JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Detection of Traces of Tetracyclines from Fish with a Bioluminescent Sensor Strain Incorporating Bacterial Luciferase Reporter Genes

TEIJO PELLINEN,† GÖRAN BYLUND,‡ MARKO VIRTA,† ANNELI NIEMI,§ AND MATTI KARP*,†

Department of Biotechnology, University of Turku, Tykistökatu 6A, FIN-20520 Turku, Finland; Department of Biology, Åbo Akademi University, Artillerigatan 6, FIN-20520 Åbo, Finland; and EELA, National Veterinary and Food Research Institute, Hämeentie 57, FIN-00580 Helsinki, Finland

Bioluminescent *Escherichia coli* K-12 strain for the specific detection of the tetracycline family of antimicrobial agents was optimized to work with fish samples. The biosensing strain contains a plasmid incorporating the bacterial luciferase operon of *Photorhabdus luminescens* under the control of the tetracycline responsive element from transposon *Tn10* (Korpela et al. *Anal. Chem.* **1998**, *70*, 4457−4462). The extraction procedure of oxytetracycline from rainbow trout (*Oncorhynchus mykiss*) tissue was optimized. There was neither need for centrifugation of homogenized tissue nor use of organic solvents. The lowest levels of detection of tetracycline and oxytetracycline from spiked fish tissue were 20 and 50 μg/kg, respectively, in a 2-h assay. The optimized assay protocol was tested with fish that were given a single oral dose of high and low concentrations of oxytetracycline. The assay was able to detect oxytetracycline residues below the European Union maximum residue limits, and the results correlated well with those obtained by conventional HPLC ($R = 0.81$).

KEYWORDS: Rainbow trout; residues of antimicrobial agents; HPLC; biosensor; *Escherichia coli*; *Photorhabdus luminescens*

INTRODUCTION

Tetracycline antibiotics are intensively used in therapy and prophylactic control of bacterial infections in human and veterinary medicine and as food additives for growth promotion in the farming industry. Intense usage of antibiotics has led to a wide distribution of antibiotic resistance among bacterial species, including resistance against tetracyclines. To limit the spread of resistance, unnecessary dosing of antibiotics should be minimized. Control of usage in animal farming is possible by monitoring antibiotic residues in different biological samples. In addition to limiting the spreading of resistance, monitoring of residues also prevents the access of possible allergenic antibiotics into finished food products and ensures that the residues do not interfere with food production processes.

Traditional approaches for the detection of tetracycline residues include microbial inhibition tests, immunoassays, and chromatographic methods. Widely used microbial inhibition methods are relatively slow, insensitive, and nonspecific to tetracyclines, but immunometric methods provide selectivity and sensitivity. However, even specific tetracycline immunoassays are not able to detect all of the important tetracyclines used in veterinary medicine. Chromatographic methods, such as HPLC, provide sensitivity and specificity for tetracyclines, but certain disadvantages, for example, high price, requirement of special equipment, and sample extraction protocols by expert personnel, limit their suitability for the analysis of large numbers of samples.

Oxytetracycline residues in rainbow trout are widely measured in Finland and other European Union (EU) countries by expensive and laborious HPLC analysis. Also, fish bacteriologists need to quantify oxytetracycline when testing antimicrobial drug treatment on fish diseases. Oxytetracycline is the most often used antimicrobial drug for bacterial infections in Finnish fish farming. In 1998, 215.3 kg of oxytetracycline was used, and in 2000 the amount was ∼150 kg (source: Plant Production Inspection Center, Finland). The use of oxytetracycline for growth promotion of fish is prohibited in the EU. The use of oxytetracyclines has been associated with many complications such as development of drug-resistant pathogens, unacceptable drug residues in fish produced for human consumption, release of drugs into the environment, and also side effects on fish health (*1*). Oxytetracycline has also been shown to suppress the immune system of fish (*2*).

A modern approach to detect drugs and other compounds is to use specific bacterial cells constructed with recombinant DNA technology. These cells carry a plasmid that contains a specific promoter/operator region and reporter genes connected to it. In

* Corresponding author (telephone +358-2-333 8085; fax +358-2-333 8050; e-mail matti.karp@utu.fi).
† University of Turku.
‡ Åbo Akademi University.
§ National Veterinary and Food Research Institute.

