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A Rapid Spectrofluorometric Screening Method for Enrofloxacin in Chicken Muscle

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A simple spectrofluorometric method was developed for screening enrofloxacin (ENRO) in chicken muscle. A single-step extraction with acidic acetonitrile gave the best results without further cleanup. Following centrifugation the supernatants were excited at 324 nm and the emission was measured at 442 nm. Using this procedure, 18 chicken breast samples from 3 producers were tested. The results showed background signal levels significantly lower than those corresponding to 300 μg/kg ENRO, the FDA approved tolerance level. Statistical treatment of these data established a threshold which can be used in subsequent screening of ENRO at the tolerance level. The calibration curve revealed a satisfactory linear relationship ($R^2 = 0.9991$) in a range of 0−700 μg/kg ENRO in fortified chicken breast. ENRO-incurred samples were examined using this approach, and the results agreed with those obtained from more extensive separation followed by high-performance liquid chromatography. Because the threshold can be set at the 3σ limit, reliable screening can be accomplished with an error rate of less than 0.26%. Based on this investigation, a high-throughput screening method for ENRO in chicken tissue is proposed.

KEYWORDS: Fluoroquinolone; enrofloxacin; fluorescence; fluorometry; chicken; muscle; screening

INTRODUCTION

Fluoroquinolones (FQs) are a group of second-generation, broad-spectrum antibiotics. Among them, enrofloxacin (ENRO, **Figure 1**), has shown its high therapeutic efficacy in the treatment of severe systemic infections affecting farm animals, and it is currently the only FQ approved in the United States for use in broiler chickens. Its widespread veterinary use may, however, have contributed to the rapid emergence of drug-resistant organisms (*1*). Increasing concern for this impact on human health warrants development of reliable and sensitive analytical methods for determination of FQ residues in food of animal origin.

Liquid chromatography (LC) combined with one of several detection methods is the most frequently employed technique for multiresidue FQ analysis (*2*−*7*). Alternatively, capillary electrophoresis has been used (*8*). The complex matrix of animal tissue is a challenge to both method specificity and instrument contamination. A multistep cleanup is usually required for isolation of FQs from tissue samples, and the chromatographic separation adds a further time requirement. A sensitive spectrofluorometric method (LOD = 20 μg/kg) which does not require chromatography has been used, but it involves a lengthy series of steps, including a 15-hour extraction with dichloromethane−10% methanol, followed by additional extraction, concentration, and cleanup steps (*9*). Elimination of the need for chromatography and simplification of extraction and cleanup would provide the simple and more rapid methods required by the food industry to screen tissue samples for veterinary drug residue violations.

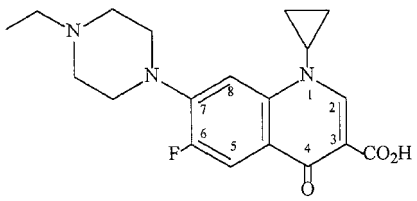

Figure 1. Molecular structure of enrofloxacin.

Here we report a simple spectrofluorometric method based on intrinsic ENRO fluorescence. In this screening method, separation of ENRO from chicken tissue involves a single-step extraction and centrifugation without further cleanup. With separation kept to a minimum, this rapid method provides adequate sensitivity and specificity for screening of ENRO in chicken muscle at the FDA tolerance level. Initially, a threshold level is established by analysis of control chicken extracts and extracts of chickens fortified at the tolerance level. Analysis of subsequent samples and comparison to the established threshold level provide positive or negative results, corresponding to an ENRO level in the samples greater or less than the tolerance (300 μg/kg), respectively. Most samples tested will typically be below tolerance (not violative). Users of this approach would then need only analyze positive samples further by other more elaborate, costly quantitative and confirmatory methods, allow-

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ing a greater number of samples to be investigated more efficiently overall.

MATERIALS AND METHODS

Reagents and Solutions. Enrofloxacin was obtained from Bayer (Kansas City, MO). All other chemicals and solvents were of analytical reagent grade. Deionized water prepared with a Barnstead (Dubuque, IA) E-pure system was used to prepare all aqueous solutions. The ENRO stock solution (100 μg/mL) was prepared in 0.03 M sodium hydroxide. Dilutions for fortification were prepared daily in 0.1 M phosphate, pH 9.

