The Analysis of Fluoroquinolones in Beef Kidney Using HPLC Electrospray Mass Spectrometry

Application

Food

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Abstract

A fast and simple screening method was validated for the analysis of three fluoroquinolone antibiotics in beef kidney. Samples were extracted with acidified methanol, centrifuged, diluted with water, and filtered. The diluted extract was analyzed directly by HPLC mass spectrometry using electrospray ionization in positive ion mode. Using an internal standard, mean recoveries were 73%–96% at spiking levels of 33 µg/kg (ppb), with statistically derived detection limits of 8–19 µg/kg. This is below the European Union maximum residue limit of 200 µg/kg for enrofloxacin and ciprofloxacin in bovine kidney. The method is evaluated relative to the requirements of the European Commission Decision 2002/657/EC for use as a confirmatory method.

Introduction

Fluoroquinolones are synthetic antibacterial compounds derived from nalidixic acid, and are useful to treat animal infections that are resistant to other antibacterial agents. They have a broad spectrum of activity, acting against both gram-positive and gram-negative bacteria. The maximum residue limit (MRL) for enrofloxacin (as the sum of enrofloxacin and ciprofloxacin) was entered into Annex 1 of Council Regulation (EEC) No. 2377/90 for kidney at 200 µg/kg in bovine and ovine species, and 300 µg/kg for porcine, poultry, and rabbits. For all other food producing species, the MRL is 200 µg/kg in kidney [1].

There are a number of methods describing the analysis of fluoroquinolones in various tissues, with HPLC coupled with fluorescence and mass spectrometric detection being very popular. Most methods involve extraction into acidic or basic organic solvents, followed by some type of cleanup, most notably solid phase extraction (SPE). The Canadian Food Inspection Agency extracts animal tissue with acidic ethanol, followed by strong cation exchange SPE cleanup, and HPLC fluorescence analysis [2]. Chen and Schneider [3] described a screening method for enrofloxacin in chicken, where extracts were detected by fluorescence without cleanup, following extraction and centrifugation.

European Community Commission Decision 2002/657/EC allows the use of HPLC coupled with fluorescence detection [4] for substances in Group B of Annex I to Directive 96/23/EC. Quinolones and other veterinary drugs fall into Group B, where three identification points are required for confirmation by Selected Ion Monitoring (SIM) using mass spectrometry (MS). With low resolution HPLC/MS, one point can be earned for each ion detected, provided that the ion ratios meet relative intensity criteria. Additional requirements of Directive 2002/657/EC, based on spiking levels of 33 µg/kg carried out in this study, are as follows:

- The internal standard (IS) shall be added to the test portion at the beginning of the extraction procedure.
- In order to allow the use of data corrected for mean recovery, the range of recoveries allowed are –20% to +10%.
- The reproducibility of coefficient variation (CV) (%) is expected to be about one-half to two-thirds of the 100 µg/kg CV, which is 23%, at a concentration of half the permitted limit.
- For liquid chromatography/mass spectrometry (LC/MS) procedures, the minimum acceptable retention time (RT) for the analyte under examination is twice the RT corresponding to the void volume of the column.
- The ratio of the chromatographic RT of the analyte to that of the IS, that is, the relative RT of the analyte, shall correspond to that of the calibration solution at a tolerance of 2.5% for LC.
- The molecular ion shall preferably be one of the selected diagnostic ions.
- The maximum permitted tolerances for relative ion intensities shall meet the criteria in the Annex, (in this case, either ±25% or 30%), as reproduced in Table 6.

Experimental

Chemicals and Materials

HPLC-grade methanol and acetonitrile were purchased from Caledon Labs (Georgetown, Ontario).

Formic acid, min. 98%, was purchased from EM Science.

Acidified methanol solution: 30% methanol in pH 3 deionized water (100 µL of formic acid per 100 mL of water).

Acidified methanol was prepared by adding 100 µL of 98% formic acid to 100 mL of methanol.

Acidified deionized water was prepared by adding 100 µL of 98% formic acid to 100 mL deionized water.

Ultra-Turrax T25 homogenizer, 50-mL polypropylene centrifuge tubes, and 13-mm polyvinylidene fluoride (PVDF) syringe filters (0.2 µm), were purchased from VWR Scientific.