10.1021/jf020402l CCC: $22.00
Published on Web 07/12/2002

such a system, the compound to be monitored turns on the reporter gene expression (*3*). Luciferase genes are commonly used as reporters, yielding light emission, bioluminescence, as the reporter signal (*4*). We have recently constructed a novel expression system for the specific detection of tetracyclines. In the approach bacterial luciferase genes (*luxCDABE)* of *Photorhabdus luminescens* (*5*) were inserted under the control of the *tetA* promoter of transposon *Tn*10 (*6*). The system has been previously used to detect seven different tetracyclines in spiked pork serum (*7*) and raw bovine milk (*8*). The sensor strain *Escherichia coli* K-12(pTetLux1) can be freeze-dried, which allows avoidance of tedious culturing of the cells. In the presence of tetracyclines the luciferase genes are specifically induced to produce light. Here we introduce a simple, fast, and cheap method for measuring tetracyclines in live fish and fish products. The method is easily customized into a portable field assay system due to the use of reagent-like lyophilized biosensor bacteria. In the assay, homogenized fish muscle tissue is directly applied to microtiter plate wells with rehydrated biosensor cells, and bioluminesence is measured with a luminometer or with a portable CCD camera after incubation.

MATERIALS AND METHODS

Reagents for the Biosensing Method. Tetracyclines (hydrochlorides) for biosensor measurements, MES (hydrate), and EDTA (disodium salt, dihydrate) were from Sigma (St. Louis, MO). Disodium hydrogen phosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$) was supplied by J. T. Baker (Deventer, Holland). Citric acid monohydrate was by Riedel-de Haën (Seelze, Germany). Tryptone, yeast extract, and agar were obtained from Hispanlab, S.A. (Alcobendas, Spain). All chemicals were of analytical grade.

Solutions for the Biosensing Method. EDTA−McIlvaine buffer solution (pH 4.1) was prepared by dissolving 15 g of $Na_2HPO_4 \cdot 2H_2O$, 13 g of citric acid monohydrate, and 37.2 g of EDTA (0.1 M) in water and diluting to 1 L. Rehydration solution was Luria Bertani (LB) medium (10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter, pH 7) containing 100 mM MES (pH 6.0).

Reagents and Solutions for HPLC. Oxytetracycline hydrochloride standard for the HPLC measurement was obtained from Sigma-Aldrich, Riedel-de Haën, VETRANAL. The standard stock solution for oxytetracycline was prepared in methanol to a concentration of 1 mg/mL, and the standard working solution for oxytetracycline was diluted with methanol from the standard stock solution to a concentration of 10 μg/mL. Hexane, dichloromethane, methanol, and acetonitrile were purchased from J. T. Baker, and *N*,*N*-dimethylformamide was from Rathburn Chemicals Ltd., Walkerburn, Scotland. All solvents were of HPLC grade. Water was purified by a Millipore Milli-Q Plus system. Disodium hydrogen phosphate, citric acid monohydrate, oxalic acid dihydrate, and trichloroacetic acid were purchased from J. T. Baker. Titriplex III was purchased from Merck, Darmstadt, Germany.

Materials for HPLC. Phase extraction columns Bond-Elut C18, 3 cm^3/500 mg, were obtained from Varian, Middelburg, The Netherlands. Filter paper 589 Black ribbon was from Schleicher and Schuell, Dassal, Germany, and Millex HV 0.45 μm filters for filtering the samples prior to the HPLC analysis were from Millipore. HVLP, 0.45 μm filters from Millipore, were used for filtering solvents for HPLC.

HPLC Apparatus. The HPLC was a combination of a Waters 717 autosampler, Waters 510 pumps, a Waters column heater module, and a Waters temperature control module. For peak detection a tunable absorbance detector, a Waters model 486, was used. The analytical column was a Hewlett-Packard Hypersil BDS-C18 reversed phase column, 250 × 4.0 mm, 5 μm i.d.. A guard column used was Lichrospher 100 RP-18, 4 × 4 mm, 5 μm i.d., Agilent Technologies. Millenium software (version 3.05, 1998, Waters) was used to handle and process chromatographic data.

Biosensing Protocol. *Fish-Spiking Experiment.* Stock solutions (5.0 mg/mL) of tetracycline (TC) and oxytetracycline (OTC) were made by dissolving to 0.1 M HCl. Working solutions (5.0 and 0.5 μg/mL) were prepared by diluting stock solution to water. The tissue samples were spiked before homogenization to concentrations of 0, 50, 100, and 500 ng of drug/g of muscle tissue.

