

Europium-sensitized luminescence determination of oxytetracycline in catfish muscle

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Abstract

An europium-sensitized time-resolved luminescence (TRL) method was developed to determine oxytetracycline (OTC) in cultivated catfish muscle. Extraction of OTC from fish muscle was performed with pH 4.0 ethylenediaminetetraacetic acid (EDTA)-McIlvaine buffer and clean up with hydrophilic–lipophilic balanced copolymer solid phase extraction (SPE) cartridges. The eluate was used without further concentration for TRL measurement in pH 9.0 micellar tris(hydroxymethyl)aminomethane (TRIS) buffer. Cetyltrimethylammonium chloride (CTACl) was used as surfactant and EDTA as a co-ligand. The excitation and emission wavelengths were set at 388 and 615 nm, respectively. The linear dynamic range was 0–1000 ng g⁻¹ ($R^2 = 0.9995$). The recovery was 92–112% in the fortification range of 50–200 ng g⁻¹ and the limits of detection (LOD) ranged from 3 to 7 ng g⁻¹. Incurred catfish samples were used to demonstrate the performance of the method around 100 ng g⁻¹, the European Union maximum residue level.

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1. Introduction

Aquaculture has expanded globally in the past 30 years and the industry has developed comprehensive plans to manage fish health; however, the need for antimicrobial therapeutics remains unavoidable in certain instances [1]. Among the most commonly used antimicrobial antibiotics is oxytetracycline (OTC), a member of the tetracycline (TC) class, and one of only three antibiotics (along with Romet 30 and sulfamerazine) currently approved by the US Food and Drug Administration (FDA). In channel catfish, OTC is approved to treat motile *Aeromonas* septicemia, *Pseudomonas* septicemia and enteric septicemia [1]. OTC residues in food may lead to increased microbial resistance in humans [2]. Evidence shows that antibiotic-resistant strains of fish pathogens have developed since the beginning of antibiotic use [1].

The risk of transferring the resistance from fish pathogens to human pathogens is under study [3]. The FDA has set a 2000 ng g⁻¹ OTC tolerance in fish muscle and a 21-day withdrawal period. The Codex Alimentarius Commission and the EU have set the OTC maximum residue level (MRL) in fish at 100 ng g⁻¹. Sensitive and selective analytical methods to monitor and regulate OTC usage are therefore in demand by the rapid growth of fish farming and trade on a global scale.

The FDA regulatory method for OTC in fish tissue is a microbial growth inhibition assay [4]. It is semiquantitative and nonspecific, and requires a long incubation time (16–18 h). A microbial receptor assay (Charm II) [5], with improved sensitivity, selectivity and throughput, has gained popularity in screening. A disadvantage with the Charm approach is its use of radioisotopes. High performance liquid chromatography (HPLC) is by far the most widely applied quantitative technique for analysis of OTC residues in fish using either UV [6] or fluorescence [7] detection.

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Alternatively, a capillary electrophoresis (CE) method has been developed for OTC determination in channel catfish [8]. Recently a novel bioluminescence method has been developed and optimized for OTC in fish muscle, in which *Escherichia coli* cells were constructed to carry a plasmid that contains a special promoter region with reporter genes [9]. When OTC residue exists in the fish sample, the expression of these genes is turned on resulting in a bioluminescence signal.

Europium-sensitized time-resolved luminescence (TRL) was applied as early as 1983 to TC analysis [10] to achieve higher sensitivity and selectivity. This method is based on the spectroscopic behavior of a TC-Eu(III) complex formed under mild alkaline conditions. TC as a ligand strongly absorbs at 388 nm and transfers the excitation energy to the Eu(III) ion. In a micellar environment this process is highly efficient resulting in a narrow, intense atomic emission peak at 615 nm and an order-of-magnitude sensitivity enhancement over intrinsic molecular fluorescence. Furthermore, selectivity is greatly improved by the exceptionally long excited state lifetime (ms time scale) and large Stokes shift (up to 300 nm). This technique has been successfully applied to analysis of TCs in biological fluids [11], calf serum [12], milk [13] and chicken muscle [14]. TRL has also been employed as a HPLC and FIA detection technique via post-column derivatization [11]. In the following we report the first application of a TRL method to OTC determination in farm-raised catfish muscle.

