Rapid Fluorescence Screening Assay for Enrofloxacin and Tetracyclines in Chicken Muscle

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A simple, rapid fluorescence assay was developed for screening both enrofloxacin (ENRO) and tetracyclines in chicken muscle at the U.S. tolerance levels (300 ng/g and 2 μg/g, respectively). Screening for both classes of antibiotics is accomplished using one extraction, thus simplifying and expediting the process. The method requires an initial extraction of chicken muscle with 1% acetic acid in acetonitrile, centrifugation, and analysis of the supernatant for ENRO fluorescence. After addition of ammonium hydroxide, magnesium chloride, and methanol, followed by centrifugation and filtration, the supernatant can be measured for tetracycline fluorescence. Chlortetracycline (CTC) was chosen as a representative tetracycline to demonstrate the method, as it displays intermediate sensitivity among the three tetracyclines approved in the U.S. Comparison of the fluorescence of control and tolerance-level-fortified samples of both ENRO and CTC shows no overlap. Setting a threshold as the average fortified fluorescence minus 3σ allows for successful screening, as illustrated with blind samples as controls or fortified with ENRO and/or CTC over a range of concentrations. This method can provide an alternative or supplemental approach to currently used microbial screening assays.

KEYWORDS: Fluorescence; screening; enrofloxacin; fluoroquinolone; tetracyclines; chicken muscle

INTRODUCTION

Antibiotics such as the fluoroquinolones and the tetracyclines are used in both medical and veterinary applications. Use of these antibiotics in food animals has generated concern due to reports suggesting this practice may contribute to increased antimicrobial resistance (*1*). Efficient methods are required to monitor the food supply to ensure that any antibiotic residues present are below the set tolerance level, thus promoting food safety and consumer confidence.

At present, enrofloxacin (ENRO) is the only fluoroquinolone antibiotic approved for use in chickens in the United States, with a tolerance level of 300 ng/g. Among the tetracyclines, tetracycline (TC), oxytetracycline (OTC), and chlortetracycline (CTC) are approved for use in chickens, with a tolerance of 2 μg/g. A number of methods currently exist for the determination of ENRO (*2−5*) and TC, OTC, or CTC (*6−8*) in poultry; however, these methods often involve time-consuming approaches or expensive instrumentation. There were 8.5 billion broiler chickens produced in the United States in 2003 (*9*), and a very low number of antibiotic violations is typically found (*10*). Given this situation, it is most efficient to perform a rapid preliminary screen for the presence of antibiotic residues. The few samples that give a positive response would then be analyzed further by a more extensive quantitative and/or confirmatory method. The majority of samples, having given a negative response, would not require any further investigation.

Screening is currently typically performed using microbial assays, such as the STOP, CAST, FAST (*11*), multiplate assays (*12−14*) or the Charm II method (*15*). Screening assays which do not rely on microbial systems or radioisotopes can provide a valuable alternative. We have developed separate rapid fluorescence screening assays for detection of ENRO (*16*) or the tetracyclines (*17*) in chicken muscle. These assays required separate extractions due to the different properties of these two classes of antibiotics. We now report a method which allows for rapid fluorescence screening of both ENRO and the tetracyclines in chicken using only one sample extract. This approach thus further reduces the time required to screen members of these two different classes, and takes advantage of their different fluorescence properties to eliminate possible interferences.

MATERIALS AND METHODS

Reagents and Solutions. ENRO (99.9%) was obtained from Bayer (Kansas City, MO), difloxacin (DIF; 89.0%) was from Abbott (North Chicago, IL), danofloxacin (DANO) was from Pfizer (Groton, CT), and ethopabate was from Fluka (Milwaukee, WI). TC (95%), OTC (95%), CTC (83%), tylosin tartrate (95%), and nicarbazin were obtained from Sigma (St. Louis, MO). Stock solutions (200 μg/ mL) of TC, OTC, and CTC were each prepared in methanol, and stock solutions (100 μg/mL) of ENRO, DANO, and DIF were each prepared in 0.03 M NaOH. Stock solutions were stored at 4 °C and prepared fresh either

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monthly (TC, OTC, CTC) or after six months (ENRO, DANO, DIF). Deionized water prepared with a Barnstead (Dubuque, IA) E-pure system was used to prepare all aqueous solutions.