Medication of Fish with OTC. Apparently healthy rainbow trouts (*Oncorhynchus mykiss*) weighing 126−594 g were obtained from Åbo Akademi University, Turku, Finland. The fish were divided into two groups (12 + 12) kept separately at 15 °C in the laboratory in 225 L fiberglass tanks supplied with aerated spring water. The flow rate was 0.5 L/min, and the pH was 7.1−7.5. The photoperiod was adjusted to 12 h of light and 12 h of darkness. The fish were fed with pelleted dry food in amounts of 1.5% of body weight per day. The fish were allowed to adapt to these conditions for at least 10 days prior to the experiment.

The fish were anesthetized with benzocaine, 50 mg/L, and weighed. One group of fish was given 40 mg of OTC/kg of body weight and the other group 75 mg of OTC/kg of body weight. The stock solution of OTC was prepared by dissolving it in water (500 mg/mL). In working solutions, the drug was suspended in 0.2% (w/v) aqueous agar to give a suitable viscosity for administration. The drug was administered orally via a stomach tube. Two fish in both groups were not given the drug, but instead used as blank fish.

From each group two fish were killed at intervals between 2 and 22 days after administration of the drug. The fish were filleted, and portions from the tail end of the fillets were frozen to −20 °C.

Homogenization of Fish Samples. Homogenization was carried out in two steps. First, the frozen samples were dry-homogenized with a kitchen blender for 25 s, and then the samples were frozen again. In the second homogenization, 2.00 ± 0.01 g of dry homogenate was measured and homogenized into 4.0 mL of EDTA−McIlvaine buffer for 25 s with a plastic piston connected to a power drill.

Luminescence Measurements. Homogenized fish tissue samples were pipetted into the wells of white microtitration plates (Labsystems Oy, Helsinki, Finland) in a volume of 200 μL. Freeze-dried *E. coli* K-12-(pTetLux1) sensor cells were rehydrated by adding 4.0 mL of rehydration solution into an ampule. Four ampule suspensions were mixed together and allowed to stand for 10 min, after which time 100 μL of suspension was added to each well. After the addition of sensor bacteria, the plates were shaken for 60 s by a plate shaker (Perkin-Elmer Life Sciences, Turku, Finland) and incubated without shaking at 37 °C for 120 min. After incubation, the luminescence was measured by using a Victor 1420 multilabel counter luminometer (Perkin-Elmer Life Sciences).

Induction coefficients were calculated using the formula $IC = L_i/L_b$, where IC is the induction coefficient, L_i is the luminescence value of the sample, and L_b is the luminescence value of a blank sample (not spiked or administered OTC or TC).

HPLC Protocol. *Sample Preparation for OTC Analysis.* The sample preparation method for OTC was a modification of the method presented earlier (*9*). Five grams of fish muscle was extracted with 20 mL of EDTA−McIlvaine buffer and 3 mL of hexane−dichloromethane (1 + 3 v/v). The mixture was homogenized and centrifuged at 4000 rpm for 10 min, and the supernatant was saved. The residue was re-extracted with 10 mL of EDTA−McIlvaine buffer, and the supernatants were combined. Three milliliters of trichloroacetic acid (0.25 g/mL) was added by mixing, and the mixture was kept on ice for 15 min, centrifuged at 4000 rpm for 3 min, and filtered through filter paper. The SPE cartridge was activated with 2 mL of methanol and 4 mL of EDTA−McIlvaine buffer. The sample was passed through the cartridge, which was flushed after that with 2 mL of water and dried with air for 5 min. OTC was eluted from the cartridge with 4 mL of 0.01 M methanolic oxalic acid. Eluted sample was then evaporated to dryness under a nitrogen stream at 50 °C and dissolved in 1 mL of HPLC mobile phase (acetonitrile/*N*,*N*-dimethylformamide/0.01 M oxalic acid, pH 2.1, 22:6:72 v/v/v) for HPLC analysis. The flow rate of the mobile phase was 0.8 mL/min and the volume injected 50 μL with detection at 355 nm.

