The objective of this study was to compare a bioassay with a liquid chromatography–fluorescence–mass spectrometry method for detection of enrofloxacin (ENRO) in incurred eggs. The bioassay developed by our laboratories involves an agar diffusion microbiological method using Klebsiella pneumoniae as an indicator organism. Results demonstrate that both methods are capable of detecting incurred fluoroquinolone residues in eggs. During the 3-day dosing period of hens (Days 1–3) and following drug withdrawal (Days 5, 7, and 9), both of these methods were able to detect incurred ENRO in eggs above the zero tolerance established by the U.S. Food and Drug Administration. The LC–fluorescence–MS method has the benefit of providing confirmation for fluoroquinolones, while the bioassay may be used as an effective, rapid screening method for detection of fluoroquinolone residues in eggs.

Enrofloxacin (ENRO) is a potent member of the fluoroquinolone (FQ) class of antibiotics and is approved for use by the U.S. Food and Drug Administration (FDA) for treatment of disease in meat type poultry (www.fda.gov/cvm). A tolerance of 300 ng/g ENRO residues in edible tissues is published in the U.S. Code of Federal Regulations (www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?FR=556.228). For most drugs approved by the FDA, a veterinarian has the legal right to use these drugs for nonapproved usage under the Animal Medicinal Drug Use Clarification Act (AMDUCA) of 1994. This is called extra-label usage. In the case of ENRO, a number of analytical methods have been developed for monitoring purposes in eggs (1, 3, 5–7). Unfortunately, these methods are expensive, time consuming, difficult to use, and require very costly equipment and highly skilled technical personnel. Although these methods are very accurate, routine use for residue monitoring is limited by the above stated problems. An alternative is the use of a relatively inexpensive, rapid, and technically simple bioassay (BA) for detection of ENRO residues. However, it is unknown if a bioassay recently developed by our laboratories can accurately detect ENRO residues in eggs comparable to a liquid chromatography–fluorescence–mass spectrometry method (LC–FL–MS) method (5). Therefore, the purpose of this study was to compare this bioassay with the LC–FL–MS method to determine its ability to detect incurred ENRO residues in eggs.

Experimental

Apparatus

(a) Antibiotic zone reader.—Fisher Scientific (Pittsburgh, PA).

(b) Stainless steel penicylinders.—8 mm outer diameter (Fisher Scientific).

(c) Penicylinder dispenser.—Arthur Farmer (Trenton, NJ).

(d) Petri dishes.—100 × 15 mm (Fisher Scientific).

(e) Spectrophotometer.—Spectronic Instruments (Rochester, NY).

(f) Reciprocal shaking water bath.—Precision (Winchester, VA).
(g) **Agar dispenser.**—Automated, or equivalent (Wheaton Scientific, Millville, NJ).

(h) **Paddle blender.**—Talboys Engineering (Montrose, PA).

(i) **Liquid chromatograph–fluorescence–mass spectrometer.**—Hewlett-Packard (Wilmington, DE) 1100 series binary LC pump with on-line degasser, autosampler, column heater, and fluorescence detector connected to a ThermoQuest (San Jose, CA) LCQ-Deca multiple mass spectrometer. XCalibur software version 1.2 controlled the system and mass spectrometer, and processed data from the fluorescence detector via an SS-420X module (Scientific Software, Pleasanton, CA).

(j) **Chromatographic column.**—ZORBAX Eclipse XDB-Phenyl, 3.0 × 150 mm, 3.5 μm (Agilent, Palo Alto, CA), with in-line 2 μm filter and C18, 2.0 mm I.D. Security Guard column cartridge (Phenomenex, Torrance, CA).

(k) **Mixers.**—Vortex, and Janke and Kunkel (Cincinnati, OH) IKA-VIBRAX-VXR.

(l) **Homogenizer.**—Janke and Kunkel Ultra-Turrax T25.

(m) **Centrifuge.**—DuPont Sorval RT6000B (Newtown, CT) refrigerated centrifuge (20°C) with A500 rotor.

(n) **Evaporator.**—Zymark TurboVap LV (Hopkinton, MA).

(o) **Filters.**—LC solvents were filtered through 47 mm, 0.45 μm nylon filters. All extracted egg samples were filtered through 25 mm, 0.2 μm nylon filters.

### Microorganisms, Media, and Reagents

(a) **Klebsiella pneumoniae.**—American Type Tissue Culture 10031 (Manassas, VA).

