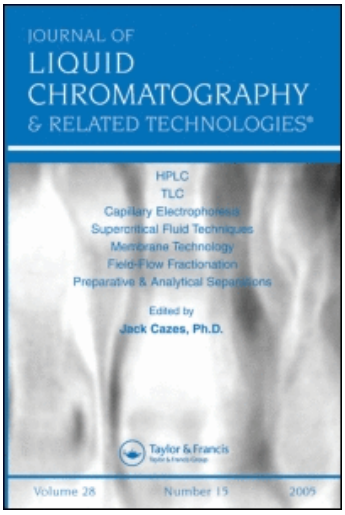

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Konstantina I. Nikolaidou[a]; Victoria F. Samanidou[a]; Ioannis N. Papadoyannis[a]
[a] Laboratory of Analytical Chemistry, Department of Chemistry, Aristotle University of Thessaloniki, Thessaloniki, Greece

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Development and Validation of an HPLC Multi-Residue Method for the Determination of Seven Tetracycline Antibiotic Residues in Bovine Liver and Kidney According to the European Union Decision 2002/657/EC

Konstantina I. Nikolaidou, Victoria F. Samanidou, and Ioannis N. Papadoyannis

Laboratory of Analytical Chemistry, Department of Chemistry, Aristotle University of Thessaloniki, Thessaloniki, Greece

Abstract: Herein an HPLC method with diode array detection, at 355 nm, is described for the determination of seven tetracyclines in liver and kidney muscle tissues: minocycline, tetracycline, oxytetracycline, methacycline, demeclocycline, chlortetracycline, and doxycycline. The examined TCs were extracted from both tissues with 20% TFA and 0.4 M oxalate buffer (pH 4). For further purification of liver and kidney tissues Discovery and LiChrolut SPE cartridges were used, respectively, with 0.01 M $C_2H_2O_4/CH_3CN/CH_3OH$ (40:30:30 v/v/v) as elution solvent.

The separation was achieved on a Kromasil ODS-3, 5 μm, 250 × 4 mm, analytical column. The mobile phase, a mixture of 0.01 M oxalic acid and CH_3CN, was delivered using a gradient program. The procedure was validated according to the Decision 2002/657/EC, determining selectivity, stability, decision limit, detection capability, accuracy, and precision. Overall recoveries ranged from 93.2–125.5% and 81.3–120.4% for liver and kidney tissues, respectively. All RSD values were lower than 9%. The decision limits CC*a* in liver tissues ranged from 311 to 321 μg/kg, while detection capability CC*b* from 324 to

Correspondence: Ioannis N. Papadoyannis, Laboratory of Analytical Chemistry, Department of Chemistry, Aristotle University of Thessaloniki, GR-54 124. Thessaloniki, Greece. E-mail: papadoya@chem.auth.gr

342 μg/kg. Respective values in kidney samples were 612–646 μg/kg for CCa and 637–690 μg/kg for CCb.

Keywords: 2002/657/EC, Bovine liver, Chlortetracycline, Demeclocycline, Doxycycline validation, HPLC, Kidney, Methacycline, Minocycline, Oxytetracycline, SPE, Tetracycline, Tetracyclines

INTRODUCTION

The contamination of products of animal origin with residues from various veterinary and human antibiotic drugs and the consequent development of antimicrobial resistance from many bacteria is already a fact, due to the excessive use of these drugs, not just for preventive and therapeutic purposes but also as feed additives for growth promotion.

Tetracyclines (TCs) belong to the most frequently used veterinary drugs in animal husbandry. They comprise a broad spectrum class of antibiotics. TCs are provided to calves, cattle, pigs, sheep, poultry, and fish for the treatment of various infections of gastrointestinal and urinary tract caused by both gram positive and gram negative bacteria, but also to fraudulently promote growth. Since TCs are absorbed from the gastrointestinal tract, due to their solubility in fat, they are well distributed to most tissues. Residual concentrations in tissues are found to be in the order of kidney > liver > skin > muscle in most animals. Polarity of each tetracycline is also important for its distribution in tissues. Doxycyxline, which is the most lipophilic tetracycline, shows the greatest degree of tissue penetration, especially in liver and kidney tissues.[1,2]

According to the E.U. regulation, which determines the use of antibiotics in stock-breading with a number of directives, three TCs (OTC, TC, and CTC) are approved for therapeutic use in animals. TCs are classified through council directive 96/23/EC and council regulation 1990/2377/EEC in group B_1 of Annex I, which includes veterinary medicines and contaminants with an MRL. The MRLs for TCs in liver and kidney tissues since their distribution there is the highest, have been set at 300 and 600 ng/g, respectively.

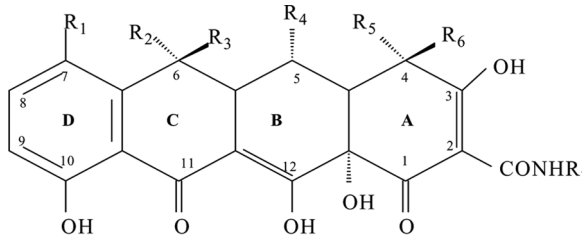
Due to the widespread use of TCs, which leads to an increasing resistant factor, a variety of analytical techniques and methods were developed for the determination of residual TCs in liver and kidney where TCs are mainly concentrated. Myllyniemi et al.[5] developed a microbiological screening method for the identification of several classes of antibiotics, among which are TCs, in kidney tissues. Another screening method for the determination of four TCs (TC, OTC, CTC, and DC) in bovine liver

and kidney was proposed by Oka et al.[6] using a TLC/FAB-MS technique.

