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Multiresidue analysis of fluoroquinolone antibiotics in chicken tissue using liquid chromatography-fluorescence-multiple mass spectrometry

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Abstract

An efficient liquid chromatographic method for the multiresidue analysis of fluoroquinolone antibiotics in chicken tissue has been developed in which quantitation using fluorescence and confirmation with multiple mass spectrometry (MS^n) was achieved simultaneously. Using this method, eight fluoroquinolones were analyzed in fortified samples of chicken liver and muscle tissue with recoveries at levels of 10–200 ng/g generally in the range of 60–93%, except for desethylene ciprofloxacin, which consistently gave recoveries ≥45%. Relative standard deviations were excellent in all cases, and the limits of detection in ng/g were determined as follows in liver and (muscle): desethylene ciprofloxacin 0.3 (0.1), norfloxacin 1.2 (0.2), ciprofloxacin 2 (1.5), danofloxacin 0.2 (0.1), enrofloxacin 0.3 (0.2), orbifloxacin 1.5 (0.5), sarafloxacin 2 (0.6), difloxacin 0.3 (0.2). Confirmation of the identities of the fluoroquinolones was achieved by monitoring the ratios of two prominent product ions in MS^2 (desethylene ciprofloxacin) or MS^3 (all others). Levels of confirmation as related to ion ratio variability criteria were established. Enrofloxacin and ciprofloxacin were also determined in enrofloxacin incurred chicken liver and muscle using this method.
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Keywords: Fluoroquinolone; Antibiotics

1. Introduction

Fluoroquinolone (FQ) antibiotics are used for a variety of human medical and veterinary applications. The use of FQs in food animals has generated growing concern as reports of microbial resistance to these drugs have increased [1]. Two FQs, enrofloxacin (ENRO) and sarafloxacin (SAR), had been approved for use in broiler chickens in the U.S., but approval for SAR has recently been withdrawn. Additional FQs are approved in Europe [2]. The U.S. Food and Drug Administration has forbidden extralabel use of FQs [3], however the potential for misuse and for additional approvals of FQs necessitates efficient methods to detect these residues in poultry.

A number of methods have been developed for the analysis of FQs in chicken tissues. Most of these rely

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on liquid chromatography with either ultraviolet or fluorescence detection [2,4–11]. Immunoaffinity chromatography [12] and capillary electrophoresis [13] methods have been reported as well. These approaches are all useful for the quantitation of FQs, but are not necessarily sufficient for confirmation purposes.

Several confirmation methods using mass spectrometric techniques have been reported. Mass spectrometric confirmation of FQs has been accomplished in milk, salmon, urine [14], catfish tissue [15,16] and pig muscle [17]. A method for confirmation of danofloxacin (DANO) at 50 ng/g in chicken and cattle liver is also available [18]. The optimum method for analysis of FQs would be one which allows confirmation as well as quantitation, has low levels of detection, and permits analysis of multiple analytes. The goal of this work is to illustrate how the combination of fluorescence with multiple mass spectrometry (MS^n) can take advantage of the strength of both techniques to allow for simultaneous quantitation, with low levels of detection, and confirmation of FQs. The usefulness of this method is shown in the multiresidue analysis of eight FQs, and its application to both fortified and incurred chicken tissue samples.

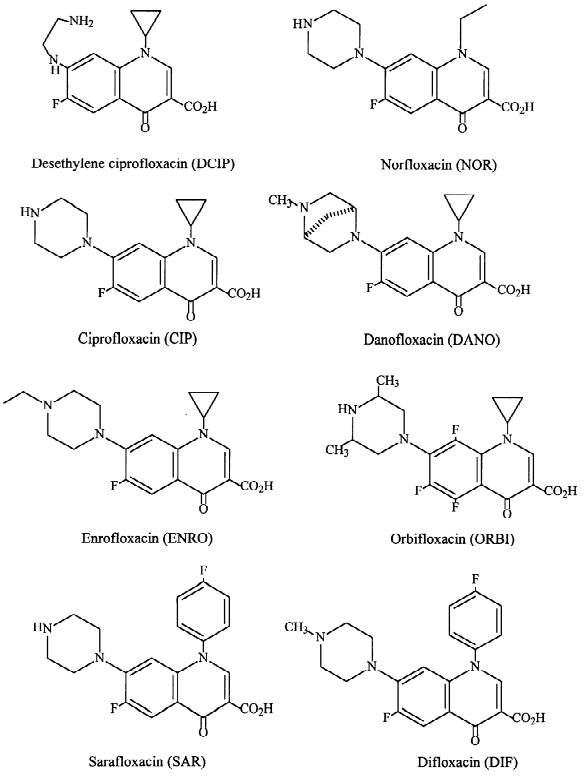

Fig. 1. Chemical structures of fluoroquinolone antibiotics.