All fluoroquinolones, including the IS, were provided as a gift from the Canadian Food Inspection Agency, Calgary, Alberta, Canada, as stock solutions of 100 ng/µL (ppm) in 1% acetic acid in methanol. Solutions were stored at 4 °C. Standard solutions at different concentrations were prepared for spiking by dilution with acidified methanol solution. The analytes ciprofloxacin, enrofloxacin, and sarafloxacin were chosen as targets since these compounds are included in the Canadian Food Inspection Agency's proficiency check samples. The spiking standard for these compounds (1 ng/µL) was prepared by diluting 100 µL of each the stock solutions to a 10-mL volumetric flask, and made to volume with acidified deionized water. A separate IS solution at 1 ng/µL was prepared the same way, except that it only contained norfloxacin and danofloxacin.

Sample Preparation

1. For beef kidney, 3 g samples were weighed directly into 50-mL polypropylene centrifuge tubes.
2. For spiked samples, 100 µL of the 1-ng/µL (100 ng) spiking solution was added, resulting in fortification levels of 33 µg/kg. Samples were allowed to stand for 1 hour before subsequent extraction.
3. For the sample blank, 100 µL of acidified methanol solution was added.
4. For all spiked samples, 100 µL of the 1-ng/µL (100 ng) IS solution was added just prior to extraction. Norfloxacin was included in this solution at the same level, to be used as an alternate IS, if required due to potential interferences for danofloxacin.
5. The samples were homogenized for 2 min with 15 mL of acidified methanol using the Ultra-Turrax homogenizer.
6. The samples were then centrifuged for 10 min, and the supernatant decanted into a clean test tube.

7. The extract was diluted with acidified deionized water 1 in 4 (250 µL of extract + 750 µL of water), filtered through a 0.2-µm PVDF filter into an autosampler vial, and analyzed directly by LC/MS.

By adding an accurately known amount of IS to the initial sample before extraction, there is no need to measure the final volume of the extracts, nor the aliquot to be diluted. The IS calculations, performed by the ChemStation, measure the relative amounts of the analytes and IS. This corrects for any concentration or dilution effects in the samples.

Standard Preparation

A 5-point calibration curve was used for the determination of each of the three target compounds, and a 1-point curve was used for norfloxacin, the alternate IS. Table 1 gives the volumes of the IS and target solutions added (1 ng/µL each) to each of five test tubes. The standards were prepared by adding 250 µL of the blank extract and 750 µL of acidified deionized water to the tubes containing the analytes, after which the solutions were filtered through 0.2-µm PVDF filters.

The final solution of each standard contained 5 ng of IS per mL of diluted extract, or 5 pg/µL. With 50 µL injected, this results in 250 pg injected. The amount of target analyte in each of the five solutions varies to produce the calibration curves, as shown in Table 1.

The correlation coefficient (R^2) for the target analytes ranged from 0.9987 to 0.9992, as shown in Table 4.

Preparation of the standards in this fashion will compensate for any ion suppression or enhancement that may occur, due to the presence of co-eluting material at the MS source, which may not otherwise occur if pure solvents alone are used.

Table 1. Preparation of Analytical Standards (50-µL Injections into LC/MSD)

Standard	IS Volume added (µL)	Target volume added (µL)	IS Amount injected (pg)	Target amount injected (pg)
1	5	1	250	50
2	5	2	250	100
3	5	5	250	250
4	5	10	250	500
5	5	20	250	1,000

LC/MS Conditions

The HPLC system was made up of an Agilent Technologies 1100 series solvent degasser, binary pump, autosampler, column oven, diode array detector (DAD), and quadrupole mass selective detector (MSD) (Table 2).

