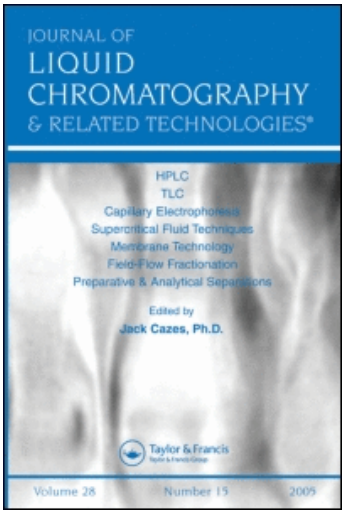

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Development and Validation of an HPLC Method for the Determination of Seven Tetracycline Antibiotics Residues in Chicken Muscle and Egg Yolk According to 2002/657/EC

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Abstract: Herein, an HPLC method with diode-array detection, at 355 nm, is described for the determination of seven tetracyclines in chicken muscle and egg yolk: minocycline, tetracycline, oxytetracycline, methacycline, demeclocycline, chlortetracycline, and doxycycline. The examined TCs were extracted from chicken muscle with 15% TFA and 0.4 M oxalate buffer (pH 4) and from egg yolk with 40% TFA and 0.4 M oxalate buffer (pH 4). LiChrolut and Nexus SPE cartridges were used for further purification of chicken tissue and egg yolk extracts, 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 92–110.1% and 89–106% for chicken tissues and eggs, respectively. All RSD values were lower than 11%. The decision limits CC_a in chicken tissues ranged from 102.98 to 109.11 μg/kg, while detection capability CC_b from 113.45 to 118.18 μg/kg. Respective values in eggs were 206.53–214.60 μg/kg for CC_a and 216.21–228.97 μg/kg for CC_b.

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Keywords: Chicken muscle, Chlortetracycline, Demeclocycline, Doxycycline validation, Egg yolk, HPLC, Methacycline, Minocycline, Oxytetracycline, SPE, Tetracycline, Tetracyclines, 2002/657/EC

INTRODUCTION

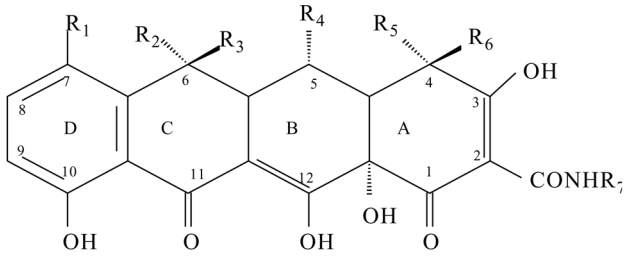
Antibiotics are commonly used all over the world as veterinary medicines, but also as feed additives to promote growth. This practice generates concerns that may contribute to the development of antimicrobial resistance and lead to the contamination of products of animal origin.[1] In order to assure food quality and protect human health European Union regulates the use of veterinary medicines through council regulation. Council Directive 96/23/EC describes the procedure for the establishment of Maximum Residue Level (MRL) values for veterinary medicinal products in foodstuffs of animal origin and regulates the residue monitoring of pharmacologically active compounds in those products. This directive classifies all residues into two groups: Group A, which comprises prohibited substances, and Group B which includes all registered veterinary drugs in conformity with Annexes I and III of 2377/90/EC and other residues.[2,3] The Decision 2002/657/EC[4] defines the performance criteria and the interpretation of results for analytical methods in the official control of residues in products of animal origin. The methods currently applied for the analysis of official samples of the substances in group B of Annex I of Council Directive 96/23/EC have to comply with the decision 2002/657/EC by 1 September 2007.[5]

Chicken meat and eggs are probably the most accepted foodstuff of animal origin and tetracyclines (TCs), a semi synthetic group of antibiotics with broad antimicrobial activity, are according to E.U. statistical data, some of the most frequently used veterinary drugs in animal husbandry. TCs are classified in group B_1 (veterinary medicines and contaminants, with an MRL) of Annex I of Directive 96/23/EC. MRL values set for the presence of tetracycline, oxytetracycline, and chlorotetracycline, the TCs approved from E.U. to be used for therapeutic purposes in poultries, in chicken tissues and eggs are 100 and 200 μg/kg, respectively.

