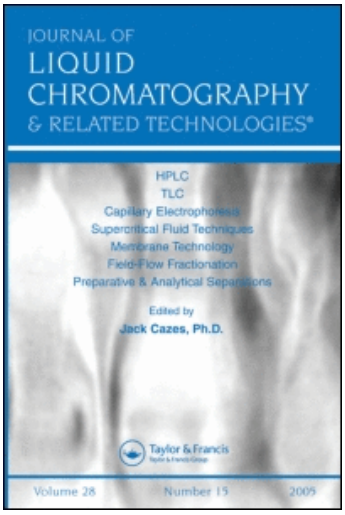

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Development and Validation of an HPLC Confirmatory Method for the Determination of Seven Tetracycline Antibiotics Residues in Bovine and Porcine Muscle Tissues According to 2002/657/EC

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Abstract: An HPLC method with diode array detection, at 355 nm, was developed and validated for the determination of seven tetracyclines (TCs) in bovine and porcine muscle tissues. Examined tetracyclines include: minocycline (MNC), tetracycline (TC), oxytetracycline (OTC), methacycline (MTC), demeclocycline (DMC), chlortetracycline (CTC), and doxycycline (DC). These were extracted from tissues using oxalate buffer (pH 4). Samples were purified by SPE on Nexus cartridges, using MeOH/ACN/0.05 M $C_2H_2O_4$ (30:30:40 v/v/v) as elution solvent. The separation was achieved on a Kromasil C_{18}, 5 μm, 250 × 4 mm, analytical column, operating at ambient temperature. The mobile phase, a mixture of A: 0.01 M oxalic acid and B: CH_3CN, was delivered using a gradient program. The procedure was validated according to the Decision 2002/657/EC, by determining selectivity, stability, decision limit, detection capability, accuracy, and precision. Overall recoveries of TCs from bovine and porcine samples ranged from 89–114.1%. All RSD values were lower than 8.5%. The decision limits CC*a* in bovine tissues ranged from 103.2 to 111.1 μg/kg, while detection capability CC*b* from 105.2 to 114.9 μg/kg. Respective values in porcine tissues were 102.5–106.4 μg/kg for CC*a* and 105.3–108.7 μg/kg for CC*b*.

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Keywords: 2002/657/EC, Bovine and porcine muscle tissues, HPLC, SPE, Tetracyclines, Validation

INTRODUCTION

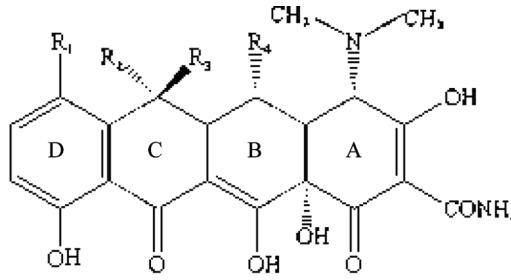
Antibiotics are used in veterinary medicine, both therapeutically to prevent and treat certain diseases and sub – therapeutically to fraudulently promote growth.[1] Out of the estimated total usage of antibiotics within the European Union (EU) plus Switzerland in 1997 (10,496 tons), antibiotics for veterinary purposes accounted for 48%. The world wide use of antibiotics for animal health purposes in 1997 was estimated at 27,000 tons with about 25% of these in the European Union (EU). Within the EU, 70% of this usage is estimated to arise for therapeutic purposes, while 30% arose from feed additive usage for growth promotion. Tetracyclines represent two thirds of the therapeutic antibiotics in veterinary medicine.[2]

Tetracycline antibiotics (TCs) are broad spectrum agents, exhibiting activity against infections caused by Gram-positive and Gram-negative bacteria, as well as chlamydia, mycoplasmas, rickettsiae, and protozoan parasites. TCs started to be used as growth promoters only four years after their discovery in 1945, and since then they remain the most used class of antibiotics, due to their broad activity, their few adverse effects, and the fact that they are relatively inexpensive. Only three TCs (TC, CTC, and OTC) are approved by the EU and FDA for therapeutic use in cattle's, pigs, sheep's, and poultries. However, four more tetracyclines, namely MNC, DC, DMC, and MTC are commercially available as human drugs and can also be used for veterinary purposes. Their chemical structures are given in Figure 1.[1–3]

The broad use of TCs for clinical medicine, veterinary, and stock breeding, during the last 40 years has lead to the emergence of resistant bacterial variants, a fact which has already eliminated the antibacterial activity of TCs. Moreover, residues of TCs in edible animal tissues can be toxic and dangerous for humans and they can potentially cause allergic reactions.[3]

The legislation of EU for drugs assures the safety and quality of products of animal origin by several council directives, some of which establish Maximum Residue Limits (MRLs) for drugs in such products (96/23/EC and 2377/90/EEC) and regulate the monitoring of the residues of drugs in animal products (657/2002/EC). TCs are classified in Group B1 (veterinary medicines and contaminants, with an MRL) of Annex I of directive 96/23/EC. The established MRL for TCs in muscle tissues have been set at 100 μg/kg.[4–6]

For monitoring the presence of TC residues in edible animal tissues, several methods have been developed: microbiological and immunoassays[7,8] as screening methods and chromatographic techniques like TLC,[9,10]



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

Figure 1. Chemical structures of examined tetracyclines.