Table 2. LC/MSD Conditions

HPLC	
Column	Zorbax Eclipse XDB-C8, 150 mm × 4.6 mm, 5 µm (P/N 993967-906)
Solvent A	0.1% Formic acid in water
Solvent B	0.1% Formic acid in acetonitrile
Gradient	t_0 = 20% B t_1 = 20% B t_8 = 90% B t_{15} = 90% B Post time = 2.0 min
Flow rate	0.4 mL/min
Injection volume	50 µL
Column temp	30 °C
MSD	
Source	Electrospray Ionization (ESI) (positive ion mode)
Ion dwell time	14 ions at 40 ms each
Fragmentation	Varies by ion, see Table 3
Drying gas flow	12 L/min
Nebulizer pressure	30 psi
Drying gas temperature	350 °C
Capillary voltage	4000 V

Table 3. Fragmentor Voltages for Acquired Ions in SIM (single acquisition group)

Compound	Ion	Fragmentor (V)
Norfloxacin (IS)	320	120
	302	200
	276	200
Ciprofloxacin	332	120
	314	200
	288	200
Danofloxacin (IS)	358	120
	340	220
Enrofloxacin	360	120
	342	220
	316	220
Sarafloxacin	386	120
	368	220
	342	220

All ions were included in a single acquisition group, which started at injection (time = 0). An alternative approach would be to set the group start time to a value around half a minute before the elution of the first compound, as this will keep the eluant stream diverted to waste as long as possible. This will reduce the amount of co-extracted material being introduced into the source, reducing contamination.

Another alternative is to add an additional time-programmed acquisition group to the method, and only include the ions for compounds eluting within the group times. This will take on more significance as the overall number of compounds in a method increases, and with three ions per compound required for identity confirmation.

Fragmentor voltages were chosen that maximized the response for each selected ion. For each fluoroquinolone, a value of 120 V produced only the protonated parent ion, while higher voltages were required to induce fragmentation to confirmatory ions. The ions monitored corresponded to the neutral losses of water and carbon dioxide in each case.

Note that although mass 342 is acquired for both enrofloxacin and sarafloxacin, it is only added to the MSD acquisition table once.

Chromatography

All compounds eluted between 5 and 9 minutes, however the total run time was set to 15 minutes with 90% organic solvent to allow co-extractives to elute from the column. Otherwise, their eventual elution could interfere with subsequent injections. This is more of a potential problem when methods with abbreviated cleanups, such as dilution-only, are used. The following figures compare the blank beef kidney sample to a sample fortified at 33 µg/kg. In each case, the selected ions are the protonated forms of the parent ion, as well as the protonated ions resulting from the loss of H_2O (M-18) and CO_2 (M-44).

The qualifier ion for danofloxacin, the compound used as the IS for this study, is mass 340. The matrix causes an interference at mass 340. The interference is shown as a small peak in the beef kidney blank as shown in Figure 1. Since a diagnostic qualifier ion is not required for the IS calculations, it had no impact on the results. It does, however, indicate that there is elution of co-extractive material in the samples, and that without further cleanup, ion suppression may result from its presence. All standards were prepared in blank beef kidney extract in order to compensate for these potential effects.