Validation of the OTC Method by HPLC. The method was validated for fish muscle by spiking at two concentration levels of 50 and 100 μg/kg for OTC. Six replicates at each concentration level and blank fish sample were prepared and analyzed. Recovery and repeatability within day were calculated from the obtained results. The average recovery was 73%. The RSD values of the repeatability within day were 1.2% at the spiking level of 50 μg/kg and 2.1% at the spiking

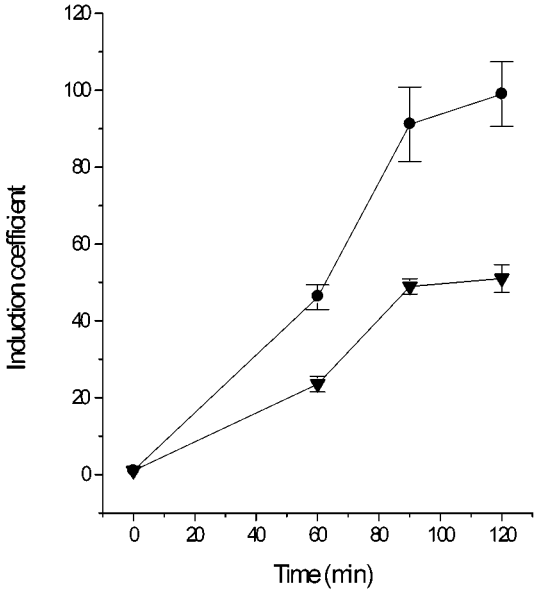

Figure 1. Time curve for tetracycline induction. Sensor cells were treated with homogenized fish spiked with 500 μg/kg of either TC–HCl (circles) or oxyteracycline (triangles) for different times and measured for light emission as described under Materials and Methods. The figure shows the response calculated as induction coefficient (light emission signal over background nonspiked homogenized fish sample) as a function of time. Bars indicate the standard error of the mean from three parallel experiments.

level of 100 μg/kg. The limit of detection (LOD) was calculated on the basis of a signal-to-noise ratio of 3:1. The limit of quantification (LOQ) was 2 times the value of the LOD. In this manner the LOD was found to be 5 μg/kg and the LOQ, 10 μg/kg.

RESULTS AND DISCUSSION

The mechanism of the action of the tetracycline biosensing approach has been described previously (*6*). In short, TC, after transportation inside the sensor cell *E. coli* K-12(pTetLux1), binds to dimeric TC repressor protein. This event causes a 5 Å conformational change (*10*) leading to displacement of the protein from the operator site and thereby transcription/translation of the reporter genes. The biosensing phenomenon commences immediately after enough TC molecules have entered the cells. Five minutes is enough for light emission to occur (data not shown). However, we used a longer incubation period to obtain a sensitive enough assay for all medically relevant TCs. A 2-h incubation period was found to be convenient as shown for the detection of oxytetracycline and TC hydrochloride in **Figure 1**.

In our optimization experiments we used freeze-dried cells and OTC as the model compound because it gives the poorest response (*11*), and therefore all of the other TC molecules are consequently detected. EDTA and low pH are known to sensitize sensor cells to minute amounts of TCs (*6*). Fortunately, these variables are also present in commonly used homogenization buffers when TC residues are extracted from fish meat for HPLC analysis. We therefore expected to obtain a very simple procedure for the analysis of TCs with the biosensing approach. Earlier we had tested the sensor with milk matrix and found that although milk is opaque, it does not disturb the light emission to the photomultiplier tube of the light-gathering instrument (*8*, *11*). Therefore, we also expected that no centrifugation would be needed after the meat homogenization step. Indeed, this is the case, and a very robust and simple procedure was developed. **Figure 2** shows experiments in which the concentration of EDTA is varied in the homogenization buffer. The result is shown where OTC was extracted from a rainbow trout fed with a high concentration of the drug. As a control we used fish that did not get any drug to serve as a fish blank in all experiments. It can be seen that a high concentration of EDTA causes a lower signal and also a lower background, but as calculated as induction coefficients, that is, signal over nontreated blank, the result is better than using low EDTA in the buffer. The trend was the same when the homogenate was centrifuged and OTC measured from the clear lysate. In this case the signals are higher and induction coefficients lower due to higher background. The result was the same for a fish that was fed with a low dose of OTC except that the light emission values and induction factors were lower. Furthermore, the response was the same after overnight incubation in an ice bath,

