ARTICLE

Screening of Danofloxacin Residue in Bovine Tissue by Terbium-Sensitized Luminescence on C18 Sorbent Strips

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ABSTRACT: Danofloxacin (DANO) residue in bovine muscle was screened at 200 ng/g by terbium-sensitized luminescence (TSL) directly measured on 10 × 6 mm C18 sorbent strips. The analyte was first adsorbed on sorbent surface by immersion in defatted homogenates. After reagent application and desiccation, TSL was directly measured on sorbent surfaces at λ_{ex} = 273 nm and λ_{em} = 546 nm. The luminescence intensity was linearly dependent on DANO concentration in the 0−1000 ng/g range (R^2 = 0.9967). A threshold was established at $x_{200} - 3\sigma_{200}$, where x_{200} and σ_{200} are the mean and standard deviation, respectively, of the DANO signals at 200 ng/g. Among 48 blind samples randomly fortified at 0−1000 ng/g, 45 were screened correctly and 3 negative samples were presumed positive. This simple screening protocol has the potential to significantly reduce sample numbers and hence improve sample throughput and save assay costs.

KEYWORDS: Terbium-sensitized luminescence, screening, danofloxacin, C18 sorbent extraction, bovine, muscle

■ INTRODUCTION

Danofloxacin (DANO) is an effective member of the fluoroquinolone (FQ) antibiotic class (Figure 1) specifically synthesized for veterinary use.[1] In the U.S. cattle industry, DANO was approved by the U.S. Food and Drug Administration (FDA) with a 200 ng/g tolerance in liver or muscle. In the European Union (EU), the maximum residue limits (MRL) were set at 200 ng/g in bovine muscle and 400 ng/g in liver or kidney. With increasing concerns on development of antibiotic resistance in pathogens,[2,3] effective monitoring methodologies are essential to ensure residue presence below safe levels, thus protecting consumer health and promoting public confidence and global trade.

So far, prominent quantitative methods for FQ residues in edible animal tissues are high-performance liquid chromatography (HPLC) coupled with ultraviolet,[4] fluorescence,[5] or mass spectrometry (MS)[6,7] detection. With high separation efficiency, capillary electrophoresis (CE) offers an alternative approach. Successful CE applications have been developed using photodiode array,[8] fluorescence,[9] and MS[10] detectors. Quantitation can also be accomplished by spectrometry such as intrinsic fluorescence[11] or terbium-sensitized luminescence.[12]. The latter, based on intrachelate energy transfer from molecular FQ donor to ionic Tb(III) acceptor, is highly specific toward FQ drugs, allowing simplified sample preparation.

Animal muscle and organs are highly complex, so successful detection of trace FQ residues must rely on effective sample preparation. Extraction, cleanup, and enrichment must be performed with minimal analyte loss to ensure data integrity, and matrix components must be thoroughly excluded to protect delicate chromatography columns and detectors such as MS. Unfortunately, sophisticated multistep procedures designed to achieve these goals often become the bottleneck of regulatory
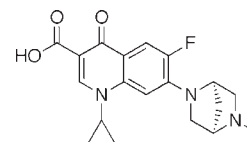

Figure 1. Molecular structure of DANO.

monitoring. To improve sample throughput, an effective strategy is binary screening,[13] which offers the distinct advantage of reducing large numbers of samples to a small fraction known as presumptive positives and then subjects them to a confirmation technique such as HPLC-MS[n]. The practical value of a screening strategy is confirmed statistically by very low numbers of antibiotic residue violations.[14] Thus far, screening methodologies include microbial growth inhibition (GI),[15,16] microbial receptor-assay (Charm II), enzyme-linked immunosorbent assay (ELISA),[17] and steady-state fluorescence.[18]

A unique approach was developed previously in this laboratory that combined sorbent extraction and europium-sensitized luminescence (ESL) and later applied to screening of oxytetracycline (OTC) residue in catfish muscle.[19] The key element of this approach was a small-format (25 × 10 mm) sorbent strip cut from a glass-backed C18 thin-layer chromatography (TLC) plate with the sorbent layer trimmed to 10 × 6 mm in the center. This strip functioned as a reverse phase sorbent to carry out extraction, cleanup, and enrichment. Following reagent application and desiccation, luminescence was directly measured on the sorbent surface. In this work, certain technical and procedural improvements enabled extension of

this approach to screening of DANO residue in bovine muscle at 200 ng/g.

