Microchim Acta 151, 67–72 (2005)
DOI 10.1007/s00604-005-0372-1

Microchimica Acta
Printed in Austria

Original Paper

Residue Analysis of Oxytetracycline in Water and Sediment Samples by High-Performance Liquid Chromatography and Immunochemical Techniques

Markus Himmelsbach and **Wolfgang Buchberger***

Institute for Analytical Chemistry, Johannes-Kepler-University Linz, Altenbergerstrasse 69, A-4040 Linz, Austria

Received February 2, 2005; accepted April 26, 2005; published online July 18, 2005

Abstract. The performances of an HPLC method and an enzyme-linked immunosorbent assay (ELISA) have been compared with respect to residue analysis of oxytetracycline in water and sediment samples of fish farms where this antibiotic is used for treating diseases. The ELISA employed in this study was a commercially available kit for determination of tetracycline in food. Due to its cross-reactivity towards oxytetracycline the kit turned out to be suited for water samples down to approximately 1 μg oxytetracycline L^{-1} without sample pretreatment. This concentration level could be lowered by a factor of at least 200 using preconcentration by solid-phase extraction (SPE) on Oasis HLB cartridges with elution by 10 mM oxalic acid in methanol. HPLC separation was carried out using a C8 reversed-phase column and a gradient of methanol and water containing 10 mM oxalic acid. The detection limit for water samples after 250-fold preconcentration by SPE was about 0.24 μg L^{-1} using UV detection at 360 nm. Recoveries of 98.7% were found at a concentration level of 2 μg L^{-1}. Satisfactory correlation was observed between the results of HPLC and ELISA. Both methods were also suited for measuring residues of oxytetracycline in sediment samples in the low μg g^{-1} range.

* Author for correspondence. E-mail: wolfgang.buchberger@jku.at

Key words: Tetracycline antibiotics; oxytetracycline; water analysis; aquaculture; HPLC; ELISA.

Tetracycline antibiotics such as tetracycline (TC), oxytetracycline (OTC), doxycycline (DC) and chlortetracycline (CTC) are pharmaceutical drugs frequently used in various veterinarian applications. Therefore, residues of these antibiotics have to be monitored in agricultural products such as eggs, milk, honey, or meat. Analytical techniques suited for this purpose include high-performance liquid chromatography (HPLC) [1–11] and immunochemical methods [11–16]. The structures of these compounds are given in Fig. 1.

Excessive use of tetracycline antibiotics may also lead to the occurrence of traces of these compounds in the aquatic environment. Liquid manure employed as fertilizer can act as a source for introduction of tetracycline antibiotics into the environment [12]. The strong binding behavior to soil results in low mobility so that aquatic systems may be less affected [17]. A different way of how these antibiotics may directly enter surface waters is their use in fish farms [18], where fish feed containing tetracycline drugs is often used to treat diseases. Since effluents of fish ponds hardly undergo any further treatment, residues of antibiotics can easily contaminate a wider range of the water system. Nowadays, oxytetracycline is the preferred tetracycline drug for aquacultures. Therefore,