2. Experimental

2.1. Materials

Desethylene ciprofloxacin (DCIP, 89.8%), ciprofloxacin (CIP), and ENRO (99.9%) were obtained from Bayer (Kansas City, MO, USA), DANO was obtained from Pfizer (Groton, CT, USA), SAR (88.5%) and difloxacin (DIF, 89.0%) were obtained from Abbott (North Chicago, IL, USA) and norfloxacin (NOR) and lomefloxacin were obtained from Sigma (St Louis, MO, USA). Chemical structures of the FQ analytes included for analysis in this study are shown in Fig. 1. Ammonium hydroxide (redistilled) and formic acid (88%, double distilled) were from GFS Chemicals (Columbus, OH, USA). Acetonitrile and hexane were from Burdick & Jackson (Muskegon, MI, USA). Anhydrous ethyl ether, sodium chloride, sodium phosphate dibasic heptahydrate and sodium phosphate monobasic were from Mallinckrodt (Paris, KY, USA). Deionized water prepared with a Barnstead (Dubuque, IA, USA) E-pure system was used to prepare all aqueous solutions. All solutions prepared for liquid chromatography were filtered through a 0.45-μm nylon filter before use. Control (antibiotic free) chicken liver and breast muscle (Bell and Evans brand, Fredericksburg, PA, USA) were purchased fresh, cut into small pieces, and ground into a homogeneous sample using a food processor. This material was then kept frozen at −80 °C until use.

2.2. Standard solutions

Stock solutions (100 μg/ml) in 0.03 *M* sodium hydroxide were prepared for each of the eight FQs in actinic volumetric flasks. These solutions were stored at 4 °C and prepared fresh every 6 months. A fortification solution containing a mix of each FQ at 2000 ng/ml was prepared by dilution of the stock solutions with 0.1 *M* phosphate, pH 9. The fortifica-

tion solution was stored at 4 °C in an amber container and prepared fresh monthly.

2.3. Incurred chicken tissue

Eight broiler chickens were treated with a solution of ENRO at the FDA approved dose (50 μg/ml in drinking water) for 7 days. Two birds were sacrificed on days 8 (first day post-dose), and 10. Two birds were also sacrificed on days 5 and 7, to establish concentrations of ENRO during the dosing period. Liver and breast muscle samples were harvested, shipped in dry ice and stored at −80 °C. Liver or breast muscle samples from two birds sacrificed on the same day were combined, partially thawed, diced, homogenized in a food processor, and then stored at −80 °C. An initial extraction and analysis of a single portion of each sample were carried out to determine the approximate ENRO concentrations. Incurred tissue samples were then diluted with the corresponding control tissue to ensure ENRO concentrations measured would fall within an optimum range (10–200 ng/g). Diluted samples were then homogenized with a food processor prior to extraction and analysis.

2.4. Sample preparation

Tissue samples (1.0 g) were placed in 50-ml centrifuge tubes and a portion of fortification solution (for preparation of fortified samples) or 0.1 *M* phosphate, pH 9 buffer (for preparation of a tissue control and incurred samples) was added. The samples were mixed for 30 min (IKA-VIBRAX-VXR, Janke and Kunkel, Cincinnati, OH, USA) and then stored in reduced light for an additional 30 min. Samples were homogenized (Ultra-turrax T-25, Janke and Kunkel) with acetonitrile (3 ml) and concentrated ammonium hydroxide (0.25 ml). The tubes were centrifuged (5 min, 2205 *g* for liver and 2791 *g* for muscle) and the supernatants decanted into fresh 50-ml centrifuge tubes. The pellet was re-extracted twice, as before. Hexane (3 ml), ethyl ether (3 ml) and 1 *M* sodium chloride (0.25 ml) were added to the combined supernatants. The tubes were mixed with a vortex mixer (15 s) and the upper layer discarded by pipet. The lower layer was transferred to a glass tube and evaporated to dryness under a stream of nitrogen at 40 °C with a TurboVap LV evaporator (Zymark, Hopkinton, MA, USA). Additional acetonitrile (~1–3 ml) was added periodically when nearing completion of the evaporation to facilitate this process. The residues were redissolved in 2.0 ml of 0.1 *M* phosphate buffer, pH 9, vortex mixed (15 s), and filtered through a 25-mm, 0.2-micron nylon syringe filter into amber autosampler vials for analysis. Although phosphate buffer is generally not used in mass spectrometry applications, it is an excellent solvent and is used in this case to selectively redissolve fluoroquinolones in the evaporated samples. The use of a divert valve for the first few minutes of the chromatographic run serves to minimize any phosphate in the small amount of sample injected entering the mass spectrometer.

2.5. Liquid chromatography-fluorescence-multiple mass spectrometry

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

A ZORBAX Eclipse XDB-Phenyl 3.0×150 mm, 3.5 μm chromatography column (Agilent, Palo Alto, CA, USA) was used, with an in-line 2-μm filter and a Phenomenex (Torrance, CA, USA) Security Guard column (C_{18}, 2.0 mm I.D. cartridge) A gradient was used with the mobile phase, combining Solvent A (1% formic acid, adjusted to 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). Depending on the column lot used, the gradient needed slight modification. Thus, for analysis of incurred liver samples, a modified gradient was used as follows: 14%B (10 min), 14–16%B (8 min), 16%B (6 min), 16–80%B (2 min), 80%B (2 min), 80–14%B (3 min), 14%B (3 min). The flow-rate was 0.5 ml/min, and the column heater was set at 30 °C. 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 components entering the mass spectrometer. Fluorescence detection used λ_{ex} 278 nm and λ_{em} 440 nm. Quantitation was achieved using an external standard curve generated daily using dilutions of the fortification solution in either buffer or control matrix extract, and measurement of fluorescence peak height. Linearity was excellent, typically with $R^2 >$ 0.999.