(b) **Mueller Hinton (MH) and Tryptic Soy Broth (TSB; Difco, Sparks, MD).**—Prepared according to Bacteriological Analytical Manual (www.cfsan.fda.gov/~ebam/bam_mi.html).

(c) **Solvents and chemicals.**—Ammonium hydroxide (redistilled) and formic acid (88%, double distilled; GFS Chemicals, Columbus, OH); acetonitrile and hexane (Burdick & Jackson, Muskegon, MI); anhydrous ethyl ether, sodium chloride, potassium phosphate monobasic, dipotassium hydrogen phosphate, sodium phosphate dibasic heptahydrate, and sodium phosphate monobasic (Mallinkrodt, Paris KY). Deionized water prepared with a Barnstead (Dubuque, IA) E-pure system was used to prepare all aqueous solutions.

(d) **Standards.**—Desethylfloxacin ciprofloxacin (DCIP, 89.8%), ciprofloxacin (CIP), and ENRO (99.9%; Bayer, Kansas City, MO); danofloxacin (DANO; Pfizer, Groton, CT); sarafloxacin (SAR, 88.5%) and difloxacin (DIF, 89.0%; Abbott, North Chicago, IL); orbifloxacin (ORBI; Schering Plough, Kenilworth, NJ); norfloxacin (NOR; Sigma, St. Louis, MO).

(e) **1% Phosphate buffer.**—Add 2 g potassium phosphate monobasic and 8 g dipotassium hydrogen phosphate to 1 L beaker, along with water, to a volume of 900 mL. Adjust pH to 9.0 with 1M NaOH. Transfer to 1 L volumetric flask and dilute to volume with water.

(f) **0.2M sodium dihydrogen phosphate.**—Transfer 27.8 g sodium dihydrogen phosphate to a 1 L volumetric flask. Dissolve and dilute to volume with water.

(g) **0.2M sodium hydrogen phosphate.**—Transfer 53.6 g sodium hydrogen phosphate heptahydrate to a 1 L volumetric flask. Dissolve and dilute to volume with water.

(h) **0.1M phosphate buffer, pH 9.0.**—Transfer 26.5 mL 0.2M sodium dihydrogen phosphate and 473 mL 0.2M sodium hydrogen phosphate to 1 L beaker, along with water, to a volume of 900 mL. Adjust pH to 9.0 with 1M NaOH. Transfer to 1 L volumetric flask and dilute to volume with water.

(i) **1% Formic acid, pH 3, with ammonium hydroxide.**—Transfer 11.4 mL 88% formic acid to a 1 L beaker, along with water, to a volume of 900 mL. Adjust pH to 3.0 with concentrated ammonium hydroxide. Transfer to 1 L volumetric flask and dilute to volume with water.

(j) **0.03M NaOH.**—Add 1.2 g NaOH to 1 L volumetric flask. Dissolve in water, and then dilute to volume with water.

### Standard Solutions

(a) **Stock solutions.**—BA: To separate 50 mL actinic volumetric flask, add 5.0 mg ENRO, dissolve each in MeOH, and then dilute to volume with MeOH. Store refrigerated. Prepare fresh every 6 months. LC–FL–MS<sup>n</sup>: To separate 50 mL actinic volumetric flask, add 5.6 mg DCIP, 5.0 mg NOR, 5.0 mg CIP, 5.0 mg DANO, 5.0 mg ENRO, 5.0 mg ORBI, 5.6 mg SAR·HCl, and 6.1 mg DIF·HCl. Dissolve each FQ in 0.03M NaOH, and then dilute to volume with 0.03M NaOH. Store refrigerated. Prepare fresh every 6 months.

(b) **Fortification solution.**—To 50 mL actinic volumetric flask, add 1.00 mL of each FQ stock solution. Dilute to volume with 0.1M phosphate, pH 9.0. The resultant solution contains each FQ at 2000 ng/g. Store refrigerated. Prepare fresh monthly.