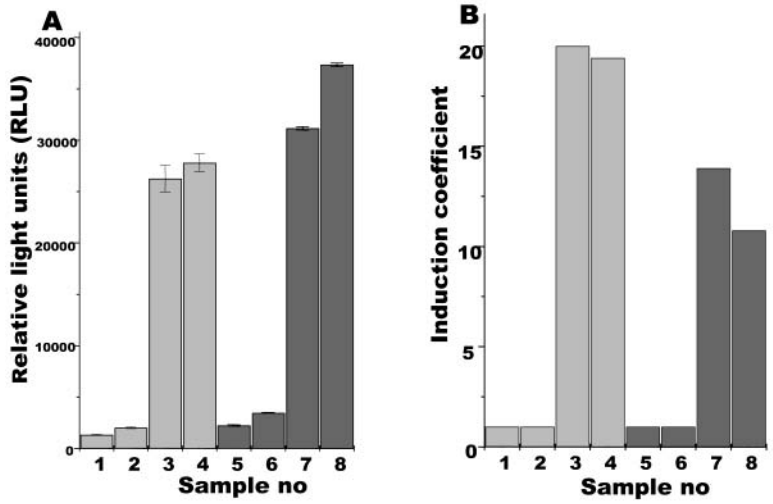

Figure 2. Effect of EDTA for the measurement of tetracycline residues from fish meat. Fish was treated with oxytetracycline as described under Materials and Methods. Panel A shows the results with relative light emission units (RLU); panel B shows the calculated induction coefficiences. Sample 1 is the control for sample 3 (0.1 M EDTA) and sample 2 is the control for sample 4 (0.01 M EDTA). Similarly, sample 5 is the control for sample 7 (0.1 M EDTA) and sample 6 is the control for sample 8 (0.01 M EDTA). Samples 1–4 (light gray) show the results obtained from measurements directly from buffer/fish homogenate and samples 5–8 (dark gray) for the corresponding centrifuged lysates.

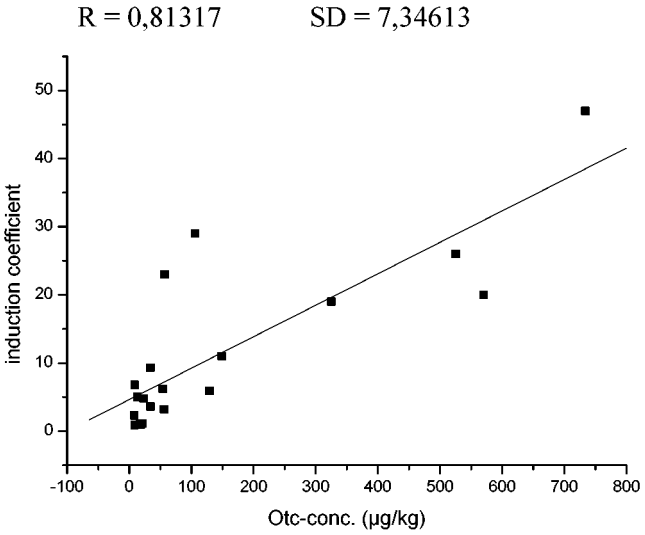

Figure 3. Correlation between the tetracycline biosensing approach and HPLC in the detection of oxytetracycline from fish fed with the drug. Altogether 20 fish samples divided into high- and low-dose groups (10 + 10) were measured, and the correlation between the sensor and HPLC methods was 0.81. The standard deviation (SD) was 7.35. The dosing, growth, sample preparation, and sensor and HPLC measurements were done as described under Materials and Methods.

indicating that the buffer stabilizes OTC very well (data not shown).

On the basis of the optimization experiments a solvent- and centrifugation-free extraction procedure could therefore be developed. We tested this methodology by feeding 10 fish with low doses and 10 fish with high doses of OTC. Two fish for both groups that were not fed served as controls. At different time points two fish from each group were sampled, and a portion of meat was frozen for subsequent analysis of drug residues. The homogenized and noncentrifuged fish muscle samples were analyzed by the biosensing approach and compared with the results taken by conventional HPLC analysis from the same muscle samples. The result is shown in **Figure 3**. The correlation between the two methods is good ($R = 0.80$). The detection limit of HPLC is at 5 μg/kg, being somewhat more sensitive than our very simple and robust approach. Previous studies showed that when low levels of the drugs are spiked in LB cultivation medium, the detection limits for various TCs were 1.25 μg/L for TC−HCl and OTC and even less for chlortetracycline, doxycycline, an minocycline (*6*). When the drugs were spiked in cow's milk, the corresponding figures were from 3 μg/L (TC−HCl) to 12 μg/L (OTC) and 2−4 μg/L for the rest of the TCs tested (*11*). Here it was found that the level of detection from spiked fish muscle tissue was ∼20 μg/kg for TC and slightly higher for OTC. The extraction procedure from fish fed with the drug was shown to liberate lower than HPLC quantification levels (10 μg/kg) of OTC as measured by the biosensing method (**Figure 3**). The coefficients of variation as measured by the induction coefficients at low drug levels were of the order of 10% and below 5% at higher levels of 500 μg/kg. The absolute light emission measurements from different ampules varied considerably, but the induction coefficients by constant amount of OTC spiking were always between 10% from day to day. The recoveries of spiked TCs were ∼80% (data not shown). The EU maximum residue limit (MRL) is set at 100 μg/kg level, and therefore our approach fulfills this regulatory requirement. The freeze-dried sensor cells showed excellent stability because they were 100% active after 9 weeks of storage at 4 °C. The ampules stored at room temperature and at 37 °C lost their activity to 33% in 9 or 7 days, respectively (data not shown).