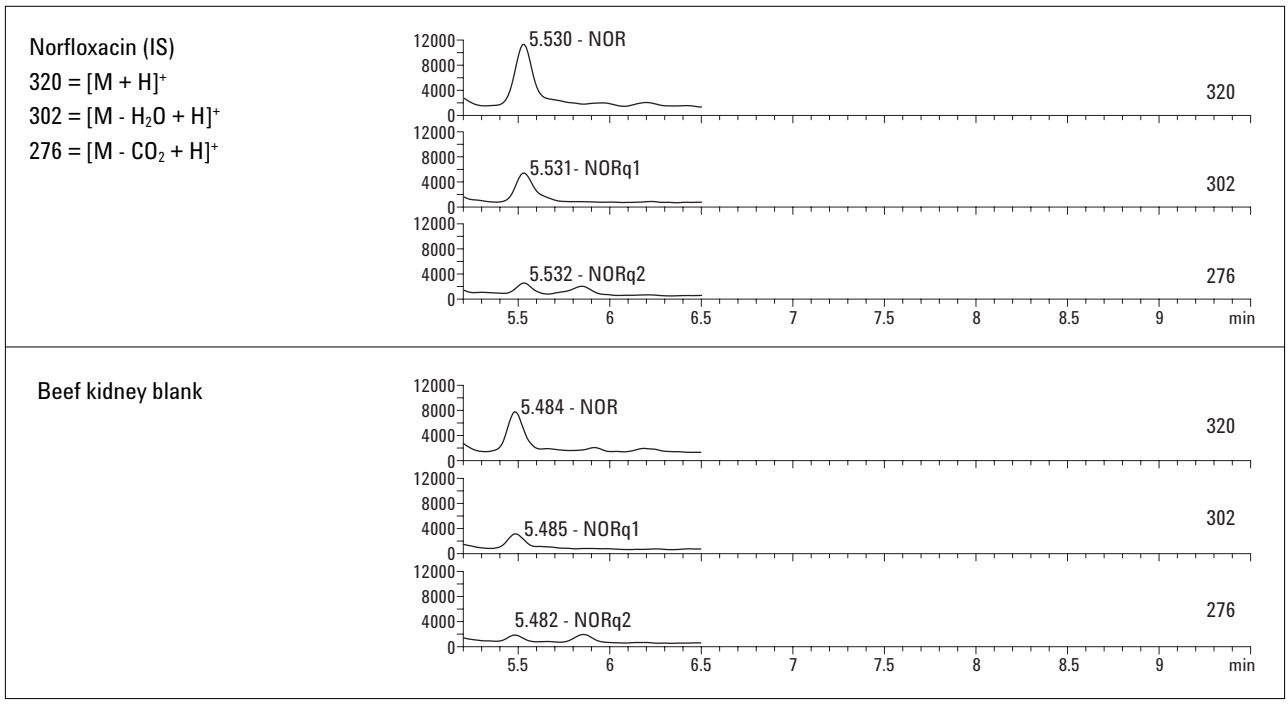
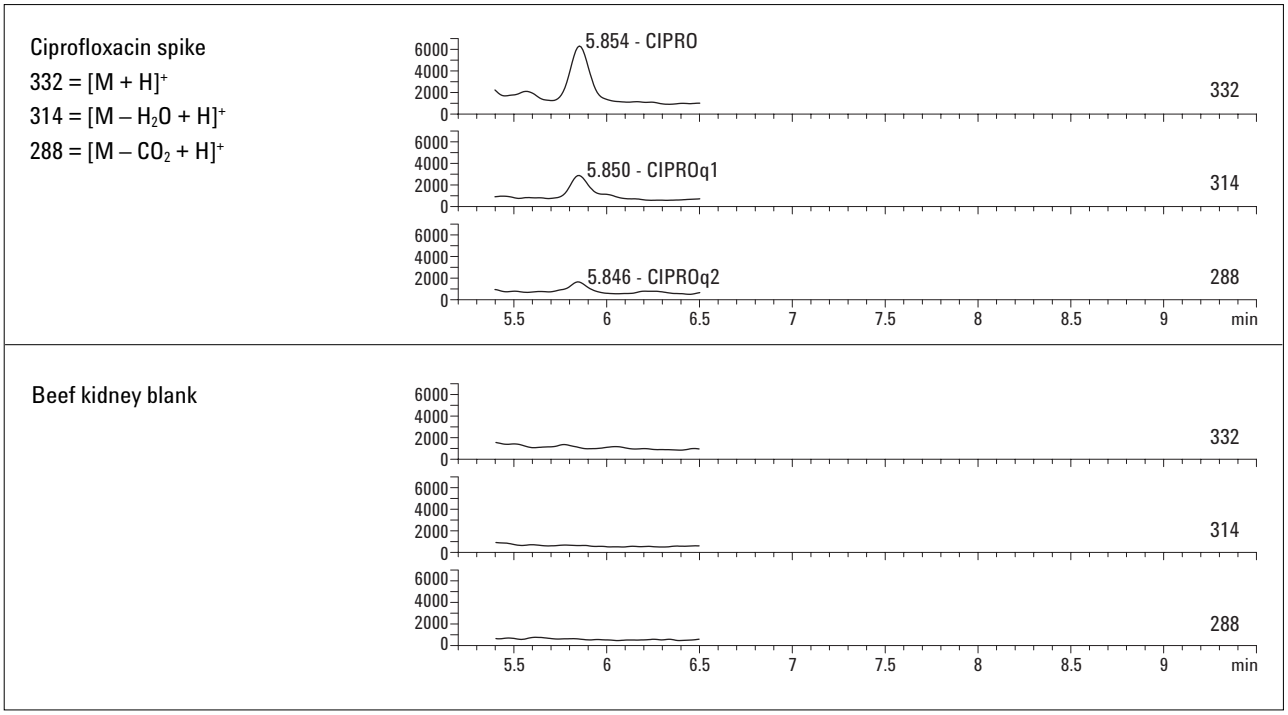


Figure 1. Comparative extracted ion chromatograms for fluoroquinolones spiked into beef kidney.