For the monitoring of the presence of TCs residues in chicken tissues and eggs only a limited number of articles can be found in literature, most of which use chromatographic techniques. Okerman et al.[6] and Schneider[7] propose a microbiological and a fluorescence method, respectively, as screening methods for the presence of TCs in chicken meat. Another screening method for residual TCs both in chicken and eggs was developed by Oka et al. based on a TLC method. They use

HPTLC plates developed with a mixture of $CH_3Cl/CH_3CH_3/Na_2EDTA$ (60:20:20 v/v/v).[8] De Wash et al.[9] correlate results taken from a microbiological and an HPLC method, using a PLRP column and oxalic acid with CH_3CN as mobile phase, for analysis of TCs residues in chicken. For the same purpose, another confirmatory method using HPLC with UV detection is proposed by Cooper et al.[10] For analysis of residual TCs in eggs, four confirmatory methods were developed using HPLC coupled with UV,[11] fluorescence,[12] or MS detectors.[11,13,14].

In order to isolate TCs from the matrix, either chicken tissues or eggs, most of the proposed methods in literature use acidic buffers like acetate,[7] succinate,[9,12] citrate,[10,11,13] and McIlvaine with Na_2EDTA.[8] In most cases, the extraction procedure was followed by an extra



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-monohydrochloride	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-7chloro-tetracycline	Cl	H	OH	H	$N(CH_3)_2$	H	H

Figure 1. Chemical structures of examined tetracyclines.

cleaning-up step, like Metal Chelating Affinity Chromatography in combination with exchange membranes[9,12] and SPE off[8] and on-line with HPLC.[11]

The objective of this work was the development of a simple, reliable, multi-residue method for the determination of TCs in chicken tissues and egg yolk.

Apart from tetracycline (TC), oxytetracycline (OTC), and chlortetracycline (CTC), which can be used in chicken husbandry, we included in our work four more TCs (minocycline (MNC), methacycline (MTC), demeclocycline (DMC), and doxycycline (DC), which are commercially available as human drugs and can also be used for veterinary purposes, since the so far published works deal mostly with OTC, TC, and CTC. Their chemical structure is given in Figure 1. The proposed method was fully validated according to the criteria enacted by E.U. through commission decision 2002/657/EC.

EXPERIMENTAL

Chemicals and Reagents

TC, CTC, and internal standard, colchicine, were obtained from Fluka (www.sigmaaldrich.com/Brands/Fluka_Riedel_Home.html, Buchs SG, Switzerland), while 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, hydrochloric acid, and Na_2ETDA were purchased from Merck (www.merck.com, Darmstadt, Germany), while citric acid monohydrate, sodium citrate trihydrate of analytical grade were supplied by Riedel-de-Haen (www.sigmaaldrich.com/Brands/Fluka_Riedel_Home.html, Seezle, Germany). Finally, trichloroacetic and trifluoroacetic acid were supplied by Acros Organics (www.acros.com, Geel, Belgium). High purity water obtained by a Milli-Q purification system (www.millipore.com, Millipore, Bedford, MA, USA) was used throughout the study. Chicken and egg samples were supplied by the 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

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 (Rheodyne, www.rheodyne.com, Cotati, California, USA) injection valve equipped with a 20 μL loop for sample injection. Detection was achieved by an SPD-M10A_{VP} photodiode array detector, complied with Data acquisition software LabSolutions-LCsolutions by Shimadzu. Degassing of the mobile phase was achieved by helium sparging in the solvent reservoirs by a DGU-10B degassing unit.

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 (www.whatman.com) Whatman Laboratory Division, Maidstone, England) cellulose-nitrate 0.2 μm membrane filters.

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 samples.

The SPE study was performed on a Supelco vacuum manifold column processor (www.sigmaaldrich.com/Brands/Supelco_Home.html, Bellefonte, PA, USA). 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 (www.varianinc.com, Harbor City, CA, USA). All evaporations were performed with a Supelco 6-port Mini-Vap concentrator/evaporator (Bellefonte, PA, USA).

Chromatographic Conditions

Separation of the seven TCs was performed on a Kromasil C-18, 5 μm, 250 × 4 mm analytical column, supplied from MZ-Analysentechnik (www.mz-at.de, Mainz, Germany).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, operated at ambient temperature, according to a gradient program, shown in Table 1. The flow rate was also changing during the analysis, as can also be seen in Table 1. An equilibration time of 3 min was required between runs. Column effluent was monitored at 355 nm for all analytes.

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

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		

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 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 chicken and egg yolk 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_2ETDA/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. TFA and HCl solutions were manufactured by dissolving appropriate amounts from stock TFA and HCl solutions in water.