Chicken Tissue. Chicken breast samples were obtained from three different suppliers: Bell & Evans (Fredericksburg, PA), Perdue (Salisbury, MD), and Tyson (Springdale, AR). For each supplier, breasts from six individual chickens were removed of bone, skin, and excess fat, cut into small pieces, and blended with a food processor to a homogeneous consistency. The individual homogenized chicken breast samples were then stored at −20 °C until analysis. ENRO-incurred chicken breast samples were supplied by D. Donoghue as previously described (*7*). Incurred sample 1 represented day 8 (first day post dose), whereas incurred sample 2 represented day 5 of dosing, and was diluted 1:20 with control chicken breast as described previously (*7*).

Fortification and Extraction of Fluoroquinolones from Chicken Muscle. Homogenized chicken breast samples (2.0 g) were placed into 50-mL centrifuge tubes. Appropriate volumes (totaling 100 μL) of ENRO stock solution and 0.1 M phosphate, pH 9, were added to achieve the desired fortification level. After addition of 1% acetic acid in acetonitrile (6 mL), the samples were mixed with an Ultra-Turrax T-25 homogenizer (Janke and Kunkel) and centrifuged (4170×*g*, 5 min). The supernatant was decanted and recentrifuged as before. The resultant supernatant was then transferred to a cuvette for fluorometric analysis. For liquid chromatographic analyses, samples of control and incurred tissue were extracted by the previously developed procedure (*7*).

Spectrofluorometry. Fluorescence spectrometry was performed on a model LS50B luminescence spectrometer (Perkin-Elmer, Norwalk, CT). Approximately 3 mL of supernatant was placed inside a 1 × 1 cm quartz cuvette with four polished sides. Instrument operation and basic spectrum manipulation were performed with FL WinLab software (Perkin-Elmer, Norwalk, CT). The instrument was allowed to stabilize for 1 h before data acquisition. All spectra were recorded at room temperature (~23 °C). Between samples, the cuvettes were washed with methanol, rinsed with deionized water, and dried using a vacuum washer (VWR, Pittsburgh, PA). The excitation and emission spectra were obtained on a randomly selected chicken breast before and after ENRO fortification. For assay measurements, the excitation wavelength was set at 324 nm and the emission intensity was measured at 442 nm. The excitation and emission slit widths were set at 3.0 μm and 7.5 μm, respectively. Stability of the instrument was tested with repeated readings on six samples before and after 100 μg/kg ENRO fortification. The reproducibility was found acceptable with RSDs in the range of 0.61−2.64% for 16 readings over a 2-h period. A calibration curve was obtained by fortification of chicken muscle samples as described in the prior section, at 5 levels from 0 to 700 μg/kg. Fluorescence of these fortified extracts was then measured as described above. Such a calibration curve, generated without subtraction of background, was used to provide an estimate of ENRO concentration in incurred samples.

Liquid Chromatographic Conditions (*7*). An Agilent 1100 LC quaternary pump equipped with an on-line degasser, diode array detector, model 1050 autosampler, and Zorbax Eclipse XDB-phenyl column (3.0 × 150 mm, 3.5 μm) was used for LC separation. The flow rate was 0.5 mL/min with a gradient of solvent A (1% formic acid, pH 3 with ammonium hydroxide) and solvent B (acetonitrile) as follows: 15% B (10 min), 15−20% B (8 min), 20% B (2 min), 20−80% B (2 min), 80% B (2 min), 80−15% B (3 min), 15% B (3 min). The eluate was monitored with a Jasco FP 1520 fluorescence detector via a 35900E interface.

RESULTS AND DISCUSSION

Extraction of ENRO from Chicken Muscle. A goal of this work was to develop a simple and rapid method for extraction of ENRO from chicken muscle. The method would not necessarily achieve a high degree of purification, but should provide a sample suitable for spectroflourometric analysis. FQs have been extracted using organic (acetonitrile, methanol, or dichloromethane) and/or aqueous solvents, in either acidic or basic conditions (*2*−*9*). Three solvents (acetonitrile, methanol, and water) were thus chosen for investigation in this study. In addition, the effect of acid and base in each solvent was tested. In each case, the tissue was extracted with the chosen solvent or with added acid (1% formic acid in solvent) or base (0.5 mL of concentrated ammonium hydroxide in 6 mL of solvent), and centrifuged. The decanted supernatant at this point generally required a second centrifugation to provide a clear solution suitable for spectrofluorometric analysis.

