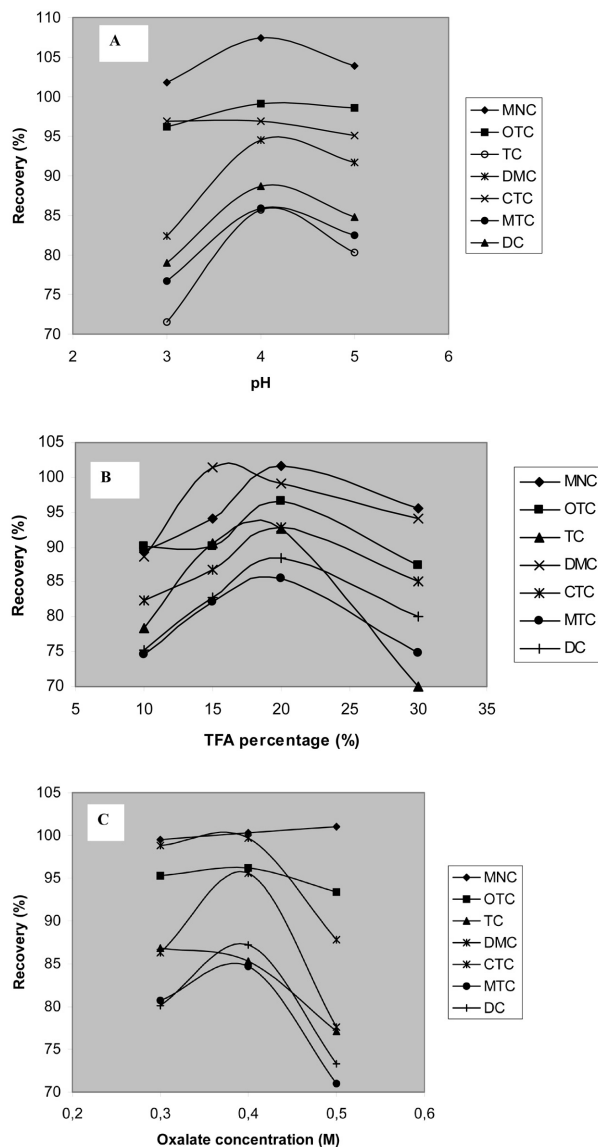


Figure 3. TCs extraction efficiency from milk studied at different (A) pH values, (B) TFA (%) concentration, and (C) oxalate buffer concentration levels.

3.3.2 Selectivity

The application of the whole procedure to ten blank milk samples in order to verify the method selectivity demonstrates that no interferences were detected. Two significant peaks and a number of smaller unknown endogenous peaks appear in the chromatogram of all samples, but as they elute at quite different retention times from the examined TCs and the IS they cause no further problem.

3.3.3 Precision and accuracy

The precision of the method based on within-day repeatability was assessed by replicate ($n = 5$) measurements from three spiked milk samples at 50, 100, and 200 $\mu\text{g}/\text{kg}$. Relative recovery rates from the spiked samples were determined at three different concentrations by comparing the peak area ratios for extracted TCs and the values derived from the respective calibration curve.

The between-day precision of the method was established using milk samples at the same concentration range as above. A triplicate determination of each concentration was conducted during routine operation of the system over a period of five consecutive days. RSD values for all the examined TCs were lower than 8.5%, while mean recovery rates were for MNC: 93.8–100.9%, for OTC: 96.8–103.7%, for TC: 96.3–101.8%, for DMC: 99.4–107.2%, for CTC: 99.4–102.9%, for MTC: 96.3–102.7%, and for DC: 94.6–102.1%.

The results of the within-day repeatability and the between-day precision of the method applied to fortified matrix samples are illustrated in Table 6.

3.2.4 Stability

TC stock standard solutions in methanol are stable for a period of 2 months at 4°C in the dark. Working solutions were analyzed after 1, 2 and 4 and 8 wk and the instrumental responses were compared with the initial peak area ratios to IS and found to be stable only for 1 wk in the dark at 4°C. The acceptance criterion was a response comprised between 95 and 105% of the initial one.

