Luminescence Screening of Enrofloxacin and Ciprofloxacin Residues in Swine Liver after Dispersive Liquid–Liquid Microextraction Cleanup

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ABSTRACT: A luminescence method was developed to screen residues of enrofloxacin (ENRO) and its metabolite, ciprofloxacin (CIPRO), in swine liver. Target analytes were extracted in acetonitrile/1.5% trifluoroacetic acid/NaCl, cleaned up by dispersive liquid–liquid microextraction (DLLME), and finally detected by terbium-sensitized luminescence (TSL) using a time-resolved luminescence photometer. CIPRO yielded slightly lower TSL response than ENRO, so a common threshold was derived from CIPRO and applied to both fluoroquinolones. Among 37 samples randomly spiked with CIPRO or ENRO up to 1 μg/g, all 19 samples spiked above the 500 ng/g tolerance were correctly screened as positive with no false negatives, but 3 of 18 samples spiked below 500 ng/g were classified as positive. This method minimized the use of chlorinated solvents and significantly improved sample throughput.

KEYWORDS: enrofloxacin, ciprofloxacin, fluoroquinolone, screening, swine liver, DLLME, terbium-sensitized luminescence

■ INTRODUCTION

Fluoroquinolones (FQs) are highly effective synthetic antibiotics. Their therapeutic actions are based on inhibition of DNA gyrase in Gram-negative species and of topoisomerase IV in Gram-positive species.[1] After merely two decades of extensive usage, however, FQ-resistant Gram-negative pathogens have emerged worldwide.[2] To ensure food safety, both FQ usage and residue limits in foods of animal origin are regulated in most countries. In the United States, the Food and Drug Administration (FDA) set a 500 ng/g tolerance for the sum of enrofloxacin (ENRO) and its metabolite, ciprofloxacin (CIPRO), in swine liver.[3] Effective analytical methodologies are essential to uphold the regulation and protect public health.

In general, analytical methodologies fall into two categories: quantitative versus screening. Quantitative information is crucial in certain fields such as pharmacokinetics and depletion studies. Currently, quantification of FQ residues in animal tissues is dominated by high-performance liquid chromatography (HPLC) with ultraviolet (UV),[4] fluorescence,[5] or mass spectrometric (MS^n)[6,7] detection. Screening, on the other hand, relies upon rapid and cost-effective protocols to identify the presence of target analytes. Simple binary results, negative versus presumptive positive, adequately serve regulatory purposes. Statistically, violative samples are the minority in regulatory practice. By reducing a large batch of samples to a small fraction, screening improves productivity and reduces overall assay cost. Presumptive positives are then confirmed by proven quantitative methodologies, in particular, HPLC-MS^n.[8]

Screening methods for FQs in foods of animal origin include microbial growth inhibition,[9,10] microbial receptor assay (Charm II), enzyme-linked immunosorbent assay (ELISA),[11] thin-layer chromatography (TLC),[12] and steady-state fluorescence.[13] Terbium-sensitized luminescence (TSL) was applied to screening FQ residues in certain food matrices such as muscle and milk. Although chromatography was eliminated, cleanup still relied upon solid phase extraction (SPE) in cartridge[14] or strip[15] format. Inevitably, slow SPE steps including loading, washing, elution, and immersion became the bottlenecks of productivity.

A novel approach known as dispersive liquid–liquid microextraction (DLLME) was developed in 2006 to fulfill both cleanup and enrichment using only a minute amount of chlorinated solvent.[16] Since then, it has been applied to FQ drugs prior to HPLC determination in swine muscle,[17] pharmaceutical wastewater,[18] and chicken liver.[19] Rapidity and low cost make DLLME an attractive approach in screening. In this work, a simple DLLME-TSL protocol was developed for the first time to target FQ residues. As model analytes, ENRO and CIPRO in swine liver were screened at 500 ng/g tolerance level.