Chicken Tissue Samples. Six individual chicken breasts were obtained from each of three different sources (Bell and Evans, Fredericksburg, PA; Tyson, Springdale, AR; Perdue, Salisbury, MD). After removal of bone and skin, each breast was cut into small pieces and then ground to homogeneity in a food processor (Robot coupe, Jackson, MS). The tissue was then stored at −80 °C.

Fortification of Chicken Muscle. A 10 μg/mL solution of ENRO, DANO, or DIF was prepared from the corresponding stock solution by dilution in methanol. This dilution (60 μL) was then added to a 2.0 g sample of chicken tissue in a disposable 50 mL centrifuge tube to achieve a fortification level of 300 ng/g. TC, OTC, or CTC stock solution (20 μL) was added to achieve a fortification level of 2 μg/g. Methanol solutions (20 μg/mL) of tylosin or ethopabate were added (20 or 50 μL) to provide fortification at 200 or 500 ng/g, respectively. A dimethylformamide solution of nicarbazin (200 μg/mL, 40 μL) was added to provide fortification at 4 μg/g. In each case, a volume of methanol was also added to the tissue to provide a constant total volume added (100 μL).

Extraction of Fluoroquinolone and Tetracycline Residues from Fortified Chicken Muscle. A solution of 1% acetic acid in acetonitrile (6 mL) was added to each centrifuge tube containing either fortified or control chicken muscle (2.0 g), and the samples were homogenized (Ultra-turrax T25, Janke and Kunkel, Cincinnati, OH). Following centrifugation (3716g, 5 min), the supernatants were decanted into 15 mL disposable centrifuge tubes. The fluorescence of these supernatants was measured to determine the levels of ENRO (or DANO or DIF) present. After fluorescence measurement, the supernatant samples were returned to the centrifuge tubes and concentrated NH$_4$OH (100 μL), methanol (0.75 mL), and 0.1 M MgCl$_2$ (60 μL) were added with mixing. The samples were allowed to sit for 10 min to complete precipitation, and then centrifuged (3716g, 5 min). The supernatants were then syringe filtered (0.2 μm, nylon, 25 mm), and their fluorescence was measured to determine the levels of TC, OTC, or CTC present.

Fluorescence Analysis. Fluorescence measurements were performed with a Cary Eclipse fluorescence spectrometer (Varian, Walnut Creek, CA). Cary Eclipse software controlled the instrument operation and signal processing. The spectrometer was allowed 30 min to warm before use. Samples were placed in 3 mL quartz cuvettes for fluorescence measurement. These cuvettes were washed between samples with deionized water and acetone. Fluoroquinolone fluorescence was monitored with λ_{ex} = 320 nm, slit$_{ex}$ = 5 nm, and λ_{em} = 440 nm, slit$_{em}$ = 20 nm. Tetracycline fluorescence was monitored with λ_{ex} = 375 nm, slit$_{ex}$ = 10 nm, and λ_{em} = 515 nm, slit$_{em}$ = 20 nm.

Preparation of Blind Samples. A protocol was developed in which 20 concentrations of ENRO were randomly selected between 0 and 300 ng/g, and 20 between 300 and 600 ng/g. Similarly, 20 concentrations of CTC were randomly selected between 0 and 2 μg/g, and 20 between 2 and 4 μg/g. In addition, 10 concentrations for ENRO and 10 for CTC were designated as 0 ng/g (controls). Concentrations for ENRO and for CTC were randomly paired, and these combinations were then each randomly paired with one of the eighteen chicken breast samples. On a day of analysis, the analyst prepared 50 mL disposable centrifuge tubes containing 2.0 g samples of the designated chicken breast and another chemist performed the fortifications of these samples using the above protocol. The analyst then proceeded to independently extract and analyze the fortified samples. A total of 12−14 samples could easily be extracted and analyzed per day by an analyst using this method.