2. Experimental

2.1. Reagents and solutions

Oxytetracycline was purchased from Sigma (St Louis, MO). All other chemicals and solvents were from Aldrich (Milwaukee, WI) and were of analytical reagent grade. Deionized water prepared with a Barnstead (Dubuque, IA) E-pure system was used to prepare all aqueous solutions. The OTC stock solution ($200 \mu\text{g g}^{-1}$) was prepared in methanol and stored at 4°C . Dilutions, as needed, were performed daily. Ethylenediaminetetraacetic acid (EDTA)-McIlvaine buffer was prepared by the combination of 0.1 M citric acid (1000 ml), 0.2 M sodium phosphate dibasic (625 ml) and disodium EDTA dihydrate (60.5 g). All glassware after cleaning was soaked in 10% nitric acid overnight, and then rinsed repeatedly with deionized water.

2.2. Apparatus

Time-resolved luminescence was performed on a Cary Eclipse fluorescence spectrometer (Varian, Walnut Creek, CA) equipped with a xenon flashlamp. Both the operation and signal processing were handled by Cary Eclipse Lifetime software. Between samples, the cuvettes were washed

with deionized water, rinsed with acetone, and dried using a vacuum washer (VWR, Pittsburgh, PA). The spectrometer was allowed to warm up for 30 min before use.

2.3. Procedure

2.3.1. Control catfish tissue

Farm-raised catfish were obtained from local grocery stores as fillets, or were supplied from experimental channel catfish stocks raised at the Harry K. Dupree Stuttgart National Aquaculture Research Center, Stuttgart, AR. These latter fish were produced from a single lot as previously described [15], filleted and shipped to the USDA Eastern Regional Research Center on dry ice. The muscle was cut into small pieces and blended with a food processor to a homogeneous consistency. The homogenized fish samples were stored at -80°C until analysis.

2.3.2. Preparation of OTC medicated diet

OTC was incorporated into a commercial production feed ration (Arkat, Dumas, AR) to provide $100 \text{ mg OTC kg}^{-1}$ fish per day when fish are fed 3% of their body weight (BW) per day. The commercial diet was ground to less than 0.5 mm in a hammermill (Model F21M, W-W Grinder, Wichita, KS). Pulverized ration with antibiotic added was thoroughly blended in a V-mixer (Blendmaster Lab Blender, Patterson-Kelly, Stroudsburg, PA) for 15 min. To avoid heating during V-mixing, the program included three 3-min periods of intensifier bar rotation separated by two 3-min periods during which the V-mixer shell rotated without intensifier bar operation. Dry ingredients were then mixed with distilled water in a commercial food mixer (Model A-200, Hobart, Troy, OH) until a uniform mixture was obtained. The moistened mixture was passed through a meat grinder equipped with a 3 mm die to obtain uniform pellets. The same method described was used to prepare basal diet (no OTC added). Pelleted diets were air-dried for 12–24 h at room temperature and then frozen at -18°C until needed. Small quantities of diet were thawed and refrigerated at 4°C until fed [16].

2.3.3. Preparation of incurred fish

Three flow-through tanks (700 l) with supplemental aeration were each stocked with a group of 16 control fish $1996 \pm 105 \text{ g}$ (mean $\pm$ standard deviation). The fish were allowed to acclimate in a 7-day period, which was followed by a 10-day OTC medication period and a 14-day withdrawal period. During the 10-day medication period the fish were given $100 \text{ mg OTC kg}^{-1}$ BW per day (fed at 3% BW), while during the acclimation and withdrawal periods the fish were given basal diet at the same rate (3%). The choice of 3% BW feeding rate rather than 1% was used to allow medication of all the fish [17]. The following water quality parameters were measured every 6 day (Hach DR/2010, Hach, Loveland, CO) and maintained at dissolved oxygen levels of $7.83 \pm 0.31 \text{ mg l}^{-1}$, total ammonia nitrogen $0.73 \pm 0.30 \text{ mg l}^{-1}$ and temperature $22.64 \pm 0.38^\circ\text{C}$.

2.3.4. Sampling of incurred fish

Immediately prior to the first feeding of the OTC medicated diet, four fish were randomly sampled as controls from each tank and the rest of the fish were weighed to calculate the weight of diet to be fed. This first sampling was followed by three samplings during the 10-day medication period (after days 3, 7 and 10 of medication) and three samplings during the 14-day withdrawal period (after days 3, 7 and 14 post medication). At each sampling one fish was removed from each tank, (three replicates per sampling), and weighed. All fish sampled were filleted and the fillet was frozen at -80°C . After each tank sampling the amount of feed administered was adjusted to account for the new BW of the group.