The mass spectrometer was operated in positive ion atmospheric pressure chemical ionization (APCI) mode, with automatic gain control on, maximum injection time 400 ms and ion targets for MS^1 and MS^n 5×10^7 and 2×10^7, respectively. FQ fragmentation patterns, tuning parameters, and MS^n parameters were established by infusing a 10 ppm solution of each FQ in mobile phase into a 0.5 ml/min flow of 15% acetonitrile in 1% formic acid, adjusted to pH 3 with ammonium hydroxide. The following optimum tuning parameters were common for all FQs: isolation width (1.2 m/z), vaporizer temperature (450 °C), sheath gas flow (65), auxiliary gas flow (0), discharge current (4.5 μA), capillary temperature (160 °C). The capillary voltage and tube lens offset were set semiautomatically and multipole 1 & 2 offsets, lens voltage, multiple RF amplitude and entrance lens voltage were set automatically. MS^2 and MS^3 parameters established for the FQs are summarized in Table 1. Wide band activation was used only for DCIP. Scan ranges were generally 200–400 m/z, except for ORBI, SAR and DIF, which were monitored to 450 m/z. Retention time windows for each FQ were checked daily with a mixture of the eight FQs and the method was adjusted as needed. Confirmation was achieved by examination of the ratios of two major MS^3 product ions (MS^2 for DCIP).

Table 1
MS^n acquisition parameters for FQs

		Precursor ion (m/z)	Normalized collision energy (%)[a]	Q	Confirmation ions (m/z)
DCIP	MS^2	306.0	40	0.35	286, 268
NOR	MS^2	320.0	25	0.25	
	MS^3	276.2	35	0.25	233, 256
CIP	MS^2	332.0	40	0.25	
	MS^3	288.2	35	0.25	245, 268
DANO	MS^2	358.0	25	0.25	
	MS^3	314.0	30	0.25	283, 294
ENRO	MS^2	360.2	45	0.25	
	MS^3	316.2	35	0.25	288, 245
ORBI	MS^2	396.0	30	0.25	
	MS^3	352.0	30	0.25	332, 295
SAR	MS^2	386.0	30	0.25	
	MS^3	342.0	35	0.25	299, 322
DIF	MS^2	400.0	40	0.25	
	MS^3	356.0	35	0.25	336, 299

[a] 100% = 20 V.

3. Results and discussion

Extraction of FQs from chicken tissue followed the general method used previously for eggs [19], with some modifications. Extraction of the tissue

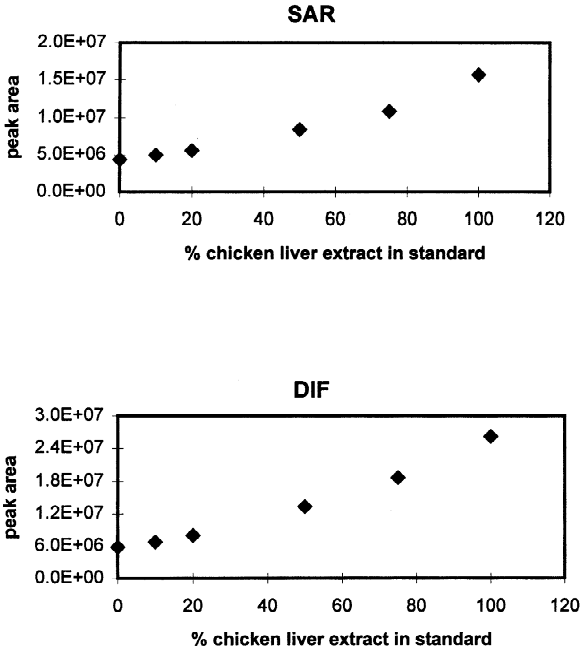

Fig. 2. Matrix enhancement of FQ MS^2 response; 50 ng/g samples of FQs were prepared in 0.1 *M* phosphate, pH 9 solutions containing varied percentages of control chicken liver extract.

three times rather than twice led to slightly increased yields, and use of an evaporation apparatus which created a vortex of nitrogen gas significantly decreased evaporation time. Samples were dissolved in 0.1 *M* phosphate buffer, pH 9, however the use of a divert valve for the first 4.4 min of a chromatographic run minimized the levels of phosphate and matrix components entering the mass spectrometer.

Excellent separation of the FQs was achieved using an Eclipse XDB-phenyl liquid chromatography column. The mobile phase used 1% formic acid, rather than 0.1% formic acid, as it provided better peak shape. FQ retention times varied between different lots of these columns, necessitating slight modification of the gradient when switching column lots.