Most of the confirmatory methods, which can be found in literature use HPLC with UV detection for the simultaneous analysis of three[7,8] or four[9,10] TCs in both tissues, liver and kidney, mainly bovine or porcine. An HPLC method coupled with fluorescence detection was announced by Croubels[11] and deals with four TCs. The only article which proposes a method for the determination of six TCs in bovine kidney was published by Degroodt et al.[12] using a C_8 column with a mixture of 0.01 M oxalic acid with CH_3CN as mobile phase and photodiode detection. Recoveries were at the range of 44 to 77%. Caro et al.,[13] based on an HPLC UV method, synthesized an MIP for the determination of two TCs (TC and OTC) in pig kidney tissues. HPLC methods coupled with MS detectors have also been developed for the determination of residual TCs in liver and kidney tissues. Two ESI-MS/MS[14,15] and two APCI-MS/MS[16,17] methods can be found. Cherlet et al.[14] determined not only four TCs, but also their 4-epimers. Goto et al.[18] announced an HPLC-ESI-MS/MS method for the simultaneous determination of three TCs and three penicillins.

Isolation of TCs from liver and kidney samples is a quite demanding procedure, since TCs and especially DC, bind strongly with proteins and divalent metal ions, and liver and kidney tissues are quite complex matrices containing several proteins and enzymatic systems. Most of the isolation methods found in literature propose extraction procedures with buffers such as succinate,[9,11,12,14] McIlvaine with Na_2EDTA [6,10,15,16,18], H_3PO_4,[7] oxalic acid,[8] and glycerin-HCl.[17] In most cases, extraction procedures were followed by a precipitation of proteins steps[7,8,11,14] and an extra cleaningup step, like liquid-liquid extraction,[7] SPE,[6,8,10,12,14–18] and MCAC in combination with exchange membranes.[9,11] Isolation of OTC and TC from kidney extracts was achieved using a molecularly imprinted solid phase extraction (MISPE) according to the work of Caro et al.[13]

The aim of the present work was to develop a simple, reliable, multiresidue method for the determination of TCs in liver and kidney tissue samples. Apart from TC, OTC, and CTC, which can be used in veterinary medicine legally, other TCs, commercially available as human drugs, may also be used in animal husbandry. MNC is the most biological active TC and is also active against TCs resistant bacteria; DC, MTC, and DMC are more stable than the other TCs and give higher and more sustained blood level concentrations with smaller doses.[19] Our work is the only so far proposed work, which determines seven TCs in both liver and kidney tissues based on a simple extraction and SPE procedure for the isolation of TCs from the matrices. Chemical structures of examined analytes are shown in Figure 1. The proposed method was validated



Compound		R_1	R_2	R_3	R_4	R_5	R_6	R_7
TC	Tetracycline	H	CH_3	OH	H	$N(CH_3)_2$	H	H
OTC	Oxytetracycline	H	CH_3	OH	OH	$N(CH_3)_2$	H	H
CTC	Chlortetracycline	Cl	CH_3	OH	H	$N(CH_3)_2$	H	H
DC	Doxycycline	H	CH_3	H	OH	$N(CH_3)_2$	H	H
MNC	Minocycline	$N(CH_3)_2$	H	H	H	$N(CH_3)_2$	H	H
MTC	Methacycline	H	$=CH_2$		OH	$N(CH_3)_2$	H	H
DMC	Demeclocycline	Cl	H	OH	H	$N(CH_3)_2$	H	H

Figure 1. Chemical structures of examined tetracyclines.

according to the criteria enacted by European Union by commission decision 2002/657/EC.

EXPERIMENTAL

Chemicals and Reagents

Oxytetracycline (OTC), Minocycline (MNC), Demeclocycline (DMC), Methacycline (MTC), and Doxycycline (DC) were purchased from Sigma (St. Louis, MO, USA), while tetracycline (TC) and chlortetracycline (CTC) from Fluka (Buchs SG, Switzerland). All TCs were of analytical grade. HPLC grade methanol and acetonitrile were supplied by Carlo Erba (Milano. Italy). Sodium hydroxide, oxalic acid, and hydrochloric acid were supplied by Merck (Darmstadt, Germany), and citric acid monohydrate and sodium citrate trihydrate of analytical grade were obtained from Riedel-de-Haen (Seezle, Germany). Trichloroacetic (TFA) and trifluoroacetic (TCA) acid were supplied by Acros Organics (Geel, Belgium). High purity water was provided by a Milli-Q purification system (Millipore, Bedford, MA, USA) and was used throughout the study. Bovine liver and kidney tissue samples were supplied by the local market.

Instrumentation

A Shimadzu (Kyoto, Japan) quaternary low pressure gradient system was used for chromatographic determination of the examined TCs. The solvent lines were mixed in an FCV-10AL mixer and an LC-10AD pump, equipped with a Shimadzu SCL-10ALVP System Controller, permitting fully automated operation, was used to deliver the mobile phase to the analytical column. Sample injection was performed via a Rheodyne 7125 injection valve equipped with a 20 μL loop for sample injection. Detection was achieved by an SPD-M10A photodiode array detector, in compliance with data acquisition software LabSolutions-LCsolutions by Shimadzu. Degassing of the mobile phase was achieved by continuous helium sparging in the solvents reservoirs by a DGU-2A degassing unit.

Oxalic acid solutions, used as constituents of the mobile phase, were filtered through Whatman cellulose-nitrate 0.2 μm membrane filters on a glass vacuum filtration apparatus obtained from Alltech Associates For the pre-treatment of tissue samples, a Glasscol small vortexer (Terre Haute, IN, USA) and a Hermle centrifuge, model Z 230 (B. Hermle, Gosheim, Germany) were used.