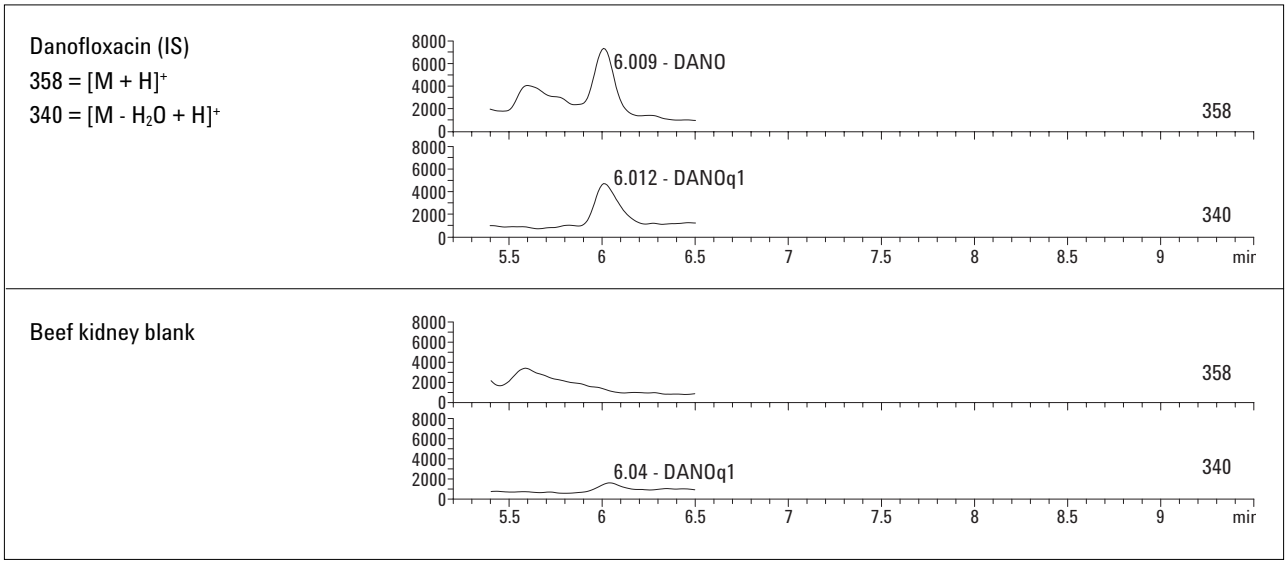
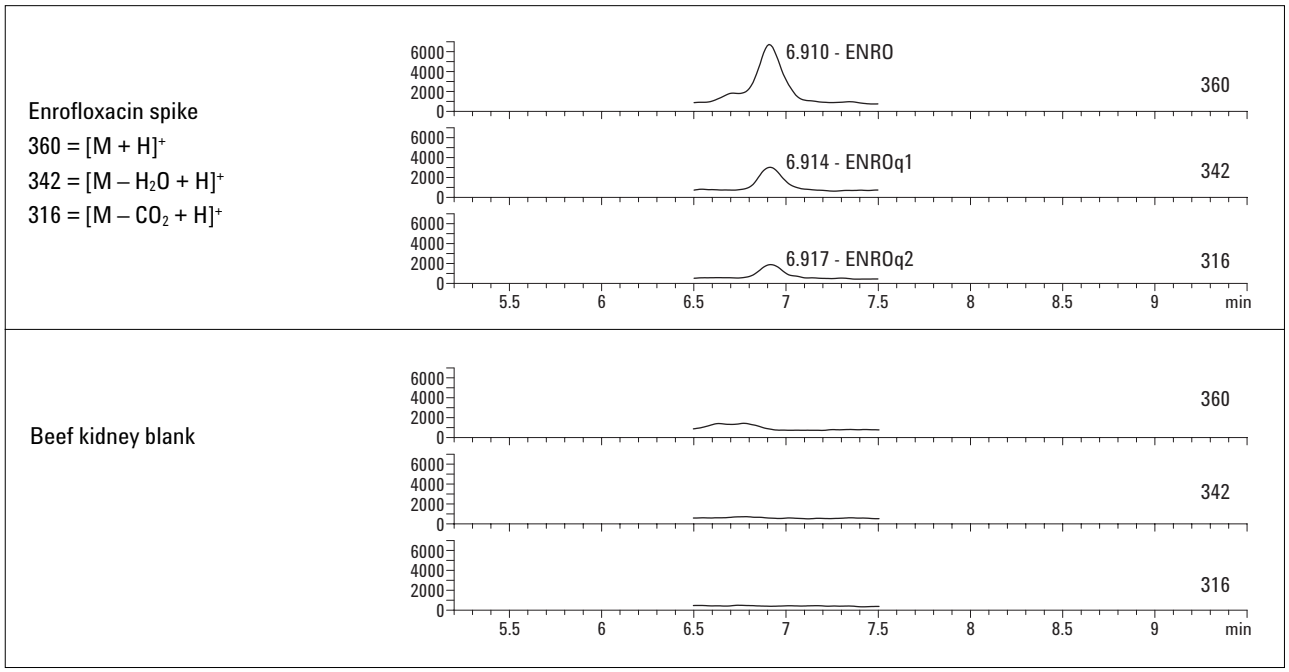