■ EXPERIMENTAL PROCEDURES

Reagents and Solutions. All analytical reagent grade chemicals and solvents were purchased from Aldrich (Milwaukee, WI). DANO stock solution (0.100 mg/mL) was prepared monthly in 0.03 M NaOH solution and stored at 4 °C; dilution was made daily with water. A 0.2 M acetate buffer, with pH adjusted to 7.4 with glacial acetic acid, was prepared within 3 days of use. The reagent solution was 1 mM $Tb(NO_3)_3$ in a 0.2 M sodium acetate solution. Deionized water, prepared with a Barnstead E-pure system (Dubuque, IA), was used to prepare all of the above solutions. As described previously in detail,[19] C18 sorbent strips (25×10 mm) were cut from a glass-backed TLC plate (50031, Analtech, Newark, DE), and the sorbent layer was trimmed with a razor blade to 10×6 mm in the center.

Instrumentation. TSL was measured with a Cary Eclipse fluorescence spectrophotometer (Varian, Walnut Creek, CA) equipped with a pulsed xenon flash lamp. Its operation and signal processing were controlled by Cary Eclipse Lifetime software. Inside the sample compartment, the original cuvette holder was replaced by a strip holder to accommodate sorbent strips, as described previously.[19] The only exception was that the exposure window was expanded to 11×7 mm to avoid contact with the sorbent layer.

DANO Extraction and Cleanup. Beef muscle from food stores or slaughterhouses was minced and stored at −80 °C before use. Thawed samples (2.00 ± 0.03 g) were placed in 50 mL polypropylene centrifuge tubes and fortified at desired levels by adding appropriate amounts of DANO standard solutions. The samples were then vortex mixed for 10 s, allowed to stand in the dark for 30 min, and homogenized in 15 mL of 0.2 M, pH 7.4, acetate buffer using an Ultra-Turrax T-25 homogenizer (Janke and Kunkel, Cincinnati, OH). After centrifugation at 3176g for 10 min, the supernatants were transferred to another 50 mL centrifuge tube. About 10 mL of hexane was added to each tube, followed by 10 s of vortex mixing and 10 min of centrifugation at 3176g. The top hexane layers were discarded, and five tubes were arranged in a row in a foam-plastic tube holder.

Fifteen C18 sorbent strips were securely clipped on a five-clip hanger, three per clip, as described previously.[19] The hanger was first lowered to immerse the strips in aqueous sample solutions for 30 min DANO adsorption, and then raised to allow extra water to drain to the strips' lower edges and absorbed by paper towel.

Terbium-Sensitized Luminescence (TSL). The strips were next dipped into five centrifuge tubes, each containing 20 mL of reagent solution, for 6 min. The hanger was then raised and extra reagent solution was absorbed similarly by paper towel. Released from the clips, the strips were laid orderly on a perforated plastic tray (some pipet tip trays serve this purpose nicely) and desiccated in a vacuum oven (model 5831, National Appliance Co., Portland, OR) for 20 min at room temperature. The sorbent layers turned bright white when desiccation was complete. Next, a strip was mounted using a document clip onto the strip holder, which was then installed inside the sample chamber of the spectrometer. Luminescence was measured immediately at 546 nm under 273 nm excitation. Both excitation and emission slit widths were set at 20 nm, and the excitation and emission filters were set at auto and open, respectively. Each strip was measured only once with 10-flash averaging to improve the signal-to-noise ratio (S/N). For each sample, triplicate readings were acquired on three strips. With delay time set at 10 μs, the signals were integrated over a 0.1−1.5 ms time interval.

■ RESULTS AND DISCUSSION

Extraction of DANO from Beef Muscle. Due to DANO's propensity to bind proteins in biological matrices, deproteinization is typically performed with acids, organic solvents, or their
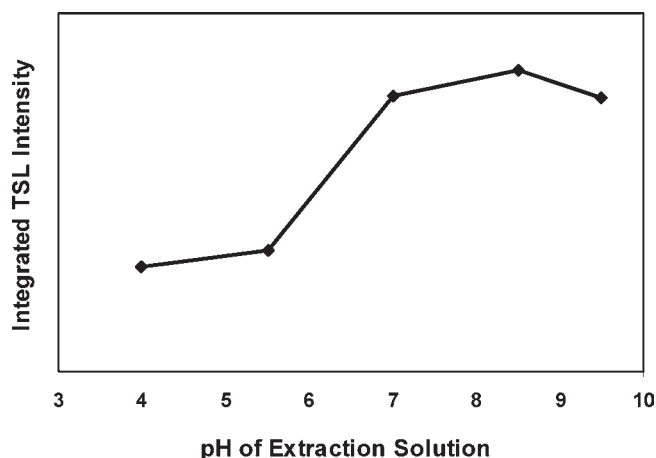