The SPE study was carried out on a Vac-Elut vacuum manifold column processor, purchased from Analytichem International. SPE cartridges studied for the optimum isolation of analytes from interferences were: Li-chrolut RP-18 (100 mg/cm^3) supplied by Merck, Discovery (500 mg/3 mL) by Supelco, and polymeric Nexus cartridges (30 mg/cm^3) by Varian (Harbor City, CA, USA). All evaporations were performed with a Supelco 6-port Mini-Vap concentrator/evaporator (Bellefonte, PA, USA).

Chromatography

A Kromasil C_{18}, 5 μm, 250 × 4 mm, analytical column, supplied from MZ-Analysentechnik (Mainz, Germany) was used for the separation of the seven TCs. The mobile phase, consisted of a mixture of 0.01 M oxalic acid as solvent A and CH_3CN as solvent B, was pumped to the analytical column, operated at ambient temperature, at a flow rate according to the gradient program shown in Table 1. An equilibration time of 3 min was required between runs. Column effluent was monitored at 355 nm for all analytes.

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

t (min)	A: $C_2H_2O_4$ (0.05 M)	B: CH_3CN	t (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		

Preparation of Standards

Stock standard solutions of each TC at a concentration of 100 ng/ μL were prepared by dissolving the appropriate amount of the analyte in methanol. Working methanolic solutions were prepared from stocks by the appropriate dilution at 0.5, 0.8, 1, 2, 5, 8, 10, and 15 ng/ μL with colchicine as internal standard at a concentration of 5 ng/ μL. All solutions were wrapped in aluminium foil in order to protect them from light during use.

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

In order to extract TCs from liver and kidney samples, various buffer solutions 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, oxalate buffer (0.4 M, pH 4) by mixing 0.4 M oxalic acid and 0.1 M sodium hydroxide, and TFA, TCA, and HCl aqueous by appropriate dilution from concentrated reagents.

Method Validation According to European Commission Decision 2002/657/EC

The herein proposed method for the determination of residual TCs in bovine liver and kidney samples was validated in terms of linearity, accuracy, precision, sensitivity, stability, decision limit (CC_α), and detection capability (CC_β) in compliance with the requirements set by E.U. through European Commission Decision 2002/657/EC. Validation was performed using spiked liver and kidney tissue samples following the criteria enacted by E.U.

Linearity was examined for both tissues by construction of calibration curves after analysing a series of spiked samples with mixed standard solutions of the examined TCs at various concentrations (from 20 to 300 μg/kg tissue). Calibration curves were obtained for each sample, by least squares linear regression analysis of the peak area ratio of each analyte to IS.

The method's limits of detection (LOD) and limit of quantitation (LOQ) were calculated according to the equations $LOD = 3.3\,\sigma/s$ and $LOQ = 10\,\sigma/s$, respectively, where σ represents the SD of y-intercepts and s the slope from regression analysis.

Since no certified reference materials were available for bovine liver tissues, recovery, which expresses the accuracy of the method, was tested after replicate analyses of spiked liver samples at three concentration levels (50, 100, 200 μg/kg), and calculated as the percentage of the found

mass of the analyte on the spiked sample toward the added mass. Intra-assay precision was estimated by six replicate measurements at these concentration levels, while inter-assay precision was conducted during routine operation of the system over a period of six consecutive days. Precision and accuracy of the method were determined simultaneously.

For the calculation of decision limits (CC_α), twenty samples from each tissue were spiked at the MRL enacted by E.U. for each analyte and each tissue, and analyzed. The mean measured concentration at the MRL (300 μg/kg for liver tissues and 600 μg/kg for kidney tissues) plus 1.64 times the SD of within-day precision at this concentration, equals the CC_α values. In order to estimate detection capability (CC_β) values, twenty samples from each tissue were spiked at the CC_α levels calculated before. CC_β were calculated as the mean measured concentration at the CC_α level plus 1.64 times the SD of within-day repeatability at the CC_α. Statistical analysis was performed at the 95% confidential level.

The selectivity of the method was assessed by the absence of interference peaks from endogenous compounds coeluted with the investigated analytes, and was checked by the analysis of ten different blank liver and kidney tissue samples. Peaks purity and comparison of their spectrum were checked by means of PDA detector.

Finally, in order to check stability of TCs on spiked frozen liver samples, homogenized blank liver tissues were divided into five aliquots of 1 g and each aliquot was spiked with TCs at 100 μg/kg. One sample was analysed immediately, while the remaining samples were stored at −18°C and analyzed after 2, 4, and 6 weeks. Stability was also investigated after several freezing–defrosting cycles. Frozen spiked liver samples were left at room temperature to thaw and analyzed after four freezing–defrosting cycles.

Sample Preparation

Liver and kidney tissue samples were homogenized, separated in aliquots of 1 g, and stored at −18°C. Each aliquot was placed into a 10 mL centrifuge tube and was spiked with 100 μL of TCs standard working solutions at different concentration levels (1, 2, 5, 8, 10, 15 ng/μL) containing the internal standard at the concentration of 5 ng/μL. Mixtures were then subsequently homogenized in a vortex mixer for 2 min and left to rest for 15 min. Subsequently, a volume of 0.5 mL of a 15% TFA solution was added. After vortex mixing for 2 min, 5 mL of 0.4 M oxalate buffer (pH 4) were added. These mixtures were vortexed for 5 min, left to stand for 15 min, and centrifuged at 4000 rpm for 15 min. The supernatants were decanted and the residues were re-extracted twice.

After the extraction of TCs from liver and kidney tissues, a purification step by solid phase extraction (SPE) was followed. The combined supernatants after filtration were applied to SPE cartridges preconditioned with 2 mL of CH_3OH and 2 mL of water. Li-Chrolut RP-18, and Nexus sorbents were tested in order to optimize the purification protocol for liver extracts, while Discovery and Li-Chrolut RP-18 cartridges were examined for kidney extracts. Elution was performed with a mixture of $CH_3OH/CH_3CN/0.01$ M $C_2H_2O_4$ (30:30:40 v/v/v) according to our previous experience on TCs analysis in both cases. The eluents were evaporated to dryness at 35°C under a steam of nitrogen in a water bath. The dry residues were reconstituted in 100 μL of methanol and 20 μL were injected into the HPLC system.