2.3.5. Extraction of OTC from fish muscle

Homogenized fish muscle samples (1.5 g) were placed in 50 ml screw-capped polypropylene centrifuge tubes. Appropriate volumes of OTC standard solution (at 5 and $10\text{ }\mu\text{g g}^{-1}$) were added to control fish muscle samples to achieve the desired fortification levels. Methanol was added to make the total volume added in this step $100\text{ }\mu\text{l}$. The samples were then extracted with 6 ml of Na_2EDTA -McIlvaine buffer (pH 4.0) using an Ultra-Turrax T-25 homogenizer (Janke and Kunkel, Cincinnati, OH) and centrifuged ($2791 \times g$, 5 min). The supernatants were immediately decanted and kept in an ice bucket to minimize precipitation. The muscle pellet was extracted and centrifuged ($3446 \times g$, 5 min) a second time; the two supernatants were combined and filtered with a $5.0\text{ }\mu\text{m}$ nylon syringe filter.

2.3.6. Solid phase extraction (SPE) cleanup

SPE clean-up was done using 3 ml, 60 mg hydrophilic-lipophilic balanced copolymer Oasis HLB cartridges (Waters, Milford, MA). Following preconditioning with 5 ml of methanol and 5 ml deionized water, the cartridges were loaded with the supernatant and washed with 6 ml deionized water, which was discarded. Then the cartridges were dried thoroughly by vacuum and eluted with 1.0 ml of methanol into a small test tube, taking the SPE cartridge to dryness. It was not necessary to concentrate the eluate prior to TRL measurement.

2.3.7. Time-resolved luminescence measurements

In the small test tube, TRL reagents were added in the following sequence with vortex mixing following each addition: $1620\text{ }\mu\text{l}$ of 0.15 M tris(hydroxymethyl)aminomethane (TRIS), pH 9.0, $120\text{ }\mu\text{l}$ of $1 \times 10^{-5}\text{ M}$ $\text{Eu}(\text{NO}_3)_3$, $120\text{ }\mu\text{l}$ of $1 \times 10^{-5}\text{ M}$ Na_2EDTA and $40\text{ }\mu\text{l}$ of 25 wt.% cetyltrimethylammonium chloride. The resultant mixture was stored in a dark cabinet for 10 min, and then transferred to a 3 ml quartz cuvette for TRL measurement at room temperature. The excitation and emission wavelengths were set at 388 and 615 nm, respectively. Both slits were set at 20 nm. For each measurement, data were collected and averaged over 10 flash cycles to improve signal-to-noise ratio. The resulting signals

were integrated over a 20–800 μs time interval. Calibration curves were obtained daily. Recovery data were calculated based on the calibration curve obtained from control and matrix-matched samples at five levels over the 0–300 ng g^{-1} range. To evaluate the working range of the method, a calibration curve was obtained from control and fortified catfish over the 0–2000 ng g^{-1} range. In incurred fish analysis control catfish provided by the Harry K. Dupree Stuttgart Aquaculture Research Center was used to prepare the calibration curve. In all other studies store-bought catfish was used.

3. Results and discussion

3.1. OTC extraction and cleanup

Animal tissues are complex matrices. OTC chelates multivalent cations and forms conjugates with proteins in the matrices. Therefore, isolation of OTC from fish muscle deserves special attention [18]. Generally, extraction methods for OTC involve aqueous solutions that contain both deproteinization and chelating agents. Approaches to remove protein include heating, ultrafiltration, ionic strength, pH conditions such as HCl [18] or trichloroacetic acid (TCA) [19], and organic precipitation agents like acetonitrile [20]. Some of these methodologies were evaluated with TCs and were found to provide poor recovery [20]. Another approach is on-line dialysis, which has been employed to separate OTC from bio-macromolecules in salmon muscle [7]. EDTA-McIlvaine buffer extraction and C18 SPE clean up were devised for TC residues in animal liver [21] and were later applied to many matrices including fish tissue [6]. In this buffer medium, the acidic pH denatures proteins, and both EDTA and citric acid compete with TCs to chelate matrix cations, thereby releasing TCs to the solution. Phosphate in this buffer must be removed by SPE because it forms a precipitate with Eu(III) , the sensitizing agent in TRL.