Both electrospray and APCI mass spectrometer probes gave good response with the FQs. However, APCI was chosen as the more stable option due to the interference of 1% formic acid with the spray current in electrospray mode. FQ fragmentation patterns were examined under MS^2 and MS^3 conditions. In general, MS^3 produced more than one product ion of significant abundance, leading to more easily measured ratio values for confirmation purposes, than observed with MS^2, in which one product ion was often significantly present, but others were much less intense. Thus, MS^3 was

Table 2
FQ recovery from fortified chicken tissue

Tissue	Fortification level (ng/g)	Analysis set	*n*	Recovery %(RSD)							
				DCIP	NOR	CIP	DANO	ENRO	ORBI	SAR	DIF
Liver	10	1	3	45.6 (5.6)	41.7 (7.0)	74.2 (3.4)	75.5 (4.5)	74.9 (4.7)	93.1 (3.1)	50.9 (11.6)	81.9 (3.8)
	10	2	3	44.7 (2.2)	53.0 (3.9)	63.7 (12.7)	71.6 (2.4)	71.0 (4.4)	64.4 (3.9)	54.7 (3.9)	80.6 (1.5)
	10	Ave 1&2	6	45.2 (4.0)	[a]47.3 (13.9)	69.0 (11.4)	73.6 (4.3)	72.9 (5.0)	[a]78.8 (20.2)	52.8 (8.5)	81.3 (2.7)
	25	1	3	50.1 (1.0)	68.3 (2.1)	62.8 (3.3)	79.8 (1.9)	82.4 (1.8)	73.7 (2.4)	80.4 (2.6)	90.4 (1.9)
	25	2	3	48.1 (2.6)	59.0 (4.2)	69.8 (3.6)	74.6 (2.2)	74.4 (3.3)	71.5 (3.2)	70.7 (5.2)	81.9 (2.5)
	25	Ave 1&2	6	49.1 (2.9)	[a]63.6 (8.5)	[a]66.3 (6.6)	[a]77.2 (4.2)	[a]78.4 (6.0)	72.6 (3.0)	[a]75.6 (7.8)	[a]86.2 (5.8)
	50	1	3	49.1 (7.0)	67.2 (5.5)	68.8 (5.3)	79.0 (2.0)	79.2 (2.8)	81.2 (1.6)	73.8 (3.1)	89.6 (2.0)
	50	2	3	47.0 (2.0)	66.3 (1.3)	63.7 (5.0)	80.2 (0.6)	81.6 (0.2)	88.4 (0.8)	80.0 (0.9)	91.4 (0.7)
	50	3	3	51.2 (1.2)	59.2 (2.2)	66.4 (2.6)	74.8 (1.6)	74.7 (1.4)	85.5 (1.9)	72.4 (2.6)	84.1 (2.6)
	50	4	3	48.8 (5.7)	59.5 (4.8)	71.8 (14.0)	72.6 (3.3)	73.1 (4.0)	77.7 (4.2)	68.8 (4.8)	82.2 (3.0)
	50	Ave 1–4	12	49.0 (5.1)	[a]63.0 (7.0)	67.7 (8.5)	[a]76.6 (4.5)	[a]77.1 (5.1)	[a]83.2 (5.5)	[a]73.8 (6.3)	[a]86.8 (4.9)
	100	1	3	62.6 (3.5)	75.1 (3.6)	72.5 (1.1)	82.5 (1.2)	82.8 (1.9)	88.5 (0.9)	84.2 (2.1)	91.5 (2.1)
	100	2	3	55.0 (2.4)	65.0 (0.9)	67.8 (2.1)	76.4 (1.6)	75.3 (1.7)	81.3 (0.9)	76.2 (2.0)	84.9 (1.4)
	100	Ave 1&2	6	[a]58.8 (7.6)	[a]70.0 (8.3)	[a]70.2 (4.0)	[a]79.4 (4.4)	[a]79.1 (5.4)	[a]84.9 (4.7)	[a]80.2 (5.8)	[a]88.2 (4.4)
	200	1	3	61.4 (3.4)	69.9 (3.4)	72.3 (3.1)	81.5 (0.9)	80.9 (1.4)	87.4 (2.0)	80.4 (2.8)	91.2 (1.5)
	200	2	3	58.0 (4.0)	68.9 (2.7)	68.0 (3.9)	79.9 (1.5)	81.3 (0.6)	88.4 (0.6)	81.0 (0.5)	90.3 (0.3)
	200	Ave 1&2	6	59.7 (4.6)	69.4 (2.9)	70.2 (4.6)	80.7 (1.6)	81.1 (1.0)	87.9 (1.5)	80.7 (1.8)	90.8 (1.1)
Muscle	10	1	3	56.9 (2.4)	64.6 (4.1)	50.6 (5.1)	72.5 (10.9)	69.8 (9.0)	79.8 (3.2)	72.0 (2.6)	76.2 (7.3)
	10	2	3	48.8 (3.6)	57.4 (5.2)	42.6 (11.0)	66.7 (0.6)	63.2 (0.9)	73.8 (1.3)	70.6 (2.6)	75.8 (3.8)
	10	Ave 1&2	6	[a]52.8 (8.8)	[a]61.0 (7.6)	46.6 (11.9)	69.6 (8.5)	66.5 (8.1)	[a]76.8 (4.8)	71.3 (2.6)	76.0 (5.2)
	25	1	2	53.6 (2.4)	62.6 (3.2)	57.4 (9.1)	77.8 (3.6)	75.6 (2.4)	82.0 (2.0)	74.2 (2.4)	84.0 (2.2)
	25	2	3	52.6 (7.7)	62.0 (7.4)	53.5 (12.2)	68.4 (8.6)	63.6 (10.6)	76.6 (7.8)	69.9 (9.0)	71.8 (9.6)
	25	Ave 1&2	5	53.0 (5.6)	62.3 (5.5)	55.1 (10.4)	72.2 (9.4)	68.4 (11.9)	78.7 (6.6)	71.6 (7.1)	76.7 (10.8)
	50	1	3	50.8 (3.7)	64.6 (3.5)	63.3 (3.7)	75.6 (3.0)	74.5 (1.8)	83.0 (0.7)	76.0 (3.8)	83.0 (2.0)
	50	2	3	52.5 (8.0)	59.5 (6.7)	55.5 (7.8)	66.8 (4.3)	63.8 (2.2)	73.4 (3.5)	65.8 (1.4)	67.1 (14.2)
	50	3	3	56.7 (1.7)	64.5 (1.6)	61.2 (2.8)	72.8 (1.0)	68.9 (2.8)	78.5 (1.3)	73.9 (3.8)	74.5 (7.8)
	50	Ave 1–3	9	53.3 (6.6)	62.8 (5.5)	[a]60.0 (7.2)	[a]71.7 (6.0)	[a]69.1 (7.0)	[a]78.3 (5.6)	71.9 (7.1)	74.9 (11.9)
	100	1	3	59.9 (2.5)	65.6 (5.5)	66.6 (6.2)	75.9 (3.4)	74.6 (3.4)	81.8 (3.7)	73.0 (8.4)	82.5 (3.0)
	100	2	3	58.7 (2.2)	66.3 (2.7)	65.6 (1.9)	76.2 (0.3)	72.9 (0.2)	82.2 (0.6)	74.7 (1.9)	80.0 (0.9)
	100	Ave 1&2	6	59.3 (2.4)	65.9 (3.9)	66.1 (4.2)	76.0 (2.1)	73.8 (2.5)	82.0 (2.4)	73.8 (5.5)	81.2 (2.6)
	200	1	3	65.9 (3.2)	71.9 (3.3)	69.5 (1.4)	78.7 (2.1)	76.8 (2.2)	87.3 (1.6)	81.7 (1.7)	85.2 (1.6)