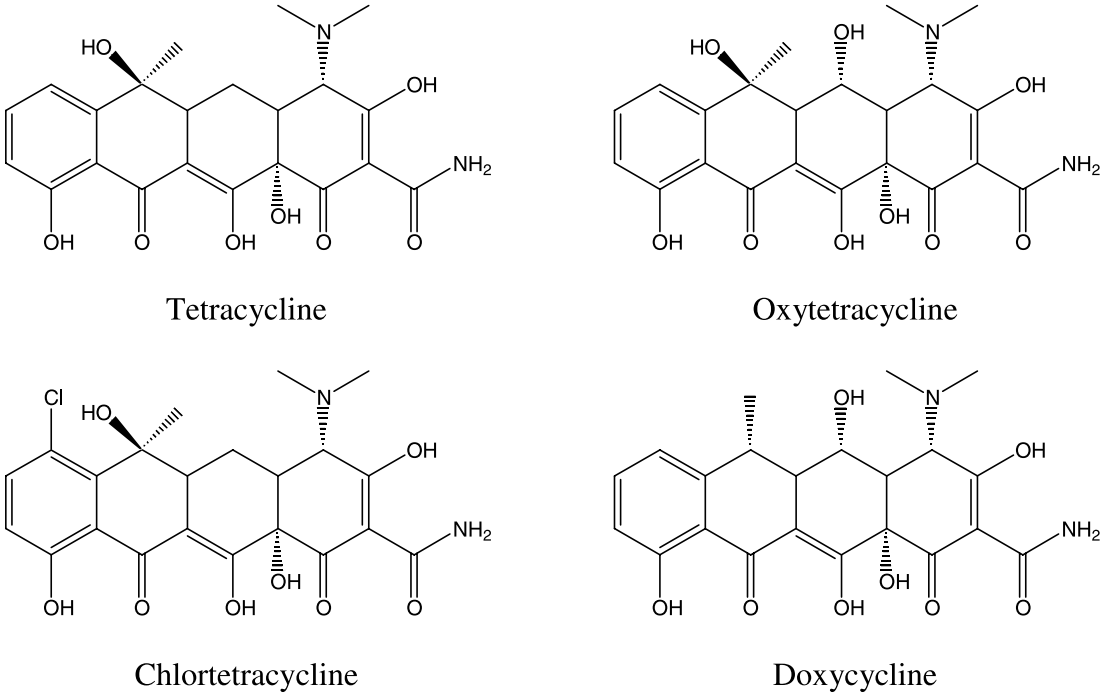

Fig. 1. Structures of tetracycline antibiotics included in this study

reliable analytical methods are required for monitoring residues of this compound in the aquatic environment.

As mentioned above, HPLC techniques are among the most efficient methods for determination of traces of tetracycline antibiotics. Their tendency to bind irreversibly to silanol groups of stationary phases requires special attention. Mobile phases containing oxalic acid can improve peak shapes considerably [19–24], but careful optimization of all chromatographic parameters is essential. In addition, tetracycline compounds may form strong complexes with multivalent metal ions, implying that the addition of complexing agents during sample preparation may be advantageous and result in noticeably higher recoveries [25, 26]. Such problems may be circumvented by using immunoassays which require little sample pretreatment and exhibit good detection limits. Nowadays, test kits are commercially available that have been optimized for determination of tetracycline in food [11, 13–15, 27]. Their applicability to environmental samples of water and manure has recently been investigated [12]. The pronounced cross-reactivity of these kits towards oxytetracycline might also make them suited for analysis of samples containing residues of oxytetracycline. Therefore, the aim of the work presented in this paper was to modify a commercial ELISA kit with regard to the requirements of quantification of oxytetracycline in water and sediment samples. To verify the results, an HPLC method was optimized for separation of tetracycline compounds in this type of samples in combination with a sample preconcentration step based on solid-phase extraction (SPE).

Experimental

Reagents

HPLC reagent grade methanol and ethylacetate for organic residue analysis were purchased from J. T. Baker (Deventer, The Netherlands). Oxalic acid, sodium chloride, sodium dihydrogen phosphate monohydrate, di-sodium hydrogen phosphate dihydrate and EDTA disodium salt dihydrate were of analytical reagent grade obtained from Merck (Darmstadt, Germany). Analytical reagent grade citric acid was purchased from Fisher Scientific (Loughborough, UK). Tween 20 (polyoxyethylene-sorbitanmonolaurate), tetracycline and oxytetracycline hydrochloride were supplied by Sigma-Aldrich (Steinheim, Germany). Doxycycline hydrochloride and chlortetracycline hydrochloride were obtained from Fluka (Buchs, Switzerland). 18 MΩ water was obtained from a Milli-Q water purification system (Millipore, Molsheim, France).

Stock solutions of all analytes were prepared by dissolving 10 mg of each substance in 10 mL methanol. Stock solutions were stored at −20 °C, and working solutions were prepared on the day of use.

The ELISA employed was a RIDASCREEN tetracycline kit from R-Biopharm (Darmstadt, Germany). This kit contained all necessary reagents and solutions except the washing buffer which was prepared by dissolving 0.55 g sodium dihydrogen phosphate monohydrate, 2.85 g di-sodium hydrogen phosphate dihydrate, 9 g sodium chloride, and 1 g Tween 20 in 1000 mL water.

HPLC Conditions

The instrumentation consisted of an Agilent Series 1100 HPLC instrument (Agilent, Palo Alto, USA) equipped with a vacuum degasser, a quaternary pump, an auto sampler (50 µL injection volume), and a UV-vis diode array detector. For separation an Agilent Zorbax Eclipse XDB-C8 column, 150 × 4.6 mm I. D., particle size 5 µm, was used. Detection was carried out at 360 nm. The optimized mobile phase consisted of water/methanol containing 10 mM oxalic acid. A gradient was run from 10% methanol to 60% methanol within 20 min. The flow rate was 1 mL min^{-1}.