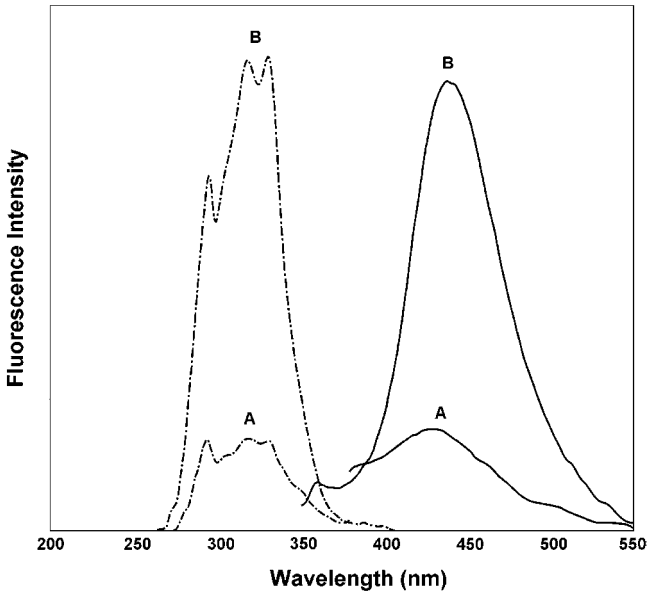

Figure 2. Excitation (dotted curves) and emission spectra (solid curves) of control chicken extract in acidic acetonitrile (A) and chicken extract fortified with ENRO at 1000 μg/kg (B): λ_{ex} = 324 nm, λ_{em} = 442 nm, excitation slit width 3.0 μm, emission slit width 7.5 μm.

Extraction of homogenized chicken breast tissue with acetonitrile and methanol provided useable clear supernatants. However, extraction with water alone, or aqueous acid or aqueous base, gave emulsions which were not readily separable. Basic extraction using concentrated ammonium hydroxide in either acetonitrile or methanol gave supernatants with very high background in control samples, relative to that of fortified samples. Neutral acetonitrile or methanol (no acid or base added) gave useable results, but addition of 1% formic acid to these solvents provided supernatants with greater sensitivity (greater difference between control and fortified samples) due to the enhanced fluorescence quantum yield discussed below. Acetonitrile gave greater sensitivity than methanol, thus acidic acetonitrile was chosen as the preferred extraction medium for this method. No difference was apparent between 1% formic acid/acetonitrile and 1% acetic acid/acetonitrile, so the latter was chosen for analysis of the 18 chicken breast samples. A good ENRO recovery (72%) was obtained using this solvent combination.

Fluorescence Spectra. The excitation and emission spectra shown in **Figure 2** were obtained on a randomly selected chicken breast before and after fortification with 1000 μg/kg ENRO. When monitored at 442 nm, there were two excitation peaks at 290 and 325 nm, the latter peak slightly more intense. When excited at 325 nm, the broad emission band was at 442

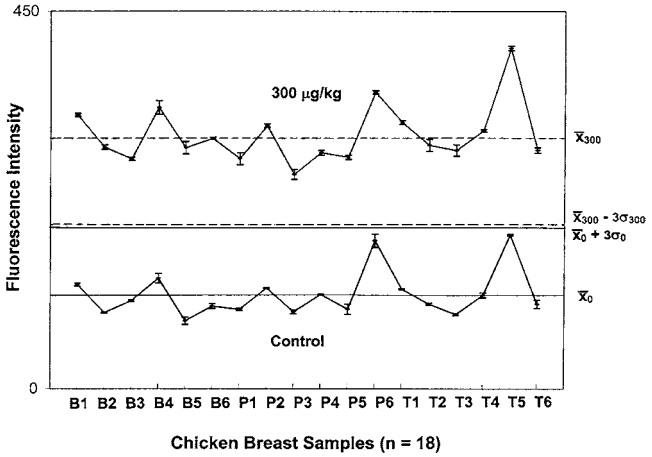

Figure 3. Fluorescence intensity of eighteen chicken breast samples measured before and after ENRO fortification at 300 μg/kg.

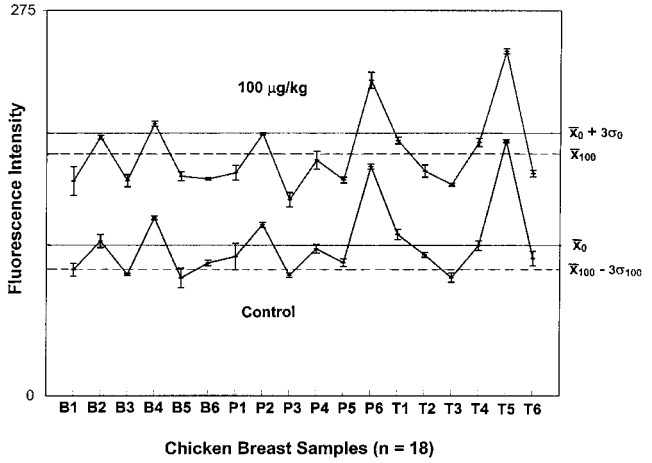

Figure 4. Fluorescence intensity of eighteen chicken breast samples measured before and after ENRO fortification at 100 μg/kg.

