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Residue Analysis of Tetracyclines and Their Metabolites in Eggs and in the Environment by HPLC Coupled with a Microbiological Assay and Tandem Mass Spectrometry

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Tetracyclines are widely used in farm animals. This can cause drug residues in products of animal origin and, after excretion of these substances, in animal slurry and in soil fertilized with that slurry. In this paper, we present a method based on a microbiological assay coupled with HPLC for the detection of oxytetracycline, tetracycline, and chlortetracycline in eggs. After a simple liquid extraction of the samples and HPLC separation, fractions were collected on microtiter plates, and the tetracyclines were analyzed using the *Staphylococcus aureus* assay. This method was able to identify residues of tetracyclines in eggs at a level set by regulatory agencies (i.e., 200 μg/kg). In addition, it was shown that the described microbiological method can be used as a screening assay for the detection of tetracyclines and possible biologically active metabolites in animal slurry and soil samples. Employing the same extraction procedure, it was demonstrated that LC−MS−MS allowed the quantification of 20−400 μg/kg in eggs with recoveries ranging from 71 to 109% and RSDs of 3−15%.

KEYWORDS: Tetracyclines; microbiological assay; LC−MS−MS; eggs, environmental samples

INTRODUCTION

Tetracyclines (TCs, molecular structures shown in **Figure 1**) are widely administered to farm animals as veterinary drugs because of their broad spectrum of activity and cost-effectiveness. The use of these substances may cause residues to be found in meat and other products of animal origin. For this reason, the European Union has established maximum residue limits (MRLs) for oxytetracycline (OTC), tetracycline (TC), and chlortetracycline (CTC) (e.g., 200 μg/kg in eggs). TCs are poorly metabolized in animals (*1*). Therefore, significant amounts of drug residues can also occur in animal slurry. Recently, it was shown that the application of contaminated slurry as fertilizer can result in drug residues in soil (*2*).

For the rapid detection of antibiotics in food, microbiological assays are routinely used because they are easy to perform and inexpensive. The main disadvantage of these tests is their lack of specificity; that is, they often detect growth inhibition of a sensitive test strain, whether a substance with antimicrobial activity is present or not. More sophisticated test systems allow the detection of at least a substance group, but there is still no possibility for identification or quantification of the residue. The screening of egg samples for the occurrence of antibiotic residues requires an appropriate sample pretreatment step, since lysozyme (an endogenous antimicrobial substance in eggs) causes growth inhibition in microbiological screening tests. Such an assay was described for the detection of OTC in yolk and albumen, where the limits of detection were 300 and 70 μg/kg, respectively (*3*).

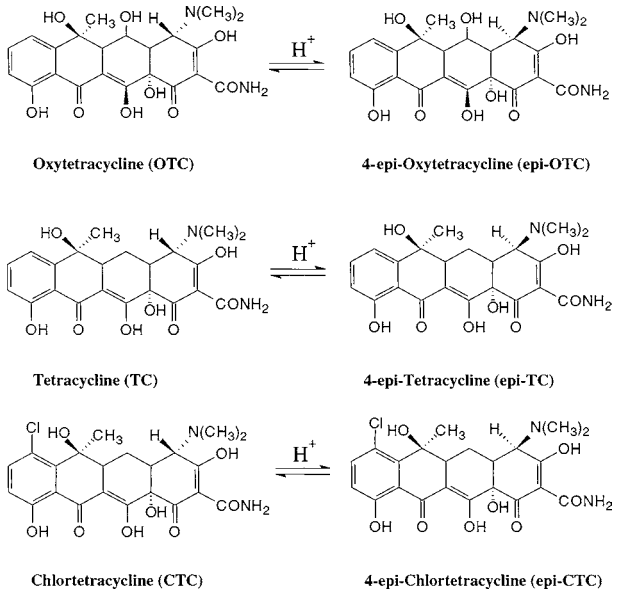

Figure 1. Molecular structures of the three investigated tetracyclines (left panel) and their corresponding reversible epimers (right panel).

The method of choice for the identification and quantification of tetracycline residues in eggs is HPLC coupled with either

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UV (*4*−*6*), fluorescence detection (*5*−*7*), or mass spectrometry (*8*). These methods require sample preparation of eggs mainly based on a liquid extraction step followed by solid-phase extraction. For this purpose, EDTA-McIlvaine buffer (*8*, *9*) as well as sodium succinate (*10*) or glycerine-HCl buffer (*7*) are recommended. Aside from this, liquid−liquid extraction can be performed using citrate buffer followed by ethyl acetate (*4*). Solid-phase extraction is mainly performed with reversed-phase materials or by metal chelate affinity chromatography (*4*, *7*). These sample preparation procedures are time-consuming and labor-intensive. A sample pretreatment without these disadvantages involves an automated sample preparation based on online dialysis coupled with solid-phase extraction (*5*, *6*, *11*).