RESULTS AND DISCUSSION

The goal of this work was to develop a method enabling a rapid fluorescence screening assay of both ENRO and TC, OTC, or CTC in the same chicken muscle extract. The simplest approach would be to choose conditions for the ENRO assay, and see if the tetracyclines would fluoresce, or vice versa. However, tetracyclines did not display a significant fluorescence

response under the acidic conditions of the ENRO assay. Furthermore, ENRO in the presence of chicken muscle extract did not significantly fluoresce under the ammoniacal acetonitrile conditions of the tetracycline assay.

The next approach was to start with the conditions of one of the two assays, measure fluorescence for that analyte, and then add acid or base to change the pH to enable measurement of the fluorescence of the second analyte. The tetracycline assay was not chosen as the starting point for this approach, as the presence of Mg^{2+}, required for optimum tetracycline fluorescence, was found to decrease the fluorescence of ENRO under acidic conditions. Thus, both ENRO and CTC were extracted from chicken muscle according to the ENRO assay procedure (*16*), and ENRO fluorescence was measured. Concentrated ammonium hydroxide (100 μL) was then added to the sample to change to a pH range of 9−10. The volume of ammonium hydroxide to be added was varied between 90 and 110 μL. The larger volume of ammonium hydroxide provided a greater fluoresence response for CTC; however, the readings were somewhat less stable, with formation of iso-CTC over time, as evidenced by an increase at 420 nm (λ_{ex} = 340 nm). Thus, 100 μL of ammonium hydroxide was chosen as an optimum volume. Water (3 mL) was then added to solubilize the second layer that developed on basification, and then 0.1 M magnesium chloride (60 μL). The volume of magnesium chloride was varied between 30 and 90 μL. The smaller volume gave a less intense fluorescence response, and there was no significant difference observed in resultant fluorescence between 60 and 90 μL; thus, the smaller of the two (60 μL) was chosen. Centrifugation of the resultant precipitate allowed the CTC fluorescence of the supernatant to be measured. Despite the potential demonstrated by this experiment, a major difficulty was that the difference in fluorescence between control extracts and those fortified with 2 μg/g CTC was not as large as desired (2 μg/g fortified sample fluorescence signal approximately 1.5× that of the control), given the potential for variation between chickens.

A reevaluation of the basification procedure led to the use of methanol, rather than water, to solubilize the basified layers. In this way, a smaller volume of methanol could be added (0.75 μL), decreasing the dilution of the resultant solution, and reducing potential quenching of the fluorescence by the added water. Following this revised procedure, the fluorescence of 2 μg/g CTC fortified samples was now approximately 4× that of the control samples, providing an improved differentiation. The volume of methanol to be added in the basification step was varied between 0.5 and 1.0 mL, with no significant difference observed in the resultant fluorescence of the samples.

The linearity of the response of the assay was illustrated with chicken muscle samples fortified with three different fluoroquinolones and three different tetracyclines over a range of concentrations (0−1 and 0−5 μg/g, respectively). Results are shown in **Figure 1a** for ENRO, DANO, and DIF, and in **Figure 1b** for TC, OTC, and CTC. Limits of detection were calculated as 3$\sigma_{control}$/slope for ENRO (155 ng/g), DANO (76.5 ng/g), DIF (226 ng/g), TC (0.150 μg/g), OTC (0.327 μg/g), and CTC (0.250 μg/g).

Recoveries were determined for the above three fluoroquinolones and three tetracyclines by extracting control and chicken muscle extracts fortified at 300 ng/g and 2 μg/g, respectively, and measuring their respective fluorescence responses. Matrix-matched samples were prepared by spiking control chicken muscle extracts at the corresponding levels immediately prior to fluorescence analysis. Recoveries were calculated as follows: (fortified sample − control)/(matrix-