Figure 1. Comparative extracted ion chromatograms for fluoroquinolones spiked into beef kidney (Continued).



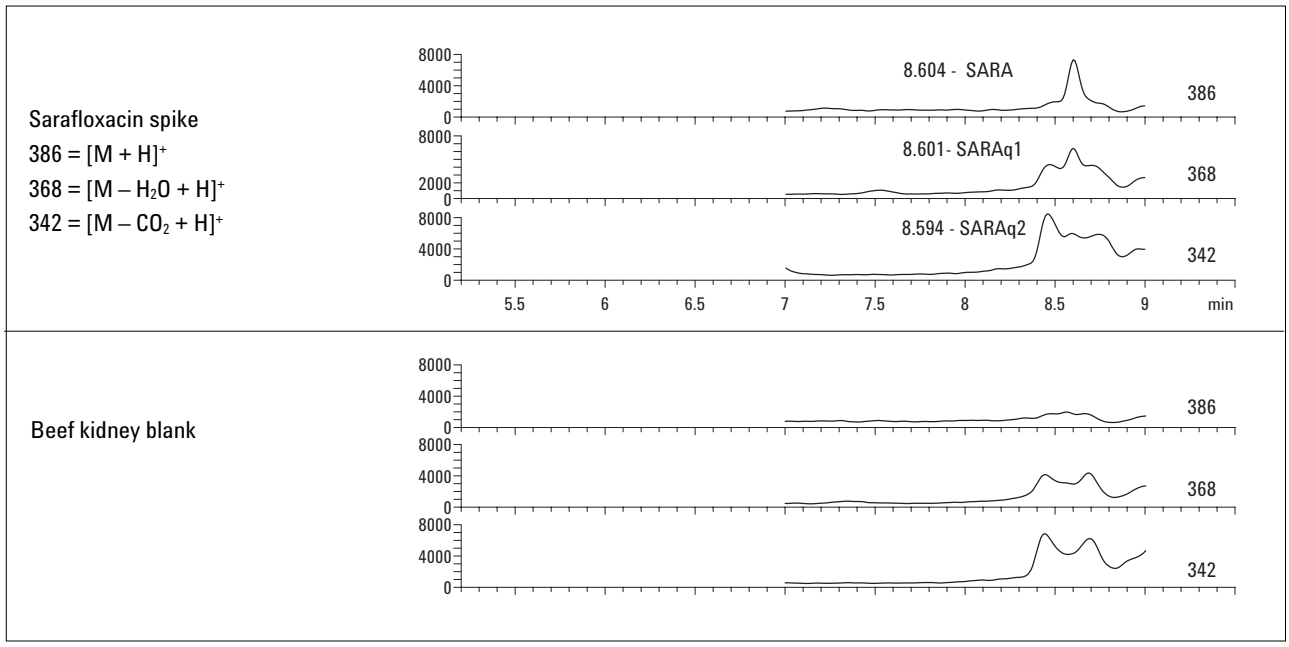


Figure 1. Comparative extracted ion chromatograms for fluoroquinolones spiked into beef kidney (Continued).

