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Time-Resolved Luminescence Screening of Antibiotics in Tissue Matrices without Centrifugation and Filtration: Spiked Recovery Studies

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Analyses of chemical residues in animal tissue matrices require multistep sample preparation. To simplify this process, a methodology was developed that combines sorbent extraction and solid-matrix time-resolved luminescence (TRL); it was applied to tetracycline screening in milk. Reported here is an effort to extend its application to tissue matrices, illustrated by oxytetracycline (OTC) screening in catfish muscle. Extraction and enrichment are accomplished by immersing small C18 sorbent strips into tissue homogenates for 20 min, followed by a 3 min rinse in water and a 2 min dip in a reagent solution. After desiccation, TRL is measured directly on the sorbent surface. Tissue particulates no longer interfere via attenuation or scattering, rendering centrifugation and filtration unnecessary. The integrated TRL intensity shows a linear dependence on OTC concentration in the 0−8 μg/g range ($R^2 = 0.9992$) with a 0.026 μg/g limit of detection. To screen OTC at 2 μg/g, the U.S. regulatory tolerance level, a threshold is established at $\bar{x}_2 - 3\sigma_2$, where $\bar{x}_2$ and σ_2 are the mean and standard deviation, respectively, of the TRL signals from 15 samples fortified at 2 μg/g. Among 45 blind samples randomly fortified at 0−4 μg/g, 41 were screened correctly and 4 negative samples were presumed positive. This method has the potential to improve throughput and save assay costs by eliminating acids, organic solvents, centrifugation, and filtration.

KEYWORDS: Time-resolved luminescence; screening; antibiotic; oxytetracycline; sorbent extraction; fish; tissue

INTRODUCTION

Thorough sample preparation, needed for trace residue analysis in foods of animal origin, reduces sample throughput and incurs significant assay costs. To improve efficiency, it can be integrated into the overall analytical scheme, for example, absorptiometry carried out directly on ion-exchange resin (*1*) or C18 surface (*2*) after extraction and enrichment. On the basis of this concept, we developed a methodology that combines sorbent extraction and solid-matrix TRL (SMTRL) using a small-format C18 layer that functions as a sorbent medium and a TRL matrix (*3*), demonstrated by a simple protocol to screen tetracycline in milk (*4*). With minor modifications its application can be extended from liquid to tissue matrices that cover broader food categories at a higher overall volume, demonstrated here by oxytetracycline (OTC) screening in catfish muscle.

OTC (**Figure 1**) is a highly effective member of the tetracycline (TC) class of antimicrobial agents, and one of only three antibiotics (along with Romet 30 and sulfamerazine) currently approved by the U.S. Food and Drug Administration (FDA) for use in channel catfish farming to treat motile *Aeromonas* septicemia, *Pseudomonas* septicemia, and enteric septicemia (*5*). Its extensive use may lead to residues in fish tissues and, therefore, the concern of increased microbial resistance (*6*). To protect public health, the FDA has set a 2 μg/g OTC tolerance in fish muscle and a 21-day withdrawal period. Effective assay methods to monitor and regulate OTC usage are therefore in demand by the rapid growth of fish farming and global trade.

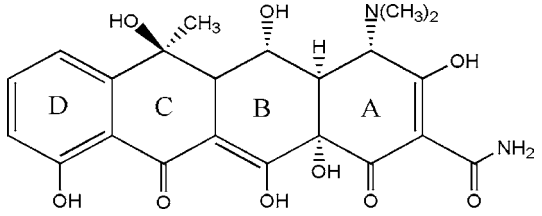

Figure 1. Molecular structure of oxytetracycline.

The FDA regulatory method for OTC in fish tissue is a microbial growth inhibition assay (*7*); its usage, however, is limited by its semiquantitative and nonspecific nature, as well as the required long incubation (16−18 h). Microbial receptor

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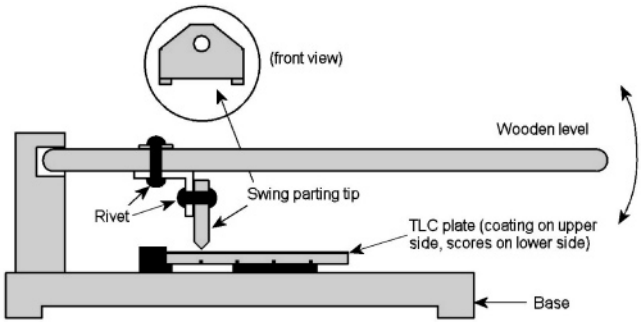