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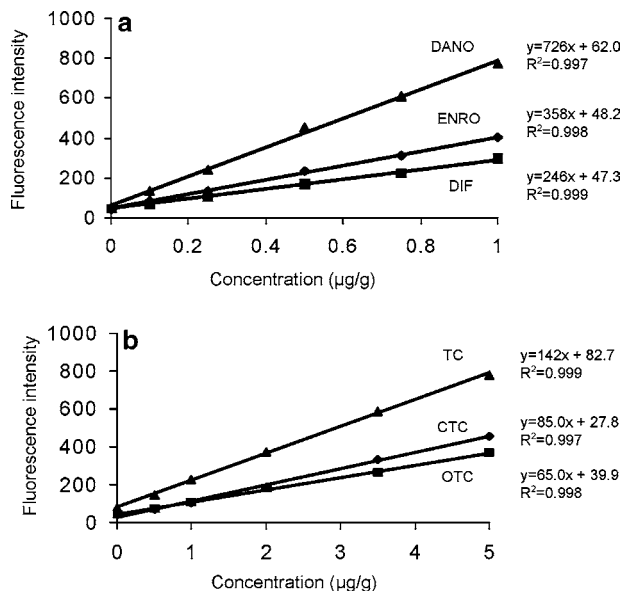
Figure 1. Linear response of fluoroquinolones and tetracyclines in chicken muscle.

Figure 2. Establishing threshold levels for ENRO and CTC.

matched sample − control) × 100%, and the values ranged from 68% to 76% for the three fluoroquinolones, which were comparable to what had been observed previously (*16*). Recoveries for the tetracyclines ranged from 22% to 36%, again comparable to previous observations (*17*).

The next step was to determine if the observed difference between control and fortified chicken muscle samples would be maintained after taking into account variations among chickens and producers. From **Figure 1**, it can be seen that different antibiotics display different sensitivities in the assay. For this study, ENRO was chosen from the fluoroquinolones, as it is the only one approved for use in chickens in the United States. CTC was chosen from the tetracyclines, as it displays intermediate sensitivity among the three approved tetracyclines in the assay. Six individual chicken breasts were obtained from each of three different suppliers. These samples were each analyzed using the assay as control samples and as samples fortified at their respective tolerance levels (300 ng/g for ENRO and 2 μg/g for CTC). The results are shown in parts **a** and **b**, respectively, of **Figure 2**. Each data point in this figure represents the average of three replicate samples. The average within day variation (repeatability) among replicates for control and fortified samples was 1.4% RSD for ENRO and 2.4% RSD for CTC. A measure of day-to-day variation (reproducibility) was obtained by extracting and analyzing control and fortified samples of a standard mix of chicken breast samples each day analyses were performed; variation ranged from 1.5% to 2.8% RSD for the control, and from 1.3 to 4.1% RSD for fortified ENRO and CTC. Analysis of such standard control and fortified mixed samples could provide a correction for instrumental drift over time, although, in this study, such a correction was not needed. In this study, two chicken breast samples (P3 and T5) appeared to have somewhat higher control fluoroquinolone fluorescence levels than the others (**Figure 2a**). Samples of these chicken breasts were extracted and analyzed for fluoroquinolones using liquid chromatography with fluorescence detection (*3*); no fluoroquinolones were found to be present. **Figure 2** includes solid lines indicating average values ($\bar{x}$) for control and fortified samples, as well as dashed lines indicating $\bar{x}_0 + 3$ times the standard deviation ($\bar{x}_0 + 3\sigma_0$) for the control data and $\bar{x}_2 - 3\sigma_2$ (CTC) or $\bar{x}_{300} - 3\sigma_{300}$ (ENRO) for the fortified data. According to normal distribution theory, for both ENRO and
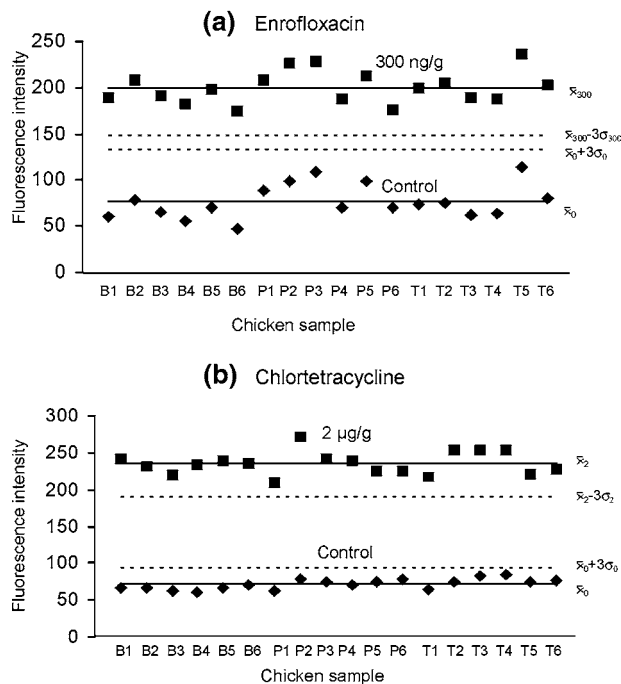
CTC, the area below $\bar{x}_0 + 3\sigma_0$ would represent 99.87% of the control samples, and the area above $\bar{x}_{300} - 3\sigma_{300}$ for ENRO or above $\bar{x}_2 - 3\sigma_2$ for CTC would represent 99.87% of the samples above their respective fortification (tolerance) levels. A clear difference thus exists between control and fortified samples within each class of antibiotics, as illustrated with ENRO and CTC, and as confirmed by ANOVA ($p < 0.01$). These differences, which still occur when different chickens or producer samples are examined, can be used in a screening assay in which samples are determined to be above or below the tolerance level for ENRO and/or CTC.