The analysis of TCs in soil in a milligrams per kilogram range has been reported in only a few methods. In particular, the application of microbiological assays for the analysis of CTC, following a simple liquid extraction of soil with aqueous methanol (pH 8) or acidified acetone, has been described (*12*, *13*). The sensitive detection of OTC, CTC, and TC in sandy soil at a detection limit of 1−2.5 μg/kg and employing a liquid−liquid extraction with citrate buffer and ethyl acetate combined with tandem mass spectrometry has been recently described (*14*).

This paper reports on a new method for the detection of TCs in eggs, animal slurry, and soil based on a microbiological assay coupled with HPLC. There are two main advantages of this procedure: first, HPLC allows the identification of the three tetracyclines via their specific retention times, and second, the microbiological assay allows for quantification via the selective detection of their antimicrobial activity. In addition, when using a microbiological detection system, a reduced sample pretreatment is necessary in comparison to HPLC−UV or HPLC−fluorescence detection. Therefore, the microbiological assay can be used as a sensitive and selective screening test for various TCs in eggs within the MRL. For further confirmation and a more sensitive quantification of TC residues in food and environmental samples with LC−MS−MS, the described sample cleanup is also recommended.

MATERIALS AND METHODS

Standard Substances. TC, CTC, and OTC (in hydrochloride form) were obtained from Sigma (Deisenhofen, Germany). Stock solutions of these antibiotics were made by dissolving 1 mg/mL in methanol. These solutions were stored at −80 °C and were stable over a period of at least 2 months. Working solutions in a concentration range of 1−6 ng/10 μL in methanol were prepared freshly on the day of use for the microbiological assay. Standards for LC−MS−MS were used at concentrations of 0.1−10 ng per injection. These standards were also freshly prepared on the day of use. To compensate for matrix effects during the analysis of TCs in eggs using LC−MS−MS, standards were prepared by adding 10% of an extract of residue-free eggs.

Preparation of the Microbiological Assay. Assay medium was prepared by dissolving 37 g of brain−heart broth (Merck, Darmstadt, Germany) in 1 L of distilled water prepared in-house by a Milli-Q system (Millipore, Eschborn, Germany). The broth was sterilized at 133 °C and 3 bar for 15 min. The microbiological assay was carried out using a cryopreserved *Staphylococcus aureus* strain resistant to colistin (*S. aureus* RU 4220) cultured in brain−heart broth with 0.011% colistin (a generous gift of Alpharm, Denmark) and adjusted to pH 5.9 with acetic acetate (Sigma). For the preparation of *S. aureus*, one well-isolated colony of the strain grown on blood agar was suspended in the above-described medium and incubated for 24 h at 37 °C. This bacterial growth was subcultured after 24 h by adding 300 μL of the initial broth culture to a second sterile aliquot of brain−heart broth (20 mL). This procedure was essential to ensure that the culture was pure. From the second culture, 2 mL was added to 40 mL of sterile media and incubated at 37 °C. After 6 h, an equal volume of sterile glycerol (Merck, 800 mL/L of H_2O) was added to the culture and mixed thoroughly. The culture was then dispensed in 0.5-mL aliquots into sterile cryovials, shock-frozen in liquid nitrogen, and stored at −80 °C prior to use. These cultures were stable for up to 2 years.

For the preparation of the assay, a 200-μL vial of rapidly thawed *S. aureus* stock culture was added to brain−heart medium (100 mL). After thorough mixing with a magnetic stirrer, 100 μL of the inoculated medium was dispensed to each well of a 96-well microplate. Eight blank controls were created by dispensing medium without bacteria in the first well of each lane of the microplates. Each plate was covered with a plate sealer (Dunn Labortechnik, Asbach, Germany), mixed by inversion, and incubated for 18 h at 37 °C. The growth of the bacteria was determined photometrically by measurement at 595 nm with a microplate reader (Modell Benchmark, Bio-Rad, Munich, Germany) connected to a computer system (Microplate Manager version 4.0, Bio-Rad). Prior to measurement, the test plates were intensively shaken by hand.

Standard Curve of the Microbiological Assay. The calibration of the assay was performed using standards in a range of 1−6 ng on the HPLC column, equivalent to 100−600 ng/mL (injection volume 10 μL). Each concentration was measured six times. Standard curves were calculated from the mean at each concentration. To control the calibration curve prior to analysis, standards at concentrations of 200, 300, and 600 ng/mL were measured.