Figure 2. Sorbent strip parting tool.

assay (Charm II), with its improved sensitivity, selectivity, and throughput, has gained popularity in screening (*8*). The assay kit, however, requires radioisotopes and freezing temperature for transportation and storage. For quantitative OTC in fish assay the most widely used technique is high-performance liquid chromatography (HPLC) with UV (*9*), fluorescence (*10*), or mass spectrometric (*11*) detection. Alternative methods are capillary electrophoresis (*12*) and bioluminescence (*13*). A time-resolved luminescence (TRL) method, based on highly efficient energy transfer within Eu−TC complexes, was developed for OTC determination in catfish muscle (*14*). Higher selectivity and sensitivity are possible by implementing both spectroscopic and temporal resolutions. Multistep sample separation, nevertheless, is required, including homogenization, centrifugation, filtration, and solid-phase extraction (SPE) cleanup.

Besides quantitative assay, another strategic approach is to screen a large pool to distinguish few potentially violative samples and subject them to a quantitative method for confirmation. The sorbent extraction−SMTRL methodology (*4*), being rapid and semiquantitative, was developed to serve this purpose. Unlike conventional liquid-phase TRL, SMTRL is directly performed on a solid surface, rendering a clear sample solution unnecessary. Tissue particulates in the homogenate cause no spectrometric interfere via light attenuation and scattering; consequently, centrifugation and filtration become unnecessary, and sample preparation is greatly simplified.

EXPERIMENTAL PROCEDURES

Reagents and Solutions. All chemicals and solvents are of analytical reagent grade and were purchased from Aldrich (Milwaukee, WI). Deionized water was prepared with a Barnstead E-pure system (Dubuque, IA) and used to prepare all aqueous solutions. The 1 mg/mL OTC stock solution is prepared monthly in methanol and refrigerated at 4 °C; from this, dilution is made daily for lower level fortification. The TRL reagent solution, 0.5 mM $Eu(NO_3)_3$−3.0 mM Na_2EDTA−2.5% (w/v) cetyltrimethylammonium chloride, is prepared daily in a 0.15 M tris(hydroxymethyl)aminomethane buffer at pH 8.5.

C18 Sorbent Strips. The sorbent strips are prepared from glass-backed, C18-impregnated silica plates (Z26547-0) made by Analtech (Newark, DE). First, a 200 × 50 mm TLC plate is placed face down on a clean piece of white copy paper. Its length is scored into eight 25-mm fractions using a diamond glass cutter and its width into five 10-mm fractions. To break strips apart without finger contact, a simple parting tool (**Figure 2**) is used to apply leverage pressure on the opposite side of the scores through two contact points 2 mm apart on a swing tip. Contact damages, limited to the edges of the strip, do not affect the central sorbent layer, which is trimmed to 10 × 6 mm with a trimming tool (**Figure 3**). The platform of the tool is tilted at 30° to improve viewing, on which a strip is secured by a vice. A rectangular aluminum column, vertically adjustable by a pinion-gear mechanism, defines the desired sorbent area and position. At its lowest position a 0.1-mm gap prevents the bottom of the column from contacting the sorbent surface. Guided by the column's 10 × 6 mm footprint, a razor blade is used to trim the sorbent layer to its final dimensions. Gloves should be worn throughout the process; and finished strips are examined visually. Occasionally cracks and edge damages develop due to the brittle nature of the C18 coating. If defects are minor, the strips are still usable as long as the central zone is intact. It was found that to a certain extent area variation does not affect the TRL signal. The finished strips are stored in a desiccator before use.