Solid-Phase Extraction of Water Samples

All glassware used during sample preparation was rinsed with a saturated solution of Na_2EDTA in methanol before use. The water samples were filtrated through 0.8 µm glass microfibre filters (Whatman, Maidstone, UK). Prior to SPE, Na_2EDTA was added to each sample, resulting in a concentration of 2 g L^{-1}. SPE cartridges investigated were OASIS HLB 500 mg (Waters, Milford, MA, USA), Lichrolut RP-18 endcapped 500 mg (Merck, Darmstadt, Germany), Bondesil C-18 1000 mg (Varian, Palo Alto, CA, USA), and Chromabond Tetracycline 500 mg (Macherey-Nagel, Düren, Germany). The cartridges were preconditioned with 10 mL of methanol and 10 mL of water containing 2 g L^{-1} Na_2EDTA. Afterwards, 250 mL of sample were passed through the cartridge at a flow rate of 10 mL min^{-1}, followed by 5 mL water to remove excess Na_2EDTA. The cartridge was dried under vacuum, and elution was carried out with 8 mL methanol containing 10 mM oxalic acid. The eluate was dried under a stream of nitrogen, redissolved in 1 mL water, and analysed by HPLC or ELISA.

Sample Preparation for Sediment Samples

1 g of wet sediment (equalling 0.72 g sediment after drying at 110 °C) was thoroughly mixed with 1.5 mL of 1 M citrate buffer using an ultrasonic bath. Subsequently, this suspension was extracted with 7 mL ethyl acetate for 10 min in an ultrasonic bath, centrifuged, and the organic phase was separated. This procedure was repeated twice. The combined extracts were evaporated to dryness under vacuum and redissolved in 10 mL methanol. Prior to analysis 1 mL of this solution was dried in a gentle stream of nitrogen and redissolved in 1 mL water.

Optimized ELISA Procedure

Standard solutions were prepared from 0.05 to 4.0 µg L^{-1} for tetracycline and from 1 to 40 µg L^{-1} for oxytetracycline. 50 µL of each standard solution or sample solution was added to separate duplicate wells of the microtiter plate. After addition of 50 µL anti-tetracycline antibody solution, the plate was incubated at room temperature for 60 min. The solutions were discarded and the wells washed with 250 µL washing buffer three times. After addition of 100 µL of enzyme conjugate solution to each well, the plates were incubated for 15 min, followed by another three washing steps with 250 µL washing buffer. Subsequently, 50 µL of substrate solution and 50 µL of chromogen solution were added to each well. After 15 min, 100 µL of stop solution was added, and the absorbance was measured at 450 nm with an HT2 Microplate Reader (Anthos Labtec Instruments, Wals, Austria). The sigmoidal calibration curves were set up using Rodbard's four-parameter function and were plotted in the form of $B/B_0 \times 100$ (%) against log c (where B and B_0 are the values of absorbance measured at the concentration of a standard solutions and at zero concentration, respectively).

Results and Discussion

SPE of Water Samples and Extraction of Sediment

Four different types of cartridges were tested for SPE of water samples to determine the most efficient extraction method for tetracycline compounds. Bondesil C18 and LiChrolut RP-18 are well-established silica-based reversed phase materials, Oasis HLB is known to retain both hydrophobic and hydrophilic compounds with high capacity and Chromabond TC is particularly designed to give high recoveries for tetracyclines. Prior to the enrichment, Na_2EDTA was added to circumvent the well-known problem of complex formation with metal ions. Elution with methanol containing 10 mM oxalic acid yielded higher recoveries than using pure methanol or acetonitrile, especially for chlortetracycline and doxycycline when using the Bondesil and LiChrolut material. This can be attributed to interactions of tetracyclines with silanol groups. The recoveries (measured by HPLC as described below) of all four analytes at different SPE materials are shown in Table 1. With LiChrolut RP-18 all recoveries were below 25%, so this material can be considered unsuitable for the enrichment of tetracyclines. Bondesil C18 showed poor recoveries when elution was carried out with 8 mL of methanol containing 10 mM oxalic acid, but the results improved

Table 1. Comparison of recoveries of tetracycline compounds (mean ± s.d.) at different SPE materials for water samples fortified with 500 ng of each analyte and elution with 8 mL 10 mM oxalic acid in methanol