[a] Inter-day variation is statistically significant compared to intra-day variation.

chosen as the method of choice for all FQs except DCIP, for which MS^2 conditions were more satisfactory.

Initially, quantitation of FQs was attempted using mass spectrometry. The presence of matrix significantly enhanced MS^2 response. Responses of SAR and DIF relative to % chicken liver extract present are shown in Fig. 2, illustrating this effect. The use of matrix-matched standards (adding standards to control tissue extracts) did not fully overcome the non-linear or variable results observed. Lomefloxacin was tested as an internal standard and was not entirely successful in compensating for the observed variations. Fluorescence results, however, were consistently reliable.

As fluorescence is a highly sensitive, reproducible, and non-destructive method for quantitation of FQs, it was decided to use this technique for quantitation and use mass spectrometry for simultaneous confirmation. Such a combination would effectively take advantage of the strengths of both techniques. With fluorescence for quantitation, matrix had no effect on response. Thus, standard curve samples no longer needed to be matrix matched, simplifying the sample preparation.

This method was tested using both chicken liver and muscle samples which had each been fortified with several levels of the standard FQ mixture. The extraction recovery results are shown in Table 2. Sample chromatograms are provided in Fig. 3. Each analysis set for a given concentration represents an experiment conducted on a separate day (Table 2). Good recoveries were generally obtained in both liver and muscle for seven of the eight FQs tested at

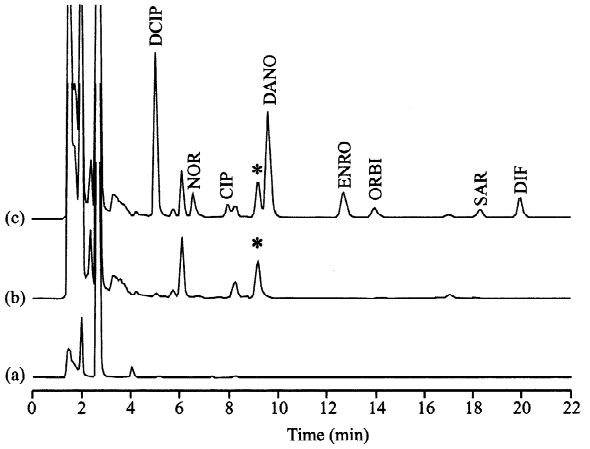

Fig. 3. Liquid chromatograms of an extract of (a) control chicken muscle; (b) control chicken liver; (c) chicken liver fortified with 50 ng/g DCIP, NOR, CIP, DANO, ENRO, ORBI, SAR and DIF; *50 ng/g lomefloxacin added.