RESULTS AND DISCUSSION

Chromatography

Separation of the studied TCs and the IS was achieved in less than 10 min using a Kromasil C_{18}, 5 μm, 250 × 4 mm, analytical column and a mixture

Table 2. Precision and accuracy of retention times of TCs in bovine liver and kidney

	Within-Day (n = 6)		Between-Day (n = 6)	
Analyte	Average time ± SD	RSD	Average time ± SD	RSD
LIVER				
MNC	2.72 ± 0.05	1.9	2.73 ± 0.01	0.4
OTC	4.71 ± 0.02	0.4	4.68 ± 0.05	1.0
TC	5.43 ± 0.02	0.4	5.39 ± 0.05	0.9
DMC	6.46 ± 0.02	0.4	6.43 ± 0.05	0.7
CTC	7.78 ± 0.03	0.4	7.72 ± 0.05	0.6
MTC	8.22 ± 0.03	0.3	8.17 ± 0.05	0.6
DC	8.77 ± 0.03	0.3	8.72 ± 0.04	0.4
IS	9.68 ± 0.01	0.2	9.62 ± 0.06	0.7
KIDNEY				
MNC	2.87 ± 0.05	1.7	2.93 ± 0.08	2.7
OTC	4.78 ± 0.03	0.7	4.77 ± 0.03	0.7
TC	5.48 ± 0.04	0.7	5.54 ± 0.08	1.4
DMC	6.52 ± 0.04	0.6	6.50 ± 0.05	0.7
CTC	7.85 ± 0.06	0.7	7.92 ± 0.06	0.8
MTC	8.30 ± 0.06	0.7	8.38 ± 0.05	0.6
DC	8.88 ± 0.06	0.7	8.89 ± 0.06	0.7
IS	10.03 ± 0.02	0.2	10.00 ± 0.04	0.4

of 0.01 M oxalic acid with CH_3CN as mobile phase, according to the gradient program shown in Table 1. Retention times of the analytes are shown in Table 2.

Sample Preparation

The main target of sample pretreatment is to obtain a homogeneous liquid phase which contains the whole amount of drugs free from interference. Two acidic buffers, citrate and oxalate at pH = 4, were tested for the extraction of TCs from bovine liver and kidney samples. Since these are quite complex matrices, consisted of a variety of proteins and enzymes, and they also contain important amounts of blood, an extra deproteinizing step is required before the extraction of TCs. For this purpose CH_3CN, TFA, and TCA solutions of various concentrations were examined. Despite the addition of those reagents, the extracts obtained could not be directly injected onto HPLC. An extra purification procedure such as SPE was necessary. In order to purify liver extracts, Nexus

Table 3. Recoveries of the examined Tcs after solid-phase extraction in liver extracts using various extraction protocols

		Recovery (%)						
		MNC	OTC	TC	DMC	CTC	MTC	DC
Discovery	15% TFA/Oxalate (0.4 M. pH 4)	91.1	94.8	92.3	93.3	103.7	92.1	101.3
	15% TCA/Oxalate (0.4 M. pH 4)	50.2	92.8	85.5	90.6	90.5	61.6	73.7
	AcN/Oxalate (0.4 M. pH 4)	86.9	90.6	72.3	76.9	88.1	60.5	70.9
	AcN-Oxalate mixture (0.4 M. pH 4)	31.9	31.4	28.8	39.7	94.0	74.2	74.6
	15% TFA/Citrate (0.4 M. pH 4)	71.2	73.7	31.3	37.7	37.9	38.8	43.7
	15% TCA/Citrate (0.4 M. pH 4)	–	99.4	56.2	47.3	65.0	62.1	67.1
	AcN/Citrate (0.4 M. pH 4)	112.6	99.4	61.8	65.4	64.2	68.1	64.7
	AcN/Oxalate (0.4 M. pH 4)	21.6	15.0	13.7	27.3	97.9	57.1	55.6
Nexus	15% TFA/Oxalate (0.4 M. pH 4)	135.4	79.6	125.5	119.1	118.3	85.0	89.2
	15% TCA/Oxalate (0.4 M. pH 4)	–	85.3	145.4	114.5	113.6	92.9	90.8

and Discovery cartridges were tested, while for kidney extracts Lichrolut and Discovery sorbents were examined.

The clearest chromatograms are obtained with TFA aqueous solution or CH_3CN using oxalate buffer (pH 4, 0.4 M) for the extraction and Discovery cartridges for the SPE cleaning up procedure. However, TFA solution was preferred since CH_3CN yielded low recovery rates as shown in Table 3. When using Nexus cartridges instead of Discovery more unknown peaks appear, one at 5.5 min, where TC is eluted. For this reason, Discovery cartridges were preferred. The use of citrate buffer (pH 4, 0.4 M) as an extraction agent gave similar results with oxalate buffer. According to the recovery rates summarized in Table 3, in combination with the purity of chromatograms obtained, 20% TFA solution with 0.4 M oxalate buffer was chosen for the extraction of TCs from liver tissues, and Discovery cartridges for the purification of the extracts.