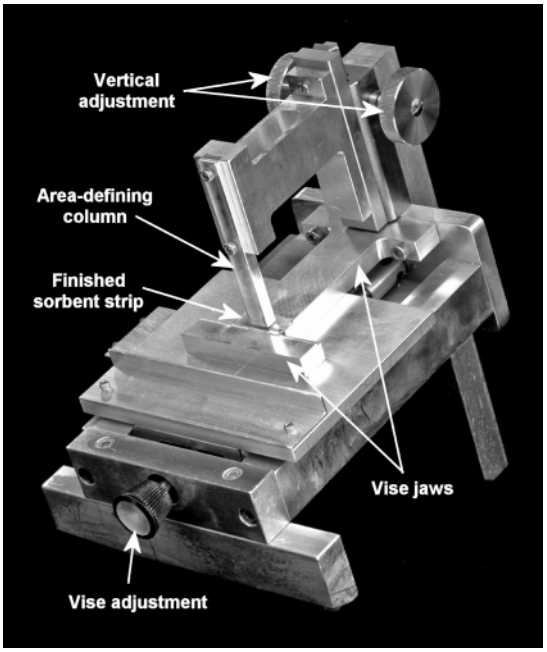

Figure 3. Sorbent strip trimming tool.

Instrumentation. SMTRL is measured with a fluorescence spectrophotometer, model Cary Eclipse, equipped with a pulsed xenon flash lamp (Varian, Walnut Creek, CA). Its operation and signal processing are controlled by Cary Eclipse Lifetime software. The original cuvette holder was converted to a sorbent strip holder as described previously (*4*), on which a 10 × 6 mm window is cut for excitation beam exposure.

OTC Extraction and Cleanup. Skin-free catfish fillets were purchased from a local food store, minced, and stored at −80 °C before use. Thawed samples (3.0 ± 0.02 g) were placed in 50-mL screw-capped polypropylene centrifuge tubes with conical bottoms and fortified to the desired levels by adding the appropriate amount of OTC standard solutions. The samples are then kept in the dark for 10 min. Homogenization is performed with an Ultra-Turrax T-25 homogenizer (Janke and Kunkel, Cincinnati, OH) in 7 mL of 0.01 M Na_2EDTA solution, followed by the addition of 0.5 mL of 20% (w/v) NaCl solution and vortex mixing. Next, five tubes are installed in a foam-plastic holder (**Figure 4**) that comes with centrifuge tubes. To handle multiple strips simultaneously, a five-clip hanger is used. The clips are tied with thin nylon lines to fully submerse the C18 layers. On each clip three strips are installed back to back, with one C18 layer facing one direction and two facing the other. Prior to immersion, the sorbent strips are first sprayed with methanol and next with water while they are still wet. Water is drained and then absorbed by contacting the lower strip edges with a paper towel. The strips are then immersed into tissue homogenates for 20 min, raised, and immediately immersed in a water bath big enough for all five clips. Three minutes later the strips are raised and subjected to a gentle one-path water spray using a squeeze bottle to remove any tissue particulates from C18 surfaces. Caution should be exercised not to damage the C18 layer with too much force. Again, water is drained and absorbed by contacting the lower strip edges with a paper towel.

Time-Resolved Luminescence. Next the strips are immediately dipped into a set of five centrifuge tubes, each filled with 11 mL of reagent solution. Two minutes later the strips are raised; the fluid is drained and absorbed by contacting the lower strip edges with a paper towel. Next the strips are released from the clips and laid orderly on a

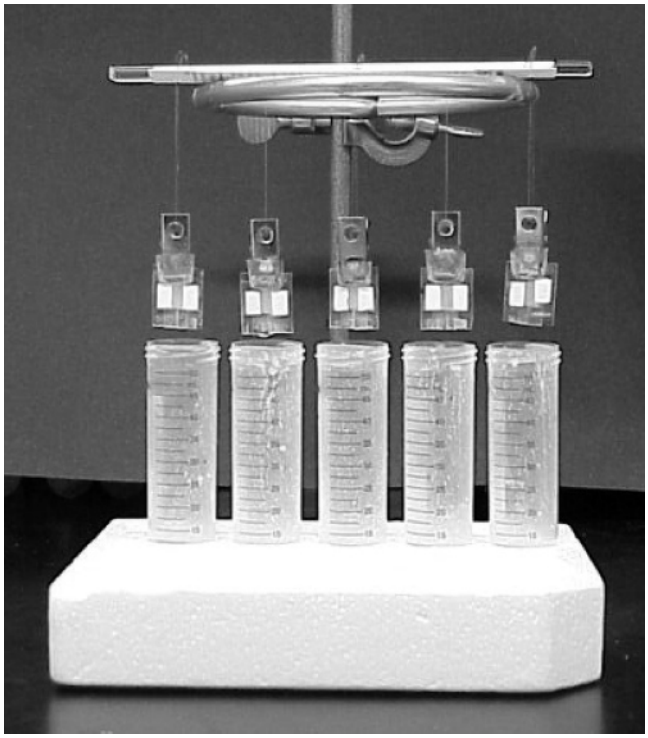

Figure 4. Five-clip sorbent strip hanger.