In such a screening assay, setting of a threshold for decision making is required. If analysis of an unknown sample gives a result higher than the threshold, the sample is considered as a positive (violation), and would then be examined by more extensive analytical methods. If the analysis gives a result lower than the threshold, the sample is determined to be negative (no violation), and no further testing is required. In such a system, it is important to minimize false negative results, as violative samples (those above the tolerance level) could then be missed. To keep such false negatives to a practical minimum, the threshold of this assay could be set at $\bar{x}_{300} - 3\sigma_{300}$ for ENRO and $\bar{x}_2 - 3\sigma_2$ for CTC, thus allowing for detection of 99.87% of violative samples according to normal distribution statistics.

This assay could also be used to screen for the simple presence or absence of ENRO and/or CTC. False positive results would be minimized by setting a threshold at $\bar{x}_0 + 3\sigma_0$ for each antibiotic. Samples giving results higher than the threshold would be considered as positive (antibiotic present), while those providing results below the threshold would be negative (antibiotic absent). False negative results would be possible when concentrations of the antibiotic are below the limit of detection for the assay.

To establish that these thresholds would be effective at identifying violations or the presence/absence of the antibiotic, a series of blind samples were prepared containing ENRO and/ or CTC over a range of concentrations above and below tolerance. These analytes provided a linear reponse in the assay, as shown in **Figure 3**.

Table 1. Results from Blind Samples

analyte	assay purpose	threshold (fluorescence intensity)	no. of false negatives	no. of false positives	no. of samples tested	concn range (μg/g)
ENRO	above/below tolerance	$\bar{x}_{300} - 3\sigma_{300}$ (147)	0	8	50	0–1
ENRO	present/absent	$\bar{x}_0 + 3\sigma_0$ (132)	9[a]	0	50	0–1
CTC	above/below tolerance	$\bar{x}_2 - 3\sigma_2$ (189)	0	9	50	0–4
CTC	present/absent	$\bar{x}_0 + 3\sigma_0$ (93)	5[a]	0	50	0–4

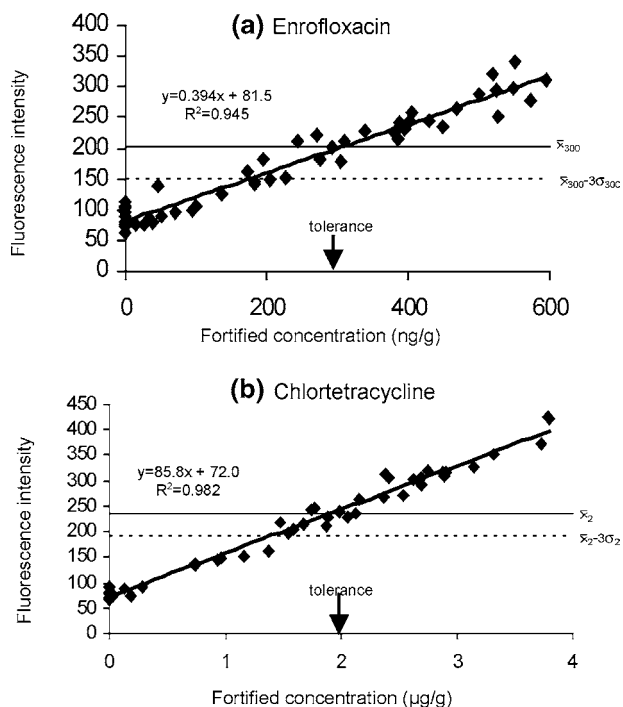[a] All but one below the limit of detection.