10–200 ng/g. DCIP consistently gave lower recovery $\geq$45%, which may reflect its relatively high polarity. CIP in muscle provided lower recoveries at $\leq$25 ng/g, and NOR and SAR in liver were low at 10 ng/g. All other recoveries were in the range 60–93%. Relative standard deviations (RSDs) were excellent in all cases tested. Average recovery values for combined analysis sets at each fortification level were determined, with *n* ranging from 5 to 12. ANOVA calculations showed a number of instances, indicated in Table 2, where inter-day variation was statistically significant compared to intra-day variation. However, the RSDs associated with the average recovery values, combining both inter- and intra-day variation, are still very good, being predominant-

Table 3
FQ levels from incurred chicken tissue

Tissue	Day	Measured ENRO (ng/g)(RSD)	Measured CIP (ng/g)(RSD)	Dilution	Corrected ENRO (ng/g)	Corrected CIP (ng/g)
Liver	5	102 (6.5)	48.2 (7.3)	1:50	5100	2410
	7	103 (4.8)	39.1 (7.5)	1:50	5150	1960
	8	148 (2.0)	74.4 (2.8)	1:5	740	372
	10	70.8 (3.2)	25.1 (4.8)	–	70.8	25.1
Muscle	5	138 (5.1)	4.08 (1.41)	1:20	2760	81.6
	7	134 (5.8)	4.62 (3.62)	1:20	2680	92.4
	8	85.2 (1.2)	2.48 (5.94)	1:4	341	9.92
	10	28.8 (3.8)	–	–	28.8	–

