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Determination of quinolones and fluoroquinolones in fish tissue and seafood by high-performance liquid chromatography with electrospray ionisation tandem mass spectrometric detection

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Abstract

A reversed-phase high-performance liquid chromatographic method with tandem mass-spectrometric detection was developed and validated for the simultaneous analysis of eight quinolones and fluoroquinolones (oxolinic acid, flumequine, piromidic acid, enrofloxacin, ciprofloxacin, danofloxacin, sarafloxacin and orbifloxacin) in trout tissue, prawns and abalone. The analytes were extracted from homogenised tissue using acetonitrile and the extracts subjected to an automated two-stage solid-phase extraction process involving polymeric reversed-phase and anion-exchange cartridges. Good recoveries were obtained for all analytes and the limit of quantification was 5 μg/kg (10 μg/kg for ciprofloxacin). The limit of detection was 1–3 μg/kg, depending on the analyte and matrix. Confirmation of the identity of a residue was achieved by further tandem mass-spectrometric analysis. A procedure for estimating the uncertainty associated with the measurement is presented.

Keywords: Fish; Food analysis; Quinolones; Fluoroquinolones

1. Introduction

There is increasing concern internationally that indiscriminate use of antibiotics has led to increased bacterial resistance to many important drugs. As well as being a result of misuse of antibiotics and antibacterials by humans, bacterial resistance arises from use of the drugs in food-producing animals, leading either to ingestion of residues by humans or to development of drug resistance in bacteria in the animal, which is then passed on [1,2].

Quinolones and fluoroquinolones are important synthetic antibacterials, used in human and veterinary medicine [3,4]. In humans they are used to treat a range of diseases, including urinary tract infections. Around the world, various quinolones find application in most types of farmed animals, and in aquaculture. Some are also used to treat diseases in companion animals. In Australia, none of these compounds are registered for use on farmed fish without a specific permit or prescription.

According to the Australia and New Zealand Food Standards Code, residues of quinolones and fluoroquinolones must not be detectable in any foodstuff for domestic consumption. The only exception is that a maximum residue limit is set for oxolinic acid in

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pacific salmon at 0.01 mg/kg. Oxolinic acid, piromidic acid and flumequine are members of the older generation of quinolone drugs which are less efficient but still regularly used in veterinary medicine. Enrofloxacin, ciprofloxacin, danofloxacin and sarafloxacin are later-generation fluoroquinolones, some of which are used in human medicine; and orbifloxacin, one of the newest drugs, is currently registered in Australia for use in cats and dogs. The structures of these compounds are shown in Fig. 1. The aim of this study was to develop a multiresidue method for detection of these eight compounds, incorporating the detection of nalidixic acid as a surrogate, to monitor the extraction process. Nalidixic acid is one of the earliest-known members of the quinolone class and is no longer used due to its limited activity [4].

Methods of analysis of quinolones and fluoroquinolones previously reported in the literature generally consider only one or two analytes [5–9], involve liquid–liquid extraction (often using chloroform) [7–11] and use fluorescence or UV detection [6,8–12]. Some multiresidue methods have been described for analysis of quinolones [10,13] and fluoroquinolones [14] but very few that detect both quinolones and fluoroquinolones. Those that do require either two extraction procedures or three sets of chromatographic conditions to deal with the chemically different subgroups [11,12]. A number of MS methods have been reported for detection of various combinations of quinolones, fluoroquinolones and other antibacterials [14–20] but none is reported in conjunction with a suitable extraction for quinolones and fluoroquinolones from fish or seafood [5].