Processing of the Plate Data. Initially, the mean of the blank controls was subtracted from the measurements of the test wells. These data were then calculated as percent growth inhibition, and a chromatogram was constructed. Finally, tetracycline concentrations were calculated using calibration curves described as a polynome of three degrees.

Extraction of Tetracyclines from Whole Egg, Slurry, and Soil Followed by HPLC Separation and Microbiological or Tandem Mass Spectrometry Detection. All glassware used was heated at 450 °C, cooled, rinsed with HPLC gradient-grade methanol (Sigma) saturated with EDTA (Sigma), and then air-dried. Citrate buffer (1 M, pH 5 for egg samples, pH 4.7 for environmental samples) was prepared by dissolving 192 g of citric acid in approximately 800 mL of distilled water, adjusting the pH with sodium hydroxide, and making up to 1 L with water.

Whole egg was homogenized using an Ultra Turrax homogenizer (T25 basic, IKA Labortechnik, Staufen, Germany). One gram of the homogenate was mixed with 1.2 mL of 1 M citrate buffer (pH 5) in 25-mL plastic tubes (Beckman, Munich, Germany) using a magnetic stirrer. Ten milliliters of acetonitrile (HPLC gradient grade, J.T. Baker, Gross-Gerau, Germany) was then added, and the whole mixture was stirred for another 15 min. The sample was centrifuged for 10 min at 3000*g*. The resulting supernatant was transferred into glass flasks, and the residue was mixed with 1.2 mL of distilled water. A further 10 mL of acetonitrile was added to the sample, and the extraction procedure was repeated. The supernatants were combined and evaporated to dryness under vacuum at 40 °C. The dried sample was reconstituted with 400 μL of methanol.

Sample preparation of soil and slurry samples was performed with a liquid−liquid extraction. A 1-g sample was mixed with 1.2 mL of citrate buffer (pH 4.7) and extracted twice with 6 mL of ethyl acetate as previously described (*14*). Ethyl acetate was evaporated to dryness, and samples were reconstituted with 200 μL of 90% acetonitrile/10% 10 mM ammonium acetate.

HPLC separation was carried out on a Puresil C18 column (Waters Corp., Milford, MA) with a gradient solvent system consisting of 0.5% formic acid (Riedel-de Haen, Selze, Germany) in water containing 1 mM ammonium acetate (Merck) (solvent A, pH 2.5) and acetonitrile (solvent B) using the method recently described (*14*). An injection volume of 10 μL for egg samples, 8 μL for soil samples, and 1−2 μL for slurry samples was used.

For detection, the microbiological assay was used, and tandem mass spectrometry was carried out employing an LCQ ion trap with an electrospray ionization source (Finnigan MAT, San Jose, CA). The source polarity was set positive for all compounds, the spray needle voltage was 5 kV. Drying gas was nitrogen generated from pressurized air in an Ecoinert 2 ESP nitrogen generator (DWT-GmbH, Gelsen-

kirchen, Germany). The optimized conditions were as follow: sheath gas flow was set at 100 units, the auxiliary gas was turned off, and the capillary temperature was 150 °C (for further details, refer to ref *14*). After HPLC separation, the solvent was split: 10% was used for tandem mass spectrometry, and 90% was fractionated in aliquots of 300 μL into microplates containing 30 μL of 2% Tween 80 (Sigma) in water in each well. After fractionation, the plates were covered with the original plastic covers and immediately frozen at −80 °C and lyophilized overnight.

Recovery Studies. Recovery studies for the LC−MS−MS method were carried out with eggs from untreated laying hens at concentrations of 20, 50, 100, 200 (the MRL), and 400 μg/kg. The recovery rates for TC and CTC were calculated as the sum of the epimer and its parent drug as an average of three individual experiments.

To demonstrate the performance of the microbiological assay coupled with HPLC, TCs stock solutions were added to homogenized egg to obtain final concentrations of 100, 150, and 200 μg/kg. Sample extraction and measurement with the microbiological assay were carried out as described above. The procedure was repeated four times at each concentration, and samples were analyzed in duplicate.

Samples. Lyophilised egg samples from an interlaboratory test under the supervision of the University of Wuppertal (Prof. Dr. Petz, Department of Food Chemistry) were used in the initial testing of the sample extraction and measurement procedure. Due to the establishment of the final procedure, these samples were stored for 1 year at −80 °C prior to analysis. The eggs were taken from hens fed with TC (series C) or TC, OTC, and CTC (series E). In addition, eggs from untreated hens were spiked with TC (series B). During reconstitution of the samples, they were mixed with water and then allowed to soak for 2 h at 4 °C. After shaking, samples were then handled like fresh whole egg samples.