■ EXPERIMENTAL PROCEDURES

Reagents and Solutions. All analytical reagent grade chemicals and solvents were purchased from Sigma-Aldrich (Milwaukee, WI, USA) except ENRO, which was provided by Bayer, and CIPRO, provided by U.S. Pharmacopeia (Rockville, MD, USA). Both FQ stock solutions (100 μg/mL) were prepared monthly in 0.03 M NaOH solution and refrigerated at 4 °C; 10-fold dilution was made weekly with water. The extraction solutions included acetonitrile, 1.5% (w/v) trifluoroacetic acid (TFA), and saturated NaCl solution. Supernatants were neutralized with 1.00 M NaOH. Trichloromethane was used as extractant in DLLME. The TSL reagents included 10 mM $Tb(NO_3)_3$

in methanol and a 1 M acetate buffer at pH 5.5 that was prepared monthly. Deionized (DI) water, prepared with a Barnstead E-pure system (Dubuque, IA, USA), was used throughout this work.

FQ Extraction. Swine liver, purchased from local food stores, was pureed in a food processor (RSI2y-1, Robot Coupe, Ridgeland, MS, USA) and immediately stored at −80 °C in small plastic bags. Partially thawed samples (2.0 ± 0.03 g) were weighed into 50 mL polypropylene centrifuge tubes, fortified to the desired levels with 10 μg/g ENRO or CIPRO standards, and allowed to stand in the dark for 15 min. After the addition of extraction solutions including 2.8 mL of acetonitrile, 1.2 mL of 1.5% (w/v) TFA, and 72 μL of saturated NaCl solution, the tubes were vortex mixed for 30 s and centrifuged at 3500 RPM for 10 min at room temperature. The supernatants were decanted into amber vials and allowed to age at room temperature for 24 h before cleanup.

DLLME Cleanup. After aging, 3.0 mL of supernatant was pipetted into a 4 mL glass vial and neutralized by the addition of 22 μL of 1.00 M NaOH. Then, 450 μL of $CHCl_3$ was added, and the vials were capped tightly. Next, 11 mL of DI water was transferred using a bottle-top dispenser to 15 mL polypropylene centrifuge tubes with conical bottoms. The vial was reversed four or five times by hand to mix the contents immediately before withdrawal into a 10 mL glass syringe with a 1.5 in. 22G blunt-tipped needle. Then, the contents were injected rapidly into water with the needle tip below water surface. The resulting cloudy solutions were centrifuged at 4150 rpm for 10 min. The lower $CHCl_3$ phase, laden with FQ analytes, was used in luminescence detection after the aqueous phase was discarded under vacuum.

Instrumentation. The luminescence photometer used in this work is published elsewhere,[20] so only a brief description is presented here. Among multiple excitation sources, a 2 W miniature xenon flask lamp RSL3100-10 (Perkin-Elmer, Santa Clara, CA, USA) was used as flexible source for general fluorophores. For FQ detection, excitation wavelength was selected using a glass filter (ZWB3, Optima, Elk Glove Village, IL, USA) and a 280 nm interference bandpass filter 280FS25-25 (Andover, Salem, NH, USA) with 25 nm full width at half-maximum (fwhm). The pulsed excitation beam, roughly 1 μs fwhm in duration, was loosely focused by a quartz planoconvex lens (d = 12 mm, fl = 20 mm) on a 10 × 10 mm quartz cuvette. The luminescence signal beam, collected at 90° using two glass convex lenses (d = 25 mm, fl = 25 mm), was projected through a 547 nm interference filter 547BP20 (Omega Optical, Brattleboro, VT, USA) with a 20 nm fwhm onto the cathode of a photomultiplier tube (PMT) module H10304-02 (Hamamatsu, Bridgewater, NJ, USA). Fluctuation of flash energy was compensated by monitoring the beam using a silicon carbide (SiC) UV photodiode SG01L-5 (Boston Electronics, Brookline, MA, USA). The instrument operation and data processing were controlled by an IBM ThinkPad T30 laptop computer running a custom LabVIEW (National Instruments, Austin, TX, USA) program. A data acquisition card 6062E (National Instruments), plugged into T30's PCMCIA slot, provided the needed hardware functions. Several techniques were implemented in this spectrometer to enhance TSL performance over desktop fluorescence spectrometer, as discussed below.