The method described here detects a wide range of quinolones using a single extraction and chromatographic analysis with mass spectrometric detection. Confirmation of all residues is possible using the same chromatographic conditions and the same

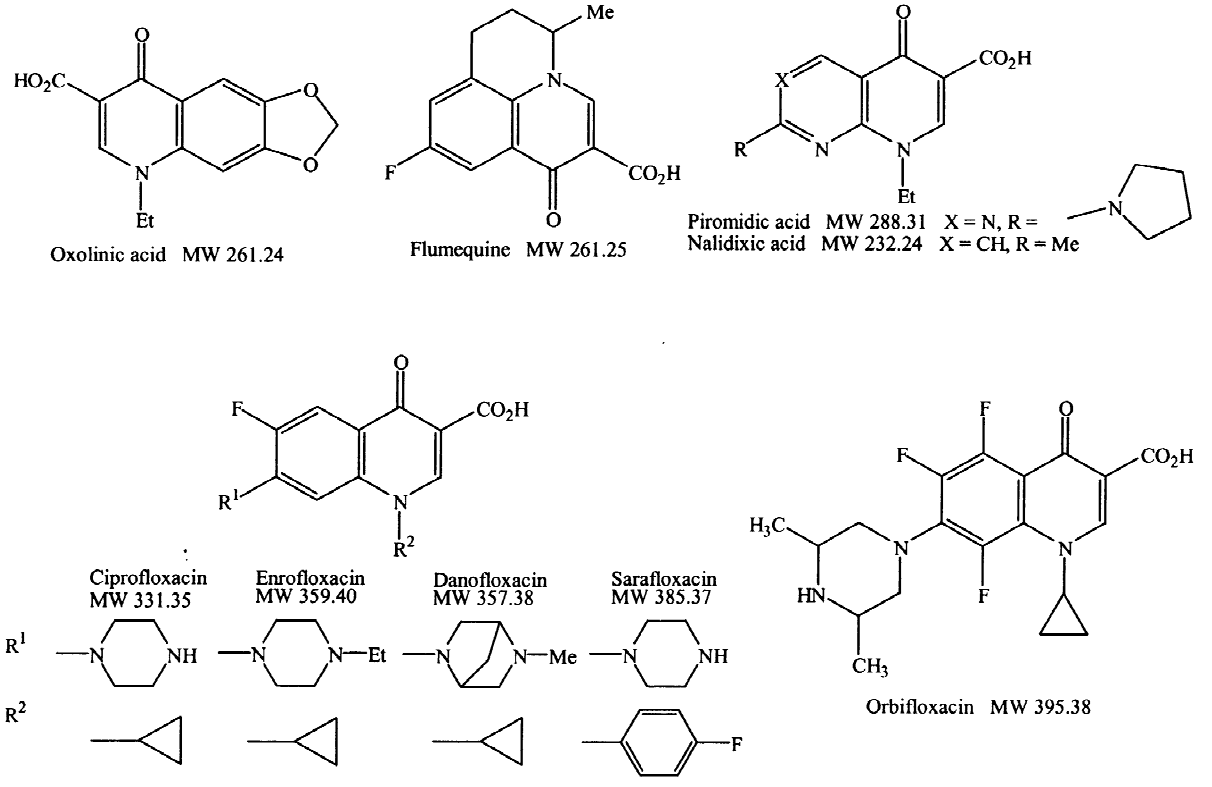

Fig. 1. Molecular structures of some quinolones and fluoroquinolones.

extract. An automated solid-phase extraction (SPE) process is used to clean up extracts, using minimal solvent and avoiding the use of chlorinated solvents, which are toxic, expensive and expected eventually to be phased out by law. The use of tandem MS, as described here, is highly selective. Strict European Union (EU) guidelines for confirmatory techniques state that LC–MS–MS monitoring two or three transition-product ions gives sufficient data to confirm the identity of a residue. Other methods, such as LC with UV or visible-wavelength spectrophotometric detection, must be combined with another technique to satisfy the criteria [21]. The confirmation method reported here complies with these guidelines.

2. Experimental

2.1. Chemicals and reagents

Methanol (LC grade) was obtained from Mallinckrodt (Paris, KY, USA), acetonitrile (LC grade) was obtained from EM Scientific (Merck, Darmstadt, Germany). Formic acid (98%, analytical reagent grade) was supplied by Ajax Chemicals (Sydney, Australia). Sodium hydroxide pellets (analytical reagent grade) were supplied by BDH (Kilsyth, Australia). Ultrapure water was filtered through a Milli-Q system (Millipore, Bedford, MA, USA).