(c) **Standard curve.**—BA: Matrix matched standards were prepared by taking 10 g of control egg homogenate per standard and adding the appropriate volume of stock solution to prepare 5 standard solutions encompassing the incurred concentration for each day (25–3200 ng/g). These matrix matched standards were then diluted 1:3 (v/v) with 1% phosphate buffer, pH 9.0, centrifuged at 1500 × g for 20 min at 5°C and the supernatant decanted. Supernatants were stored at –80°C until analysis. Standard curves are linear over the range 25–3200 ng/g (r<sup>2</sup> > 0.999). LC–FL–MS<sup>n</sup>: Dilute fortification solution with 0.1M phosphate, pH 9.0, to prepare 6–8 standard solutions encompassing the concentration of diluted incurred samples (1–100 ng/g) each day. Standard curves are linear over the range 1–100 ng/g (r<sup>2</sup> > 0.999).

### Egg Samples

(a) **Control eggs.**—Eggs were either collected from undosed hens (University of Arkansas) or obtained from a local store (Giving Nature brand, Westfield Farms, New Holland, PA), homogenized with a paddle mixer (ca setting 7, 15 s), pooled, aliquoted, and stored at –80°C until needed.

(b) **ENRO-incurred eggs.**—Seven hens were individually caged and dosed with a single daily bolus of 0.34 mL ENRO (Baytril<sup>®</sup>, 32.3 mg/mL) for 3 consecutive days. This bolus dose corresponds with the FDA-approved label dose of 50 ppm in the water. Eggs were collected for 11 days. Each day,
eggs from all hens were homogenized with a paddle mixer (ca setting 7, 15 s), pooled, aliquoted, and stored at –80°C.

**Sample Preparation**

BA: Mixed eggs (1 g) were diluted 1:3 (v/v) with 1% phosphate buffer, pH 9.0, centrifuged at 1500 × g for 20 min at 5°C, and the supernatant decanted and stored at –80°C until analysis. The recoveries were 98%.

LC–FL–MS\(^n\): Transfer 1.0 g incurred egg homogenate to each of six 50 mL disposable centrifuge tubes. Transfer 1.0 g control egg homogenate to 2 additional centrifuge tubes. To one of the control egg tubes, add an appropriate volume of fortification solution to achieve a fortification level of 100 ng/g. This fortified sample was used to confirm that recoveries of ENRO and CIP were greater than or equal to 60%. To all remaining tubes, add the same volume of 0.1M phosphate, pH 9.0. Mix all tubes on VIBRAX mixer for 30 min and then store all tubes in reduced light for additional 30 min. To each tube, add 3 mL acetonitrile and 0.25 mL concentrated ammonium hydroxide, and homogenize. Centrifuge for 5 min at 2205 × g, and decant supernatant into new disposable 50 mL centrifuge tubes. Add 750 μL water to each pellet. Repeat addition of acetonitrile and ammonium hydroxide, homogenize, and centrifuge. To each of the combined supernatants, add 3 mL hexane, 3 mL ethyl ether, and 0.25 mL 1M NaCl. Mix on a Vortex mixer 15 s; remove and discard upper layer. Transfer lower layers into 18 × 150 mm glass disposable test tubes, rinsing in with 4 × 1 mL acetonitrile. Evaporate samples at 40°C under a Vortex of nitrogen. Add additional 1 mL portions of acetonitrile, as needed, to facilitate evaporation. Dissolve residues in 2.00 mL 0.1M phosphate, pH 9.0, and mix on a Vortex mixer 10 s. Pass through 25 mm, 0.2 μm nylon syringe filters into amber autosampler vials for analysis. Incurred egg samples with high levels of ENRO were diluted initially with control egg homogenate prior to FQ extraction.

**Bioassay Procedure**

On the test date, working cultures of *Klebsiella pneumoniae* were grown in TSB to a turbidity of 0.809 absorbance. This corresponds with approximately 9.2 × 10\(^8\) *Klebsiella pneumoniae/mL*. An appropriate amount of media was made, autoclaved, and allowed to cool to 49°C in a shaking water bath. The cooled agar was inoculated with *Klebsiella pneumoniae* to a concentration of 1 × 10\(^8\)/mL. The flask was thoroughly swirled and 8 mL was dispensed into each Petri dish. Each plate was gently tilted and swirled to ensure even coverage and minimize air bubbles. The plates were allowed to solidify for 30 min on a level surface. Six penicylinders were then evenly placed on the agar using the penicylinder dispenser. Each standard concentration was pipetted onto 3 plates. On each plate, 3 alternative penicylinders were filled with the standard (200 μL/cylinder) and the other 3 cylinders were filled with a reference concentration (200 μL/cylinder). The reference concentration on each plate corrects for any potential plate to plate variation (internal plate standard). The reference concentration is a standard with ENRO concentrations in the mid range of the standard curve.