In conclusion, a robust and streamlined approach for the detection of TC residues in fish meat was developed. The method utilizes living *E. coli* sensor bacteria that have been genetically modified to respond specifically for trace amounts of TCs. The method does not necessitate the use of centrifugation or solvents; only homogenization of frozen fish tissue is needed for sample preparation. The assay can be performed in high throughput using microtitration plate readers or with extremely cheap manual luminometers. The sensor cells do not need to be cultivated because we showed that throughout the assay development freeze-dried indicator cells could be used. This results in reagent-like behavior and hence lower variation between results from day to day.

LITERATURE CITED

(1) Van der Hejden, M. H.; van Muiswinkel, W. B.; Grondel, J. L.; Boon, J. H. Immunomodulating effects of antibiotics. In *Chemotherapy in Aquaculture: from Theory to Reality*; Michel, C., Alderman, D. J., Eds.; Office International des Epizooties: Paris, France, 1991; pp 219−230.

(2) Lunden, T. Effects of antimicrobial drugs on the immune response of rainbow trout (*Oncorhynchus mykiss*). Ph.D. Thesis, Åbo Academi University, Åbo, Finland, 2001.

(3) Lewis, J. C.; Feltus, A.; Ensor, C. M.; Ramanathan, S.; Daunert, S. Applications of reporter genes. *Anal. Chem.* **1998**, *70*, 579A−585A.

(4) Pazzagli, M.; Devine, J. H.; Peterson, D. O.; Baldwin, T. O. Use of bacterial and firefly luciferases as reporter genes in DEAE-dextran-mediated transfection of mammalian cells. *Anal. Biochem.* **1992**, *204*, 315−323.

(5) Frackman, S.; Anhalt, M.; Nealson, K. H. Cloning, organization, and expression of the bioluminescence genes of *Xenorhabdus luminescens*. *J. Bacteriol.* **1990**, *172*, 5767−5773.

(6) Korpela, M.; Kurittu, J.; Karvinen, J.; Karp, M. A recombinant *Escherichia coli* sensor strain for the detection of tetracyclines. *Anal. Chem.* **1998**, *70*, 4457−4462.

(7) Kurittu, J. S.; Karp, M. T.; Korpela, M. T. Detection of tetracyclines with luminescent bacterial strains. *Luminescence* **2000**, *15*, 291−297.

(8) Kurittu, J. S.; Lönnberg, S.; Virta, M.; Karp, M. T. Qualitative detection of tetracycline residues in milk with a luminescence-based microbial method: the effect of milk composition and assay performance in relation to an immunoassay and a microbial inhibition assay. *J. Food Prot.* **2000**, *63*, 953−957.

(9) Sokol, J.; Matisova, E. Determination of tetracycline antibiotics in animal tissues of food-producing animals by high-performance liquid chromatography using solid-phase extraction. *J. Chromatogr. A* **1994**, *669*, 75−80.

(10) Hinrichs, W.; Kisker, C.; Duvel, M.; Muller, A.; Tovar, K.; Hillen, W.; Saenger, W. Structure of the Tet repressor-tetracycline complex and regulation of antibiotic resistance. *Science* **1994**, *264*, 418−420.

(11) Kurittu, J. S.; Lönnberg, S.; Virta, M.; Karp, M. T. A group-specific microbiological test for the detection of tetracycline residues in raw milk. *J. Agric. Food Chem.* **2000**, *48*, 3372−3377.

Received for review April 4, 2002. Revised manuscript received May 27, 2002. Accepted May 28, 2002. We gratefully acknowledge financial support from AboaTech Ltd. M.K. acknowledges support from the Academy of Finland (Grant SA54229).

JF020402L