HPLC coupled with UV,[11–21] fluorescence,[22–25] and MS[26–31] detectors, as confirmatory methods. The main problem for the determination and separation of TCs by HPLC arise from their capability to bind to the free silanol groups of silica based materials ($C_8$and C_{18}) in the analytical columns. This tendency results in a severe peak tailing and low column efficiency. In order to face the problem, most analysts add oxalic acid to the mobile phase.[9,11,13,17,21–23,26–28,31] Alternative solutions proposed include the use of end-capped modified silica based materials[13,20,22,29] and the use of polystyrene-divinylbenzene copolymer (PS-DVB) columns.[12,14,17,22,23,26,30]

Since all kinds of animal tissues are quite complex matrices, the isolation of TCs from those before analysis, demands extraction, deproteinizing, and cleanup steps. Moreover, the chemical structure and chemical properties of TCs make their isolation more difficult. TCs have a fused, partially aromatic 4 ring structure, with various substituents. They are amphoteric compounds, soluble in acids, bases, and alcohols. Due to their polar character, TCs bind strongly with proteins and chelate with divalent metal ions, like calcium. Consequently, acidic solvents are considered to be suitable for the extraction of TCs from tissues. However, TCs, which are quite unstable compounds, degrade in low pH values ($pH < 2$), so mild acidic buffers are mainly used for their extraction, like McIlvaine buffer,[9–11,14,18,19,25,27,29] succinate,[16,22,23,26,31] acetate,[24]

citrate,[12] oxalate,[17,21] and phosphate[13] buffer, while trichloroacetic acid,[11,19,26,31] hydrochloric acid,[20,28] acetonitrile,[13,17,24] and methanol[23,25] are used as deproteinizing agents. Goto et al. use ultra pure water in order to extract TCs from bovine tissues.[30]

In the majority of the proposed methods a cleanup procedure is required after the extraction of TCs and before HPLC analysis. Sample purification can be achieved by liquid-liquid extraction[13,20,24] as well as by SPE using C_8,[16,26] C_{18},[9–11,15,18,19,21,25,27,29] or polymeric SDB[17,28] cartridges conditioned with metal blocking agents, like EDTA and matrix solid phase dispersion (MSPD).[14] Techniques like metal chelate affinity chromatography,[12,22,23,16] exchange membranes,[21–22,30] and XAD-2 resins,[16] have also been proposed as cleaning up steps, however, these methods are characterized by complicated procedures and poor recoveries.

In the present work, a rapid, reliable, and sensitive method for quantitative simultaneous determination of residues of seven TCs most often used in stock breeding: i.e., MNC, TC, OTC, DMC CTC, MTC, and DC, in bovine and porcine muscle tissues is proposed. The isolation of TCs from the matrices is achieved with a simple extraction protocol using oxalate buffer (pH = 4) without a precipitation step, which is followed by an SPE procedure, using a hydrophilic-lipophilic sorbent. Separation and determination of seven TCs is then performed by an HPLC method using a C_{18}analytical column and oxalic acid in the mobile phase. Peak detection, identification, and quantitation are achieved by means of a diode-array detector. Validation of the method was based on the Commission Decision 2002/657/EC.

Three tetracycline: TC, OTC, and CTC, are approved by EU and FDA for therapeutic use in cattle and pig husbandry. However, four more TCs are commercially available as human drugs and can also be used illegally for veterinary purposes. Since so far published works determine mainly OTC, TC, and CTC, the rest are included in this work as well. Validation of the method was based on the Commission Decision 2002/657/EC and decision limits and detection capabilities of the seven TCs were calculated. Additionally, to the best of our knowledge, it is the only method validated according to the Commission Decision 2002/657/EC for all seven TCs.

EXPERIMENTAL

Chemicals and Reagents

TC, CTC, and internal standard, colchicine, were purchased from Fluka (http://www.sigmaaldrich.com/Brands/Fluka_Riedel_Home/About_Fluaka_and_Riedel.html, Buchs SG, Switzerland), OTC, MNC, DMC,

MTC, and DC from Sigma (www.sigmaaldrich.com, St. Louis, MO, USA). HPLC grade methanol and acetonitrile were supplied by Carlo Erba (www.carloerbareagenti.com, Milano, Italy). Sodium hydroxide, oxalic acid, hydrogen sodium phosphate, and Na_2EDTA were obtained from Merck (www.merck.com, Darmstadt, Germany), while citric acid monohydrate and sodium citrate trihydrate of analytical grade were supplied by Riedel-de-Haen (http://www.sigmaaldrich.com/Brands/Fluka___Riedel_Home/About_Fluka_and_Riedel.html, Seezle, Germany). High purity water obtained by a Milli-Q purification system (www.millipore.com, Millipore, Bedford, MA, USA) was used throughout the study. Bovine and porcine muscle tissue samples *longissimus dorsi* were supplied by a local market.

Instrumentation

A Shimadzu (www.shimadzu.com, Kyoto, Japan) quaternary low pressure gradient system was used for chromatographic determination of the examined TCs. The solvent lines were mixed in an FCV-$10AL_{VP}$-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. Sample injection was performed via a Rheodyne 7725i injection valve (Rheodyne, www.rheodyne.com, Cotati, California, USA) equipped with a 20 μL 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 continuous helium sparging in the solvents reservoirs by a DGU-10B degassing unit.

The analytical column, a Kromasil C_{18}, 5 μm, 250 × 4 mm, was purchased from MZ-Analysentechnik (www.mz-at.de, Mainz, Germany).

A glass vacuum filtration apparatus obtained from Alltech (Alltech Associates, www.alltechweb.com, Deerfield, IL, USA) was employed for the filtration of the buffer solution, using Whatman cellulose-nitrate 0.2 μm membrane filters, (www.whatman.com, Whatman Laboratory Division, Maidstone, England).