In a further step, the described method was applied to slurry, dried slurry aggregates, and soil samples in order to build up a screening method for TCs in samples of environmental impact. Soil samples were taken from an agricultural field in northern Germany at a depth of 0−30 cm and divided further into subsamples of 0−10, 10−20, and 20−30 cm. Soil was also sampled at a depth of 0−90 cm, with subsamples ranging from 0−30, 30−60, and 60−90 cm. Two weeks before sampling, the field was fertilized with pig slurry, which was also investigated. Dried slurry aggregates were picked up from the topsoil of an agricultural field previously fertilized approximately 4 weeks before. All environmental samples were obtained from the same area.

RESULTS AND DISCUSSION

Microbiological Assay. The microbiological assay described here is performed in microplates, and the bacteria are grown as a broth culture. This is an important difference from other described microbiological methods for the detection of antibiotic residues, which are based on agar diffusion tests (*3*, *15*). These tests detect substances with antimicrobial activity by a growth inhibition zone surrounding the sample spot (*16*) or by alteration in the color of the growth agar due to changes in the pH caused by the production of bacterial waste. The growth inhibition of bacteria used in an agar diffusion test depends not only on the concentration of the antibiotic but also on the substance class investigated. In addition, the employed test strains usually even show different sensitivities to members of the same substance class (e.g., chlortetracycline, oxytetracycline, and tetracycline). Consequently, the accurate quantification of antimicrobial residues is not possible.

The newly developed assay based on a photometric measurement of the bacterial growth allows for this quantification of the residue. Therefore, we used a spreadsheet (Microsoft Excel) to plot (nanograms per well on the *y* axis, and growth inhibition on the *x* axis) the calibration for the microplates. We could show that the calibration curves were nonlinear and calculated a polynomial (third degree) equation (see **Figure 2**). A similar approach has recently been undertaken for the analysis of microbiological folate in erythrocytes (*17*).

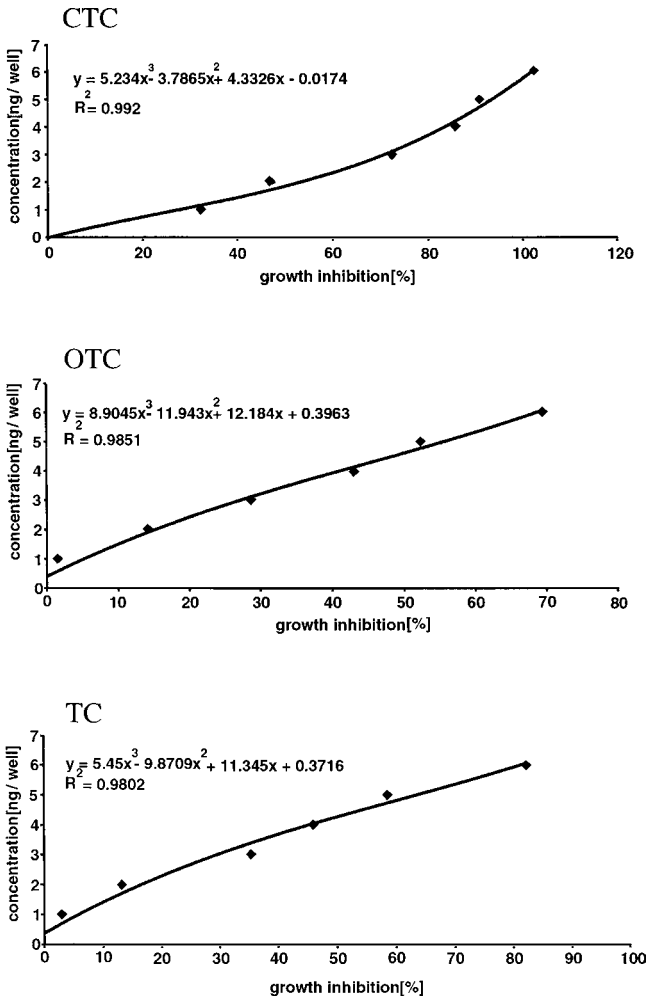

Figure 2. Calibration curves of the microbiological assay for chlortetracycline (CTC), oxytetracycline (OTC), and tetracycline (TC). The standard deviations were 2–8% for chlortetracycline, 3–9% for oxytetracycline, and 6–12% for tetracycline.

There are two additional advantages of the described assay: the simple assay preparation procedure and the use of cryopreserved cultures, which eliminates serial subculturing of the bacterial strain.