SPE material	n	Sample volume (mL)	TC (%)	OTC (%)	CTC (%)	DC (%)
1 g Bondesil C18	8	250	67.8 ± 12.0	78.2 ± 9.6	78.0 ± 6.8	65.6 ± 4.1
0.5 g LiChrolut RP-18	4	250	7.8 ± 1.0	6.6 ± 0.6	12.3 ± 1.8	25.3 ± 1.4
Oasis HLB 500	10	250	98.3 ± 3.8	98.7 ± 2.9	88.9 ± 4.7	84.0 ± 7.4
Oasis HLB 500	4	1000	90.9 ± 4.6	93.7 ± 2.9	89.0 ± 2.2	88.6 ± 3.1
Chromabond TC	10	250	76.2 ± 9.7	80.5 ± 12.5	79.2 ± 6.7	73.6 ± 8.9

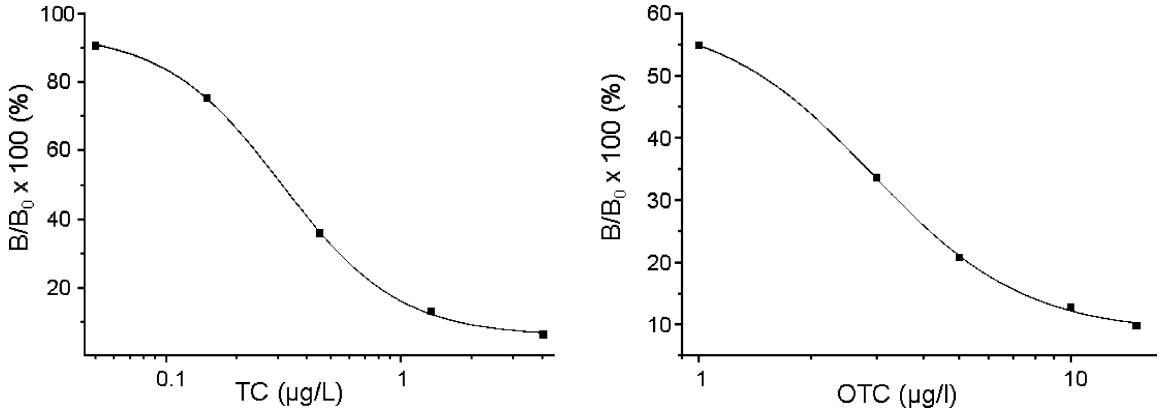

Fig. 2. ELISA calibration curves for tetracycline (*TC*) and oxytetracycline (*OTC*)

when the volume was raised up to 15 mL. Oasis HLB 500 mg delivered higher recoveries than Chromabond TC and better repeatability. Furthermore, there was no dependence of recovery upon sample volumes in a range of 250 to 1000 mL. To demonstrate the applicability of this procedure to the environmental samples in this study, the recovery for OTC was also determined at LOQ levels ($0.4\,\mu g\,L^{-1}$), resulting in a satisfying value of $93.3 \pm 2.1\%$. Therefore the Waters Oasis HLB 500 mg cartridges were used for all subsequent analytical work.

To determine the recoveries for the sediment extraction procedure, two sediment samples (mainly mud samples) without any tetracycline contamination were fortified with $5\,\mu g\,g^{-1}$ of each analyte and extracted as described in the experimental part. Recoveries were 78.1% for tetracycline, 90.4% for oxytetracycline, 82.2% for chlortetracycline and 81.4% for doxycycline.

ELISA

The RIDASCREEN Tetracycline kit used in this study is a competitive enzyme-linked immunosorbent assay developed for the quantitative determination of tetracycline in milk, honey, or meat. When used for water and sediment samples, the procedure applied is very similar to the one described for milk. Calibration curves measured with tetracycline showed an IC_{50} of $0.31\,\mu g\,L^{-1}$. The cross-reactivity towards oxytetracycline is approximately 10%. Therefore, in samples where only oxytetracycline is expected, this kit can also be used for quantitation of oxytetracycline as long as the poorer sensitivity is still sufficient. An IC_{50} of $2.9\,\mu g\,L^{-1}$ was found for oxytetracycline. Typical calibration curves are shown in Fig. 2. As discussed below, real samples from fish farms contained oxytetracycline concentrations of up to $5\,\mu g\,L^{-1}$. In such cases the ELISA can be used without any sample preparation. To measure concentrations in the $ng\,L^{-1}$ range, a preconcentration step by SPE in the same way as for HPLC can be used. Actually, the preconcentration factors obtained by SPE with subsequent HPLC are higher than those necessary for ELISA. For practical reasons, only one SPE procedure was carried out and the resulting solution used directly for HPLC or, after appropriate dilution, for ELISA.