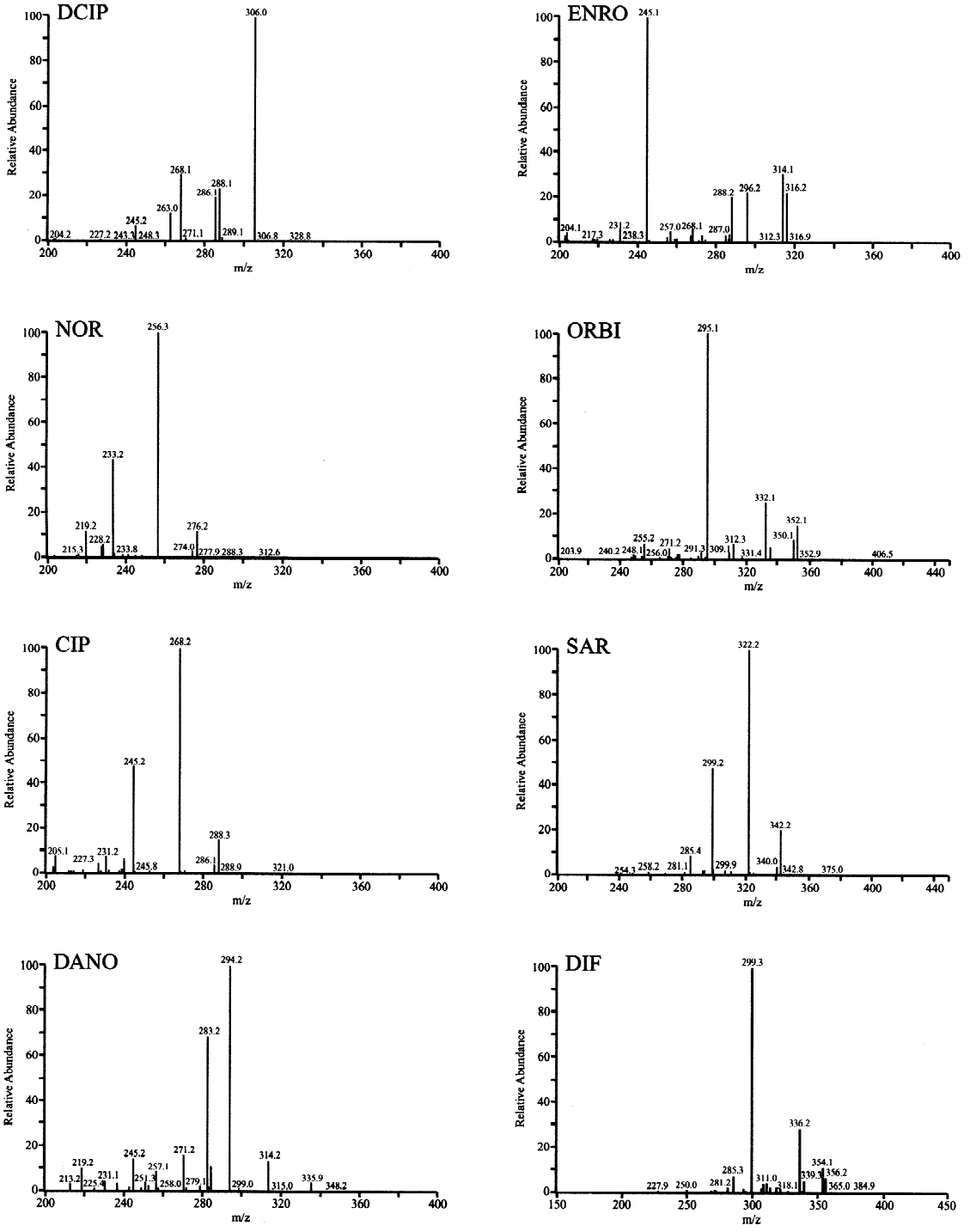

Fig. 4. MS^2 spectrum for DCIP; MS^3 spectra for NOR, CIP, DANO, ENRO, ORBI, SAR and DIF. These spectra are from a sample of chicken liver, fortified with a standard FQ mix at 50 ng/g.

Table 4
Confirmation of CIP and ENRO in chicken tissue samples

Tissue	Sample	CIP Peak ratio %245/268 (%RSD)	*n*	ENRO Peak ratio %288/245 (%RSD)	*n*
Liver					
	std FQ mix	46.0 (11.9)	32	18.1 (25.0)	24
	control liver	–	1	–	1
	Day 5 incurred	47.3 (6.4)	3	15.9 (20.6)	3
	Day 7 incurred	50.1 (11.9)	3	19.9 (13.3)	3
	Day 8 incurred	50.1 (10)	3	18.0 (9.5)	3
	Day 10 incurred	47.9 (3.6)	3	19.4 (9.6)	3
Muscle					
	std FQ mix	46.3 (11.4)	32	17.0 (14.9)	24
	control muscle	–	1	–	1
	Day 5 incurred	47.8 (0.2)	3	17.1 (3.8)	3
	Day 7 incurred	49.6 (11.3)	3	16.7 (4.8)	3
	Day 8 incurred	41.0 (30.4)	3	15.5 (16.4)	3
	Day 10 incurred	54.5 (71.7)	3	18.5 (48.8)	3

ly <10%. Fluorescence limits of detection for the FQs were determined as three times the root mean square of the noise divided by the slope of the standard curve and are as follows in liver and (muscle), in ng/g: DCIP 0.3 (0.1), NOR 1.2 (0.2), CIP 2 (1.5), DANO 0.2 (0.1), ENRO 0.3 (0.2), ORBI 1.5 (0.5), SAR 2 (0.6), DIF 0.3 (0.2). Standard curves were linear over the range of 2–125 ng/g.

This method was also tested with incurred tissue samples. The results are shown in Table 3 The incurred samples contained high enough levels of ENRO that dilution with control tissue was required for the ENRO levels to fall within the range of the calibration curve for this method. The ''corrected'' values in the last two columns of Table 3 represent the actual levels in the original sample after taking the dilution into account. The levels of ENRO remained high during days 5 and 7 of dosing. They dropped dramatically by the third day post-dose (day 10), in keeping with what would be expected for the required 2-day withdrawal period for the use of ENRO in chickens [20]. As in our previous work [11], the ENRO metabolite CIP was also detected in these samples using this method.

Analysis of incurred liver samples was performed using a new column (different lot), which required a slight modification of the chromatographic gradient. With this new column, two matrix peaks were not completely resolved from the FQs, resulting in a higher limit of detection for CIP and SAR (9 and 12 ng/g, respectively). All other limits of detection were comparable to those observed with fortified samples.

Confirmation of FQs was accomplished using peak ratios of prominent MS^3 (MS^2 for DCIP) product ions (Table 1). MS^n spectra for the eight FQs are shown in Fig. 4, and Table 4 outlines the confirmation of ENRO and CIP in ENRO-incurred samples of chicken liver and muscle, respectively. Data for days 8 and 10 in muscle are associated with quite high RSDs, due to the low levels of CIP and ENRO present.

Fig. 5 illustrates the generally decreasing trend of peak ratio variability with increasing sample concentration. The data in this figure represent peak ratios generated from standard curve samples run on the same day as incurred samples were analyzed. A limit of confirmation could be determined dependent upon a desired RSD value. Such limits of confirma-

Table 5
FQ limits of confirmation versus RSD of confirmation ion ratios

% RSD	Limit of confirmation (ng/g)							
	DCIP	NOR	CIP	DANO	ENRO	ORBI	SAR	DIF
10	75	10	50	50	50	125	50	125
15	20	10	50	50	50	50	20	50
20	10	5	2	20	50	5	20	20
30	10	2	2	20	50	5	5	20