Coupling HPLC with a Microbiological Assay. The successful coupling of HPLC with a microbiological assay has some special requirements in the assay procedure. The HPLC eluent consists of significant amounts of formic acid and acetonitrile, which are both highly toxic to the test organism. Therefore, the solvents need to be completely removed, for example, by lyophilization. Dispensing 2% Tween 80 into the wells prior to eluent fractionation prevents the adsorption of TCs to the surface of microplates and also facilitates the redissolving of freeze-dried fractions in the growth medium. Therefore, the identification of TCs is achieved on the basis of not only their retention times but also the specific growth inhibition of a sensitive strain.

Assay Calibration. Typical calibration curves of CTC, OTC, and TC are shown in **Figure 2**. The three control samples (corresponding to 2, 3, and 6 ng on the HPLC column per 10-μL injection) demonstrated the fitting of the calibration curve in each sample series. The limited concentration range of the calibration lines did not allow for the quantification of residues at concentrations of more than 6 ng per 10 μL injection. Using the described sample preparation for eggs, 5 ng on the HPLC

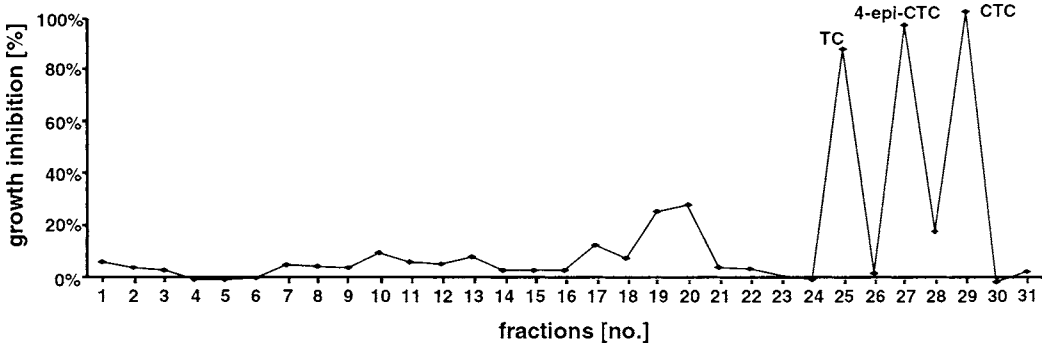

Figure 3. HPLC coupled with the microbiological assay. The analysis of a dried slurry aggregate containing tetracycline (TC), chlortetracycline (CTC), and 4-epi-chlortetracycline (4-epi-CTC) is shown.

Table 1. Recoveries ± Standard Deviations and the Corresponding Relative Standard Deviations (RSD) of Oxytetracycline (OTC), Tetracycline (TC), and Chlortetracycline (CTC) in Whole Egg Samples Spiked with 20, 50, 100, 200, and 400 μg/kg Using LC–MS–MS[a]

	OTC		TC + 4-epi-TC		CTC + 4-epi-CTC	
concn (μg/kg)	recovery (%)	RSD (%)	recovery (%)	RSD (%)	recovery (%)	RSD (%)
20	84 ± 8	10	85 ± 11	13	88 ± 3	4
50	71 ± 11	15	91 ± 3	3	73 ± 9	12
100	84 ± 8	9	109 ± 9	8	89 ± 11	13
200	98 ± 10	10	105 ± 16	15	90 ± 13	14
400	84 ± 5	6	88 ± 5	6	80 ± 4	4

[a] Values are the means of three independent determinations at each concentration.

column was equivalent to the MRL (200 μg/kg). The measurement of higher concentrations required an appropriate dilution or a reduction of the injection volume.

Quantification of 4-epi-TC and 4-epi-CTC was not possible because of their low antimicrobial activity. Nevertheless, various chromatograms illustrated some growth inhibition, with fractions corresponding to 4-epi-CTC (as shown in **Figure 3**). This can be explained by the fact that after 4-epi-CTC is separated via HPLC, fractionated into the microplate, and then incubated, the 4-epimer is partially converted back to the parent drug. Consequently, growth inhibition at the retention time of 4-epi-CTC is caused mainly by CTC itself. This growth inhibition was not used for quantification because the ratio between the epimer and its parent drug in the well was not known.

LC−MS−MS and Recovery Studies. Calibration curves constructed for OTC, TC, and CTC ranged from 0.1 to 10 ng per injection (10 μL) and were linear, with $r^2 = 0.9998$ for OTC, $r^2 = 0.9985$ for TC, and $r^2 = 0.9971$ for CTC for the MS−MS procedure. Quantification was obtained by comparing the peak areas of the samples with those of the external calibration curves, and all data were corrected for recovery. To compensate for the signal enhancement effects resulting from co-extracted matrix compounds, standards were prepared in methanol containing 10% of a tetracycline-free egg extract.