Nalidixic and piromidic acids (>99%) and flumequine (99.9%) were purchased from Sigma (St. Louis, MO, USA). Ciprofloxacin (99.9%) was supplied by Bayer (Leverkusen, Germany), danofloxacin mesylate (74.5%) by Pfizer (Groton, CT, USA), sarafloxacin hydrochloride (88.5%) by Abbott (North Chicago, IL, USA) and orbifloxacin (99.8%) by Schering-Plough (Union, NJ, USA). Oxolinic acid (99%), enrofloxacin (99.0%), penbutolol sulfate (100%) and clenbuterol hydrochloride (98%) were obtained through NARL-AGAL (Sydney, Australia).

Supelclean ENVI Chrom P cartridges (3 ml, 0.25 g) and empty 3-ml filtration tubes and frits for preparing cartridges were obtained from Supelco (Bellefonte, PA, USA). AG MP-1 resin (200–400 mesh) was obtained from Bio-Rad (Hercules, CA, USA). AG MP-1 cartridges were prepared by packing 0.25 g of resin into the empty 3-ml tubes between two frits.

2.2. Equipment

A Sonifier 450 sonic probe (Branson, Danbury, CT, USA), an Ystral X1020 disperser (Ystral, Dottingen, Germany), a REAX2 end-over-end rotator (Heidolph, Germany) and a 2161 Midispin R centrifuge (LKB, Bromma, Sweden) were used for sample extraction. A Turbovap LV Evaporator (Zymark, Hopkinton, MA, USA), Aspec XL4 automated SPE equipment (Gilson, Villiers-le-Bel, France) and a Vortex Genie 2 vortexer (Scientific Industries, Bohemia, NY, USA) were used in sample clean-up. An AE240 analytical balance (Mettler, Greifensee, Switzerland) and an ultrasonic bath (Unisonics, Sydney, Australia) were used in the preparation of standard solutions.

A 2690 Alliance Separations Module (Waters, Milford, MA, USA) integrated autosampler, solvent delivery system and column heater and a Quattro LC triple-quadrupole mass spectrometer (Micromass, Manchester, UK) were used for LC–MS–MS analysis.

2.3. Standard solutions

Stock solutions of clenbuterol at 500 μg/ml and penbutolol at 250 μg/ml were prepared in methanol–water (1:1). These were diluted and combined to prepare a mixed internal standard (I.S.) solution containing 0.5 μg/ml clenbuterol and 0.05 μg/ml penbutolol in acetonitrile–water (1:4).

Stock solutions of the quinolones and fluoroquinolones at 100 μg/ml were prepared by dissolving the standard in a solution of methanolic sodium hydroxide (1 ml of 1 *M* aqueous sodium hydroxide/l methanol), with sonication. Stock solutions were stored in the dark at 4 °C for up to 3 months. Under these conditions, most of these analytes have been reported to be stable [11]. Mixed-analyte working and calibration standard solutions were freshly prepared for each analysis.

2.4. Sample preparation and spiking

Whole, cleaned fish was filleted, the skin and

bones removed, and the tissue pureed in a food processor. Whole abalone or prawns with the heads and tails removed were pureed with a hand-held food mixer. Portions (1.5±0.01 g) were weighed into 50-ml polypropylene centrifuge tubes and kept frozen until analysis. Fortified samples were prepared by adding the appropriate volume (15–120 μl) of mixed-analyte spiking solution (5 μg/ml) to thawed or partially thawed samples then refreezing overnight before analysis.

2.5. Extraction procedure

To extract residues from trout or abalone, the frozen sample was thawed and nalidixic acid added at 200 μg/kg as a surrogate. To provide sufficient extract to prepare the series of calibration standards, at least two blank samples not spiked with nalidixic acid were required. Acetonitrile (5 ml) was added to the sample and the tissue disrupted using the sonic probe (3 min, 30% duty cycle, 40% power) prior to mixing by rotation for 10 min and centrifuging at 3000 rev./min for 5 min. A second extraction was performed using additional acetonitrile (5 ml) and 10 min rotation. The combined acetonitrile extracts were evaporated to ~4 ml under nitrogen at 45 °C.