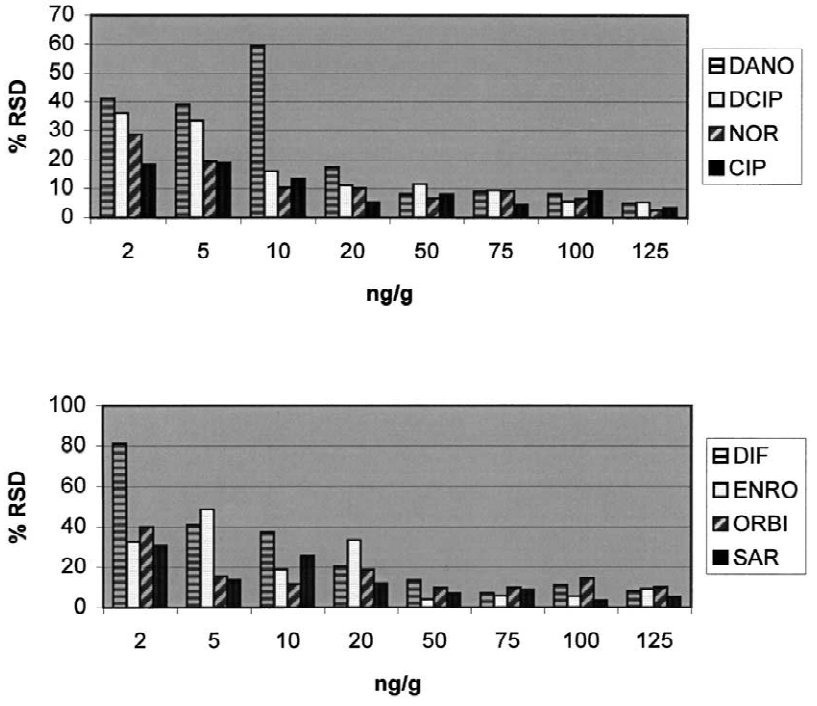

Fig. 5. FQ MS^n peak ratio variability dependence on concentration.

tion for the eight FQs are listed in Table 5. For example, selection of 20% RSD as an acceptable confirmation criterion would correspond to the following limits of confirmation (ng/g): CIP (2), NOR and ORBI (5), DCIP (10), DANO, SAR and DIF (20) and ENRO (50). Comparison of the ratios of incurred or unknown samples with those of standards can give valuable confirmatory information to accompany the quantitation obtained via fluorescence.

4. Conclusions

The coupling of on-line fluorescence with MS^n analysis allows for the simultaneous quantitation and confirmation of FQ antibiotics. This approach was developed for the efficient analysis of eight FQs in chicken liver or muscle tissue. Good recoveries from fortified samples were obtained over a range of 10–200 ng/g, with low limits of quantitation and excellent RSDs. MS^n peak ratio data were generated to allow for FQ confirmation, and limits of confirmation dependent on acceptable RSDs were determined. This method was successfully used to analyze ENRO-incurred chicken muscle and liver tissue samples.

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References

[1] World Health Organization Report No. WHO/EMC/ZDI/98, Geneva, Switzerland, 1998.
[2] M.D. Rose, J. Bygrave, G.W.F. Stubbings, Analyst 123 (1998) 2789.
[3] Fed. Reg. 62 (1997) 27944.
[4] T.B. Waggoner, M.C. Bowman, J. AOAC 70 (1987) 813.
[5] M. Horie, K. Saito, N. Nose, H. Nakazawa, J. Chromatogr. B 653 (1994) 69.
[6] R. Charriere, W. Leiser, R. Dousse, Trav. Chim. Aliment. Hyg. 87 (1996) 223.
[7] T.J. Strelevitz, M.C. Linhares, J. Chromatogr. B 675 (1996) 243.
[8] J.C. Yorke, P. Froc, J. Chromatogr. A 882 (2000) 63.
[9] C. Maraschiello, E. Cusido, M. Abellan, J. Vilageliu, J. Chromatogr. B 754 (2001) 311.
[10] E. Cohen, R.J. Maxwell, D.J. Donoghue, J. Chromatogr. B 724 (1999) 137.
[11] M.J. Schneider, J. Chromatogr. Sci. 39 (2001) 351.
[12] C.K. Holtzapple, S.A. Buckley, L.H. Stanker, J. Agric. Food Chem. 47 (1999) 2963.
[13] D. Barron, E. Jimenez-Lozano, J. Cano, J. Barbosa, J. Chromatogr. B 759 (2001) 73.
[14] D.A. Volmer, B. Mansoori, S.J. Locke, Anal. Chem. 69 (1997) 4143.
[15] J.B. Schilling, S.P. Cepa, S.D. Menacherry, L.T. Bavda, B.M. Heard, B.L. Stockwell, Anal. Chem. 68 (1996) 1905.
[16] S.B. Turnipseed, C.C. Walker, J.E. Roybal, A.P. Pfenning, J.A. Hurlbut, J. AOAC Int. 81 (1998) 554.
[17] B. Delepine, D. Hurtaud-Pessel, P. Sanders, Analyst 123 (1998) 2743.
[18] R.P. Schneider, J.F. Ericson, M.J. Lynch, H.G. Fouda, Biol. Mass Spectrom. 22 (1993) 595.
[19] M.J. Schneider, D.J. Donoghue, J. AOAC Int. 83 (2000) 1306.
[20] Code of Federal Regulations. Title 21, 6:520.813.