For the extraction of TCs from kidney tissues, the same buffers were tested in combination with TFA and TCA water solutions. Nexus cartridges gave more unknown peaks in chromatograms of liver samples, with low recoveries, for the purification of kidney extracts. Discovery and Lichrolut cartridges were examined. From the recovery results of Table 4, it can be concluded that Lichrolut sorbents in combination with

Table 4. Recoveries of the examined Tcs after solid-phase extraction kidney extracts using various extraction protocols

		Recovery (%)						
		MNC	OTC	TC	DMC	CTC	MTC	DC
LiCcrolut	15% TFA /Oxalate (0.4 M. pH 4)	81.3	120.4	102.7	101.5	97.3	104.7	95.9
	15% TCA/ Oxalate (0.4 M. pH 4)	–	114.3	51.8	88.5	74.2	114.5	103.5
	15% TFA/Citrate (0.4 M. pH 4)	89.7	114.3	85.5	102.0	94.2	97.0	101.6
	15% TCA/Citrate (0.4 M. pH 4)	–	102.8	88.8	133.3	108.0	95.1	103.8
Discovery	15% TFA / Oxalate (0.4 M. pH 4)	69.5	120.2	83.0	110.0	108.4	114.2	106.0
	15% TCA/Oxalate (0.4 M. pH 4)	–	103.3	88.2	106.3	102.2	89.3	76.2
	15% TFA/Citrate (0.4 M. pH 4)	89.7	121.3	95.1	113.3	90.7	106.6	103.5
	15% TFA/Citrate (0.4 M. pH 4)	–	126.9	88.2	101.1	68.0	81.6	75.2

20% TFA and oxalate 0.4 M buffer as extraction medium yields optimum recovery rates, providing the clearest chromatograms with two unknown peaks at 3.78 and 4.21 min.

Typical chromatograms of blank and spiked liver and kidney tissue samples are presented in Figures 2 and 3. The unknown peaks on chromatograms of both matrices are well resolved from vicinal peaks. Resolution factors are within the range 0.9–3.8.

Method Validation

To check linearity of the proposed method, calibration curves were constructed for both liver and kidney samples by least squares linear

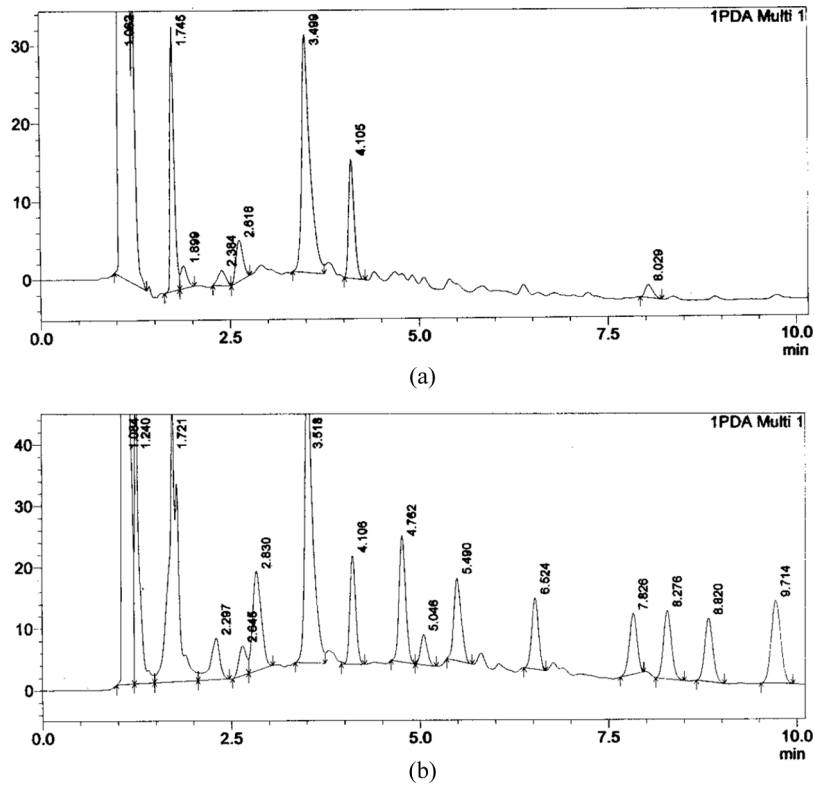


Figure 2. High performance liquid chromatogram of (a): Blank liver tissue and (b): spiked liver tissue at 600 μg/kg, after SPE using the conditions described in text. Peaks: (1) MNC 2.799 min, (2) OTC 4.753 min, (3) TC 5.481 min, (4) DMC 6.538 min, (5) CTC 7.853 min, (6) MTC 8.316 min, (7) DC 8.863 min, and colchicine (IS) 9.721 min.