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

The herein proposed method for the determination of residual TCs in chicken tissues and egg yolk was fully validated according to the performance characteristics specified in European Commission Decision 2002/657/EC. Linearity, accuracy, precision, sensitivity, stability, decision limit (CC_α), and detection capability (CC_β), were assessed using spiked chicken and egg yolk samples following the criteria enacted by E.U.

Linearity response was examined in both matrices by analysing series of those samples spiked with mixed standard solutions of the examined

TCs and the internal standard, at concentrations covering a broad range from 20 to 300 μg/kg. Calibration curves were constructed for each sample, by least squares linear regression analysis of the peak area ration 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.

Precision and accuracy expressed in terms of TCs recovery from chicken and egg yolk samples were studied by analyzing spiked samples at three concentration levels (50, 100, 200 μg/kg), including the MRL. Intra-assay precision was estimated by five 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. Since no certified reference materials were available for chicken tissues and eggs, 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.

The two novel criteria enacted by E.U. for the determination of residual antibiotics in various matrices are decision limit (CC_α), which expresses the limit at and above which it can be concluded with an error probability of α that a sample is non compliant and detection capability (CC_β), the smallest content of the substance that may be detected, identified, and/or quantified in a sample with an error probability of β. The decision limit 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, while the detection capability as CC_α plus 1.64 times the SD of within-day repeatability at CC_α. Statistical analysis was performed at the 95% confidential level and the number of replicate analyses was 20.

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 chicken and egg yolk 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 was tested only on spiked deep frozen chicken tissues, since eggs are a product which is consumed fresh. For that purpose, homogenized blank chicken tissues samples were divided into five aliquots of 1 g. Each aliquot was spiked with TCs at 100 ng/g. One aliquot was analysed immediately, while the remaining aliquots were stored at −18°C and analyzed after 2, 4, and 6 weeks. Stability was also investigated after several freezing-defrosting cycles. Aliquots of frozen

spiked chicken samples were left at room temperature to thaw and analyzed after four freezing–defrosting cycles.

Sample Preparation

Chicken tissue samples, as well as egg yolk samples, were homogenized, separated in aliquots of 1 g, and stored at −18°C. Through the sample preparation protocol, each aliquot 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 a concentration of 5 ng/μL. Mixtures were then subsequently homogenized in a vortexer for 2 min, and after 15 min without vortexing 5 mL of 0.4 M oxalate buffer (pH = 4) were added. In order to succeed the optimum deproteinization of egg yolk samples 0.5 mL of 40% TFA solution was added to those samples before the oxalate buffer. 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 reextracted twice.

After extraction of TCs from both chicken and egg yolk samples, a purification procedure, like solid phase extraction (SPE), was considered necessary. Thus, the combined supernatants after filtration were applied to SPE cartridges preconditioned with 2 mL of CH_3OH and 2 mL of water. Three different sorbents, Li-Chrolut RP-18, Discovery, and Nexus were tested in order to optimize the purification protocol. 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. 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

Chromatographic Conditions

Under the assay conditions described in Chromatographic Condition section, the seven studied TCs and colchicine used as internal standard were well separated in 10 min. Retention times of the examined analytes were 2.68 for MNC, 4.76 for OTC, 5.49 for TC, 6.54 for DMC, 7.87 for CTC, 8.33 for MTC, 8.88 for DC, and 9.78 for colchicine. As can be concluded from the resolution factors (R_s) which were found to be 4.0 for MNC-OTC, 2.3 for OTC-TC, 3.1 for TC-DMC, 3.6 for DMC-CTC, 1.2 for CTC-MTC, 1.5 for MTC-DC, and 2.9 for DC-IS, the examined analytes were well separated.

Sample Preparation

The optimization of the sample preparation procedure both for chicken tissues and egg yolk samples, had two targets, to find first, the most efficient extraction buffer and second, the optimum SPE protocol to clean up the extract.

According to our previous experience on TCs analysis, medium acidic buffers (citrate, oxalate, and McIlvaine with Na_2EDTA buffers) were tested for the extraction of TCs from chicken tissues. Citrate and oxalate buffers were chosen for the extraction of TCs from egg yolk. Addition of a deproteinizing agent, like TFA solution, did not improve recovery rates in the case of chicken tissues. However, in order to isolate TCs from egg yolk, precipitation of egg proteins before adding the extraction buffer was considered crucial. CH_3CN, TFA, and HCl solutions were examined as deproteinizing agents. When using CH_3CN, precipitation was insufficient, while with HCl even in high concentrations like 10 M, an emulsion was formed. Addition of aqueous solutions of 15 or 20% TFA yielded low recoveries, which, however, were increasing by an increase of TFA concentration.