TSL Measurement. TSL reagents were added to 5 mL volumetric flasks including 20 μL of 10 mM $Tb(NO_3)_3$ in methanol and 100 μL of 1 M sodium acetate buffer at pH 5.5. The flasks were filled with isopropanol to 1 cm below the mark using a disposable transfer pipet. Next, the $CHCl_3$ bottom layer was transferred to flasks using a 1 mL syringe and a 1.5 in. 20G blunt-tipped needle. The syringe barrel was rinsed three times with liquid in the flasks. Finally, the flasks were filled to the mark with isopropanol. TSL was measured at λ_{ex} = 280 nm and λ_{em} = 547 nm. Triplicate readings were acquired, each on a roughly 1.5 mL fresh aliquot. Delay time was set at 60 μs; the resulting TSL signals were integrated over a 60−3500 μs interval. Averaging was performed over 20 flashes to improve the signal-to-noise ratio (S/N).

RESULTS AND DISCUSSION

Extraction. To overcome FQ's propensity to bind proteins, acids and organic solvents are used to precipitate proteins and to release FQ drugs. Many extraction media proved effective, as extensively reviewed by Hernández-Arteseros et al.,[21] such as HCl, 0.05 M phosphate buffer at pH 7.4, acetonitrile, dichloromethane, 0.2% metaphosphoric acid/acetonitrile, and 5% trichloroacetic acid (TCA)/acetonitrile. Acetonitrile proved effective for FQ extraction; it also functioned as a dispersant in the subsequent DLLME. Therefore, it constituted the majority (70% v/v) of the extraction medium. Several acids were tested for maximal analyte extraction while keeping coextracted interference manageable: hydrochloric acid, phosphoric acid, perchloric acid, TCA, and TFA. Experimental data indicated that 1.5% (w/v) TFA outperformed the others for both FQs. At higher concentrations, protein precipitated rapidly and thoroughly, leading to a clear supernatant. Unfortunately, analyte loss was also observed, especially for ENRO, likely due to entrapment by a dense protein pellet. A possible solution is to add extraction medium dropwise under constant agitation at the cost of productivity. Fortunately, it was found that both FQs could be extracted reasonably well by reducing the TFA concentration to 1.5%. NaCl was added to promote FQ partitioning into the acetonitrile phase by the salting-out effect.

Optimization of DLLME Cleanup. *(1) Acetonitrile/ Aqueous Phase Ratio.* The dependence of TSL intensity on the acetonitrile-to-aqueous phase ratio was studied while the TFA amount was maintained constant. A 7:3 ratio resulted in optimal TSL signal and hence was assumed in this work.

(2) pH Adjustment. FQs exist as cations in acidic pH, as zwitterions in neutral pH, or as anions in basic pH. Among these entities, zwitterions possess zero net change and therefore partition better in nonpolar solvents. This was fulfilled by neutralizing the remaining TFA in supernatants with 1.00 M NaOH to approximately pH 7 (Figure 1).
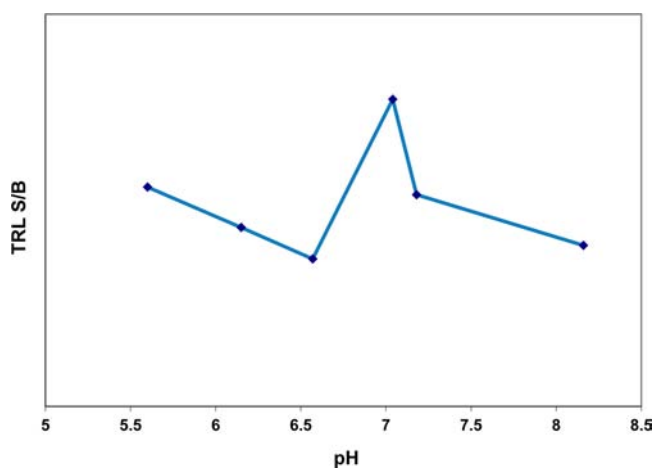

Figure 1. Dependence of TSL signal-to-background ratio (S/B) on supernatant pH (swine liver spiked with CIPRO at 500 ng/g).