The procedure for extraction of residues from prawn tissues differed only in that the Ystral disperser (1 min, 20% power) was used to disrupt the tissue.

SPE clean-up of the acetonitrile extracts was automated, using a Gilson Aspec XL4. The acetonitrile extracts were passed through ENVI Chrom P cartridges conditioned with methanol (2 ml) and acetonitrile (2 ml), and the cartridges eluted with a further 1 ml of acetonitrile. The acetonitrile extracts were then diluted to 40 ml with aqueous sodium hydroxide (0.008 *M*) and this basic solution loaded onto AG MP-1 resin cartridges (previously conditioned with methanol, water and aqueous sodium hydroxide (0.008 *M*, 5 ml)). After washing with water (2 ml) and methanol (2 ml), the cartridges were dried with a stream of nitrogen and eluted with acetonitrile–aqueous 2% formic acid (1:4, 3 ml). Mixed I.S. (90 μl) was added to the final extracts.

2.6. LC and MS–MS conditions

Chromatographic separation was achieved on a Zorbax Extend C_{18} column (Agilent, 150×2.1 mm, 5 μm) maintained at 30 °C. Mobile phase A was aqueous 2% formic acid, mobile phase B was acetonitrile (filtered, 0.2 μm PTFE filter) and mobile phase C was Milli-Q water (filtered, 0.2 μm cellulose nitrate filter). The gradient program consisted of a constant 10% mobile phase A with 20% mobile phase B, 70% mobile phase C for 3 min, increasing to 55% B, 35% C by 3.1 min, holding at 55% B until 10 min, returning to 20% B by 10.1 min and holding at 20% until 17 min. The flow-rate was 0.2 ml/min and the injection volume was 10 μl. All analytes eluted in less than 12 min.

The mass spectrometer was operated in positive-ion mode, with ultra-high purity (UHP) nitrogen as the nebuliser and drying gas (~100 and 700 l/h, respectively). UHP argon was used as the collision gas with a collision-cell gas pressure of 1×10^{-3} mBar. The source block and desolvation temperatures were set at 110 and 350 °C. The MS–MS transitions were monitored in two functions, the first with six and the second with five channels. Details are given in Table 1. Dwell time was 0.15 s for all transitions, with an interchannel delay of 0.03 s.

2.7. Calibration and calculations

I.S.s were added to the extract before LC–MS analysis to correct for run-to-run variations in injection volume and instrument response. Clenbuterol was used as the I.S. for the fluoroquinolones eluting in the first 6 min, and penbutolol was used as the I.S. for the remaining analytes eluting between 6 and 12 min (see Fig. 2). Matrix-matched calibration standards at six levels from 2 to 200 ng/ml were used to prepare quadratic calibration curves for all analytes, by plotting the ratio of the analyte response to the appropriate I.S. response against concentration. The dilution factor was taken into account when calculating the concentration of analytes in the samples. Nalidixic acid surrogate recovery for a sample should be better than 60% to ensure that the extraction was performing within expected parameters.

2.8. Confirmation procedure

LC and MS–MS conditions were identical to those used for the quantification analysis except that three

Table 1
Conditions for the MS–MS detection of quinolones and fluoroquinolones

Retention window: 0 to 6 min				Retention window: 6 to 12 min			
Analyte	Transition (m/z)	Cone voltage (V)	Collision energy (eV)	Analyte	Transition (m/z)	Cone voltage (V)	Collision energy (eV)
Clenbuterol (I.S.)	277.00>203.00	20	15	Nalidixic acid	233.09>187.05	28	28
Ciprofloxacin	332.15>288.07	40	16	Oxolinic acid	262.12>160.02	28	40
Danofloxacin	358.08>95.91	38	22	Flumequine	262.14>202.03	28	34
Enrofloxacin	360.24>316.14	40	16	Piromidic acid	289.17>243.06	34	28
Sarafloxacin	386.20>299.06	40	28	Penbutolol (I.S.)	292.27>236.13	28	16
Orbifloxacin	396.36>352.11	32	16				