The overall reference concentration is determined by averaging the recovery concentrations from each standard curve plate. Triplet plate averages for each standard point are then corrected to the overall reference concentration. Unknowns are treated in a similar manner; except only one plate was used for each unknown sample. To increase the assay’s sensitivity, the plates were allowed to pre-incubate at room temperature for 3 h. Following this, plates were incubated 15–18 h at 37°C. Following incubation, the growth inhibition zone was measured using the antibiotic zone reader. A best fit regression line using the diameter of growth inhibition zones vs the logarithm concentration of ENRO was calculated by the method of least squares. The lower limit of assay sensitivity (9 mm zone size) was calculated at 15 ng/g.

**Liquid Chromatography–Fluorescence**

A gradient composed of 1% formic acid, pH 3.0, with ammonium hydroxide (Solvent A) and acetonitrile (Solvent B) was used: 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); flow rate 0.5 mL/min; column heater 30°C; fluorescence detector λ\(_{ex}\) 278 nm, λ\(_{em}\) 440 nm. An external standard curve was generated daily and fluorescence peak height was used for quantitation.

![Detection of incurred fluoroquinolone (FQ) residues in mixed eggs by either a bioassay or LC–FL–MS\(^n\) method (mean ± 99% confidence interval).](image)
Mass Spectrometry

The mass spectrometer was operated in positive ion atmospheric pressure chemical ionization mode, with automatic gain control on, maximum injection time 400 ms, and ion targets for MS\(^1\) and MS\(^n\) 5 \(\times\) 10\(^7\) and 2 \(\times\) 10\(^7\), respectively. FQ fragmentation patterns, tuning parameters, and MS\(^n\) parameters were established by infusing a 10 \(\mu\)g/mL solution of each FQ in mobile phase into a 0.5 mL/min flow of 15% acetonitrile in 1% formic acid, pH 3, with ammonium hydroxide. FQ tune parameters: isolation width 1.2 \(m/z\), vaporizer temperature 450\(^\circ\)C, sheath gas flow 65, auxiliary gas flow 0, discharge current 4.5\(\mu\)A, capillary temperature 160\(^\circ\)C. Capillary voltage and tube lens offset were set semiautomatically and multipole 1 and 2 offsets, lens voltage, multiple RF amplitude, and entrance lens voltage were set automatically. MS\(^n\) precursor ions \((m/z)\), collision energies (%): DCIP MS\(^2\) 306.0, 40; NOR MS\(^2\) 320.0, 25, MS\(^3\) 276.2, 35; CIP MS\(^2\) 332.0, 40, MS\(^2\) 288.2, 35; DANO MS\(^2\) 358.0, 25, MS\(^3\) 314.0, 30; ENRO MS\(^2\) 360.2, 45, MS\(^3\) 316.2, 35; ORBI MS\(^2\) 396.0, 30, MS\(^3\) 352.0, 30; SAR MS\(^2\) 386.0, 30, MS\(^3\) 342.0, 35; DIF MS\(^2\) 400.0, 40, MS\(^3\) 356.0, 35. The value of Q was set at 0.25 for all FQs, except DCIP (0.35), which also used wide band activation. Scan ranges were generally 200–400 m/z, except for ORBI, SAR, and DIF, which were monitored to 450 m/z. A divert valve was used for the first 4.4 min and the last 10 min of the chromatographic run to minimize phosphate and matrix contamination in the mass spectrometer. Retention time windows were checked daily with a mixture of the 8 FQs and adjusted as needed. Confirmation was achieved by examination of the ratios of 2 major MS\(^2\) (DCIP) or MS\(^3\) (NOR, CIP, DANO, ENRO, ORBI, SAR, DIF) product ions.

Statistical Analysis

Data were analyzed by analysis of variance using the Statistical Analysis System (SAS) general linear models program (8). Treatment means were partitioned by Tukeys analysis (8). A probability of \(p < 0.05\) was required for statistical significance.

Results and Discussion

In this study, we compared a relatively inexpensive and simple bioassay with a more elaborate LC–FL–MS\(^n\) method for the detection of ENRO residues in eggs. For this comparison study, we assayed eggs with incurred ENRO residues (produced from treated hens) as opposed to simply spiking eggs with ENRO. Incurred ENRO residues may have different matrix binding characteristics and, unlike spiked samples, would be representative of samples collected for monitoring.