(3) Injection Conditions. The goal of the DLLME is to promote phase contact by creation of an emulsion with fine droplets that collectively possess a large surface area. Injection is a simple and convenient approach to fulfill such a goal. In this highly dynamic process, the gauge of the syringe needle played an important role. A smaller needle with a larger gauge number produced a finer jet stream that interacted more efficiently with

the water phase, but it was also vulnerable to clogging by residual tissue particulates or protein precipitates. Extraction efficiency also depended on the injection flow rate leading to a certain degree of signal variation. Reproducible injection conditions are therefore highly recommended to make variation manageable. The needle finally chosen in this work was a blunt-tipped 22G stainless needle.

(4) Supernatant Aging. It was found that after the supernatant was separated from the protein pellet, a large portion of FQ analytes was still bound to soluble proteins. Protein denaturation was considerably slower under mild acidic conditions. As shown in Figure 2, free FQs were gradually
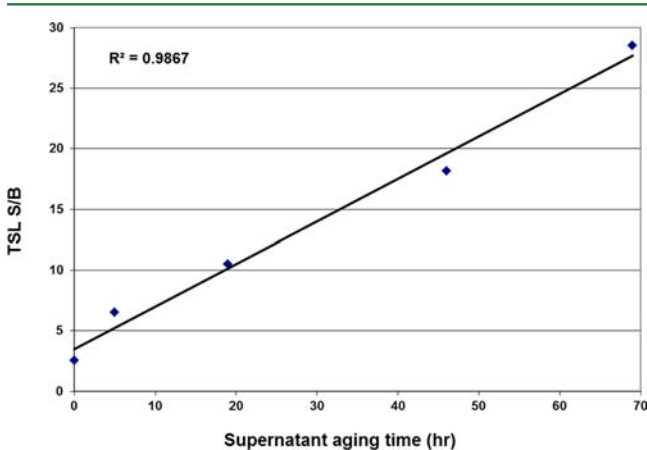

Figure 2. Dependence of TSL S/B on supernatant aging time at room temperature (swine liver spiked with CIPRO at 500 ng/g).

released. Various measures were implemented to speed this process. Higher (2.5, 5, and 10%) TFA concentrations, unfortunately, led to considerable degrees of analyte entrapment. A water bath at 50−70 °C sped the process, but the needed thermostatic water bath and extra human attention would translate into higher assay cost. Furthermore, the higher the temperature, the worse the FQ degradation. In fact, degradation was observed within merely 2 h. Fortunately, 24 ± 1 h of aging at room temperature provided a convenient and labor-free approach. As seen from the slope of Figure 2, aging consistency is crucial for the integrity of within-run and between-run data. Prolonged aging beyond 24 h was considered to be unjustifiable from the standpoint to balance signal and throughput. In comparison, aging at 4 °C took much longer to achieve the same effect.

(5) Selection of DLLME Extractants. Four chlorinated hydrocarbons, $CHCl_3$, CH_2Cl_2, CCl_4, and $C_2H_4Cl_2$, were compared as DLLME extractants on the basis of extraction efficiency. $CHCl_3$ was finally selected, and its amount was further optimized. Considerable solubility in aqueous phase accounts in part for the inferior performance of CH_2Cl_2 and $C_2H_4Cl_2$. On the other hand, CCl_4 was excluded due to its poor extraction efficiency and its worse role (class I) in damaging the ozone layer than its hydrohalogenated counterparts (class II).

TSL. All FQ drugs reveal similar TSL behaviors except decay rates and intensities. The differences, nevertheless, are insufficient to distinguish one FQ from the other. If multiple FQs are allowed in a food matrix, quantification is impossible unless prior separation is performed, for example, by HPLC.[22] However, the presence of any FQ beyond its tolerance is detectable due to TSL's sensitivity and class specificity to FQs.

This is the basis of this screening method that obviates separation and thus improves sample throughput. To take advantage of TSL's long excited-state lifetime, measurement was made in time domain, and a 60 μs delay was introduced to effectively reject short-life interferences. In this photometer design, PMT gating was implemented[20] to further reduce background noises and enhance sensitivity. As a result of DLLME cleanup, a heavier-than-water $CHCl_3$ phase was laden with FQ analytes. If followed by reverse-phase HPLC, evaporation of the sedimented phase to dryness must be performed because $CHCl_3$ is not compatible with common mobile phases. In this work, this step was eliminated because TSL can be measured in alcohols miscible with $CHCl_3$. Four alcohols miscible to both water and trichloromethane were compared on the basis of TSL signal intensity: methanol, ethanol, isopropanol, and tertiary butanol. Isopropanol and ethanol outperformed the others; isopropanol was finally selected. At very low water content, quenching by water molecules was not observed, rendering a synergistic agent unnecessary.