A Glasscol small vortexer (www.glascol.com, Terre Haute, IN, USA) and a Hermle centrifuge, model Z 230 (B. Hermle, www.hermle.de, Gosheim, Germany) were employed for the pretreatment of muscle tissue samples.

The SPE study was performed on a Vac-Elut vacuum manifold column processor, purchased from Analytichem International. SPE cartridges LiChrolut 100 mg/cm^3 were supplied by Merck, Discovery

Table 1. Gradient program for the elution of the examined TCs

t (min)	A: $C_2H_2O_4$ (0.01 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		

500 mg/3 mL by Supelco(www.sigmaaldrich.com/Brands/Supelco_Home.html, Bellefonte, PA, USA), and Nexus cartridges 30 mg/cm^3 by Varian (www.varianinc.com, Harbor City, CA, USA). All evaporations were performed with a Supelco 6 port Mini-Vap concentrator/evaporator.

Chromatography

A Kromasil C_{18}, 5 μm, 250 × 4 mm analytical column, operated at ambient temperature, was used for the separation of the seven tetracyclines. The mobile phase, a mixture of 0.01 M oxalic acid as solvent A and CH_3CN as solvent B, was delivered to the analytical column according to a gradient program, shown in Table 1, with a flow rate which was also changing during the analysis. An equilibration time of 3 min was required between runs. The monitoring of the examined TCs was performed at 355 nm.

Preparation of Standards

Stock standard solutions of each TC at a concentration of 100 ng/μL were prepared by dissolving the appropiate amount of the analyte in methanol. These solutions were found to be stable for 2 months, when stored refrigerated at 4°C and wrapped in aluminium foil. Working methanolic standards were prepared from stocks by the appropriate dilution at the following concentrations: 0.5, 0.8, 1, 2, 5, 8, 10, and 15 ng/μL. All working standards contained colchicine as internal standard at a concentration of 5 ng/μL. All solutions were protected 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.

Buffer solutions used for the extraction of TCs from bovine and porcine 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 (0.4 M, pH 4) by mixing 0.4 M oxalic acid and 0.1 M sodium hydroxide. McIlvaine buffer was prepared by mixing 0.1 M citric acid and 0.5 M hydrogen sodium phosphate in a ratio of 38.5:61.5 v/v and 0.1 M Na_2EDTA/McIlvaine buffer by dissolving 1.5 g hydrogen sodium phosphate, 1.3 g citric acid, and 0.372 g Na_2ETDA in 100 mL of water.

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

In the present paper, the proposed method for the determination of residual TCs was validated according to the European Commission Decision 2002/657/EC, using spiked bovine and porcine muscle samples, since a certified reference material (CRM) was not available for TCs in muscles. From the performance characteristics enacted by EU, linearity, accuracy, precision, sensitivity, and stability were examined using spiked bovine and porcine samples at various concentrations.

Linearity and Sensitivity

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. Linearity response was then examined in bovine and porcine muscle tissue samples by analysing a series of those samples spiked with mixed standard solutions 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 calculations for the limits of detection (LODs) were based on the standard deviation of y intercepts of regression analysis (σ) and the slope (S), using the following equation $LOD = 3.3\ \sigma/S$. Limits of quantitation (LOQs) were calculated by the equation $LOQ = 10\ \sigma/S$.

Precision and Accuracy

Precision and accuracy expressed in terms of TCs recovery from porcine and bovine samples was studied by analyzing spiked samples at three concentration levels (40, 100, 200 μg/kg). 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 the period of six 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 replicate analysis of spiked samples in various concentrations.

Limits of Decision (CC*a*) and Detection Capability (CC*b*)

The decision limit, CC*a*, was calculated as the mean measured concentration at the MRL (100 μg/kg) 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 of spiked samples at CC*a*. Statistical analysis was performed at the 95% confidential level and the number of replicate analyses was 20.

Selectivity and Stability

The selectivity of the method was assessed by the absence of interference peaks from endogenous compounds and was investigated by the analysis of ten different blank bovine and porcine muscle tissue samples. Peak purity was checked by means of a PDA detector, using the 3 point mode. Comparison of spectra at up-slope, apex, and down-slope provides data required for peak purity evaluation.

TCs stability in muscles was investigated as follows: homogenized blank bovine and porcine muscle tissue samples were divided into five aliquots of 1 g. Each aliquot was spiked with TCs at 200 μg/kg. One aliquot was analyzed immediately, while the remaining aliquots were stored at –18°C and analyzed after 0.5, 1, and 2 months. Stability was also investigated after several freezing defrosting cycles. Aliquots of frozen spiked muscle samples were left at room temperature to defrost and analyzed after four freezing defrosting cycles.

Sample Preparation

Isolation from the Matrix

Bovine or porcine muscle tissue samples were minced and homogenized in a porcelain mortar. Aliquots of 1 g were accurately weighed and fortified 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. Mixtures were subsequently homogenized in a vortexer for 2 min and after 15 min in calm, 5 mL of 0.4 M oxalate buffer (pH = 4) were added. These mixtures were vortexed for 1 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.

Citrate buffer (0.3 M, pH = 4), Citrate buffer (0.4 M, pH = 4), oxalate buffer (0.3 M, pH 4), oxalate buffer (0.4 M, pH 4), 1% AcN-Citrate

(0.3 M. pH 4), 2% AcN-Citrate (0.3 M. pH 4), McIlvaine buffer (pH 4), and McIlvaine/Na_2EDTA (pH 4), were tested as extraction solvents in order to optimize the isolation procedure.