nm. In general, features observed here agreed with those observed in the literature (*9*−*12*): red shift of the emission band in acetonitrile solvent and higher quantum yield in acidic medium. The red shift is attributed to reverse solvatochromism which results from interaction between excited-state intermediates and the solvent environment (*10*). Under acidic conditions the protonated nitrogen atom in the C−N bond stabilizes the planar piperazine group (**Figure 1**), contributing both to the red shift and the fluorescence quantum yield enhancement (*11*, *12*). In some cases, the emission intensity doubles when pH changes from 4.8 to 8.0 (*11*). From the analytical point of view, sensitivity is improved with a higher fluorescence quantum yield.

Analysis of Chicken Muscle Data. To test the linearity of response in this assay, a calibration curve was obtained with a randomly selected chicken breast sample fortified with ENRO at 0, 100, 300, 500, and 700 μg/kg levels. The results showed a satisfactory linear relationship ($R^2 = 0.9991$). Subsequent calibration curves were also linear ($R^2 \geq 0.998$) and had consistent slopes. A considerable background fluorescence level exists at 0 μg/kg ENRO.

To establish a threshold level for use of this screening assay, extracts of 18 chicken breast samples were measured before and after ENRO fortification at 300 and 100 μg/kg. The results are shown in **Figures 3** and **4**. Subgroups B, P, and T, 6 data points each, represent samples from 3 producers. Each data point is the mean of 3 replicate samples, with the error bar representing 1 standard deviation (SD) above and below the mean. Two points in the same column are from the same sample.

Table 1. Comparison of Raw and Net Fortification Fluorescence Data

sample ($n = 18$)	mean fluorescence intensity (arbitrary units)	RSD (%)
control	109.1	24.4
100 ppb	172.2	15.8
300 ppb	304.9	11.6
100 ppb – control	64.1	8.5
300 ppb – control	191.3	7.9

For the unfortified samples, the scatter of data indicates the fluctuation of background fluorescence intensity. The major source of this background is expected to be fluorescent compounds that were not completely excluded by the one-step extraction employed in this method. It was observed from **Figures 3** and **4** that 2 out of 18 samples (P6, T5) showed much higher backgrounds, which contributed to the large standard deviations. Repeated experiments on two additional days confirmed that the higher levels of background signal were reproducible ($n = 3$, RSD = 2.74% for P6, RSD = 0.82% for T5). One possibility for the high background level in these two samples was that they contained ENRO or another FQ. More extensive separation was performed on these two samples followed by HPLC with fluorescence detection using a previously developed method (*7*). The resulting chromatogram clearly showed the absence of ENRO as well as the absence of any additional peak in the region in which FQs would typically elute. The additional fluorescence in these samples, thus, may be coming from an early eluting component which overlaps with the normal early eluting background material present in chicken extracts. No further attempts were made to identify these background components.

Potential interference from other drugs administered to chickens is an issue to consider. Many types of drugs are not inherently fluorescent under these conditions, and would not be expected to interfere in the assay. Preliminary investigation of chicken samples fortified with sample drugs at their respective tolerance levels (tetracycline, 2000 μg/kg; tylosin, 200 μg/kg; and nicarbazin, 4000 μg/kg) gave no fluorescence above the control samples in the assay. Other FQs, however, having a similar structure, would be expected to fluoresce under these conditions. Any such signal from FQs other than ENRO would actually be useful, as the presence of these unapproved FQs would then be detected through further quantitative/confirmation analysis. Further studies of the use of this screening method to detect additional FQs are planned.

Compared to the variations observed between individual chickens, noise contributions from day-to-day variations related to operator and instrumentation conditions are relatively minor. This was examined in more detail by statistical analysis of the net fluorescence signals from the added ENRO (fortified level minus control level), represented by the distances between data points in the same columns in **Figures 3** and **4**. Lower RSDs were obtained, as expected, without inclusion of the variability between chickens (**Table 1**). Results from a paired *t* test analysis confirm very consistent nonzero differences ($p < 0.0001$) exist between the control and fortified levels for both the 100 and 300 μg/kg experiments. This is an indication of the reproducibility of the method and instrumentation, taking into account the day-to-day variations.