HPLC Separation

For optimization of separation and peak shapes, mobile phases containing acetonitrile or methanol and the addition of organic acids such as formic acid or oxalic acid were investigated. Chromatograms obtained with mixtures of methanol/water or acetonitrile/water showed a strong fronting of the analyte peaks. Addition of formic acid did not improve peak shapes, whereas the addition of oxalic acid improved peak shapes considerably. Mobile phases containing acetonitrile did not yield satisfactory separation of the critical peak pair tetracycline and oxytetracycline. Therefore, a methanol/water gradient containing 10 mM oxalic acid was used in all further experiments. A typical chromatogram of a standard mixture and of a fish pond water sample after SPE preconcentration is shown in Fig. 3.

The detection limits of all four tetracycline antibiotics were about $60\,\mu g\,L^{-1}$ (signal-to-noise ratio 3), which corresponds to $0.24\,\mu g\,L^{-1}$ after 250-fold preconcentration by SPE.

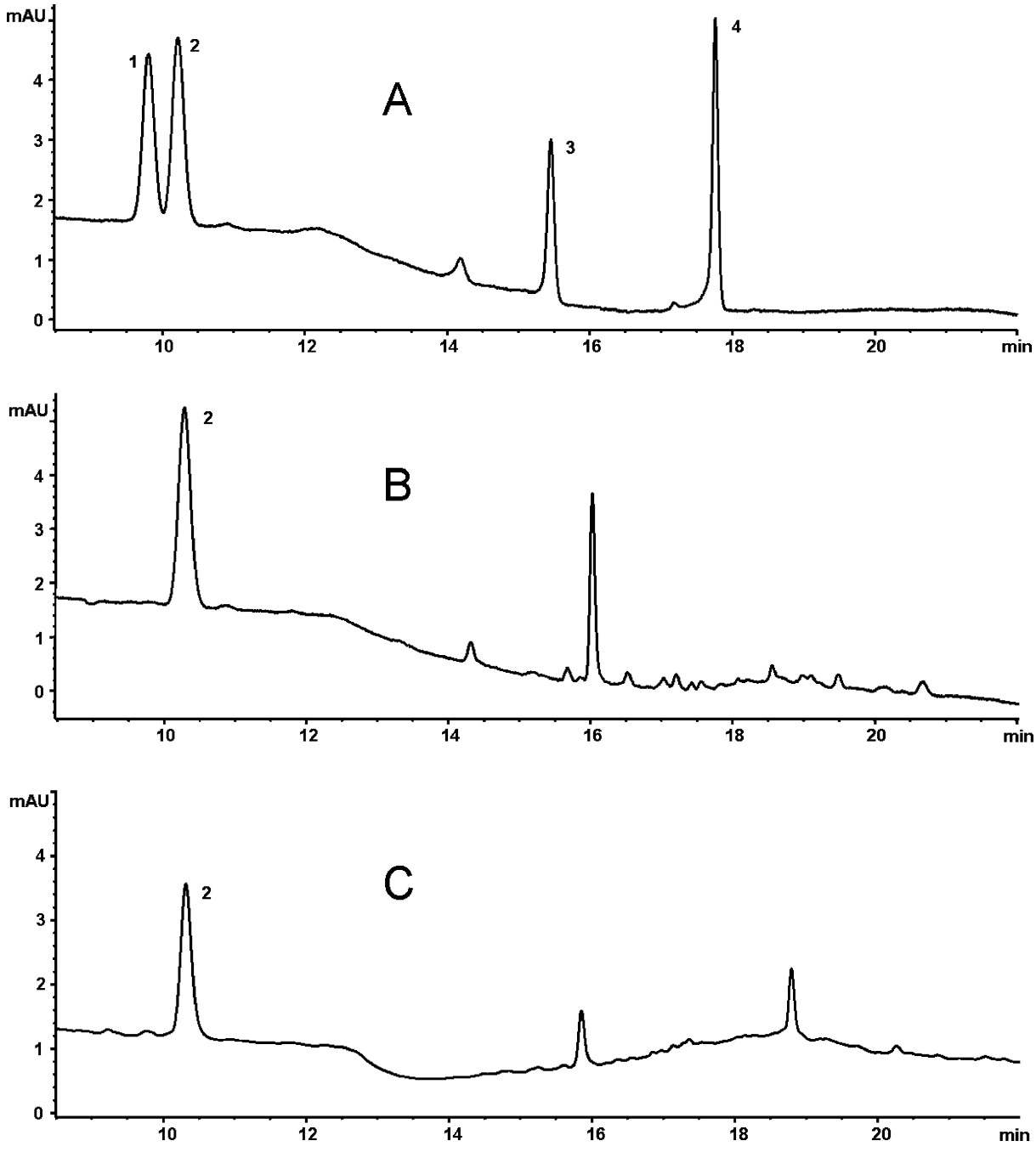

Fig. 3. HPLC separations of tetracycline antibiotics. (A) Standard (1 mg L^{-1} each); (B) Fish pond water sample after SPE preconcentration containing 4.3 µg L^{-1} oxytetracycline; (C) Sediment sample after extraction containing 5.0 µg g^{-1} oxytetracycline. Peaks: (*1*) tetracycline, (*2*) oxytetracycline, (*3*) chlortetracycline, (*4*) doxycycline. Chromatographic conditions see Experimental

Analysis of Environmental Samples

Five samples taken from fish ponds within the periods of oxytetracyline application contained concentrations between 0.5 and 4.2 µg L^{-1} oxytetracycline. In sediment samples, concentrations between 1.5 and 5 µg g^{-1} could be detected. A comparison of the results obtained by HPLC and ELISA for environmental samples was done based on polluted fish pond water samples, different river water samples and lake water samples (containing no oxytetracycline and spiked at a level of 0.50 to 2.50 µg L^{-1}), a sewage treatment plant effluent sample (containing no oxytetracycline and spiked at a level of 1.5 µg L^{-1}), and a polluted

Table 2. Comparison of the results for the determination of oxytetracycline in different matrices by HPLC and ELISA

Sample	Content ($\mu g\,L^{-1}$ for water samples $\mu g\,g^{-1}$ for sediment samples)	