Solid Phase Extraction

Solid phase extraction was chosen to be used as a purification step after extraction of TCs from tissues. SPE protocol development was tested using standard solutions prior to the application of the method to edible tissues. Three different sorbents were tested: Abselut Nexus by Varian, Discovery by Supelco, and Lichrolut by Merck. After preconditioning the cartridges by flushing 2 mL of methanol and 2 mL of water, 100 μL from a standard solutions of TCs were applied by allowing them to pass through the bed sorbent without suction. Elution efficiency was examined using three eluting solvents MeOH/AcN/$C_2H_2O_4$ (30:30:40), MeOH/AcN/$C_2H_2O_4$ (25:25:50) and MeOH/AcN/$C_2H_2O_4$ (35:35:30), which were selected according to our previous experience on TC analysis.[21] The samples were subsequently evaporated to dryness under a nitrogen steam in a water bath at 35°C and the residues were dissolved in 100 μL of methanol.

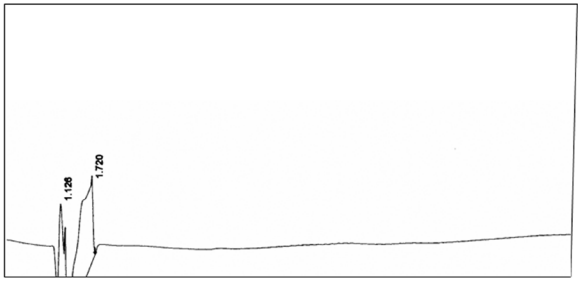
The optimum protocol was then applied as a purification method to the combined extracts from the spiked bovine or porcine muscle samples. The supernatants were applied to preconditioned Nexus cartridges after filtration through 0.2 μm Whatman filter papers.

RESULTS AND DISCUSSION

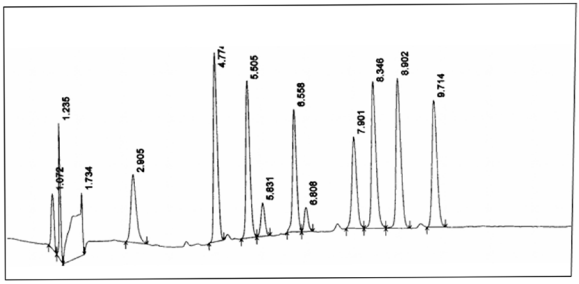
Chromatography

The seven studied TCs and the internal standard are well separated in 10 min. Retention times of the examined analytes are 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). 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 are found to be 3.4 for MNC–OTC, 2.0 for OTC–TC, 3.0 for TC–DMC, 3.5 for DMC–CTC, 1.0 for CTC–MTC, 3.2 for MTC-DC, 1.5 for DC-IS, indicating a satisfactory separation. The flow rate was initially 1.65 mL/min and it was decreased to 1.2 mL/min after 2 min. for optimum analysis time and peak shape.

Typical high performance liquid chromatograms of blank and spiked samples of bovine and porcine tissues are shown in Figures 2a–2d, respectively. No peaks from endogenous compounds were noticed.



(a)



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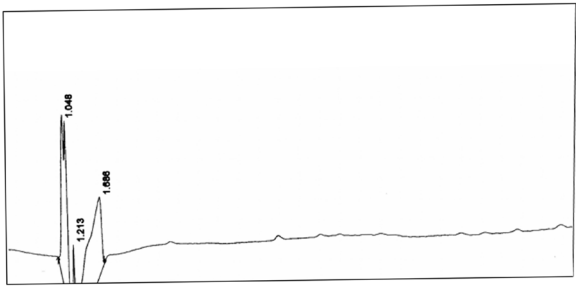

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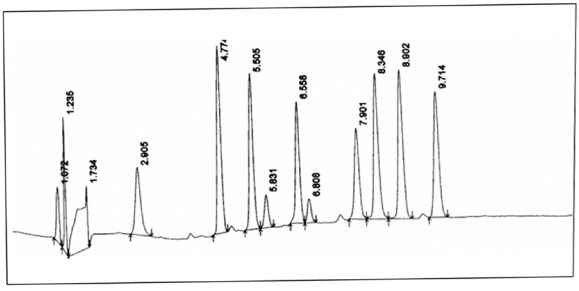

(d)

Sample Preparation

SPE in Standard Solutions

Three different sorbents (Nexus, Discovery, and LiChrolut) and three different protocols with different elution solvents were tested, in order to find the most efficient SPE as a clean up method to the extracts from bovine and porcine muscle tissue samples. Recovery rates, calculated by comparing the observed analyte concentration in extracted standard solutions to those of non-processed standard solutions, of each protocol can be seen in Table 2. From the recovery results shown in this table it can be concluded that the highest efficiency in TCs extraction (63.4–105.5%) is achieved by Nexus cartridges, using a mixture of MeOH/ACN/0.01 M $C_2H_2O_4$ (30:30:40 v/v/v) as elution solvent.

Extraction from Tissues

As described in section above various buffer solutions and deproteinizing agents were tested in order to optimize extraction of TC from bovine tissues. As shown from the results summarized in Table 3, the use of oxalate buffer (0.4 M, pH = 4) provides better recovery rates than McIlvaine, McIlvaine with Na_2EDTA or citrate buffer. Moreover, the use of AcN as a deproteinizing agent before the addition of the buffer solution didn't enhance recovery.

Extraction efficiency of TCs from bovine samples was also studied using three different oxalate buffer concentration levels, and three pH values. Results are shown in Figures 3A–B. Optimum recovery rates were obtained using 0.4 M oxalate buffer at pH 4.