plastic perforated tray, which is gently moved into a 30 × 30 × 28 cm ($h \times w \times d$) stainless steel desiccator. The glass door and side panels of the desiccator are covered with aluminum foil to minimize OTC photodecomposition by room light (*15*). Desiccation, indicated by transition of sorbent color from dim to bright white, is typically achievable in ~30 min with sufficient desiccant (calcium sulfate). Next, a dry strip is mounted with two document clips on the strip holder to achieve optimal C18 layer-window alignment. The holder is then installed in the sample chamber and TRL measured immediately. To minimize the effect of humidity on the TRL signal, desiccant is placed inside the sample chamber. The excitation and emission wavelengths are set at 388 and 615 nm, respectively, and both excitation and emission slit widths are set at 20 nm. The excitation and emission filters are set at auto and open, respectively. For each sample, three strips are used to obtain triplicate readings. Each strip is measured once with 10-flash averaging to improve the signal-to-noise ratio (S/N). The delay time is set at 10 μs, and the signals are integrated over a 0.1−1.0 ms time interval.

RESULTS AND DISCUSSION

C18 Sorbent Strips. Unlike conventional liquid-phase spectrometry, SMTRL involves uncertainties related to solid surfaces. Commercial C18 plates are fabricated for optimal TLC performance; their chemical compositions and physical parameters such as thickness, particle size, and porosity may vary from manufacturer to manufacturer and from lot to lot. Highly luminescent mineral particles were observed microscopically embedded in the sorbent layer. These plates must be kept in a desiccator to avoid adsorption of airborne contaminants, and gloves must be worn throughout preparation to prevent fingerprints. On each strip, all of the important tasks including extraction and measurement occur on the central 10 × 6 mm area. It was found that finger contact on this zone, even with gloves on, somehow alters surface conditions, contributing to signal fluctuation. This obstacle was overcome by using the parting tool and the trimming tool; the latter also significantly improved trimming speed and area consistency over visual control using graph grids (*4*). In addition, the cost of a C18 strip, estimated at ~$0.20, is only a small fraction of that of a SPE cartridge.

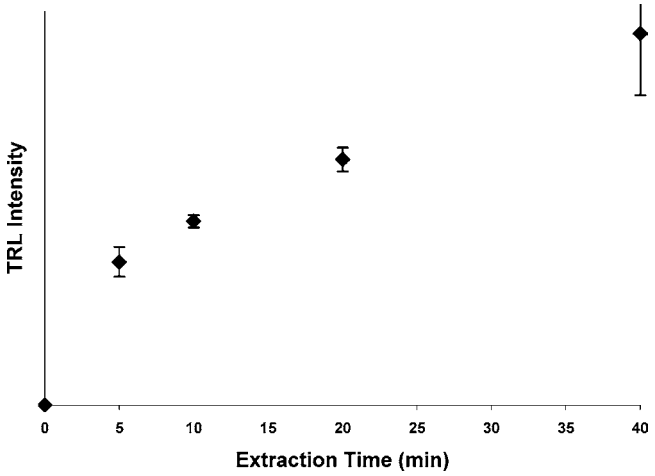

Figure 5. TRL intensity versus extraction time. Triplet data were collected using three sorbent strips and are expressed as mean ± 1 SD.