In order to optimize the SPE procedure, which follows the extraction step and avoid the presence of unknown peaks from endogenous compounds from both chicken tissue and egg yolk, different cartridges were studied: Discovery and LiChrolut for chicken extracts and Discovery, LiChrolut, and Nexus cartridges for egg yolk extracts. With both Discovery and Lichrolut cartridges, unknown peaks did not appear in chromatograms of blank chicken samples. Yet, LiChrolut provide higher recovery rates in combination with 15% TFA and oxalate buffer (0.4 M, pH 4) for the extraction, as can be seen from Table 2.

In order to clean up egg yolk extracts the same protocol used for chicken samples was applied. However, in chromatograms of blank samples endogenous peaks appear at 4.06 and 8.12 min. Thus, Discovery and Nexus cartridges were also tested. From the recovery results summarized in Table 2, it can be concluded that Nexus cartridges in combination with 40% TFA and oxalate buffer (0.4 M, pH 4) as an extraction system, yields the optimum recovery rates but also provides the most clear chromatograms, with one unknown peak at 4.06 min.

Typical chromatograms of blank and spiked chicken and egg yolk samples are given in Figures 2 and 3. No peaks from endogenous compounds were noticed on chicken chromatograms, while the unknown peak at 4.06 min on egg chromatograms was well resolved from analytes peaks.

Table 2. Recoveries of the examined TCs after solid-phase extraction in chicken tissue and egg yolk extracts using various extraction protocols

Extraction protocol	Recovery (%)						
	MNC	OTC	TC	DMC	CTC	MTC	DC
				Chicken			
20% TFA/Oxalate buffer (0.4 M pH 4) Discovery	110.2	84.1	85.4	91.2	85.1	80.7	74.6
20% TCA/Oxalate buffer (0.4 M pH 4) Discovery	101.2	88.2	79.6	89.1	75.1	61.0	59.6
Oxalate buffer (0.3 M pH 4) Discovery	126.7	87.8	88.5	99.5	87.6	78.4	75.4
McIlvaine buffer (pH 4) Discovery	113.2	78.0	71.8	93.0	74.6	64.4	65.6
Citrate buffer (0.4 M pH 4) Discovery	107.4	95.0	78.0	86.2	87.9	75.3	73.7
15% TFA/Oxalate buffer (0.4 M pH 4) LiChrolut	**107.4**	**92.7**	**83.2**	**100.2**	**89.9**	**73.7**	**75.7**
20% TFA/Oxalate buffer (0.3 M pH 4) LiChrolut	95.8	90.4	74.6	90.3	85.4	75.6	73.2
Citrate buffer (0.4 M pH 4) LiChrolut	75.6	84.9	65.8	71.2	60.2	65.8	63.2
				Eggs			
20% TFA/Oxalate (0.4 M pH 4)/Discovery	92.0	78.0	68.4	92.6	77.3	72.0	67.2
40% TFA/Oxalate (0.4 M pH4)/LiChrolut	91.3	88.2	84.4	103.0	91.9	85.4	81.3
10 M HCl/Oxalate (0.4 M pH 4)/LiChrolut	100.9	87.9	78.5	100.8	94.7	78.6	75.3
40% TFA/Oxalate (0.4 M pH 4)/Nexus	**103.2**	**88.8**	**81.2**	**99.3**	**89.6**	**87.6**	**79.9**

Method Validation

To check linearity of the proposed method calibration curves were constructed. For that purpose chicken tissue and egg yolk samples were spiked with TCs standard solutions at various concentration levels. For each calibration level three samples were prepared and each sample was analyzed in triplicate. Calibration curves constructed for both chicken and egg samples were obtained by least squares linear 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