Since porcine muscle tissues are slightly different from the bovine ones, the two most efficient extraction buffers (0.4 M oxalate, pH 4 and 0.4 M citrate pH 4) were tested in order to find the optimum extraction protocol for porcine muscle tissue samples. From the results shown in Table 3, the two buffers present similar extraction efficiencies, with the recovery rates from oxalate buffer being slightly better. Consequently, 0.4 M oxalate buffer at pH 4 was used for the extraction of TCs from both bovine and porcine muscle tissue samples.

Figure 2. High performance liquid chromatogram of a: Blank bovine tissue. b: spiked bovine tissue at 10 ng/ μL, after SPE, using the conditions described in text. Peaks: (1) MNC 2.905 min, (2) OTC 4.774 min, (3) TC 5.505 min, (4) DMC 6.558 min, (5) CTC 7.901 min, (6) MTC 8.346 min, (7) DC 8.902 min, and colchicine (IS) 9.714 min. c : Blank porcine tissue and d: spiked porcine tissue at 8 ng/ μL, after SPE, using the conditions described in text. Peaks: (1) MNC 2.986 min, (2) OTC 4.906 min, (3) TC 5.637 min, (4) DMC 6.679 min, (5) CTC 8.017 min, (6) MTC 8.464 min, (7) DC 9.008 min, and colchicine (IS) 9.614 min.

Table 2. Recoveries of the examined TCs after solid-phase extraction in standard solutions using various extraction protocols

SPE sorbent	Elution solvent	MNC	OTC	TC	DMC	CTC	MTC	DC
Discovery	MeOH/ACN/$C_2H_2O_4$ (30:30:40)	**52.4**	**95.1**	**94.7**	**90.0**	**74.8**	**78.4**	**64.5**
	MeOH/ACN/$C_2H_2O_4$ (25:25:50)	37.6	66.5	47.2	66.4	34.9	22.2	11.1
	MeOH/ACN/$C_2H_2O_4$ (35:35:30)	36.7	75.6	68.4	70.6	69.0	70.6	55.7
LiChrolut	MeOH/ACN/$C_2H_2O_4$ (30:30:40)	**77.3**	**86.2**	**85.5**	**94.5**	**88.4**	**85.5**	**77.6**
	MeOH/ACN/$C_2H_2O_4$ (25:25:50)	51.3	52.0	48.7	54.4	56.1	53.8	59.0
	MeOH/ACN/$C_2H_2O_4$ (35:35:30)	94.2	81.3	69.0	89.3	83.1	99.9	89.1
	MeOH/ACN/$C_2H_2O_4$ (40:40:20)	52.3	43.6	40.1	49.5	46	42.7	44.3
	MeOH/ACN(50:50)	<15	<15	<15	<15	<15	<15	<15
	MeOH	<20	<20	<20	<20	<20	<20	<20
	MeOH/ACN/$C_2H_2O_4 + 10^{-4}$M Na_2EDTA (30:30:40)	71.1	97.3	70.6	97.6	98.7	89.1	92.3
Nexus	MeOH/ACN (50:50)	60.1	41.4	60.6	78.2	68.8	53.9	57.7
	ACN/$C_2H_2O_4$ (50:50)	69.9	55.3	59.4	85.4	73.3	56.8	62.1
	MeOH/$C_2H_2O_4$ (50:50)	73.2	66.1	64.3	90.2	86.9	70.1	76.5
	MeOH/ACN/$C_2H_2O_4$ (30:30:40)	85.6	98.2	68.7	93.2	91.9	108.3	96.1
	MeOH/ACN/$C_2H_2O_4$ (25:25:50)	25.5	85.6	98.3	68.6	93.2	91.9	108.3
	MeOH/ACN/$C_2H_2O_4$ (35:35:30)	79.4	96.5	56.4	98.9	96.7	109.2	98.9

Table 3. Recoveries of the examined TCs after solid-phase extraction in extracts from bovine samples using various extraction protocols

Extraction solvent	Recovery (%)						
	MNC	OTC	TC	DMC	CTC	MTC	DC
Citrate (0.3 M, pH 4)	90.1	82.2	53.6	64.0	56.6	79.4	126.2
Citric acid (0.3 M, pH 4)	116.1	89.8	51.3	74.9	66.7	91.2	126.7
1% ACN-Citrate (0.3 M, pH 4)	69.9	82.8	67.4	64.5	71.8	52.3	83.1
2% ACN-Citrate (0.3 M, pH 4)	67.8	77.6	68.7	57.1	60.1	63.2	79.5
Oxalate (0.3 M, pH 4)	83.9	85.4	82.3	85.8	86.8	84.1	89.1
McIlvaine (pH 4)	<50	<50	<50	<50	<50	<50	<50
McIlvaine/Na_2EDTA (pH 4)	<60	<60	<60	<60	<60	<60	<60
Citrate (0.4 M, pH 4)	77.9	95.5	85.6	83.4	71.5	77.8	83.1
Citrate Buffer* (0.4 M, pH 4)	88.1	73.4	95.2	99.9	112.5	89.7	96.3
Oxalate (0.4 M, pH 4)	84.6	87.2	80.2	93.4	95.6	91.2	97.8
Oxalate* (0.4 M, pH 4)	80.2	85.9	85.6	89.8	92.3	95.4	90.1

*assay on porcine tissues.

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.

Linearity and Sensitivity

Calibration curves were constructed both for standard solutions and for bovine and porcine muscle tissue samples and were obtained by least squares linear regression analysis of the peak area ratio of analyte to internal standard versus analyte injected amount. The method was linear up to 15 ng/ μL for all TCs in both standard solutions and muscle samples. All calibration data as well as LOD and LOQ values are presented in Table 4, both for standard solutions and for muscle tissues.