Figure 2. Dependence of integrated TSL intensity on pH of extraction solution.

mixtures. If solid phase extraction (SPE) is part of the cleanup procedure, deproteinization also serves a secondary purpose to lower the sample solution's viscosity, hence facilitating loading on a sorbent bed. So far, many extraction media have proved effective including water,[7] HCl,[20] 0.05 M phosphate buffer at pH 7.4,[21] acetonitrile,[22] dichloromethane,[4] 7:3 (v/v) 0.2% metaphosphoric acid/acetonitrile,[23] and 7:3 (v/v) 5% trichloroacetic acid/acetonitrile.[24] The pH of the chosen extraction medium plays an important role. With $pK_{a1} = 6.03$ and $pK_{a2} = 8.56$, DANO exists as a cation in an acidic pH and as an anion in a basic pH; a net charge promotes dissolution in aqueous media. In neutral pH range, DANO exists as a zwitterion with zero net charge, leading to lower solubility in aqueous medium but higher solubility in organic solvents and better adsorption on reversed phase sorbents. To identify the pH region optimal for extraction and adsorption, a pH dependence study was carried out. On the basis of the results (Figure 2), neutral pH 7.4 was chosen. Organic solvents, such as acetonitrile and alcohols, in extraction media improve solubility and hence extraction efficiency, but adversely affect adsorption of DANO onto C18 sorbent, so they were not used in this extraction protocol. Under such conditions, solubility in aqueous media was not optimal at pH 7.4, but when adsorption took place, the equilibrium would shift to dissolve DANO from muscle matrix. As described below, extraction and adsorption in this simple medium resulted in satisfactory linear response around the FDA tolerance level at 200 ng/g. Besides cost saving, exclusion of organic solvents from extraction medium rendered this method user-friendly and environmentally responsible. Agitation was not implemented during the 30 min immersion due to concerns of possible uncertainties of this dynamic process.

DANO Cleanup. Bovine muscle is a complex biological matrix. To screen DANO residue in such a matrix at 200 ng/g, effective analyte separation and cleanup are essential. Quantitative methods typically rely upon prior extraction and cleanup, and powerful separation by HPLC or CE. In practice, a screening protocol must obviate HPLC or CE to be useful, so analyte cleanup becomes even more critical. Matrix components are typically excluded by liquid−liquid extraction (LLE)[25] or SPE.[4,6,7] Significant fat content in bovine muscle has the potential to alter surface conditions of C18 sorbent and also affect DANO's solubility and adsorption-desorption equilibrium on the C18 sorbent. A hexane-defatting step was thus necessary prior to C18 sorbent immersion. Among various types of SPE sorbents, C18 proved to be effective for DANO cleanup.[6] By replacing a C18 column with a C18 strip, this approach maintained
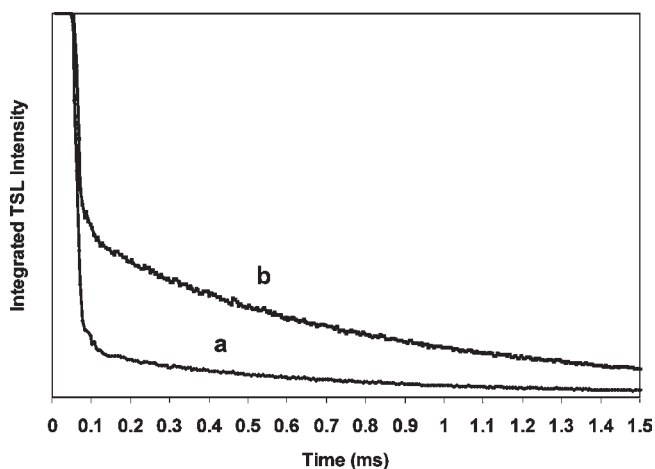
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Figure 3. TSL decay curves of blank bovine muscle before (a) and after DANO fortification at 200 ng/g (b).
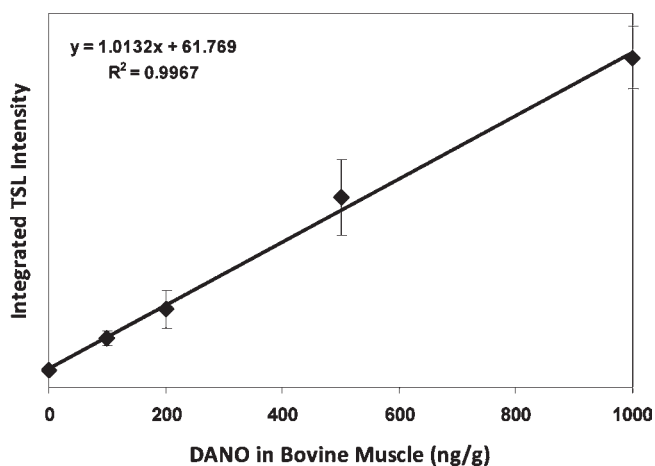

y = 1.0132x + 61.769
$R^2 = 0.9967$

Figure 4. DANO calibration curve with ±1 SD error bars at 0−1000 ng/g fortification in bovine muscle, averaged over five data sets obtained in 5 days.