	HPLC	ELISA
Fish pond sample 1	2.64	3.40
Fish pond sample 2	4.25	5.24
River water 1 spiked with $0.50\,\mu g\,L^{-1}$	0.50	0.45
River water 2 spiked with $1.00\,\mu g\,L^{-1}$	0.99	1.23
River water 3 spiked with $1.00\,\mu g\,L^{-1}$	0.89	1.02
River water 4 spiked with $1.50\,\mu g\,L^{-1}$	1.46	1.42
STP effluent spiked with $1.50\,\mu g\,L^{-1}$	1.40	1.48
Lake water 1 spiked with $2.00\,\mu g\,L^{-1}$	1.99	2.21
Lake water 2 spiked with $2.50\,\mu g\,L^{-1}$	2.68	2.83
Lake water 3 spiked with $1.00\,\mu g\,L^{-1}$	0.92	1.07
Sediment	5.03	6.13

sediment sample. The results are given in Table 2. The correlation between the two methods was reasonably good, although there was a tendency of the ELISA to produce slightly higher results. One cannot rule out a cross-reactivity of the ELISA to degradation products of oxytetracycline [12], but this would require additional detailed investigations. The correlation coefficient between the two methods calculated for all water samples was 0.98. The relative standard deviations calculated from the spiked water samples were 10.3% for the ELISA procedure and 5.8% for the HPLC method.

Conclusions

The results of this study clearly demonstrate that the use of oxytetracycline as an animal feed additive in fish farms can lead to concentrations of this compound in the low $\mu g\,L^{-1}$ range in fish pond water and in the low $\mu g\,g^{-1}$ range in sediment of fish ponds. Such concentrations can be monitored by optimized HPLC techniques after solid-phase extraction or by ELISA kits originally developed for the determination of tetracycline in food. The cross-reactivity towards oxytetracycline is sufficient to directly measure concentrations of oxytetracycline exceeding approximately $1\,\mu g\,L^{-1}$. Lower concentrations down to the $ng\,L^{-1}$ range can easily be measured if the ELISA is combined with a simple SPE step. The immunochemical technique yielded results that agreed with HPLC in a satisfactory way. Therefore, the ELISA is well-suited for screening a large amount of samples. It may help to monitor the extent of pollution by residues of oxytetracycline in surface waters in the vicinity of fish pond effluents.

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