Clean up of TCs can be achieved by liquid-liquid partitioning that extracts TCs into an organic phase such as ethyl acetate. Unfortunately, among members in the TC class this process is especially inefficient for OTC [22]. In comparison, SPE clean up performed better in removing water-soluble species including phosphate and other coextracted components [21]. For the relatively polar OTC, the Oasis HLB hydrophilic-lipophilic balanced copolymer SPE cartridge provided much better retention than typical C18 cartridges, resulting in higher recovery and better reproducibility [14]. In addition its improved wetting properties allowed thorough drying of the sorbent before methanol elution. This allowed better control of the eluate volume and composition contributing to overall data reproducibility. In our previous study [14], methanol proved to be a highly efficient eluant, with over 97% TCs eluted in the first ml. The compatibility of methanol with the TRL reagents made it unnecessary to switch back to 100% aqueous solution by evaporation to

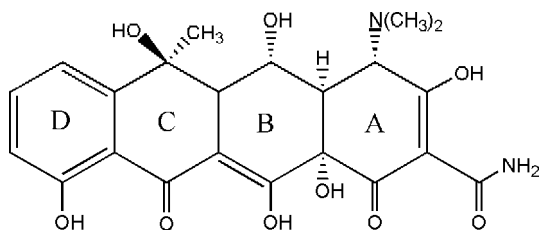


Fig. 1. Molecular structure of OTC.

dryness. By abolishing this time-consuming step, throughput was greatly improved and the potential for OTC loss during evaporation [23] was eliminated. Higher volume of methanolic eluant, however, caused eluate dilution and solubility problems for some TRL reagents and was therefore avoided. OTC can undergo photodecomposition [24], so the eluate should be measured without much time lapse to minimize light exposure.

3.2. Time-resolved luminescence

The optimum conditions for formation of a fluorescent 1:1 β -diketone:Eu(III) complex via the BCD ring hydroxyl groups of OTC (Fig. 1) occurs over the pH range of 7.7–9.7 [25]. Based on the fluorescence characteristics of this complex [26], the TRL method was developed for OTC analysis. The broad OTC excitation peak at 388 nm is typical of molecular absorption (Fig. 2). The excited ligand undergoes intersystem crossing to a triplet state, then transfers the excitation energy to Eu(III). The dominant emission peak at 615 nm (Fig. 2), results from Eu(III) $5d^0$ to $7f^2$ transition [26]. Water molecules are well known to fill the Eu(III) coordination sites and deactivate the excited state causing fluorescence quenching. EDTA was used here as a synergistic agent to shield water molecules from the central ion [12].

Under alkaline micellar conditions intrachelate energy transfer is highly efficient in comparison to intermolecular energy transfer. As a result the system is relatively immune to oxygen quenching, allowing TRL measurement to be performed in the air at room temperature. The results of this process are an order-of-magnitude enhancement in peak intensity [10], large Stokes shift (~ 230 nm), and exceptionally long luminescence lifetime (~ 1 ms) (Fig. 3). Time-delayed signal collection, therefore, can be implemented in addition to spectroscopic resolution to reject short-lived (ns time scale) fluorescence from interfering species. In this work, the delay time was chosen to be $10 \mu\text{s}$. The experimental conditions including pH, Eu(III) and cetyltrimethylammonium chloride (CTACl) concentrations were the same as those that were optimized in our previous studies on chicken muscle [14].

3.3. Analysis of catfish muscle

A calibration curve of OTC in catfish muscle was linear in the fortification range of 0 – 1000 ng g^{-1} ($R^2 = 0.9995$). Beyond 1000 ng g^{-1} , it was found that the TRL response started to level off due to SPE cartridge overload. The working range of this method is therefore limited to 1000 ng g^{-1} . If a greater working range is desired, a SPE cartridge with higher capacity must be used.

The relatively large y-intercept in calibration curves indicated that interfering species were not completely eliminated during sample preparation. This background signal, however, was quite reproducible. The limit of detection (LOD), based on three times the standard deviation of the control signal divided by the slope of the calibration curve, ranged from 3 to 7 ng g^{-1} . Limit of quantitation (LOQ) can be calculated as $3.3 \times \text{LOD}$. As summarized in Table 1, the recoveries were 92 – 112% in the 50 – 200 ng g^{-1} range. Day-to-day