Selectivity

In order to verify the method selectivity the developed method was applied to ten blank bovine and ten blank porcine samples. No interferences were detected by unknown endogenous peaks from the matrix in both muscle tissue samples, as can be seen from the chromatograms of those blank samples as shown in Figures 2a and 2c.

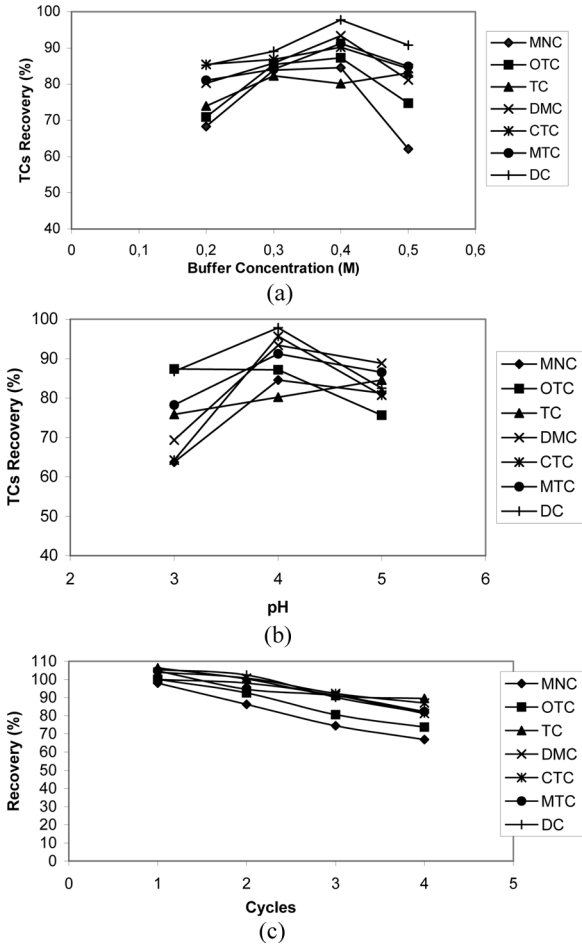

Figure 3. a: Tetracyclines' recovery from bovine muscle tissues at different oxalate buffer concentration levels. b: Tetracyclines' recovery from bovine muscle tissues at three pH values. c: Stability of the examined tetracyclines during several freezing defrosting cycles.

Precision and Accuracy

Studies using bovine and porcine muscle tissue samples spiked at three levels: 40, 100, and 200 μg/kg tissue, were carried out for the determination of the precision and the accuracy of the pretreatment protocol, by replicate injections (n = 6) from these samples. 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

Table 4. Calibration and sensitivity data of the examined Tetracyclines in standard solutions as well as in bovine and porcine samples after solid-phase extraction

TCs	Slope (ng^{-1})	intercept	r	Uncertainty of the blank (n = 7) 1.90 × SD	LOD (ng)	LOQ (ng)
	Standard Solutions					
MNC	0.0048 ± 0.0002	−0.0288 ± 0.0299	0.9941	0.0568	6	20
OTC	0.0080 ± 0.0002	0.0296 ± 0.0442	0.9952	0.084	3	10
TC	0.0066 ± 0.0002	0.0225 ± 0.0250	0.9980	0.0475	3	10
DMC	0.0046 ± 0.0001	0.0017 ± 0.0208	0.9968	0.0395	3	10
CTC	0.0040 ± 0.0001	0.0092 ± 0.0219	0.9953	0.0416	3	10
MTC	0.0064 ± 0.0002	0.0406 ± 0.0308	0.9964	0.0585	3	10
DC	0.0063 ± 0.0002	0.0317 ± 0.0282	0.9969	0.0536	3	10
	Bovine					
MNC	0.0047 ± 0.0001	−0.0153 ± 0.0100	0.9998	0.0190	7	21
OTC	0.0060 ± 0.0003	0.0665 ± 0.0451	0.9967	0.0857	14	42
TC	0.0043 ± 0.0004	0.0891 ± 0.0361	0.9924	0.0686	12	36
DMC	0.0037 ± 0.0002	0.0246 ± 0.0216	0.9973	0.0410	13	39

CTC	0.0030 ± 0.0001	0.0538 ± 0.0184	0.9970	0.0350	14	42
MTC	0.0048 ± 0.0002	0.0601 ± 0.0207	0.9989	0.0393	10	30
DC	0.0057 ± 0.0001	0.0508 ± 0.0193	0.9994	0.0367	11	33
	Porcine					
MNC	0.0052 ± 0.0002	-0.0399 ± 0.0282	0.9974	0.0536	12	36
OTC	0.0074 ± 0.0002	0.0183 ± 0.0360	0.9978	0.0684	12	36
TC	0.0050 ± 0.0001	0.0256 ± 0.0189	0.9987	0.0359	9	27
DMC	0.0044 ± 0.0001	0.0202 ± 0.0239	0.9976	0.0454	10	30
CTC	0.0035 ± 0.0001	0.0131 ± 0.0169	0.9982	0.0321	13	39
MTC	0.0056 ± 0.0002	0.0102 ± 0.0284	0.9986	0.0540	11	33
DC	0.0055 ± 0.0001	0.0723 ± 0.0246	0.9984	0.0467	9	27

derived from the respective calibration curve. For the between-day precision study bovine and porcine samples spiked at the same concentration range as above were tested. A triplicate determination of each concentration was conducted during routine operation of the system over a period of five consecutive days.