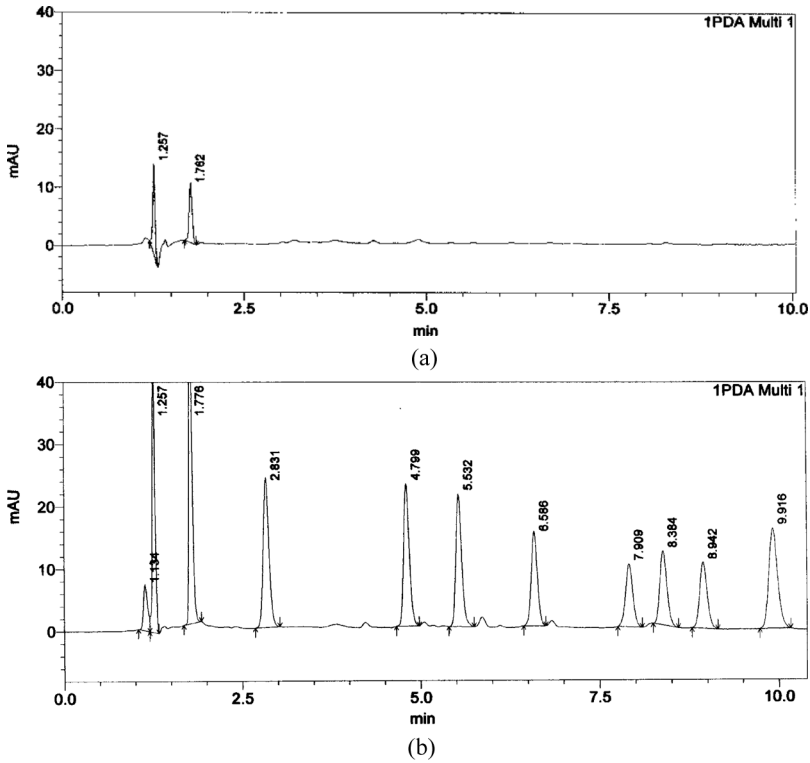


Figure 2. High performance liquid chromatogram of (a) Blank chicken tissue and (b) spiked chicken tissue at 100 μg/kg, after SPE using the conditions described in text. Peaks: (1) MNC 2.831 min, (2) OTC 4.799 min, (3) TC 5.532 min, (4) DMC 6.586 min, (5) CTC 7.909 min, (6) MTC 8.384 min, (7) DC 8.942 min, and colchicine (IS) 9.916 min.

are presented in Table 3. LODs and LOQs of the seven TCs were below the MRL value.

Ten different blank chicken tissue samples and ten different blank egg yolk samples, taken from various industrial and non-industrial producers, were analyzed in order to verify the selectivity of the proposed method. No interferences were detected by unknown endogenous peaks from the matrix.

Studies of precision and accuracy of the method based on within- and between- day repeatability and on recovery, respectively, were performed simultaneously. Chicken and egg yolk samples were spiked at three concentration levels, 50, 100, and 200 μg/kg (0.5, 1, and 2 times the MRL). At each level five different samples were prepared and analyzed.

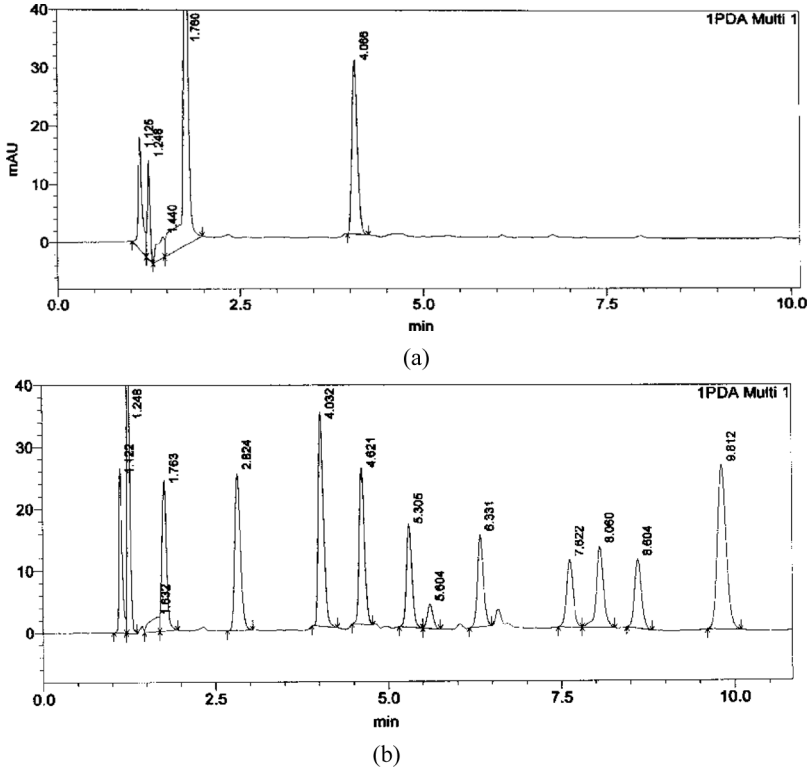


Figure 3. High performance liquid chromatogram of (a) Blank egg yolk and (b) spiked egg yolk at 100 μg/kg, after SPE using the conditions described in text. Peaks: (1) MNC 2.824 min, (2) OTC 4.621 min, (3) TC 5.305 min, (4) DMC 6.331 min, (5) CTC 7.622 min, (6) MTC 8.060 min, (7) DC 8.604 min, and colchicine (IS) 9.812 min.