MS–MS transitions were monitored for each analyte instead of one. The ratios of the peak areas of the two less-intense product ions to the strongest ion were calculated. The transitions and expected ratios (determined from replicate analysis of a 100 ng/ml standard) are shown in Table 2. The nalidixic acid surrogate and clenbuterol and penbutolol I.S.s were monitored using the reactions specified in the quantification method (see Table 1) to ensure that the chromatography was performing as expected. The ratios of the product ions in the sample were compared with the ratios in a standard or an extract of a sample spiked at a similar concentration to the sample to be confirmed. If the S/N ratio for all peaks used was $\geq$3:1 and the ratios were the same within appropriate tolerances, the sample was declared positive. Suggested tolerances are based on EU guidelines and range from $\pm$20% for peaks greater

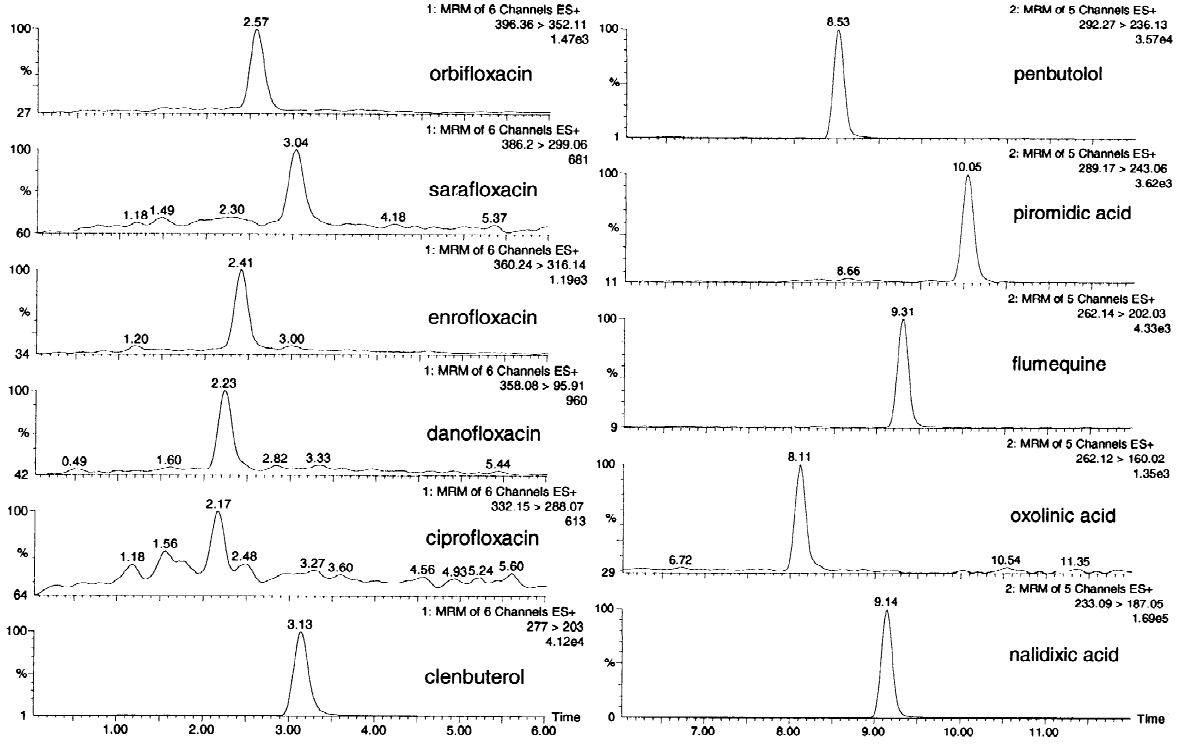

Fig. 2. LC–MS–MS chromatograms (Zorbax Extend C_{18} column) of extract of trout spiked at 5 μg/kg (LOQ/2 for ciprofloxacin, LOQ for all other analytes). Time scale in minutes.