the benefits of reverse phase SPE cleanup at a fraction of the cost (about $0.20 per strip). In this study, it was also found that data reproducibility was compromised by conditioning. By eliminating preconditioning, rinsing, and elution steps from a typical SPE protocol, and replacing loading by immersion, productivity was significantly improved. In fact, washing was accomplished in a subsequent 6 min reagent immersion, during which the majority of polar matrix components dissolved, including proteins.

TSL. TSL was chosen as a detection scheme for FQ drugs[26] due to its sensitivity and specificity that originate from efficient FQ-to-Tb(III) energy transfer.[27] Two prerequisites for this efficient mechanism are donor-acceptor proximity and matching energy levels. Even though many species chelate Tb(III) and achieve intrachelate proximity, few match Tb(III) in energy levels. Technically, sensitivity and selectivity are further enhanced by time-resolved measurement, which is enabled by extremely long excited-state lifetimes. Shown in Figure 3 are the decay curves of control muscle samples before and after DANO fortification at 200 ng/g.

Even though screening is itself a binary method, its reliability is based on certain concentration dependence, preferably a linear relationship. As shown in Figure 4, this was indeed the case for

DANO ($R^2 = 0.9967$) fortified at 0−1000 ng/g in bovine muscle. If used as a quantitative method, the limit of detection (LOD) would be 82 ng/g, calculated from the background signal plus 3 times the standard deviation and then divided by the slope of calibration curve. Despite a relatively high background from the highly scattering solid surface, background signals from 22 samples of different origins were reproducible within 8.6% relative standard deviation (RSD). In our previous work, the luminescence signal was found to be highly dependent on reagent distribution and degree of desiccation. The former was improved by replacing reagent spotting by dipping; the latter was much improved in this work by a 20 min desiccation in a vacuum oven at room temperature. Thorough desiccation avoids water quenching, rendering synergistic agent, such as EDTA, and surfactant unnecessary. Hence, the reagent solution was finally simplified to 1 mM $Tb(NO_3)_3$ sensitizer in 0.2 M sodium acetate solution. Simplicity not only saved chemical cost but also led to better data reproducibility by eliminating uncertainties. As described below, these modifications enabled DANO screening at the 200 ng/g level, an order of magnitude concentration improvement over our previous work on OTC screening in catfish muscle.

Due to similar molecular structure and spectroscopic behavior, it is expected that all FQ drugs are extracted, enriched, and detected similarly with this protocol. However, minor structural variations among members inevitably alter the efficiency of energy transfer, leading to variations in luminescence quantum yield and hence signal intensity.[27] With postcolumn derivatization, FQ analytes in food matrices can be separated by HPLC and detected by TSL.[28] Without HPLC, enrofloxacin (ENRO), another FQ member also approved in cattle, is a particular concern. Our experimental data ruled out the possibility of screening ENRO using this protocol due in part to ENRO's lower TSL response and in part to its lower residue limits, for example, 100 ng/g in bovine muscle in the EU. Therefore, prior to screening, target identity must be ascertained beyond a well-behaved TRL decay curve.

Screening of DANO in Bovine Muscle. After development of a satisfactory extraction-cleanup protocol and TSL measurement parameters that produce reliable luminescence data, successful screening depends on judicious selection of a threshold. Ideally, background TSL signals from control beef samples should be low and consistent among samples with vastly different origins and tissue compositions. Besides, the data quality should be consistent over a long time span. From 15 control beef samples, the average TSL reading was 59.1 with 13.4% RSD. This background signal had multiple origins. First, unlike liquid phase, a bright-white C18 sorbent surface is expected to generate a much higher background signal due to high reflectivity and scattering. Second, commercial TLC plates are not designed for such an application, but rather for separation of nonpolar compounds based on capillary action. Under excitation, impurities such as small mineral particles embedded in the sorbent layer during manufacturing may generate phosphorescence, contributing to the background. Finally, matrix components adsorbed onto a C18 layer were not completely removed during brief immersion in the reagent solution, especially the nonpolar ones. Elaborate cleanup would come with not only a higher cost but also a tendency to reduce recovery. Fortunately, this relatively simple cleanup protocol resulted in a reasonably reproducible signal, rendering background noise comparatively manageable.