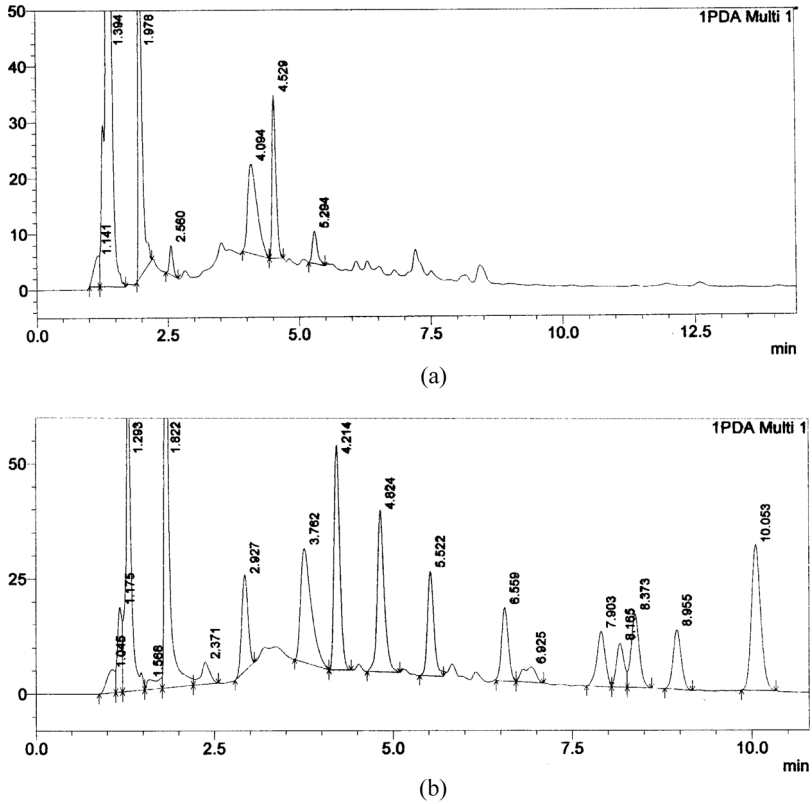


Figure 3. High performance liquid chromatogram of (a): Blank kidney tissue and (b): spiked kidney tissue at 600 μg/kg, after SPE using the conditions described in text. Peaks: (1) MNC 2.927 min, (2) OTC 4.824 min, (3) TC 5.522 min, (4) DMC 6.559 min, (5) CTC 7.903 min, (6) MTC 8.373 min, (7) DC 8.955 min, and colchicine (IS) 10.053 min.

regression analysis of the peak area ratio of analyte to internal standard versus analyte injected amount. All calibration data, as well as LOD and LOQ values are presented in Table 5. LODs and LOQs of the seven TCs were below the MRL value.

In order to check the selectivity of the proposed method, ten liver and ten kidney samples supplied from various producers were extracted and analyzed according to the developed method. All samples proved to be free from matrix interference at the elution time of all TCs and the IS.

Measurements for the study of precision and accuracy of the method were performed simultaneously on spiked liver tissues at three concentration levels 50, 100, and 200 μg/kg. Precision was expressed in terms

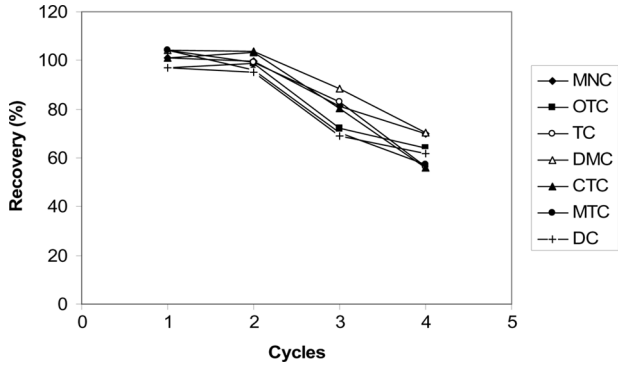


Figure 4. Stability of Tetracyclines in bovine liver after four freezing–defrosting cycles.

Table 5. Calibration and sensitivity data of the seven examined Tetracyclines in bovine liver and kidney samples after solid-phase extraction

		Within-day (n = 5)			Between-day (n = 5)		
Analytes	Added (ng)	Measured ± SD (μg/kg liver)	RSD	R (%)	Measured ± SD (μg/kg liver)	RSD	R (%)
MNC	50	52 ± 2.9	5.7	103.3	50 ± 5.6	9.2	99.7
	100	126 ± 1.7	1.3	125.5	118 ± 5.6	4.8	97.8
	200	203 ± 6.1	3.0	101.3	209 ± 10.0	4.8	104.6
OTC	50	47 ± 2.2	4.7	93.2	48 ± 3.4	7.2	96.0
	100	98 ± 2.7	2.7	98.2	98 ± 4.0	4.1	97.8
	200	202 ± 8.4	4.1	100.9	197 ± 8.9	4.5	98.7
TC	50	51 ± 2.6	5.0	102.5	51 ± 2.6	5.1	101.9
	100	102 ± 3.0	3.0	102.3	102 ± 6.5	6.4	102.5
	200	196 ± 8.8	4.5	98.2	204 ± 9.2	4.5	101.9
DMC	50	54 ± 2.4	4.5	107.1	51 ± 2.4	4.6	102.4
	100	112 ± 4.7	4.2	112.4	105 ± 3.6	3.5	104.8
	200	198 ± 10.8	5.5	98.9	204 ± 12.8	6.3	102.1
CTC	50	53 ± 3.2	6.1	105.9	52 ± 2.5	4.8	104.5
	100	113 ± 3.3	2.9	112.6	109 ± 6.6	6.0	109.2
	200	197 ± 10.2	5.2	98.3	202 ± 9.8	4.8	100.8
MTC	50	53 ± 3.6	6.9	106.2	52 ± 4.4	8.5	103.5
	100	105 ± 5.3	5.3	105.1	103 ± 5.1	5.0	102.8
	200	212 ± 12.1	5.7	106.1	213 ± 11.2	5.3	106.3
DC	50	47 ± 2.9	6.2	93.9	49 ± 2.6	5.4	97.2
	100	96 ± 3.0	3.2	96.2	94 ± 4.0	4.3	93.6
	200	188 ± 9.6	5.1	93.8	194 ± 15.6	8.0	96.9

Table 6. Within and between-day precision and accuracy of the developed method for the determination of seven TCs in liver samples after solid-phase extraction