Sarafloxacin elutes from the column in the same region as a number of other co-extractives, making identification and quantitation more difficult. However, as shown in Table 7, the qualifier ions still meet the identification criteria for relative responses of the qualifiers, and so further cleanup of the samples may not be necessary. The effect of these co-extractives will also be reduced at higher incurred residue levels, closer to those permitted by the European Union MRL.

Recoveries

In order to allow results to be corrected for recoveries, where the determined incurred levels are divided by the percent recovered from certified reference materials or spiked samples, Table 2 of the Annex requires that the recoveries for analytes at levels greater than 10 µg/kg be within the range of 80% to 110%. Table 4 shows that recoveries for ciprofloxacin and enrofloxacin meet this requirement, with 96.3% and 86.0%, respectively. However, sarafloxacin fails the requirement, with only 72.6% mean recovery. With a CV of only 8% for this compound, it looks as though the method may still produce acceptable results for screening purposes, but some additional work may be required to produce higher recoveries. Since the work presented here involves spiked samples only, recovery-correction calculations do not apply.

Norfloxacin was added along with danofloxacin as an additional IS. However, examination of the blank beef kidney used in this study shows norfloxacin to be present as an incurred residue, at a concentration approximately one half of the spiking level. Assuming a linear response through the origin, this would mean that norfloxacin was detected at approximately 15–20 µg/kg, which is about 10% of the permitted level for enrofloxacin in bovine kidney. Recoveries for norfloxacin are included in Table 4, even though they were calculated with a single point calibration, and not corrected for incurred residues. However, there is some compensation for this since the standards used for calibration were prepared by addition of the targets to the blank extracts.

Table 4. Recoveries of Fluoroquinolones from Beef Kidney

	Amount recovered (ng)			
Description	**Norfloxacin**	**Ciprofloxacin**	**Enrofloxacin**	**Sarafloxacin**
Kidney spike 1	111.8	96.3	84.9	68.5
Kidney spike 2	93.1	94.0	85.6	64.1
Kidney spike 3	88.0	89.6	83.8	77.6
Kidney spike 4	98.9	95.4	86.2	75.2
Kidney spike 5	82.2	93.8	85.4	82.1
Kidney spike 6	143.0	109.3	87.9	72.9
Kidney spike 7	102.6	101.3	83.3	73.0
Kidney spike 8	110.6	90.8	91.3	67.7
Amount spiked (ng)	100.0	100.0	100.0	100.0
Mean	103.8	96.3	86.0	72.6
SD (Precision) ng	18.9	6.3	2.5	5.8
MDL (SD × t-stat) ng	56.7	19.0	7.6	17.4
LOQ (SD × 10) ng	189.1	63.4	25.4	58.1
CV (SD/Mean) %	18.2	6.6	3.0	8.0
Accuracy (%)	103.8	96.3	86.0	72.6
Linearity (R^2)	0.9895	0.9987	0.9992	0.9987
t-stat (N = 8)	3.00	3.00	3.00	3.00

Compound Identification

For chromatographic separation, Section 2.3.3.1 of the Annex to 2002/657/EC requires that the minimum acceptable RT for the analyte under investigation be at least twice the RT corresponding to the void volume of the column (k'=1). The first compound to elute under these conditions is norfloxacin, with a k' of 2.6, therefore this condition is easily met. The second condition is that the ratio of the RT of the analyte to that of the IS, that is the relative RT, shall correspond to that of the calibration solution at a tolerance of ±2.5% for LC. Table 5 shows the RT times of each analyte in the spiked samples, compared to those of the standards, and that they are well within the allowable tolerance.