Table 4. ANOVA analysis of the recovery results obtained after extraction of the examined TCs with various extraction protocols ($p = 0.05$)

	Oxalate/citrate $F_{\text{crit}} = 4.96$	TFA/TCA $F_{\text{crit}} = 4.96$	Nexus/Lichrolut $F_{\text{crit}} = 5.99$	Nexus/Discovery $F_{\text{crit}} = 5.99$	Discovery/Lichrolut $F_{\text{crit}} = 5.99$
MNC	0.71	1.52	0.30	14	4.09
OTC	0.45	0.38	8.43	11.7	0.04
TC	3.24	0.89	0.42	0.17	0.01
DMC	0.003	1.57	9.06	0.05	1.24
CTC	0.65	1.51	3.50	1.47	0.36
MTC	0.50	0.04	63.95	21.44	0.2
DC	0.69	0.12	17.92	8.31	2.05

Table 5. Calibration and sensitivity data of the seven examined TCs in standard solutions

Analyte	Slope	Intercept	Correlation coefficient	Uncertainty of the blank matrix samples of milk ($n = 7$) $1.90 \times \text{SD}$	LOD (ng)	LOQ (ng)
Standard solutions						
MNC	0.0048 ± 0.0002	-0.0288 ± 0.0299	0.994	0.0568	6	20
OTC	0.0080 ± 0.0002	0.0296 ± 0.0442	0.995	0.0840	3	10
TC	0.0066 ± 0.0002	0.0225 ± 0.0250	0.998	0.0475	3	10
DMC	0.0046 ± 0.0001	0.0017 ± 0.0208	0.997	0.0395	3	10
CTC	0.0040 ± 0.0001	0.0092 ± 0.0219	0.995	0.0416	3	10
MTC	0.0064 ± 0.0002	0.0406 ± 0.0308	0.996	0.0585	3	10
DC	0.0063 ± 0.0002	0.0317 ± 0.0282	0.997	0.0536	3	10
Milk						
MNC	0.0042 ± 0.0001	0.0410 ± 0.0190	0.997	0.0361	30	100
OTC	0.0070 ± 0.0001	0.0033 ± 0.0220	0.999	0.0418	20	100
TC	0.0044 ± 0.0004	0.0805 ± 0.0797	0.996	0.1514	20	100
DMC	0.0041 ± 0.0002	0.0311 ± 0.0216	0.995	0.0410	20	100
CTC	0.0041 ± 0.0001	0.0209 ± 0.0120	0.996	0.0228	20	100
MTC	0.0051 ± 0.0002	-0.0011 ± 0.0309	0.994	0.0587	20	100
DC	0.0052 ± 0.0001	-0.0095 ± 0.0219	0.997	0.0416	20	100

Table 6. Within and between-day precision and accuracy of the developed method for the determination of seven TCs in milk samples after SPE

Analyte	Added (ng)	Within-day ($n = 5$)			Between-day ($n = 5$)		
		Measured $\pm$ SD (ng/g milk)	RSD	Recovery (%)	Measured $\pm$ SD (ng/g milk)	RSD	Recovery (%)
MNC	50	49 ± 1.7	3.4	98.5	49 ± 1.7	3.5	98.6
	100	94 ± 1.8	1.9	93.8	100 ± 2.1	2.1	100.9
	200	199 ± 2.9	1.5	99.8	199 ± 4.1	2.0	99.7
OTC	50	52 ± 0.9	1.7	103.7	48 ± 4.1	8.5	96.8
	100	100 ± 0.7	0.7	100.1	103 ± 2.5	2.4	102.8
	200	198 ± 1.4	0.7	98.9	201 ± 2.6	1.3	100.5
TC	50	48 ± 1.1	2.4	96.3	50 ± 2.1	4.2	100.7
	100	102 ± 1.4	1.3	101.8	99 ± 1.5	1.5	99.1
	200	193 ± 6.1	3.1	96.3	194 ± 8.1	4.2	96.9
DMC	50	50 ± 1.6	3.2	99.4	51 ± 1.7	3.4	101.6
	100	107 ± 3.5	3.3	107.2	100 ± 4.1	4.1	99.9
	200	206 ± 3.7	1.8	103.9	207 ± 3.2	1.6	103.6
CTC	50	50 ± 1.5	2.9	99.8	51 ± 2.8	5.4	102.1
	100	103 ± 1.9	1.9	102.9	99 ± 1.8	1.8	99.4
	200	204 ± 3.5	1.7	102.0	201 ± 2.1	1.1	101.6
MTC	50	50 ± 1.0	2.0	100.3	51 ± 1.3	2.4	102.7
	100	96 ± 2.3	2.4	96.3	101 ± 3.4	3.3	100.6
	200	201 ± 6.4	3.2	100.4	203 ± 2.3	1.1	101.6
DC	50	49 ± 1.3	2.7	98.1	51 ± 0.9	1.8	102.1
	100	95 ± 2.1	2.2	94.6	100 ± 2.1	2.1	100.2
	200	195 ± 3.2	1.6	97.7	201 ± 5.7	2.8	100.6