Analytes	Intercept	Slope	Correlation coefficient	LOD (μg/kg)	LOQ (μg/kg)	MRL (μg/kg)
LIVER						
MNC	-0.0113 ± 0.0274	0.0040 ± 0.0003	0.990	23	69	300
OTC	0.0569 ± 0.0409	0.0072 ± 0.0002	0.996	19	57	300
TC	0.0383 ± 0.0415	0.0056 ± 0.0002	0.993	24	74	300
DMC	0.0018 ± 0.0249	0.0041 ± 0.0001	0.995	20	61	300
CTC	0.0115 ± 0.0267	0.0032 ± 0.0001	0.992	28	83	300
MTC	0.0737 ± 0.0147	0.0048 ± 0.0001	0.9991	10	31	300
DC	0.0332 ± 0.0479	0.0056 ± 0.0003	0.990	28	86	300
KIDNEY						
MNC	0.1488 ± 0.0619	0.0038 ± 0.0003	0.990	54	163	600
OTC	0.2242 ± 0.0641	0.0046 ± 0.0003	0.990	46	139	600
TC	0.2571 ± 0.0339	0.0033 ± 0.0002	0.994	34	103	600
DMC	0.1380 ± 0.0358	0.0027 ± 0.0002	0.990	44	133	600
CTC	0.1553 ± 0.0249	0.0023 ± 0.0001	0.993	36	108	600
MTC	0.1303 ± 0.0458	0.0037 ± 0.0002	0.991	41	124	600
DC	0.1742 ± 0.0425	0.0032 ± 0.0002	0.990	44	133	600

Table 7. Calculating errors a and b, as well as the limit of decision (CC_a) and capability of detection (CC_b) at the MRL enacted by the EU at 300 μg/kg liver

Analytes	Added (μg/kg)	Measured ± SD (μg/kg)	RSD	Recovery (%)	Error α (1.64*SD)	CC_α (μg/kg)
MNC	300	306.28 ± 9.91	3.2	102.1	16.26	312.26
OTC	300	303.26 ± 6.88	2.3	101.1	11.29	311.29
TC	300	310.40 ± 14.38	4.6	103.5	23.58	323.58
DMC	300	304.98 ± 13.34	4.4	101.7	21.88	321.88
CTC	300	294.37 ± 11.33	3.9	98.1	18.59	318.59
MTC	300	285.90 ± 11.23	3.9	95.3	18.41	318.41
DC	300	276.27 ± 11.40	4.1	92.1	18.69	318.69
Analytes	Added (μg/kg)	Measured ± SD (μg/kg)	RSD	Recovery (%)	Error β (1.64*SD)	CC_β (μg/kg)
MNC	312	306.69 ± 10.74	3.5	98.3	17.62	329.62
OTC	311	305.24 ± 8.18	2.7	98.1	13.42	324.42
TC	324	322.70 ± 4.52	1.4	99.6	7.42	331.42
DMC	322	320.92 ± 8.20	2.5	99.7	13.37	335.37
CTC	319	314.64 ± 14.30	4.5	98.6	23.45	342.45
MTC	318	316.51 ± 9.87	3.1	99.5	16.19	334.19
DC	319	307.67 ± 7.99	2.6	96.5	13.11	332.11

Table 8. Calculating errors a and b, as well as the limit of decision (CC_a) and capability of detection (CC_b) at the MRL enacted by the EU at 600 μg/kg kidney

Analytes	Added (μg/kg)	Measured ± SD (μg/kg)	RSD	Recovery (%)	Error α (1.64*SD)	CC_α(μg/kg)
MNC	600	578.86 ± 22.23	3.8	96.5	36.46	636.46
OTC	600	587.97 ± 15.92	2.7	97.0	26.11	626.11
TC	600	600.81 ± 19.69	3.3	100.1	32.30	632.30
DMC	600	600.99 ± 12.07	2.0	100.2	19.80	619.80
CTC	600	589.04 ± 28.26	4.8	98.2	46.34	646.34
MTC	600	596.99 ± 13.13	2.2	99.5	21.53	621.53
DC	600	594.92 ± 7.84	1.3	99.2	12.87	612.87
Analytes	Added (μg/kg)	Measured ± SD (μg/kg)	RSD	Recovery (%)	Error β (1.64*SD)	CC_β (μg/kg)
MNC	636	627.11 ± 10.00	1.6	98.6	16.40	652.40
OTC	626	623.55 ± 10.95	1.8	99.6	17.95	643.95
TC	632	625.45 ± 9.22	1.5	99.0	15.12	647.12
DMC	620	625.80 ± 16.47	2.6	100.9	27.01	647.01
CTC	646	637.93 ± 27.31	4.3	98.8	44.79	690.79
MTC	621	594.70 ± 17.37	2.9	95.8	28.49	649.49
DC	612	615.03 ± 15.33	2.5	100.5	25.15	637.15

of recovery, which was determined at these concentrations by comparing the peak area ratios for extracted TCs and the values derived from the respective calibration curve. Examination of accuracy of the method was based on within- and between- day repeatability. Measurements for that purpose were performed according to the procedure described in Method and Valuation According to ECD 2002/657/EC.

Precision and accuracy results are presented in Table 6. Precision of the method is considered to be very satisfactory, since RSD values in liver tissues were lower than 9.2. Recovery rates obtained in liver were for MNC: 125.5–97.8%, for OTC: 100.9–93.2%, for TC: 102.5–98.2%, for DMC: 112.4–98.9%, for CTC: 112.6–98.3%, for MTC: 106.3–102.8%, and for DC: 97.2–93.6%.

Stability of TCs in liver tissues under storage of aliquots of 1 g spiked at 100 μg/kg, was tested, and proven to be stable for at four weeks using as acceptance criterion a response comprised between 90 and 100% of the initial one. The same criterion was used in order to verify stability of TCs in liver tissues after four freezing-defrosting cycles. All TCs proved to be stable for two cycles as shown in Figure 4.

Finally, limits of decision (CC*a*) were calculated in both matrices by analyzing twenty blank liver or kidney samples spiked at the MRL (300 and 600 μg/kg, respectively), while detection capability (CC*b*) values

were obtained by analyzing twenty blank samples fortified at the corresponding CC*a* value for each TC. Results are summarized in Tables 7 and 8.