The results of the recovery experiments at 20, 50, 100, 200, and 400 μg/kg using LC−ESI−MS−MS are given in **Table 1**. The mean recovery is in the range of 71−109% for all TCs in eggs. The relative standard deviations were between 3 and 15% over all concentrations. These values are acceptable for such a method and comparable to those obtained by another method recently described (*4*). The limit of quantification, defined as the lowest level at which the method is valid, was 20 μg/kg for all TCs. The limit of detection with a signal-to-noise ratio greater than 3 is approximately 5-fold lower for all TCs. Precursor and

Table 2. Retention Times and Optimized MS–MS Parameters for the Determination of Various Tetracyclines in Soil (Refer to Ref *14*) and in Whole Egg

compound	t_R (min)	precursor mass (*m*/*z*)	collision energy (%)	product ions (*m*/*z*) (relative abundance, %)
oxytetracycline	7.06	461	20	426 (8), 443 (100), 444 (8)
tetracycline	7.39	445	20	410 (6), 427 (100), 428 (5)
chlortetracycline	8.35	479	27	444 (70), 461 (56), 462 (100)

Table 3. Mean Concentrations ± Standard Deviations and the Corresponding Relative Standard Deviations (RSD) of Oxytetracycline (OTC), Tetracycline (TC), and Chlortetracycline (CTC) in Whole Egg Samples Spiked at 100, 150, and 200 μg/kg Using the Microbiological Assay Coupled with HPLC[a]

	OTC		TC		CTC	
concn (μg/kg)	measured (μg/kg)	RSD (%)	measured (μg/kg)	RSD (%)	measured (μg/kg)	RSD (%)
100	nd		nd		nd	
150	172 ± 22	13	178 ± 23	13	125 ± 20	16
200	nc		203 ± 28	14	198 ± 34	17

[a] Values are the means of eight independent determinations at each concentration. nd, not detected; nc, not calculated, outside the linear range of the calibration curve.

product ions observed under MS−MS conditions were characteristic for TCs as recently described (*14*) and enabled identification and confirmation of these compounds (see **Table 2**). In light of all method validation parameters, the newly developed method including a rapid sample cleanup provides a high degree of confidence, even at one-tenth of the actual maximum TC residue level of 200 μg/kg in eggs.

In addition, it could be shown that the microbiological assay coupled with HPLC was able to detect TC residues in eggs at a concentration range between 150 and 200 μg/kg. The mean concentrations of TCs determined in these samples are provided in **Table 3**. This demonstrates that the newly developed microbiological assay detects TC residues in eggs at the level set by regulatory agencies. The overestimation of OTC at a concentration of 200 μg/kg can be explained by a fractionation of epi-TC (epimer of TC) in the same well as OTC. During incubation, epi-TC is converted back to TC, resulting in growth inhibition at the retention time of OTC, which is caused by both substances. At a concentration of 150 μg/kg, co-fractionation of epi-TC and OTC leads to only a slight overestimation of OTC. A concentration of 100 μg/kg, which is equivalent to 2.5 ng on the column, could not be detected. Injection of a standard at the same concentration led to a distinct growth inhibition in the microbiological assay. An explanation for these differences

Table 4. Egg Samples from an Interlaboratory Test[a]

	OTC (μg/kg)	TC (μg/kg)	TC + 4-epi-TC (μg/kg)	CTC (μg/kg)	CTC + 4-epi-CTC (μg/kg)	iso-CTC (μg/kg)	4-epi-iso-CTC (μg/kg)
			Series B				
LC–MS–MS		149	192				
HPLC–MA		127					
			Series C				
LC–MS–MS		93	153				
HPLC–MA		nd					
mean interlaboratory test			166				
			Series E				
LC–MS–MS	172	195	317	120	174	288	219
HPLC–MA	163	190		167			
mean interlaboratory test	184		275		142		

[a] Comparison of LC–MS–MS and the microbiological assay coupled with HPLC results with the mean values of all participants of the interlaboratory test supervised by the University of Wuppertal. Samples from series B were spiked with 200 μg/kg TC, samples from series C were taken from hens fed with TC, and samples from series E were obtained from hens fed with OTC, TC, and CTC. nd, not detected.