Table 2
Conditions for the MS–MS confirmation of quinolones and fluoroquinolones, including peak-area ratios obtained from replicate analysis of 100 ng/ml standards

Analyte	Cone voltage (V)	Transition (collision energy) (m/z, eV)			Ratio
Oxolinic acid	28	262>244 (35)	262>160 (40)	262>215 (35)	100:23:59
Flumequine	28	262>202 (34)	262>126 (46)	262>174 (40)	100:36:15
Piromidic acid	34	289>243 (28)	289>201 (35)	289>173 (35)	100:21:9
Ciprofloxacin	40	332>314 (25)	332>245 (25)	332>288 (25)	100:94:51
Enrofloxacin	40	360>316 (16)	360>245 (30)	360>342 (30)	100:56:25
Danofloxacin	38	358>96 (22)	358>82 (35)	358>255 (40)	100:21:11
Sarafloxacin	40	386>299 (28)	386>342 (22)	386>368 (22)	100:88:69
Orbifloxacin	32	396>295 (25)	396>352 (16)	396>254 (25)	100:99:5

than 50% of the base peak to ±50% for peaks less than or equal to 10% of the base peak [21].

3. Results and discussion

3.1. LC and MS method development

During LC method development, the LC mobile phases investigated were methanol with aqueous acetic acid and acetonitrile with aqueous formic acid (both suitable for electrospray ionisation). Under these acidic conditions, the nine quinolones studied fell into two groups with regard to chromatographic behaviour. The older quinolones were well-retained on C_{18} columns but the newer fluoroquinolones were eluted quickly and with some overlap amongst the five peaks. Barbosa et al. have optimised the separations of various fluoroquinolones by careful control of the pH of phosphoric acid mobile phases [22,23]. These mobile phases are unsuitable for use with electrospray MS. However, the selectivity of the detector allows co-chromatographing compounds to be detected separately, by monitoring different ions simultaneously, and complete peak resolution is unnecessary.

A methanol–aqueous acetic acid gradient on an Alltima C_{18} column gives satisfactory separation of a subgroup of the analytes (the quinolones and enrofloxacin). However, when the group of analytes was expanded to include the fluoroquinolones, an acetonitrile–aqueous formic acid system with a Supelco Discovery C_{18} or a Zorbax Extend C_{18} column was found to give slightly better separation of the fluoroquinolones and better peak shapes. A step function rather than a smooth gradient reduced the retention times of the more strongly retained compounds so that all analytes were eluted in less than 12 min. Chromatograms of a low-level spiked sample are shown in Fig. 2.

As the analytes fall into two groups chromatographically, with the faster-eluting compounds being much more sensitive to minor variations in conditions, an I.S. was required to elute with each group. The β-agonists clenbuterol and penbutolol were chosen, as they are not expected to be found in seafood and fish tissue, and chromatograph well under the conditions developed for this method, giving sharp peaks with suitable retention times. Chromatograms for these compounds are included in Fig. 2.

MS method development was partially automated with the aid of the QuanLynx software supplied by Micromass to operate the Quattro. In determining a parent-ion to product-ion transition to monitor for each analyte, the transition resulting from loss of water was excluded from consideration as it is common to many classes of compounds. The transitions selected are given in Table 1.

3.2. Extraction and clean-up

The analytes were extracted from the matrix by sonication (or dispersion in the case of prawn tissue) with acetonitrile followed by rotation and centrifugation. Dispersion was used for prawn tissue as the sample hardened rapidly when sonicated in acetonitrile and could not be broken up. A second extraction

by rotation with another aliquot of acetonitrile was found to increase extraction efficiency. The combined acetonitrile extracts were reduced to 4 ml by evaporation and passed through a polymer SPE cartridge. Most of the coloured components of the extract were removed by this step, and probably some fatty components. Omission of this polymer SPE step led to LC column degradation, evidenced by drifting responses for most analytes and by peak doubling for some of the fluoroquinolones.