Table 5. Relative RTs of Analytes in Samples, Compared to Standards

Compound	Average RRT in standards (N = 15)	CV (%) RRT in standards (N = 15)	RRT in samples, relative to standards (N = 8)
Norfloxacin	0.922	0.12%	99.8%–100.1%
Ciprofloxacin	0.975	0.05%	99.9%–100.1%
Enrofloxacin	1.150	0.16%	99.8%–100.2%
Sarafloxacin	1.439	0.47%	99.5%–100.3%

Compound Confirmation

Section 2.3.3.2 of the Annex to 2002/657/EC gives the maximum permitted tolerances for relative ion intensities, which is reproduced in Table 6.

Table 6. Maximum Permitted Tolerances for Relative Ion Intensities Using a Range of Mass Spectrometric Techniques

Relative intensity (% of base peak)	GC/MS(EI) (relative)	GC/MS(CI), GC/MS^n, LC/MS, LC/MS^n (relative)
>50%	±10%	±20%
>20% to 50%	±15%	±25%
>10% to 20%	±20%	±30%
≤10%	±50%	±50%

Note MS^n equals MS/MS if n = 2

Table 7 shows the relative intensities for each of the qualifier ions for the three target compounds, as well as norfloxacin and danofloxacin (one ion). As expected, norfloxacin meets the criteria in each of the eight spiked samples, even though it had incurred residues. The presence of additional norfloxacin should not negatively affect this qualitative aspect of performance, and it does not. Danofloxacin, however, showed an interference for the single qualifier ion monitored, and so the relative amount of this signal would be expected to vary to a larger degree, depending upon the exact amount of blank extract used in preparing the sample dilutions and standards. As previously mentioned, the standards are prepared by accurately measuring the relative amounts of target and IS compounds into a tube or vial, followed by addition of blank kidney extract and water. The exact proportions of extract and water do not have to be known, since the IS calculations uses amount and response ratios, rather than absolute amount and response, in determining concentrations in unknowns. An accurate measurement of extract and water volumes can, however, reduce interference variability.

Table 7. Relative Intensities of Qualifier Ions for Fluoroquinolones in Beef Kidney, Compared to Permitted Tolerances

	Relative intensities (%) of qualifier ions								
	Norfloxacin		**Ciprofloxacin**		**Danofloxacin**	**Enrofloxacin**		**Sarafloxacin**	
Sample	**Q1 = 302**	**Q2 = 276**	**Q1 = 314**	**Q2 = 288**	**Q1 = 340**	**Q1 = 342**	**Q2 = 316**	**Q1 = 368**	**Q2 = 342**
Spike 1	49	15	47	17	64	44	28	50	15
Spike 2	45	15	48	17	58	46	24	41	17
Spike 3	48	16	46	20	63	44	28	45	14
Spike 4	42	15	46	17	60	43	30	43	13
Spike 5	49	17	45	21	65	44	26	45	11
Spike 6	50	19	39	17	72	45	29	43	12
Spike 7	49	17	41	18	65	46	29	46	13
Spike 8	47	17	42	19	62	39	24	46	12
Average for Stds	49	20	44	20	86	43	26	47	15
Std Dev for Stds	2	1	3	2	22	2	2	6	1
Tolerance(Table 7)	25	30	25	30	20	25	25	25	30
Lower Allowable (calculated)	**37**	**14**	**33**	**14**	**69**	**32**	**19**	**35**	**11**
Upper Allowable (calculated)	**62**	**26**	**55**	**25**	**103**	**53**	**32**	**59**	**20**

Conclusion

A fast and sensitive single quadrupole LC/ESI/MS method was validated for the detection of three fluoroquinolone antibiotics (ciprofloxacin, enrofloxacin, and sarafloxacin) in beef kidney. The detection limits ranged from 8 to 19 µg/kg (ppb), with direct analysis of sample extracts after dilution with water. All qualitative requirements were met with respect to the Annex to EU Directive 2002/657/EC for spiked samples, and recoveries of two of the three compounds met the quantitative requirements. Recovery of sarafloxacin was slightly lower than the level required to allow correction for recoveries in reported results.

References

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