Relative recovery rates from the spiked samples were determined at these concentrations by comparing the peak area ratios for extracted TCs and the values derived from the respective calibration curve. For the between-day precision study chicken and egg yolk 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 taken from the statistical evaluation of measurements are presented in Tables 4 and 5. As can be noticed, precision of the method was very good, since RSD values in chicken tissues were lower than 11.3, while in egg yolk samples lower than 11. Recoveries noticed in chicken tissues were for MNC: 97.7–107.4%, for OTC: 95.9–103.6%,

Table 3. Calibration and sensitivity data of the seven examined Tetracyclines in chicken tissues and egg yolk after solid-phase extraction

Analytes	Intercept	Slope	Correlation coefficient	LOD (μg/kg)	LOQ (μg/kg)	MRL (μg/kg)
			Chicken			
MNC	0.0791 ± 0.0299	0.0044 ± 0.00002	0.994	22	68	100
OTC	0.0330 ± 0.0206	0.0064 ± 0.0001	0.9990	11	32	
TC	0.0683 ± 0.0289	0.0049 ± 0.0002	0.996	19	59	
DMC	0.0203 ± 0.0107	0.0044 ± 0.0007	0.9992	8	24	
CTC	0.0116 ± 0.0036	0.0039 ± 0.0002	0.9999	30	73	
MTC	0.0117 ± 0.0266	0.0050 ± 0.0002	0.996	18	53	
DC	−0.0102 ± 0.0282	0.0053 ± 0.0002	0.996	18	53	
			Eggs			
MNC	0.0425 ± 0.0290	0.0040 ± 0.00002	0.993	24	73	200
OTC	0.1329 ± 0.0500	0.0057 ± 0.0003	0.9931	29	88	
TC	0.1149 ± 0.0464	0.0048 ± 0.0003	0.9933	25	76	
DMC	0.0548 ± 0.0218	0.0036 ± 0.0001	0.9992	20	61	
CTC	0.0367 ± 0.0248	0.0031 ± 0.0002	0.9999	26	80	
MTC	0.0542 ± 0.0338	0.0037 ± 0.0002	0.996	24	72	
DC	0.0806 ± 0.0409	0.0036 ± 0.0003	0.996	28	84	

for TC: 97.1–103.8%, for DMC: 96.4–106.6%, for CTC: 88.8–96.5%, for MTC: 91.9–107.5%, and for DC: 88.9–102.7%; and for egg yolk samples for MNC: 99.2–108.7%, for OTC: 97.2–106.4%, for TC: 92.2–106.8%, for DMC: 96.8–110.2%, for CTC: 98.7–106.1%, for MTC: 99.1–110.4%, and for DC: 95.3–104.1%.

Stability of TCs in chicken tissues was also tested. In order to investigate stability of TCs in chicken samples during their storage at −18°C, aliquots of 1 g from the tissues were spiked at 100 μg/kg (MRL) and analyzed after 2, 4, and 6 weeks. TCs proved to be stable for at least 6 weeks using as acceptance criterion a response comprised between 90 and 100% of the initial one. Stability was also assessed after four freezing-defrosting cycles, using the same acceptance criterion. For that purpose five chicken samples were prepared and analyzed after four freezing and defrosting cycles. All TCs proved to be stable for two cycles. Stability results are illustrated in Figure 4.

Finally, limits of decision (CC*a*) were calculated in both matrices by analyzing 20 blank chicken or egg samples spiked at the MRL (100 and 200 μg/kg, respectively), while detection capability (CC*b*) values by analyzing 20 blank chicken or egg yolk samples fortified at the corresponding CC*a* value for each TC. Results are summarized in Tables 6 and 7.