Extraction of OTC from Fish Muscle. With their multiple proton-donating groups, tetracycline antibiotics (TCs) have strong propensity to chelate multivalent cations in biological matrices. Competing chelating agents, such as EDTA (*16*), citrate (*16*), and succinate (*17*), must be used to free TC from these complexes. A commonly accepted method is to extract TC in EDTA−McIlvaine buffer at a mild acidic pH followed by C18 SPE cleanup (*16*). Recovery is also affected by formation of TC−protein conjugates in biomatrices. To denature proteins, acids are commonly used, such as hydrochloric acid (*18*), trichloroacetic acid (*11*), metaphosphoric acid (*19*), and McIlvaine buffer that contains citric acid (*16*). Higher recovery is generally possible at lower pH due to more complete deproteination; however, TCs decompose to the dehydrogenated form (*20*) at pH <2 and isomerize at pH 2−6 (*21*). Organic solvents, such as alcohol (*22*), methylene chloride (*12*), and acetonitrile (*23*), are often used in combination with acids to better denature protein.

In this study it was found that homogenization and extraction in Na_2EDTA−McIlvaine buffer generate a lower SMTRL signal relative to that from milk (*4*). This is probably caused by phosphate, an ingredient of this buffer, which leaves a residue on the sorbent surface that later forms a precipitate with europium. Extraction with other commonly used acids, unfortunately, also results in lower TRL signals, probably due to OTC isomerization and adverse effects of lower pH on sorbent integrity. Use of organic solvents, such as acetonitrile, methanol, and ethanol, in extraction media increases solvent eluotropic strength and decreases adsorption efficiency, leading to decreased TRL signal. Fortunately, using water as the extraction medium, relatively high extraction efficiency was achieved and improved further by the addition of EDTA and NaCl. EDTA releases OTC from multivalent metals present in muscle tissue, and NaCl increases the ionic strength of the homogenate, which facilitates protein denaturation as well as OTC adsorption on the nonpolar C18 sorbent. The dependence of the TRL signal on extraction time is shown in **Figure 5**: the signal increases more quickly with time at the beginning and gradually slows down. The adsorption does not reach equilibrium even at 40 min, so it is critical to maintain a consistent immersion time. In this study 20 min was set using a timer. During extraction agitation is not implemented due to the concern that dynamic parameters might cause signal fluctuation.

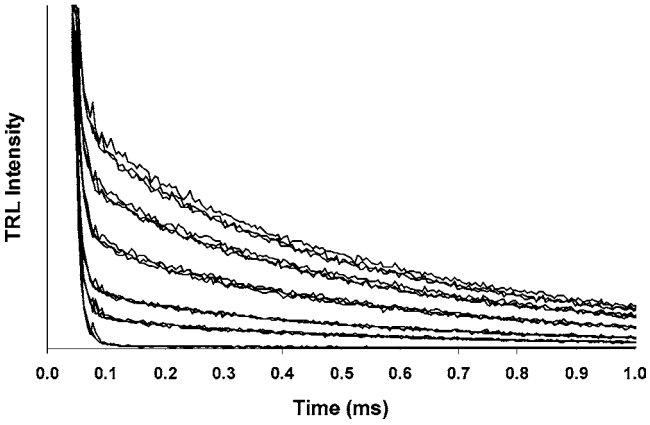

Figure 6. TRL intensity decay with time. Triplicate traces were collected using three sorbent strips for each of six catfish muscle samples fortified with OTC at 0, 1, 2, 4, 6, and 8 μg/g, respectively.

It is essential to validate a method with incurred samples. After gastrointestinal (GI) absorption, vascular distribution, and storage in tissue depots, OTC might behave differently in extraction. In our previous study, however, OTC residue in incurred catfish muscle never reached 0.15 μg/g during or after 10 days of oral medication at therapeutic dose (*14*). A similar observation was made on striped bass: on day 11 of oral therapy while GI and liver OTC concentrations peaked at 407 and 1.97 μg/g, respectively, residue in muscle never reached 0.25 μg/g (*24*). Orally prepared incurred samples are therefore not suited for this screening study at 2 μg/g.

OTC Cleanup. Liquid−liquid partitioning (LLP) is inefficient for tetracyclines and OTC in particular, due to their polar nature, unless ion pairs are formed (*25*). An alternative method, metal chelate affinity chromatography (MCAC), takes advantage of TC's chelating ability to achieve cleanup at the costs of sample throughput and an MCAC column (*26*). The most common method for TC cleanup is C18 SPE carried out in three slow steps: sample loading, washing, and elution (*16*). In fact, SPE is the essence of this method with SPE cartridges replaced by sorbent strips, loading replaced by immersion, and elution obviated by SMTRL performed directly on the sorbent surface. As a result organic solvents are eliminated for extraction and cleanup and so are their harmful effects on human health and the environment. Tissue particulates remain in the homogenate, and the few adhering to the vertical strip surfaces are easily removable by gentle water spray. For SMTRL a clear solution is unnecessary, so are centrifugation and filtration in tissue analysis, saving consumables, equipment, time, and labor. Complete protein denaturation is not achieved in an aqueous EDTA−NaCl solution at mild pH; nevertheless, adsorption of OTC on a hydrophobic surface might favor release of protein to aqueous phase.