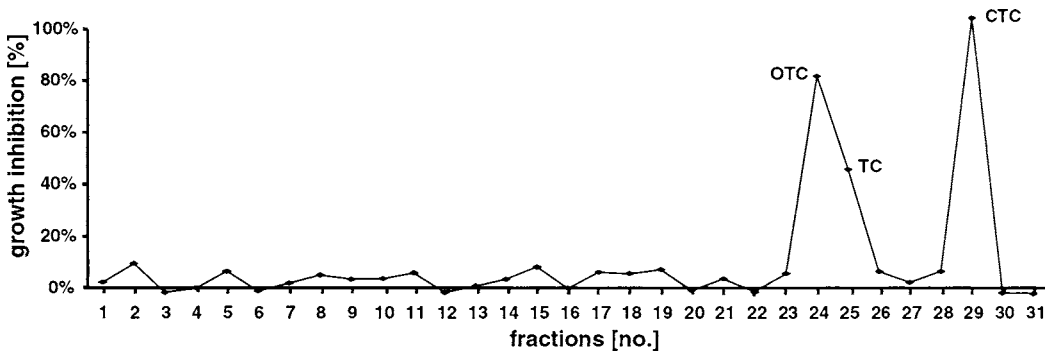

Figure 4. HPLC coupled with the microbiological assay. The analysis of an egg sample containing 200 μg/kg oxytetracycline (OTC), tetracycline (TC), and chlortetracycline (CTC) is shown.

might be the significant epimerization of the TCs during the sample preparation (refer to MS−MS data) or a lower availability of TCs in the bacterial growth medium caused by cofractionated egg substances.

Egg Samples. Egg samples from the interlaboratory test were analyzed using both described methods. Our results, as well as the mean concentration of the values of all participants of this test, are provided in **Table 4**. The findings of the MS−MS procedure are documented for the parent drug, as well as the sum for the parent drug and its epimer. In addition, the concentrations of iso-chlortetracycline (iso-CTC) and 4-epi-iso-chlortetracycline (4-epi-iso-CTC) are shown. Using the microbiological assay, only the parent compounds were quantified. Using LC−MS−MS, the egg sample of series E was measured three times to detect parent substances and their 4-epimers and isomers (refer to **Figure 5**). The detection of all compounds with antimicrobial activity required only a single HPLC separation when the microbiological assay was used as a detector (refer to **Figure 4**). In conclusion, we demonstrate that HPLC coupled with the described microbiological assay or tandem mass spectrometry is able to detect TC residues in eggs after the treatment of hens. Using the microbiological assay, detection of TC residues at 93 μg/kg in an egg sample from series C was not possible. This is in accordance with the recovery experiments mentioned previously. Measuring samples of series B with the microbiological assay coupled with HPLC and all samples using LC−MS−MS demonstrated that data generated with these methods are comparable with the interlaboratory test data. These results show that the microbiological assay can be used as a screening method and provides the additional possibility to identify TC residues in eggs. At the MRL level, the results of this assay are comparable to those of existing analytical methods. However, in contrast to most described techniques, which require labor-intensive sample preparation procedure, this assay needs only a simple sample pretreatment. The LC−MS−MS method presented here is a useful and highly sensitive method to identify and quantify TC residues and their epimers at a range of 20−400 μg/kg, without a sophisticated sample preparation procedure. In addition, it is possible to detect iso-CTC and 4-epi-iso-CTC residues in eggs after the treatment of laying hens with CTC. Both substances are the main metabolites of CTC in eggs (*18*).

Environmental Samples. The described microbiological method was also employed for the detection of TCs in slurry and soil samples. Concentrations of 3.6 mg/kg TC and 0.18 mg/kg CTC were detected in slurry, and LC−MS−MS also achieved similar values (4 mg/kg TC and 0.1 mg/kg CTC, refer to *14*). In addition, the slurry sample contained 0.5 mg/kg 4-epi-TC and less than 0.05 mg/kg each of iso-CTC and 4-epi-iso-CTC. The newly developed microbiological method was also used as a screening test to analyze soil samples taken from a field fertilized with the slurry as previously mentioned. In two samples, this screening procedure showed detectable amounts of TC. In one sample taken at a depth of 0−30 cm, a TC concentration of 214 μg/kg was found. In the other sample, consisting of soil from a depth of 10−20 cm, the TC concentration was 275 μg/kg. Quantification with MS−MS led to concentrations of 254 and 195 μg/kg for the same samples, respectively. A detailed investigation of the occurrence of TCs in this agricultural field has been recently described (*14*).

The dried slurry aggregates were of special interest because residues may accumulate therein during the drying process.