Figure 3. Linear response of ENRO and CTC blind fortified samples.

Results from the screening of the blind samples are shown in **Table 1**. In assaying for violations (threshold at $\bar{x}_{300} - 3\sigma_{300}$ or $\bar{x}_2 - 3\sigma_2$), no false negatives were found, and there were only a few false violations (positives). The potential for false positives will always be present when false negatives are minimized in such a screening assay. However, as the total number of violative samples would likely be low in an actual monitoring situation (*10*), there should also be few samples close enough to a violative level to respond as a false positive in the assay. Thus, the presence of false positives should not be a significant concern.

When blind samples were screened for the presence/absence of ENRO and/or CTC (threshold at $\bar{x}_0 + 3\sigma_0$), all control samples were identified correctly (**Table 1**). There were some false negative responses for samples containing low amounts of either ENRO (0.015−0.136 μg/g) or CTC (0.02−0.28 μg/g). In all but one case, these concentrations were below the limit of detection for the analyte in the assay.

This screening assay should also be effective for monitoring samples where TC or OTC is known to be present. Prior to analysis of such unknown samples, experiments setting an exact threshold fluorescence level would be required, such as the ones which generated **Figure 2b**. However, an estimate of the degree of separation between control and fortified samples can be obtained from the data provided. For these cases, the $\bar{x}_0 + 3\sigma_0$ upper boundary of the control region would be unchanged, while the $\bar{x}_2 - 3\sigma_2$ threshold would occur at different fluorescence intensities, due to the differing sensitivities of these analytes. This level can be approximated by examination of the fluorescence intensities at 2 μg/g for each analyte (**Figure 1**), and subtracting the value of $3\sigma_2$, as found for CTC. Thus, the $\bar{x}_2 - 3\sigma_2$ threshold would correspond to approximate fluorescence intensities of 170 and 367 for OTC and TC, respectively, providing a clear distinction from the $\bar{x}_0 + 3\sigma_0$ fluorescence intensity value of 93. In both cases, and particularly for TC, there should thus be adequate separation between control and fortified samples for the screening assay to be effective.

In this study the specific tetracycline present in the samples was known (CTC). In an actual monitoring situation, TC, OTC, and/or CTC could be present in the samples. Use of this screening assay in a monitoring situation where tetracyclines present were not identified would require setting a threshold to determine the least sensitive of the tetracyclines likely to be present (OTC). Such an approach would minimize false negative results, and would likely generate additional false violations in the case of TC. CTC would not be expected to give a significantly larger number of false violations than OTC, as the sensitivities of CTC and OTC at 2 μg/g are not greatly different.

A final consideration was to examine the effect on this screening assay of other drugs approved for use in poultry. Three additional drugs (tylosin, nicarbazin, and ethopabate) were tested at their U.S. tolerance levels (200 ng/g, 4 μg/g, and 500 ng/g, respectively). None of these drugs increased the fluorescence of fortified samples over that observed for control samples in the assay.

In conclusion, this simple, rapid fluorescence assay has been shown to be effective for screening chicken muscle samples for the presence of both ENRO and CTC at their respective tolerance levels. As the same chicken muscle extract is used for both analytes, this procedure is more efficient than analyzing for these analytes separately. This method should be effective for the two other approved tetracyclines, TC and OTC, as well. The assay represents a useful alternative to currently used microbial assays and, in combination with more extensive quantitative and/or confirmatory methods, can provide an efficient approach to monitoring chicken muscle for these analytes.

ABBREVIATIONS USED

ENRO, enrofloxacin; DANO, danofloxacin; DIF, difloxacin; TC, tetracycline; CTC, chlortetracycline; OTC, oxytetracycline; RSD, relative standard deviation.

ACKNOWLEDGMENT

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