Our results indicate that our bioassay was comparable to the ability of an LC–FL–MS\(^n\) method for detection of incurred ENRO residues in eggs (Figure 1). During the 3-day dosing
period (Days 1–3) and following drug withdrawal (Days 5, 7, and 9), both of these methods had 99% confidence intervals for the true quantity of incurred ENRO, whose lower ends were well above the zero tolerance established by the FDA. On Day 11, only the LC–FL–MSn method was able to detect residues at an extremely low concentration, which was below the sensitivity of our bioassay (Figure 1, Table 1). In conducting this study, it was not anticipated that the bioassay would provide similar quantitation of residues to that of the LC–FL–MSn method. Rather, it was thought the bioassay may accurately detect the presence of fluoroquinolone residues as a screening procedure (see ensuing discussion) with follow-up quantitation of positive samples by the LC–FL–MSn method. Although the results support the utility of the bioassay for this purpose, interestingly, the bioassay quantitation of residues was not significantly different ($p > 0.05$) from the LC–FL–MSn method except for Day 2. Neither method was adversely affected by the presence of matrix (whole mixed egg). There was no difference in quantitation between standards made with buffer or whole mixed egg.

Although both the bioassay and LC–FL–MSn methods were quantitative, only the LC–FL–MSn method allowed separation and confirmation of the individual FQ analytes. Sample chromatograms of control, incurred, and fortified eggs are shown in Figure 2. In chickens, ENRO is metabolized by the liver to CIP (9, 10). On Days 2, 3, 5, 7, 9, and 11, analysis with the LC–FL–MSn detected not only ENRO residues but also the ENRO metabolite, CIP (Table 1). On Day 1, the CIP was below the limit of quantitation (LOQ). The MS confirmation data for incurred fluoroquinolone residues is shown in Table 2. Sample MS$^3$ spectra are shown in Figure 3. Confirmation is achieved by comparing the peak height ratios of 2 major MS$^3$ product ions ($\% 245/268$ for CIP and $\% 288/245$ for ENRO) in incurred samples to those of standard samples. For both ENRO and CIP, the peak ratio values are comparable to those obtained for standard samples, confirming the presence of these 2 FQs. Higher RSDs are found for samples, which were analyzed at a lower concentration, as we have found previously (e.g., Day 11 incurred ENRO; 5). However, in all cases, the average peak ratio value reported for the incurred samples is within less than one standard deviation of that for the corresponding pooled standard.

As is the case for most analytical methods, each has its advantages and disadvantages. Although the bioassay accurately detects ENRO residues in eggs, it is unable to differentiate between ENRO and CIP. Thus, it would not be suitable as a definitive assay for regulatory action. However, as the bioassay is relatively inexpensive, easy to use, and allows for high sample throughput, it would serve as an excellent screening

![Figure 3. MS$^3$ spectra of CIP and ENRO in ENRO-incurred egg, Day 9.](image-url)
method. If any of these samples were positive, then the LC–FL–MS^n method, which is able to quantitate and differentiate between fluoroquinolones, can be used to confirm the amount and types of residues present. Another possible disadvantage of the bioassay is its higher LOQ versus the LC–FL–MS^n method (15 versus 1.5 ng/g). The LC–FL–MS^n method is able to detect extremely low concentrations of illegal ENRO residues, and there may be instances where the increased sensitivity of LC–FL–MS^n would be needed.

Conclusions

We have determined that a bioassay recently developed in our laboratories can detect incurred ENRO residues in eggs comparable to an LC–FL–MS^n method. This method is easy to use and has a high sample throughput. It was, however, not selective between different fluoroquinolone type analytes and would best be used as a screening method for ENRO residues in eggs, while the LC–FL–MS^n method can be used to provide confirmation when needed.

Acknowledgments

We thank Susan Braden (USDA-ARS-ERRC), Pam Blore, John Holliman, and Ron McNew (University of Arkansas) for technical assistance; Bayer for providing samples of DCIP, CIP, and ENRO; Pfizer for a sample of DANO; Abbott Laboratories for samples of SAR and DIF; and Schering Plough for a standard of ORBI.

References

(3) Schneider, M.J., & Donoghue, D.J. (2000) J. AOAC Int. 83, 1306–1312