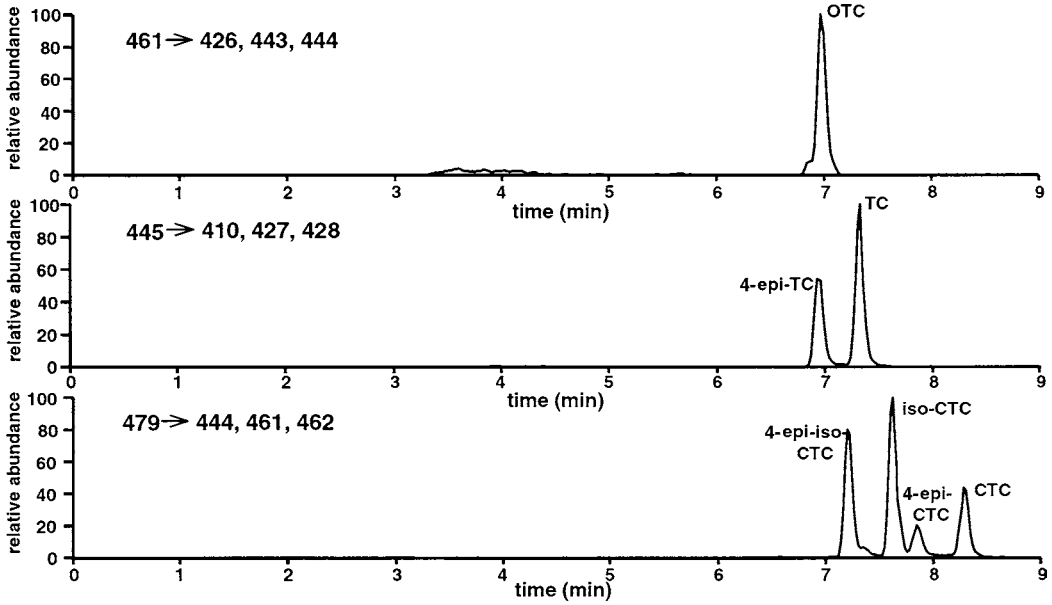

Figure 5. LC–MS–MS analysis of an egg sample of series E. Three LC runs were performed to detect oxytetracycline (OTC), tetracycline (TC), and chlortetracycline (CTC) including their characteristic epimers and isomers.

During screening of these aggregates, a total growth inhibition was seen at the retention times of TC, 4-epi-CTC, and CTC. By reducing the injection volume, a TC concentration of 280 μg/kg and a CTC concentration of 540 μg/kg were calculated. In addition, a distinct growth inhibition at the retention time of 4-epi-CTC could be seen (refer to **Figure 3**). Quantification of this compound, however, was not possible because 4-epi-CTC has very low antimicrobial activity and the ratio between 4-epi-CTC and CTC was unknown. At retention times between 5.5 and 6.1 min, further growth inhibition could be detected. To confirm these findings and identify the unknown substance, tandem mass spectrometry was performed. Using this method, the occurrence of TC, 4-epi-CTC, and CTC was confirmed. In addition, 4-epi-TC, iso-CTC, and 4-epi-iso-CTC were detected in the dried slurry aggregates. An identification of the unknown substance was not obtained; however, various MS−MS experiments showed that it seems highly unlikely that TCs or microbiologically active degradation products therof caused this specific growth inhibition.

After administration of TC and CTC to animals, these substances and their metabolites, 4-epi-TC and 4-epi-CTC, are excreted. Iso-CTC and 4-epi-iso-CTC are not known as major mammalian CTC metabolites (*18*), but CTC is known to be degraded to its isomers under alkaline conditions. This might be the reason for degradation of CTC to iso-CTC and its 4-epimer in slurry. These degradation products of CTC have no antimicrobial activity (*18*) and are therefore undetectable in the microbiological assay.

In conclusion, the newly developed microbiological assay is a powerful tool which can be employed to screen environmental samples for the occurrence of various active antimicrobial substances without a sophisticated sample preparation procedure. Microbiological screening of environmental samples without sample pretreatment is not possible for several reasons. First, the huge amount of bacteria naturally present in these samples can lead to an overgrowth of the employed test strain. Second, the low water content in soil, when that is compared to food samples, and the adsorption of TCs to soil components (e.g., humic materials) may reduce or prevent the diffusion of the analytes into the growth medium. Microbiological methods combined with a simple sample cleanup were described (*12*, *13*). After a liquid extraction of soil samples enriched with CTC, only the detection of very high concentrations was possible with an agar diffusion test. One group detected CTC in soil samples at a concentration range between 2.3 and 4.5 mg/kg (*13*). Others were able to detect CTC at a concentration of 5.6 mg/kg in soil (*12*). Both methods were not suitable to detect environmental concentrations of TCs occurring after the fertilization of soil with animal slurry containing TCs (*2*, *14*). In light of this, the screening of environmental samples at concentrations down to 100 μg/kg can be achieved using the particular microbiological assay described here. For the confirmation of these findings and the accurate quantification of tetracycline residues at much lower concentrations, LC−MS−MS is the method of choice.

NOTE ADDED AFTER ASAP

This article was released ASAP on 1/3/2003 before final corrections were made. The incorrect species name was given in the abstract and incorrect versions of Figures 1 amd 3 were included in the original posting. It has now been corrected in this version posted 1/09/03.

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