To evaluate this method for screening at 300 μg/kg (FDA tolerance), a group of four lines was drawn in **Figure 3** to

Table 2. Comparison of Results for Incurred Samples Using This Screening Method and an HPLC–Fluorescence Method (*7*)

	screening				HPLC–fluorescence (*7*)		
incurred samples	±	ENRO concentration (μg/kg)	*n*	RSD (%)	ENRO concentration (μg/kg)	*n*	RSD (%)
1	positive	418	3	2.0	341	3	1.2
2	negative	146	3	2.4	138	3	5.1

indicate signal levels corresponding to the control mean $\bar{x}_o$, fortified mean $\bar{x}_{300}$, $\bar{x}_0 + 3\sigma_0$, and $\bar{x}_{300} - 3\sigma_{300}$ (σ here is the standard deviation for 18 samples, $n = 18$). For a successful screening method, the number of false positive and false negative results should be minimized. The line corresponding to $\bar{x}_0 + 3\sigma_0$ is less than that of $\bar{x}_{300} - 3\sigma_{300,}$ indicating this method would be acceptable for screening at the 300 μg/kg level. For any measurement, the probability for a result to exceed the 3σ limit falls to 0.13% on each side of the curve. Thus, the combined errors from false positives and false negatives can be kept to 0.26%. To minimize the possibility of false negatives, the decision threshold could be set at $\bar{x}_0 + 3\sigma_0$. Thus, a sample would give a positive response in the assay if its fluorescence intensity was greater than the threshold at $\bar{x}_0 + 3\sigma_0$, and a negative response if its fluorescence intensity was less than this threshold.

For evaluation of this method at 100 μg/kg, the corresponding 4 lines are drawn in **Figure 4**, including the control mean $\bar{x}_0$, fortified mean $\bar{x}_{100}$, $\bar{x}_0 + 3\sigma_0$, and $\bar{x}_{100} - 3\sigma_{100}$. This time, the threshold set at $\bar{x}_0 + 3\sigma_0$ is greater than $\bar{x}_{100} - 3\sigma_{100}$, suggesting this method would be unreliable at this level. In fact, at this threshold, 0 false positives and 15 false negatives were obtained from the 18 samples. Interestingly, ANOVA analysis of this data set indicates the differences between the means (fortified and control) of the 18 samples are still significant. Thus, if screening was required at this level, analysis of multiple rather than single samples could still provide usable results. Otherwise, use of the method at this level on individual samples would require decreasing the standard deviation by decreasing variation between samples. This would be accomplished by more extensive cleanup, which necessitates additional extraction and concentration steps. Considering the large number of chickens consumed in the U.S., throughput is of paramount importance for any screening method, thus this work has focused on a method that functions well at the tolerance level (300 μg/kg) as opposed to a more lengthy method for a lower concentration.

In addition, it may still be possible for this method to be effective with individual chicken samples at some point between 100 and 300 μg/kg, providing a less stringent threshold were adopted, such as $\bar{x}_0 + 2\sigma_0$. With this limit, a 4.6% error rate might be expected, which could still be useful depending on the particular application.

A good way to test such a screening method is with actual incurred samples, rather than with only fortified samples. To demonstrate the feasibility of this rapid screening approach, two ENRO-incurred chicken breast samples representing two levels were analyzed. These particular samples had been analyzed previously using a more elaborate quantitative method involving extraction followed by HPLC with fluorescence detection (*7*). From the previous work, sample 1 was known to be >300 μg/kg, and sample 2 was known to be <300 μg/kg (**Table 2**). This screening method gave a positive result for sample 1 (above tolerance), and a negative result for sample 2 (below tolerance), as would be expected from our work with the fortified samples. From a fortified calibration curve run at the same time, an ENRO concentration was calculated for each sample and compared with the previously determined quantitative results (**Table 2**). The results were reasonably similar between the two methods. Although the screening assay cannot strictly be used as a quantitative method due to the potential variability in control samples, it may be possible to at least obtain such an approximate concentration using this approach. The principal use of this method, however, is clearly for screening, where it can provide a valuable aid in the preliminary evaluation of large numbers of samples.

CONCLUSIONS

A simple, rapid spectrofluorometric screening method was described for ENRO in chicken muscle. The only separation involved in this method is a single-step extraction and centrifugation without further cleanup. Reliable screening (3σ limit) can be done for ENRO in chicken muscle at 300 μg/kg, the tolerance set by FDA. The sensitivity and specificity are adequate with minimum sample preparation, and the results are reproducible.

ABBREVIATIONS USED

ENRO, enrofloxacin; FDA, U.S. Food and Drug Administration; FQ, fluoroquinolone; HPLC, high-performance liquid chromatography; LC, liquid chromatography; LOD, limit of detection; RSD, relative standard deviation; SD, standard deviation.

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