FQ Screening in Swine Liver. Ideally, spectrometric screening should be based on the foundation of a linear response. This is indeed the case for both target FQs. In comparison to ENRO, CIPRO reveals more gradual luminescence decay. Despite their different temporal behaviors, the integrated TSL intensities of both drugs reveal linear dependence on concentration below 500 ng/g, as shown in the first half of Figure 3, a calibration curve of CIPRO in swine liver in the 0−1000 ng/g region. The second half (>500 ng/g) of the curve levels off due to filter effects.
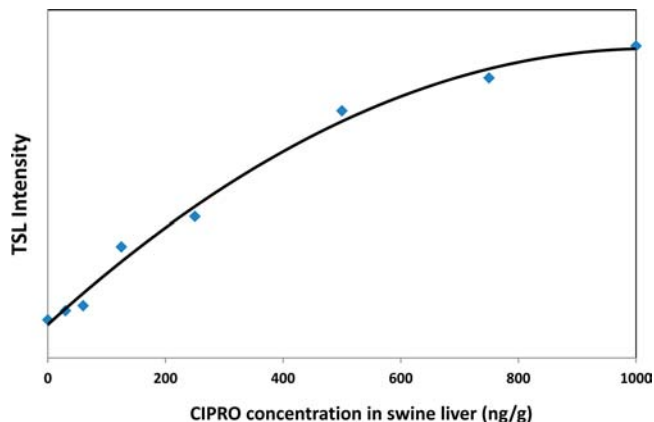

Figure 3. Calibration curve of CIPRO in swine liver at 0−1000 ng/g.

In theory, screening would be straightforward if all target analytes yielded identical responses. In reality, nevertheless, responses among target analytes vary regardless of detection techniques. Between the two target FQs involved in this work, CIPRO's TSL response was slightly lower than that of ENRO. To obtain simple binary results, a single screen threshold was derived from CIPRO and applied to both targets. On the basis of the guideline set in EU Commission Decision 2002/657/EC,[23] to screen a target residue at its maximum residue limit (MRL), the highest response of the blank should not overlap with the lowest response of the target at half of the MRL. Shown in Figure 4 are TSL responses of 20 control chicken samples as well as their spiked counterparts at 250 and 500 ng/g. These data were acquired on swine liver samples purchased from multiple stores over a period of 10 weeks, during which
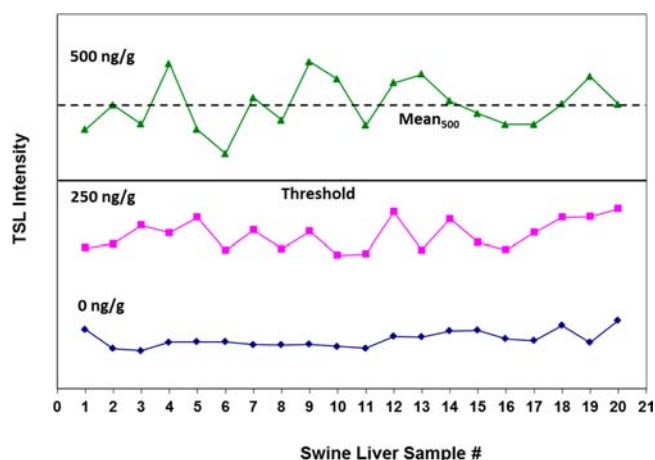
Figure 4. TSL intensity of 20 swine liver samples spiked with CIPRO at 0, 250, and 500 ng/g.