The acetonitrile eluent from the polymer SPE cartridge was diluted with aqueous sodium hydroxide and loaded onto an anion-exchange cartridge. Recovery of the fluoroquinolones from spiked acetonitrile–aqueous sodium hydroxide solutions loaded onto AG MP-1 resin began to decrease when the acetonitrile content increased beyond 15%. Hence evaporation of the combined extracts before commencing clean-up was necessary to reduce the volume of sodium hydroxide solution required to prepare a solution containing less than 15% acetonitrile. The Gilson Aspec XL4, used to automate the clean-up stages, is limited in the volumes it can handle. Our system is equipped with 10-ml syringes and sample tubes of up to 20 ml in volume. Hence 40 ml was a convenient upper limit on the volume of the diluted extracts.

After loading and washing, the anion-exchange SPE cartridge was eluted with acetonitrile in aqueous 2% formic acid. The use of 0.2% formic acid (as in the LC mobile phase) gave lower and/or variable recoveries for most analytes.

Nalidixic acid is added as a surrogate to all samples prior to addition of the extraction solvent to monitor method performance. Surrogate recovery should be better than 60% to indicate that the extraction is within expected parameters for each sample and that the automated SPE has not failed. No correction for surrogate recovery is applied to results.

LC–MS–MS chromatograms of extracts of trout spiked at 5 μg/kg and blank trout (spiked with surrogate) are shown in Figs. 2 and 3.

It became clear during the method development that the chemical natures of the eight analytes were sufficiently different that obtaining high recoveries for all analytes would be unlikely. In particular, ciprofloxacin was the most difficult analyte to extract in this multiresidue approach, and its recoveries were the lowest and most variable. However, the extraction and clean-up protocol described here gave moderate to good recoveries for all the quinolones and fluoroquinolones investigated.

During the method development process, matrix-matched standards were observed to give different responses to solvent standards for some of the analytes, particularly the fluoroquinolones. This was particularly noticeable once LC column degradation began to occur. The use of matrix-matched standards minimises the potential for problems when large numbers of samples are processed.

3.3. Confirmation

EU criteria for the confirmation of residues of antibacterial veterinary drugs by LC–MS–MS require the observation of two transition products and the correspondence of the ratios of the intensities of these two products in the sample with the ratios observed for a standard of the analyte [21]. These ratios must agree within specified tolerances. The confirmation method for this analysis, developed by choosing three transitions (Table 2) from the product-ion MS–MS spectra obtained during quantification method development using Micromass Quanlynx software, allows these criteria to be satisfied.

The repeatability of the product-ion ratios obtained in the confirmation procedure were determined by calculating the ratios for seven replicate injections of 5 and 100 ng/ml standards, and for the extracts of seven replicate trout samples spiked at 10 and 200 μg/kg. Good RSDs were obtained from analysis of 5 and 100 ng/ml standards for most analytes.

The analysis of the replicate extracts of trout spiked at 10 and 200 μg/kg (corresponding to 3–5 and 60–100 ng/ml, respectively, in the extract as a result of the method dilution factor and varying recoveries) gave good RSDs for the high-level extract, but only flumequine and piromidic acid gave RSDs less than a third of the allowed tolerances (according to EU criteria) for the low-level extracts. This variability means that for most analytes present in samples at low levels it is possible for the confirmation ratios to fall outside the expected range. The probabilities of successfully confirming the