Results of accuracy and precision studies, expressed in terms of recovery and relative standard deviation (RSD) respectively, are given in Table 5. RSD values for all the examined TCs in bovine samples were lower than 8.6%, while mean recovery rates were for MNC: 89.0–95.5%, for OTC: 95.9–100.7%, for TC: 93.7–114.1%, for DMC: 99.0–105.2%, for CTC: 93.9–108.2%, for MTC: 99.7–109.7%, and for DC: 96.7–110.5%. For porcine samples RSD values were lower than 6.7% and mean recovery rates were for MNC: 94.3–100.5%, for OTC: 94.2–104.3%, for TC: 97.8–111.1%, for DMC: 97.5–105.2%, for CTC: 102.1–104.1%, for MTC: 97.6–107.1%, and for DC: 101.3–106.7%.

Stability

In order to investigate stability of TCs in muscle bovine and porcine muscle tissue samples, spiked samples at 200 μg/kg and 100 μg/kg, respectively, were stored at –18°C. These samples were analyzed after 1/2, 1, and 2 months and stability was demostrated for at least 2 months for both matrixes. Stability was also assessed after four freezing defrosting cycles. Degradation decision was based upon −10% criterion. As shown in Figure 3c all TCs were stable for three cycles, except for OTC and MNC, which were stable for two or one respectively.

Decision Limit and Detection Capability

Moreover, the validation procedure, according to the 2002/657/EC decision, includes the determination of two novel criteria CC*a* (limit of decision) and CC*b* (detection capability). The CC*a* values were calculated by spiking and analyzing 20 blank bovine tissue samples at the MRL (100 μg/kg) established from EU. The same procedure was followed for the determination of CC*a* values in porcine muscle tissues. CC*b* values were calculated by analyzing 20 blank spiked samples at the corresponding CC*a* level for each analyte, for both matrices. Results are illustrated in Tables 6 and 7, for bovine and porcine muscle tissue samples, respectively. CC*a* values ranged from 103.2 to 111.1 μg/kg for bovine tissues and from 102.5 to 106.4 μg/kg for porcine tissues, while CC*b* values from 105.2 to 114.9 μg/kg and from 105.3–108.7 μg/kg for the two matrices, respectively.

Table 5. Within and Between-day precision and accuracy of the developed method for the determination of seven TCs in bovine and porcine samples after SPE

		Within-day (n = 5)			Between-day (n = 5)		
Analytes	Added (μg/kg)	Measured ± SD (μg/kg)	RSD	Recovery (%)	Measured ± SD (μg/kg)	RSD	Recovery (%)
Bovine							
MNC	40	36 ± 2.2	6.2	89.0	36 ± 1.9	5.5	88.8
	100	90.5 ± 7.8	8.6	90.5	95 ± 5.9	6.2	94.9
	200	179 ± 13.0	7.2	89.7	192 ± 14.9	7.8	95.9
OTC	40	40 ± 1.9	4.7	100.7	39 ± 1.6	4.0	97.6
	100	97 ± 1.1	1.1	97.4	98 ± 4.7	4.8	98.4
	200	193 ± 3.7	1.9	96.3	197 ± 1.3	0.7	98.7
TC	40	49 ± 2.6	6.5	100	38 ± 2.6	6.9	93.7
	100	114 ± 3.1	2.7	114.1	104 ± 4.3	4.2	103.6
	200	207 ± 11.8	5.7	103.2	204 ± 5.2	2.5	102.0
DMC	40	41 ± 1.0	2.5	101.5	40 ± 2.4	6.1	99.0
	100	105 ± 4.9	4.6	105.2	104 ± 7.0	6.8	104.2
	200	208 ± 13.7	5.7	104.5	198 ± 7.7	3.9	99.0
CTC	40	39 ± 3.4	8.8	96.2	38 ± 1.9	5.5	93.9
	100	108 ± 1.3	1.2	108.2	105 ± 2.2	2.1	105.0
	200	213 ± 4.0	1.9	106.5	210 ± 8.8	4.2	105.3
MTC	40	40 ± 2.1	5.2	99.7	40 ± 2.1	5.3	100.3
	100	110 ± 3.0	2.8	109.7	102 ± 6.8	6.7	102.1
	200	199 ± 8.5	4.3	99.8	199 ± 7.9	4.0	99.9
DC	40	39 ± 2.7	7.0	96.7	39 ± 2.4	6.2	98.4
	100	111 ± 1.7	1.5	110.5	109 ± 2.6	2.4	109.5
	200	206 ± 5.9	2.9	102.9	201 ± 2.1	1.1	100.5
Porcine							
MNC	40	40 ± 1.0	2.5	100.5	38 ± 1.0	2.7	96.0
	100	96 ± 1.3	1.3	96.0	97 ± 1.9	2.0	97.4
	200	189 ± 1.9	1.0	94.3	192 ± 6.1	3.2	96.1
OTC	40	41 ± 0.9	2.3	102.3	42 ± 1.0	2.3	104.3
	100	100 ± 1.3	1.3	100.2	101 ± 1.8	1.8	101.3
	200	188 ± 2.0	1.1	94.2	195 ± 4.5	2.3	97.6
TC	40	44 ± 0.8	1.8	111.1	44 ± 2.0	4.5	108.8
	100	98 ± 3.9	4.0	97.8	104 ± 6.5	6.2	103.5
	200	200 ± 7.5	3.6	104.9	215 ± 2.9	1.4	107.3
DMC	40	39 ± 1.4	3.5	97.5	39 ± 1.9	4.9	98.2
	100	101 ± 1.7	1.7	100.6	100 ± 1.4	1.5	100.0
	200	210 ± 4.7	2.2	104.9	210 ± 2.9	1.4	105.2
CTC	40	41 ± 1.9	4.7	103.0	41 ± 2.0	4.7	102.9
	100	102 ± 3.2	3.1	102.1	105 ± 1.7	1.6	104.9
	200	205 ± 2.3	1.1	102.4	208 ± 4.6	2.2	104.1