The quality of the overall screening protocol was examined over a 24 day time span based on 22 samples spiked at 200 ng/g. The within-day and between-day variations (Table 1) were statistically similar, and both were reasonably consistent with RSD below 10%. From this set of data, a threshold was

Table 1. Within-Day and Between-Days Variations of TSL Signals at 200 ng/g DANO Fortification

			TSL signal			
			av (arbitrary		RSD	calcd concn
	day	n	units)	SD	(%)	(ng/g)
within-day	1	6	261.1	21.1	8.1	196.7
	3	2	285.0	7.1	2.5	220.3
	4	2	261.9	3.0	1.1	197.6
	22	4	264.7	25.4	9.6	200.3
	23	4	261.0	24.3	9.3	196.7
	24	4	269.3	26.2	9.8	204.8
between-day	1−24	22	265.5	22.9	8.6	201.0
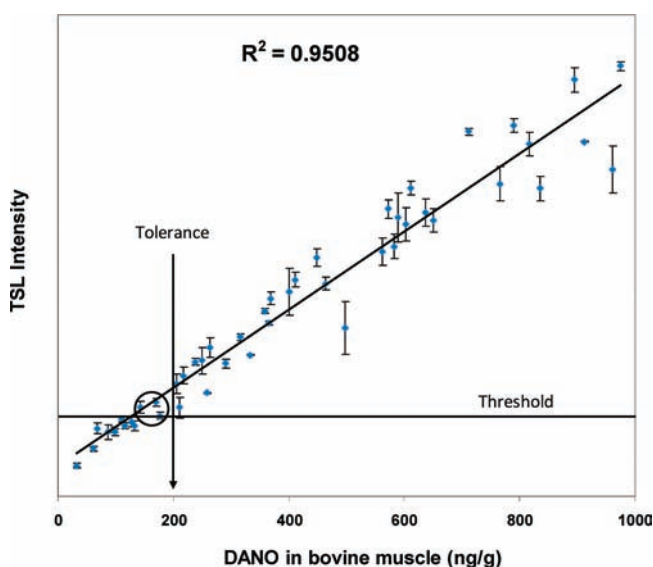
Figure 5. Screening results of 48 blind samples spiked at 0−1000 ng/g. All samples were correctly screened except three circled presumptive positives.

established at $x_{200} - 3\sigma_{200}$, where x_{200} and σ_{200} were the mean and standard deviation, respectively, of integrated TSL signal intensity. Forty-eight blind samples were prepared from 15 different beef samples known to originate from 15 individual animals spiked at random levels between 0 and 1000 ppb. The screening results against this threshold are shown in Figure 5. All 36 positives were identified correctly; 9 of 12 negatives were categorized correctly, but 3 were presumed positives. Due to the finality of a negative verdict, a useful screening program should have a minimal false-negative rate. For any random population, the odds of lying outside the 3σ limits of a normal curve fall to 0.13% on each side. Therefore, a threshold set at $x_{200} - 3\sigma_{200}$ should be statistically robust. In practice, however, both x_{200} and σ_{200} are derived from a limited instead of an infinite number of tests under experimental conditions that varied only over a limited extent. A significant bias therefore may enter threshold calculation, hence compromising screening quality.[29] From a practical standpoint, the protocol must be developed to cover all possible target variations, and the threshold must be established on a large enough test over a reasonably long time span under conditions similar to that for screening.

Besides cost saving and improved throughput, this simple screening method has the potential to reduce sample numbers to a small fraction to be confirmed further, thus promoting food safety.

■ ABBREVIATIONS USED

CE, capillary electrophoresis; DANO, danofloxacin; ELISA, enzyme-linked immunosorbent assay; ESL, europium-sensitized luminescence; EU, European Union; FDA, U.S. Food and Drug Administration; FQ, fluoroquinolone; GI, growth inhibition; HPLC, high-performance liquid chromatography; LLE, liquid−liquid extraction; LOD, limit of detection; MRL, maximum residue limit; MS, mass spectrometry; RSD, relative standard deviation; S/N, signal-to-noise ratio; SPE, solid phase extraction; TLC, thin-layer chromatography; TSL, terbium-sensitized luminescence.

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■ DISCLOSURE

Mention of trade names or commercial products in this paper is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

■ ACKNOWLEDGMENT

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