multiple analysts participated and new batches of standards and reagents were used. The relative standard deviations (RSD) of TSL signals at 0, 250, and 500 ng/g fortifications are 16.7, 14.5, and 8.9%, respectively. The threshold, T, was established using the formula

$$T = x_{500} - 3\sigma_{500}$$

where x_{500} and σ_{500} are the mean and standard deviation of 20 spiked samples at 500 ng/g. The statistical basis here is that for a large enough data set, the population outside 3σ falls below 0.13%; the screening quality is hence upheld. On the basis of this threshold, 37 blind samples randomly spiked with either CIPRO or ENRO at 0−1000 ng/g were screened. The results are summarized in Figure 5 and Table 1. Overall, 34 samples
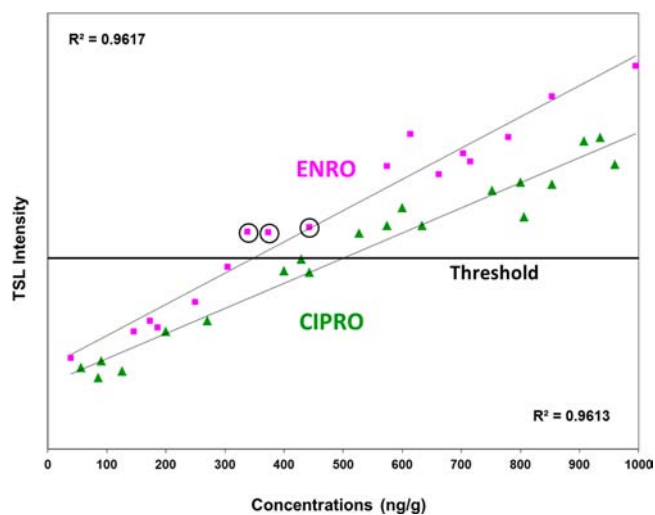

Figure 5. Screening of swine liver samples randomly spiked with CIPRO and ENRO at 0−1000 ng/g. Three false positives are circled.

were screened correctly, but 3 samples fortified with ENRO below the 500 ng/g tolerance (at 338, 373, and 443 ng/g, respectively) were classified as presumptive positives (circled in Figure 5). Because presumptive positives must be confirmed, such errors will eventually be corrected. In contrast, negative results are final, so reliable screening methods should keep the false-negative rate as low as possible.

Table 1. Screening of 37 Blind Swine Liver Samples Randomly Fortified with CIPRO and ENRO at 0−1000 ng/g

	ENRO			
	>500 ng/g fortification ($n = 11$)		<500 ng/g fortification ($n = 6$)	
	presumptive positives	false negatives	presumptive positives	negatives
results	11	0	3	3
	CIPRO			
	>500 ng/g fortification ($n = 11$)		<500 ng/g fortification ($n = 9$)	
	presumptive positives	false negatives	presumptive positives	negatives
results	11	0	0	9

The reproducibility of this screening method is fundamentally limited by the DLLME step. This highly dynamic process led to a relatively large variation, rendering threshold T in Figure 4 significantly lower than x_{500}. Factors involved in the dynamic DLLME process are many, among which droplet size distribution in the resulting emulsion cannot be easily reproduced by manual injection. Although, in general, SPE as a cleanup approach still provides better reproducibility, in this work, cost and speed advantages make DLLME useful for screening. Minimal chlorinated solvent usage and low-budget instrumentation are two other distinct advantages.

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Notes
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.
The authors declare no competing financial interest.

■ **ACKNOWLEDGMENTS**

■ **ABBREVIATIONS USED**

CIPRO, ciprofloxacin; DI, deionized; DLLME, dispersive liquid−liquid microextraction; ELISA, enzyme-linked immunosorbent assay; ENRO, enrofloxacin; EU, European Union; FDA, U.S. Food and Drug Administration; FQ, fluoroquinolone; fwhm, full width at half-maximum; HPLC, high-performance liquid chromatography; LLE, liquid−liquid extraction; MRL, maximum residue limit; MS, mass spectrometry; PMT, photomultiplier tube; RPM, revolution per minute; RSD, relative standard deviation; S/B, signal-to-background ratio; S/N, signal-to-noise ratio; SPE, solid phase extraction; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; TSL, terbium-sensitized luminescence; USDA, U.S. Department of Agriculture; UV, ultraviolet

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