(*Continued*)

Table 5. Continued

Analytes	Added (μg/kg)	Within-day (n = 5) Measured ± SD (μg/kg)	RSD	Recovery (%)	Between-day (n = 5) Measured ± SD (μg/kg)	RSD	Recovery (%)
MTC	40	43 ± 1.6	3.8	107.1	42 ± 1.3	3.1	105.1
	100	98 ± 1.6	1.6	97.6	100 ± 2.2	2.2	99.8
	200	206 ± 3.2	1.6	103.2	199 ± 7.9	3.9	99.6
DC	40	42 ± 0.9	2.2	105.2	43 ± 2.1	2.1	106.7
	100	103 ± 1.3	1.3	102.8	104 ± 0.9	0.8	104.4
	200	213 ± 2.1	1.0	106.6	203 ± 9.4	4.7	101.3

Application to Real Samples

The developed method was applied to ten bovine and ten porcine samples from the local market. None of the examined tetracyclines were detected in these samples.

Table 6. 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 100 μg/kg in bovine samples

Analytes	Added (μg/kg)	Measured ± SD (μg/kg)	RSD	Recovery (%)	Error α (1.64*SD)	CC_α (μg/kg)
MNC	100	86.82 ± 4.10	4.7	86.8	6.72	106.7
OTC	100	96.62 ± 2.81	2.9	96.6	4.61	104.6
TC	100	109.92 ± 3.83	3.5	109.9	6.28	106.3
DMC	100	106.50 ± 6.79	6.4	106.5	11.13	111.1
CTC	100	118.17 ± 2.31	2.0	118.2	3.79	103.8
MTC	100	106.89 ± 4.81	4.5	106.9	7.88	107.9
DC	100	105.59 ± 1.96	1.9	105.6	3.22	103.2
Analytes	Added (μg/kg)	Measured ± SD (μg/kg)	RSD	Recovery (%)	Error β (1.64*SD)	CC_β (μg/kg)
MNC	107	99.50 ± 2.11	2.12	93.2	3.47	110.5
OTC	105	103.58 ± 0.79	0.76	99.0	1.29	106.3
TC	106	114.65 ± 1.15	1.00	107.0	1.89	107.9
DMC	111	120.45 ± 2.32	1.93	108.4	1.80	112.8
CTC	104	113.45 ± 1.02	0.89	109.3	1.66	105.7
MTC	108	114.52 ± 2.03	1.77	106.1	3.33	111.3
DC	103	115.80 ± 1.22	1.05	112.2	2.00	105.0

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 100 μg/kg in porcine samples

Analytes	Added (μg/kg)	Measured ± SD (μg/kg)	RSD	Recovery (%)	Error α (1.64*SD)	CC_α (μg/kg)
MNC	100	91.60 ± 3.88	4.2	91.6	6.36	106.4
OTC	100	98.76 ± 2.22	2.4	98.8	3.64	103.6
TC	100	107.04 ± 1.50	1.4	107.0	2.47	102.5
DMC	100	103.65 ± 2.72	2.6	103.7	4.45	104.4
CTC	100	105.52 ± 1.88	1.8	105.5	3.08	103.1
MTC	100	96.88 ± 1.72	1.8	96.5	2.82	102.8
DC	100	99.51 ± 1.82	1.8	99.5	2.99	103.0
Analytes	Added (μg/kg)	Measured ± SD (μg/kg)	RSD	Recovery (%)	Error β (1.64*SD)	CC_β (μg/kg)
MNC	106	104.23 ± 1.04	1.0	98.3	2.68	108.7
OTC	104	104.68 ± 1.13	1.1	100.7	2.77	106.8
TC	102	109.04 ± 1.67	1.5	106.9	3.31	105.3
DMC	104	105.84 ± 2.84	2.7	101.7	4.48	108.5
CTC	103	106.08 ± 2.34	2.2	103.0	3.98	107.0
MTC	103	103.65 ± 1.99	1.9	100.6	3.63	106.6
DC	103	105.19 ± 1.00	1.0	102.1	2.68	105.7

CONCLUSIONS

In the present work, a confirmatory method for the determination and quantitation of residues of seven TCs in bovine and porcine tissues is proposed. The developed method can be characterized as fast, since the seven investigated tetracyclines are well resolved in less than 10 min, simple, as the pretreatment procedure for both matrices involves an extraction step with a common buffer and a SPE protocol as a purification step, and reliable, according to the results from the validation study.

Validation was performed according to the European Union decision 2002/657/EC for the validation of an analytical method for residues in animal products. LOQ values achieved were less than ½ of the MRL levels. Overall recoveries of TCs from spiked bovine and porcine samples at fortification levels of 40, 100, and 200 μg/kg were 89–100.5% for MNC, 94.2–104.3% for OTC, 93.7–114.1% for TC, 97.5–105.2% for DMC, 93.9–108.2% for CTC, 97.6–109.7% for MTC, and 96.5–110.5% for DC. All RSD values were lower than 8.5%. Finally the decision limits CC*a* ranged from 102.5 to 111.1 μg/kg for both matrices, while detection capability CC*b* from 105.2 to 114.9 μg/kg.

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