Table 4. Within and Between-day precision and accuracy of the developed method for the determination of seven TCs in chicken tissue after solid-phase extraction

		Within-day ($n = 5$)			Between-day ($n = 5$)		
Analytes	Added (ng)	Measured ± SD (μg/kg)	RSD	R (%)	Measured ± SD (μg/kg)	RSD	R (%)
MNC	50	53 ± 5.9	11.3	105.5	54 ± 3.8	7.2	107.4
	100	98 ± 6.2	6.3	97.5	104 ± 3.5	3.4	103.8
	200	196 ± 13.0	6.7	97.7	209.2 ± 6.4	3.1	104.6
OTC	50	48 ± 2.4	5.0	96.3	48 ± 2.7	5.7	95.9
	100	98 ± 4.3	4.4	98.1	104 ± 5.2	5.0	103.6
	200	199 ± 8.4	4.3	99.4	194 ± 4.8	2.5	97.0
TC	50	50 ± 2.1	4.2	99.1	49 ± 1.9	3.9	97.1
	100	100 ± 4.2	4.2	100.3	104 ± 4.3	4.1	103.8
	200	188 ± 7.6	4.1	93.8	195 ± 13.2	6.8	97.5
DMC	50	53 ± 2.5	4.6	106.6	50 ± 3.4	6.8	100.9
	100	100 ± 4.2	4.7	100.0	101 ± 4.8	4.7	101.3
	200	196 ± 5.2	2.6	98.2	193 ± 8.0	4.2	96.4
CTC	50	48 ± 2.0	4.3	95.0	48 ± 1.4	2.8	96.5
	100	89 ± 4.0	4.5	88.8	97 ± 7.7	8.0	96.3
	200	188 ± 4.1	2.2	93.7	184 ± 9.3	5.0	91.8
MTC	50	54 ± 2.6	4.9	107.5	53 ± 3.4	6.4	106.3
	100	95 ± 7.2	7.5	95.1	92 ± 5.1	5.5	91.9
	200	194 ± 6.0	3.1	96.8	199 ± 10.1	5.1	99.7
DC	50	51 ± 2.6	5.1	102.7	51 ± 3.8	7.5	102.2
	100	94 ± 2.6	2.8	93.5	89 ± 4.2	4.7	88.9
	200	191 ± 3.6	1.9	95.5	186 ± 5.1	2.8	92.8

CONCLUSIONS

The herein described method is a simple and reliable analytical procedure for the simultaneous determination of residues of seven tetracyclines in chicken tissues and egg yolk samples. The extraction procedure developed for the isolation of TCs from both matrices uses common buffers and similar SPE protocols. The method was fully validated for each matrix according to E.U. commission decision 2002/657/EC. Validation procedure results prove reliability of the proposed method for the residual analysis of TCs in chicken and egg samples, since the recovery rates for all TCs were higher than 89 and 92%, respectively, and LQD values were below the MRL set by the E.U.

Table 5. Within and Between-day precision and accuracy of the developed method for the determination of seven TCs in egg yolk after solid-phase extraction

		Within-day ($n = 5$)			Between-day ($n = 5$)		
Analytes	Added (μg/kg)	Measured ± SD (μg/kg)	RSD	R (%)	Measured ± SD (μg/kg)	RSD	R (%)
MNC	50	50 ± 3.6	7.1	100.8	54 ± 3.9	7.0	108.7
	100	101 ± 2.8	2.8	100.7	102 ± 6.1	6.0	101.9
	200	198 ± 3.2	1.6	99.2	205 ± 4.9	2.4	102.6
OTC	50	49 ± 1.3	2.7	97.2	53 ± 3.4	6.4	105.4
	100	99 ± 1.2	1.2	99.2	106 ± 6.2	5.8	106.4
	200	197 ± 2.0	1.0	98.7	195 ± 11.4	5.9	97.4
TC	50	49 ± 1.6	3.2	98.0	51 ± 2.0	3.9	102.5
	100	99 ± 2.0	2.0	99.8	107 ± 6.7	6.2	106.8
	200	184 ± 3.4	1.8	92.2	204 ± 20.3	9.9	102.1
DMC	50	51 ± 1.8	3.6	101.3	53 ± 2.9	5.4	106.1
	100	100 ± 2.8	2.8	99.6	110 ± 7.0	6.4	110.2
	200	194 ± 4.4	2.3	96.8	200 ± 5.5	2.7	100.0
CTC	50	52 ± 1.8	3.4	103.5	53 ± 2.9	5.4	106.1
	100	100 ± 3.4	3.4	100.4	103 ± 5.5	5.3	103.4
	200	195 ± 3.0	1.5	97.7	197 ± 5.7	2.9	98.7
MTC	50	51 ± 2.4	4.7	102.0	55 ± 3.6	6.5	110.4
	100	101 ± 2.8	2.8	100.6	108 ± 5.6	5.1	108.0
	200	198 ± 3.4	1.7	99.1	208 ± 8.1	3.9	103.9
DC	50	50 ± 2.7	5.3	100.7	50 ± 5.5	11.0	99.5
	100	100 ± 2.3	2.3	100.5	103 ± 3.9	3.9	102.5
	200	191 ± 3.6	1.9	95.5	208 ± 6.4	3.1	104.1