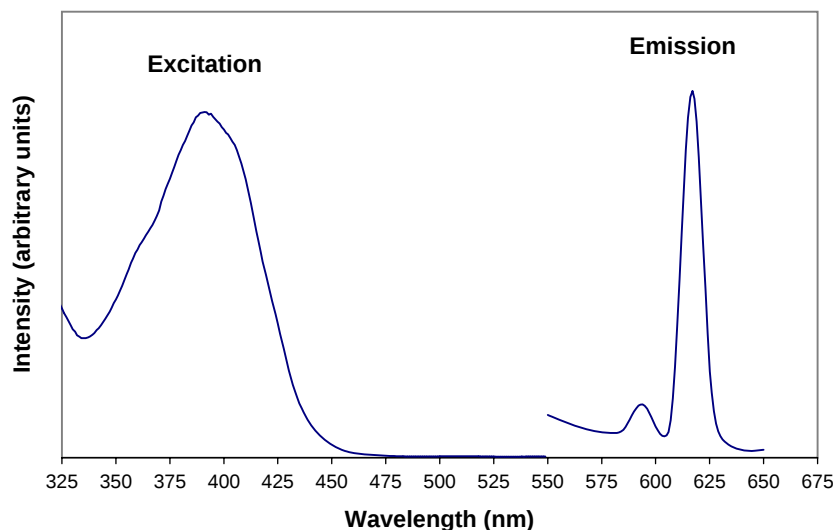


Fig. 2. Excitation and emission spectra of OTC. OTC at $5 \mu\text{g ml}^{-1}$ in matrix-matched medium; $\lambda_{\text{ex}} = 388 \text{ nm}$, $\lambda_{\text{em}} = 615 \text{ nm}$; excitation slit at 10 nm , emission slit at 5 nm .

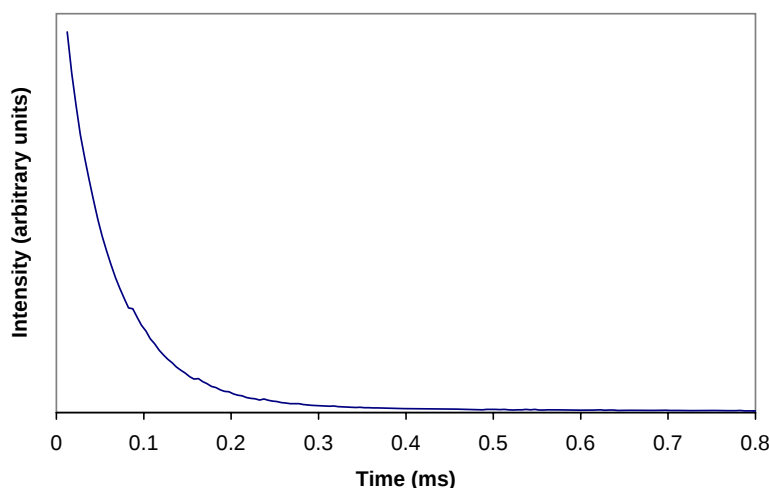


Fig. 3. Time decay curve of OTC in catfish extract. OTC at 200 ng g^{-1} ; other conditions are described in the text.

variation, showing instrument stability and method reproducibility, is 11% at the 100 ng g^{-1} level over the course of 3 days.

In method development, it is very useful to test the method with incurred tissues, if possible, as these samples (from dosed animals) are closer to what would be found in an actual monitoring situation than samples which have only been fortified. Thus, six incurred catfish samples were analyzed using this method. The data shown in Table 2 were based on pooled muscle samples, each from three incurred catfish, to average out differences in fish feeding behavior. The analytical TRL method was found to successfully detect OTC in the incurred samples, at levels ranging from 149 ng g^{-1} (day 3 dosing) to a level that is above LOD but below LOQ (day 14 withdrawal).

Table 1
Recovery of fortified OTC from catfish muscle

Concentration (ng g^{-1})	<i>n</i>	Recovery (%)	R.S.D. (%)
50	5	102	2.7
100 day 1	5	92	13
100 day 2	5	111	15
100 day 3	5	112	12
100 average days 1–3	3	105	11
200	5	101	11

Table 2
OTC from incurred catfish muscle

Day	<i>n</i>	OTC (ng g^{-1})	R.S.D. (%)
Day 3 dosing	4	149	12
Day 7 dosing	5	115	5.3
Day 10 dosing	5	140	4.1
Day 3 withdrawal	5	37	13
Day 7 withdrawal	5	10	4.3
Day 14 withdrawal	5	LOD < result < LOQ	–

4. Conclusions

In this study, we have found the europium-sensitized time-resolved luminescence method to be effective for determination of OTC levels in farm-raised catfish muscle. The method provides excellent recoveries and high sensitivity, with a LOD ranging from 3 to 7 ng g^{-1} . The utility of the method was further shown by its determination of OTC levels in incurred catfish muscle samples. This approach provides a valuable addition to current methods for detection of OTC in fish muscle.

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