SMTRL. The energy transfer within Eu−OTC complex results in an extremely long excited-state lifetime, leading to slow TRL signal decay as shown in **Figure 6** for 0−8 μg/g OTC fortification in catfish muscle**.** As expected, the integrated TRL signal (the area below the curve) correlates well with analyte concentration ($R^2 = 0.9992$). Experiments also reveal its dependence on local surface concentrations of Eu and surfactant, so the amounts and distributions of these two ingredients are controlled reproducibly. On the basis of a typical pore volume for TLC silica sorbent, 0.75 mL/g, and the C18 layer's weight and volume, a 31% porosity is obtained. The void space for a 10 × 6 × 0.22 mm C18 layer is hence 4.1 μL. In our previous work (*4*), 5 μL of reagent solution was spotted on the surface. For such a small volume, significant delivery error is inevitable. If prior to spotting the surface is not wet enough, the spotted solution forms a droplet first due to poor surface wettability. The droplet is gradually absorbed into the adjacent zone, leading to uneven distribution and hence signal fluctuation. Uniform distribution of aqueous solution is possible only on a wet C18 surface that allows free flow. Conversely, if the surface is too wet, the reagent spreads to glass substrate or even runs off the strip during handling, leading to a low reagent concentration and a low TRL signal. To provide a flow barrier on a wet C18 surface, a hydrophobic mark was created prior to spotting with a hydrophobic pen or a wax stamp. The mark effectively retained the spotted reagent solution, prevented runoff, and hence improved signal reproducibility. Unfortunately, during the slow spotting step some C18 layers still became too dry, leading to uneven reagent distribution. A logical solution is to simultaneously dip the wet sorbent strips into the reagent solution for a couple of minutes. This approach dramatically improved signal reproducibility by eliminating variations in delivery volume, surface dryness, and surface concentration. The latter, governed by dynamic adsorption, is no longer a function of sorbent area; consequently, strips with varied degrees of edge damage still yield good results. At 2 μg/g fortification, 93% of 15 samples have RSD < 10% on triplicate readings using three strips. At random fortification in the 0−4 μg/g range, 80% of 45 samples have RSD < 10%. Finally, it is important to maintain strips in a level position during desiccation to minimize the gravity effect.

Another distinct advantage of SMTRL is the minimal water quenching due to the absence of water. Thorough desiccation is generally achievable in 30 min with sufficient desiccant, or longer if a large number of strips are treated simultaneously. Desiccation can be automated, or sped up by ventilation, but elevated temperature should be avoided because it causes OTC loss (*18*). Upon removal from the desiccator, measurement must be done quickly to minimize the effects of room light and humidity. In comparison to TRL measurement in liquid phase, a TLC sorbent layer has a higher background due to surface scattering and impurities, either embedded or adsorbed from the homogenate. Judicious selection of the integration interval, 100−1000 μs in this study, excludes the early signal components from interfering species. The Eu concentration, another important parameter, must be adjusted for optimal signal-to-background ratio. The surfactant is added to improve luminescence quantum efficiency (*27*).

Screening of OTC in Fish Muscle. Although screening is the goal of this study, calibration curves were obtained to evaluate the performance of this method itself. Satisfactory linearity is observed: $R^2 = 0.9992$ in the 0−8.0 μg/g range, and $R^2 = 0.9861$ in the 0−1.0 μg/g range. The blank signal, equivalent to 0.129 μg/g with RSD = 6.9%, results in a 0.026 μg/g limit of detection (LOD).