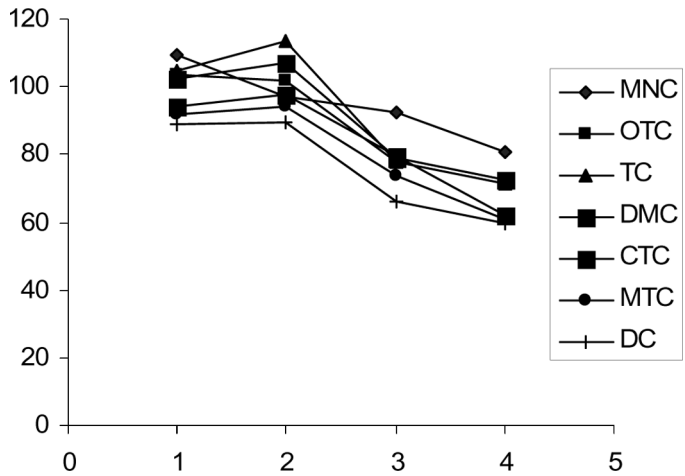


Figure 4. Stability of Tetracyclines in chicken muscle after four freezing – defrosting cycles.

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 chicken

Analytes	Added (μg/kg)	Measured ± SD (μg/kg)	RSD	Recovery (%)	Error α (1.64 ∗ SD)	CC_α (μg/kg)
MNC	100	99.68 ± 5.55	5.6	99.7	9.11	109.11
OTC	100	98.25 ± 5.12	5.2	98.3	8.40	108.40
TC	100	98.68 ± 4.81	4.9	98.7	7.88	107.88
DMC	100	102.02 ± 4.65	4.6	102.0	7.62	107.62
CTC	100	94.18 ± 4.51	4.8	94.2	7.40	107.40
MTC	100	96.81 ± 4.87	5.0	96.8	7.98	107.98
DC	100	96.83 ± 1.82	1.9	96.8	2.98	102.98
Analytes	Added (μg/kg)	Measured ± SD (μg/kg)	RSD	Recovery (%)	Error β (1.64 ∗ SD)	CC_β (μg/kg)
MNC	109	122.9 ± 3.98	3.2	112.8	6.52	115.52
OTC	108	113.90 ± 5.14	4.5	105.5	8.43	116.43
TC	108	124.16 ± 6.21	5.0	115.0	10.18	118.18
DMC	108	114.11 ± 3.32	2.9	105.7	5.45	113.45
CTC	107	109.66 ± 4.72	4.3	102.5	7.74	114.74
MTC	108	108.32 ± 5.06	4.7	100.3	8.30	116.30
DC	103	107.02 ± 5.15	4.8	103.9	8.45	114.45

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 200 μg/kg egg yolk

Analytes	Added (μg/kg)	Measured ± SD (μg/kg)	RSD	Recovery (%)	Error α (1.64 ∗ SD)	CC_α (μg/kg)
MNC	200	195.54 ± 6.13	3.1	97.8	10.06	210.06
OTC	200	207.07 ± 3.99	1.9	103.5	6.53	206.53
TC	200	198.17 ± 5.34	2.7	99.1	8.76	208.76
DMC	200	195.36 ± 7.64	3.9	97.7	12.52	212.52
CTC	200	202.39 ± 8.91	4.4	101.2	14.60	214.60
MTC	200	191.52 ± 6.52	3.4	95.8	10.69	210.69
DC	200	199.71 ± 5.31	2.7	99.9	8.71	208.71
Analytes	Added (μg/kg)	Measured ± SD (μg/kg)	RSD	Recovery (%)	Error β (1.64 ∗ SD)	CC_β (μg/kg)
MNC	210	209.62 ± 7.94	3.8	99.8	13.02	223.02
OTC	207	229.07 ± 5.62	2.4	110.7	9.21	216.21
TC	209	214.34 ± 5.23	2.4	102.6	8.57	217.57
DMC	213	209.50 ± 3.51	1.7	98.4	5.76	218.76
CTC	215	208.50 ± 8.52	4.1	97.0	13.97	228.97
MTC	211	208.10 ± 7.65	3.7	98.6	12.55	223.55
DC	209	208.43 ± 7.05	3.4	99.7	11.56	220.56

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