CONCLUSIONS

The herein developed confirmatory HPLC method proposes a simple and reliable analytical procedure for the simultaneous determination of residues of seven tetracyclines in bovine liver and kidney. The extraction procedure developed for the isolation of TCs from both matrices using common buffers and similar SPE protocols is very effective, despite the presence of unknown peaks at retention times different from that of TCs. According to the validation procedure results, the proposed method is reliable for the residual analysis of TCs in liver and kidney samples. The recovery rates for all TCs were higher than 93 and 96%, respectively, and LOD values were below the MRL set by the E.U. The new criteria of CC_α and CC_β specified by EU were also calculated.

REFERENCES

1. Bostoglou, N.A.; Fletouris, D.J. *Drug Residues in Foods, Pharmacology, Food Safety and Analysis*; Marcel Dekker: New York, U.S.A. 2001, 95–96.
2. www.fda.com (September 2007).
3. 96/23/EC: Council Directive of 29 April 1996 on measures to monitor certain substances and residues thereof in live animals and animal products and repealing Directives 85/358/EEC and 86/469/EEC and Decisions 89/187/EEC and 91/ 664/EEC. Off. J. Eur. Commun. **1996**, *L125*, 10–32.
4. 2377/90/EC: Council Regulation (EEC) of 26 June 1990 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin, Brussels, Belgium. Off. J. Eur. Commun. **1990**, *L224*, 1–124.
5. Myllyniemi, A.L.; Rintala, R.; Backman, C.; Niemi, A. Microbiological and chemical identification of antimicrobial drugs in kidney and muscle samples of bovine cattle and pigs. Food Addit. Contam. **1999**, *16* (8), 339–351.
6. Oka, H.; Ikai, Y.; Hayakawa, J.; Masuda, K.; Harada, K.; Suzuki, M.; Martz, V.; McNeil. J. Improvement of chemical analysis of antibiotics. 18. Identification of residual tetracyclines in bovine tissues by TLC/FABMS with a sample condensation technique. J. Agr. Food. Chem. **1993**, *41*, 410–418.

7. Moats, W. Determination of tetracycline antibiotics in beef and pork tissues using Ion-paired liquid chromatography. J. Agric. Food Chem. **2000**, *48*, 2244–2248.
8. Posyniak, A.; Zmudzki, J.; Semeniuk, S.; Niedzielskal, J.; Ellis, R. Determination of tetracycline residues in animal tissues by liquid chromatography. Biomed. Chromatogr. **1998**, *12* (5), 294–299.
9. Stubbings, G.; Tarbin, J. A.; Shearer, G. On-line metal chelate affinity chromatography clean-up for the high-performance liquid chromatographic determination of tetracycline antibiotics in animal tissues. J. Chromatogr. B. **1996**, *679* (1–2), 137–145.
10. Oka, H.; Matsumoto, H.; Uno, K.; Harada, K.; Kadowaki, S.; Suzuki, M. Improvement of chemical analysis of antibiotics : VIII. Application of prepacked C_{18} cartridge for the analysis of tetracycline residues in animal liver. J. Chromatogr. **1985**, *325*, 265–274.
11. Croubels, S.M.; Vanoosthuyze, K.E.I.; Van Peteghem, C.H. Use of metal chelate affinity chromatography and membrane-based ion-exchange as clean-up procedure for trace residue analysis of tetracyclines in animal tissues and egg. J. Chromatogr. B. **1997**, *690*, 173–179.
12. Degroodt, J.M.; De Bukanski, B.W.; Srebrnik, S. Multiresidue analysis of tetracyclines in kidney by HPLC and photodiode array Detection. J. Liq. Chromatogr. **1993**, *16* (16), 3515–3529.
13. Caro, E.; Marce, R.M.; Cormack, P.A.G.; Sherrington, D.C.; Borull, F. Synthesis and application of an oxytetracycline imprinted polymer for the solid-phase extraction of tetracycline antibiotics. Anal. Chim. Acta. **2005**, *552*, 81–86.
14. Cherlet, M.; Schelkens, M.; Croubels, S.; De Backer. P. Quantitative multiresidue analysis of tetracyclines and their 4-epimers in pig tissues by high-performance liquid chromatography combined with positive-ion electrospray ionization mass spectrometry. Anal. Chim. Acta. **2003**, *492* (1–2), 199–213.
15. Oka, H.; Ikai, Y.; Hayakawa, J.; Harada, K.; Suzuki, M.; Odani, H.; Maeda, K. Improvement of chemical analysis of antibiotics: XXIII. Identification of residual tetracyclines in bovine tissues by electrospray high-performance liquid chromatography–tandem mass spectrometry. J. Chromatogr. B. **1997**, *693* (1), 337–344.
16. Nakazawa, H.; Ino, S.; Kato, K.; Watanabe, T.; Ito, Y.; Oka, H. Simultaneous determination of residual tetracyclines in foods by high-performance liquid chromatography with atmospheric pressure chemical ionization tandem mass spectrometry. J. Chromatogr. B. **1999**, *732* (1), 55–64.
17. Blanchflower, W.J.; McCracken, R.J.; Haggan, A.S.; Kennedy, D.G. Confirmatory assay for the determination of tetracyline, oxytetracycline, chlortetracycline and its isomers in muscle and kidney using liquid chromatography-mass spectrometry. J. Chromatogr. B. **1997**, *692* (2), 351–360.
18. Goto, T.; Ito, Y.; Yamada, S.; Matsumoto, H.; Oka, H. High-throughput analysis of tetracycline and penicillin antibiotics in animal tissues using

electrospray tandem mass spectrometry with selected reaction monitoring transition. J. Chromatogr. A. **2005**, *100* (2), 193–199.
19. Oka, H.; Ito, Y.; Matsumoto. H. Chromatographic analysis of tetracycline antibiotics in foods. J. Chromatogr. A. **2000**, *882*, 109–131.

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