Prior to screening, a threshold is established from samples of the same matrix spiked at the tolerance level. Data points below the threshold are negative, whereas those above are presumed to be positive, to be later confirmed by a quantitative method. The finality of negative designation requires a minimal false negative rate. Shown in **Figure 7** are the TRL data from 15 blank and 15 fortified (2.0 μg/g) catfish muscle samples and lines at $\bar{x}$ and $\bar{x} \pm 3\sigma$, where $\bar{x}$ and σ are the mean and standard deviation, respectively, of the signals. Statistically for any random population, the odds to lie outside the σ, 2σ, and 3σ limits on each side of the normal curve fall to 15.87, 2.27, and 0.13%, respectively. **Table 1** shows the screening results, based

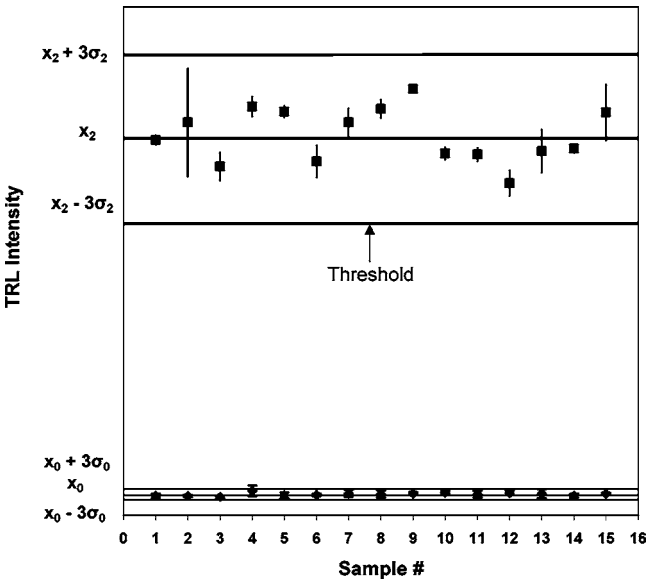

Figure 7. Establishment of a screening threshold. Triplicate data were collected using three sorbent strips and are expressed as mean ± 1 SD.

Table 1. Screening Results of 45 Random Blind Samples

fortification	positives (21)		negatives (24)	
	presumptive positives	false negatives	presumptive positives	negatives
screening results	21	0	4	20

on a threshold at $\bar{x}_2 - 3\sigma_2$, of 42 blind catfish samples randomly fortified at 0−4.0 μg/g plus 3 samples fortified at 0, 2, and 4 μg/g, respectively. All 21 positive samples were correctly designated with a zero negative rate. Among 24 negative samples, 20 were designated negative and 4, fortified at 1.97, 1.78, 1.65, and 1.50 μg/g, respectively, were presumed to be positive. Theoretically, a 0.13% false negative rate is expected with a $\bar{x}_2 - 3\sigma_2$ threshold. In practice, however, it may be slightly higher because $\bar{x}_2$ and σ_2 are derived from a limited number of samples. If so, it can be decreased by pushing the threshold lower at the cost of an increased presumptive positive rate that is manageable in case these two data groups are well separated. Typically, a threshold at $\bar{x}_2 - 3\sigma_2$ is considered to be reliable and practical. It is worth noting that **Table 1** does not correctly represent the reality in which violative samples are generally few; therefore, the false negative rate will be very low, making this screening protocol effective and practical.

On the basis of an average 7.2% RSD from 45 blind samples and a 0.026 μg/g LOD, this method is intended only for screening. To examine its potential as a quantitative method, recovery is calculated for 45 blind samples based on the calibration curves: 24.4% samples are within 95−105% recovery, 55.6% are within 90−110%, 88.9% are within 80−120%, and overall the average recovery is 99.3%. Efforts are underway to improve the sensitivity and reproducibility for quantitative analysis.

Conclusions. This methodology gains full SPE benefits without laborious SPE procedures and cartridge cost. The simple SPE and water-rinsing cleanup are compensated by TRL's high selectivity resulting from donor−acceptor energy level matching and time resolution. With centrifugation eliminated, the method is applicable to field analysis with a portable fluorometer such as the one we developed (*28*). Certain modifications will extend its application to other antibiotic classes with good TRL behavior such as fluoroquinolones (*29*) and other animal tissue matrices. As the first phase of method development, this work measured only spiked samples. Recovery of OTC in incurred samples will be studied next to establish this method in screening practice.

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