3.3.3 Decision limit and detection capability

In compliance with the 2002/657/EC decision, the validation procedure includes the determination of two novel criteria, CC_a (limit of decision) and CC_b (capability of detection), defined respectively as “the limit at and above which it can be concluded, with an error probability of a , that a sample is noncompliant” (greater than the MRL for group B substances), and “the smallest content of the sub-

stance that may be detected, identified, and/or quantified in a sample with an error probability of b .” For group B substances, errors must be $<5\%$.

The CC_a values calculated by spiking 20 blank milk samples at MRL (100 $\mu\text{g/kg}$) ranged from 101.25 to 105.84 $\mu\text{g/kg}$ for the examined antibiotics. CC_b values calculated by analyzing 20 blank spiked samples at corresponding CC_a level for each analyte were in the range

Table 7. Calculating errors *a* (A) and *b* (B) as well as the limit of decision (CC_a) (A) and capability of detection (CC_b) (B) at the MRL enacted by the EU at 100 ng/g milk

Analytes	Added (ng/g milk)	Measured $\pm$ SD (ng/g milk)	RSD	Recovery (%)	Error <i>a</i> ($1.64 \times SD$)	CC_a (ng)
(A)						
MNC	100	93.79 $\pm$ 1.80	1.9	93.8	2.95	102.95
OTC	100	100.11 $\pm$ 0.76	0.7	100.1	1.25	101.25
TC	100	101.81 $\pm$ 1.37	4.3	101.8	2.26	102.28
DMC	100	107.17 $\pm$ 3.56	3.3	107.2	5.84	105.84
CTC	100	109.89 $\pm$ 1.93	1.9	102.9	3.16	103.16
MTC	100	96.29 $\pm$ 2.35	2.4	96.3	3.85	103.85
DC	100	94.63 $\pm$ 2.10	2.2	94.6	3.44	103.44
Analytes	Added (ng/g milk)	Measured $\pm$ SD (ng/g milk)	RSD	Recovery (%)	Error <i>b</i> ($1.64 \times SD$)	CC_b (ng)
(B)						
MNC	103	114.14 $\pm$ 2.03	1.8	110.8	3.34	106.29
OTC	101	109.13 $\pm$ 1.64	1.5	108.1	2.69	103.94
TC	102	106.00 $\pm$ 2.10	2.0	103.9	3.45	105.71
DMC	106	110.96 $\pm$ 1.85	1.7	104.7	3.04	108.88
CTC	103	108.21 $\pm$ 1.85	1.7	105.0	3.04	106.20
MTC	104	104.02 $\pm$ 2.88	2.8	100.0	4.72	108.57
DC	103	101.30 $\pm$ 2.40	2.4	98.3	3.95	107.39

from 103.94 to 108.88 $\mu\text{g/kg}$. Results are presented in Table 7.

Ten milk samples from the local market were analyzed according to the developed method and no TC residues were detected.

4 Concluding remarks

The herein described confirmatory method is a simple validated assay, which can be readily adapted by any laboratory for the quality control and the quantitative determination of residues of the seven examined TCs in milk. Validation was performed according to the European Union decision 2002/657/EC for the validation of an analytical method for residues in animal products.

The results of validation process demonstrate that the method is suitable for application in European Union statutory veterinary drug residue surveillance programs.

The seven investigated TCs were well resolved in less than 10 min. LOQ values achieved were matching to MRL values.

Overall recoveries of TCs from spiked milk samples at fortification levels of 50, 100, and 200 ng/g were 93.8–100.9% for MNC, 96.8–103.7% for OTC, 96.3–101.8% for TC, 99.4–107.2% for DMC, 99.4–102.9% for CTC, 96.3–102.7% for MTC, and 94.6–102.1% for DC. All RSD values were lower than 8.5%.

The decision limits CC_a calculated by spiking 20 blank milk samples at MRL (100 $\mu\text{g/kg}$) ranged from 101.25 to 105.84 $\mu\text{g/kg}$, while detection capability CC_b from 103.94 to 108.88 $\mu\text{g/kg}$.

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