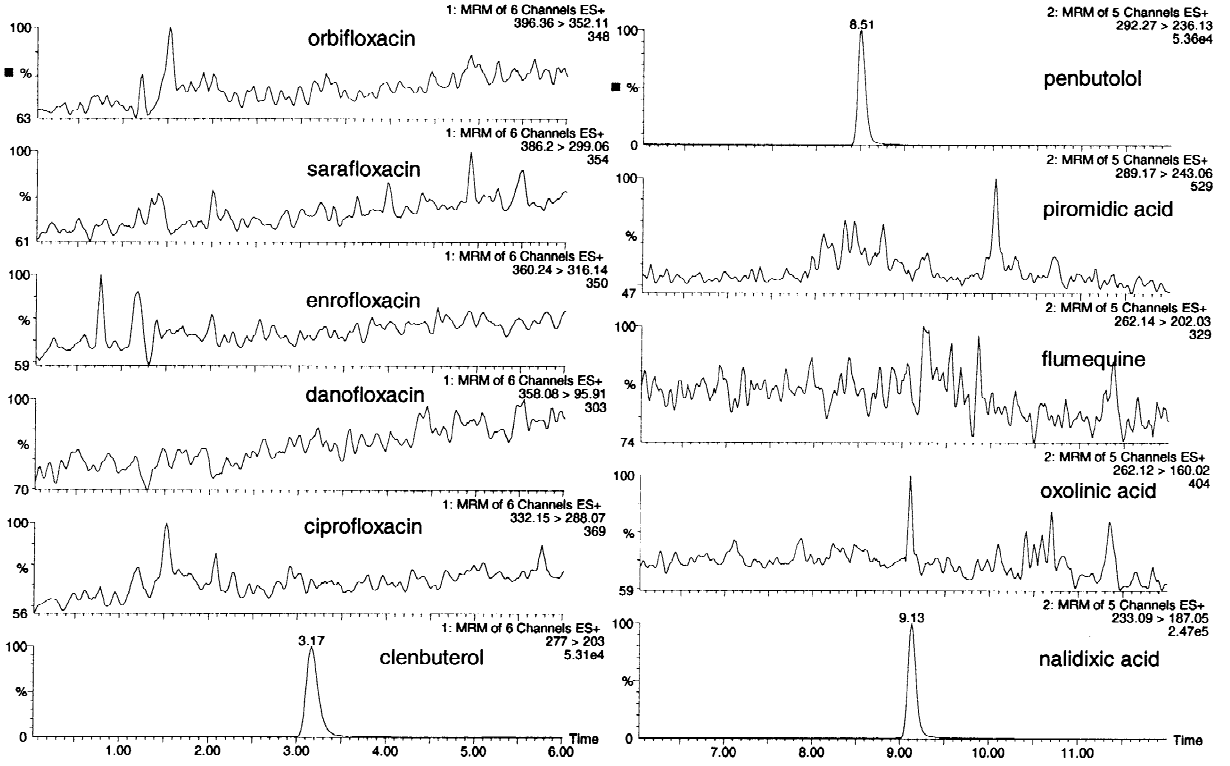

Fig. 3. LC–MS–MS chromatograms (Zorbax Extend C_{18} column) of a blank trout extract. Time scale in minutes.

presence of each analyte at the low level were calculated based on the ratio of the allowed tolerance to the RSD of the confirmation ratio for each analyte, and are shown in Table 3. For danofloxacin and orbifloxacin the RSDs were largest, and the probabilities of successful confirmation at 10 μg/kg were 61 and 77%, respectively. For the remaining analytes the probabilities were 84–100%. As the fluoroquinolones elute in a small window, it was unavoidable that many transitions be scanned simultaneously during confirmation, reducing the sensitivity of the spectrometer towards each transition and hence increasing the RSDs at low levels. To improve the limits of confirmation the mass spectrometric method could be optimised by confirming only one analyte in any retention window. In reality, as positive samples for these analytes are rare, MS–MS confirmation would most likely be carried out on only one analyte at a time and the probability of meeting the EU criteria would then be considerably higher.

Typical LC–MS–MS chromatograms of an ex-

Table 3
Probability of successful confirmation of analytes spiked in trout at 10 μg/kg (LOQ for ciprofloxacin, 2×LOQ for all other analytes)

Analyte	OXO	FLU	PIRO	CIPRO	ENRO	DANO	SARA	ORBI
Transitions	262>160	262>126	289>201	332>245	360>245	358>82	386>342	396>352
	262>244	262>202	289>243	332>314	360>316	358>96	386>299	396>295
Average ratio ($n=7$)	0.57	0.35	0.20	0.68	0.59	0.22	0.88	1.08
Tolerance (%)	20	25	25	20	20	25	20	20
RSD (%)	11	7	4	10	9	27	12	15
Prob. (%)	88	99	100	91	94	61	84	77

tracted trout sample for the confirmation of enrofloxacin are shown in Fig. 4.

3.4. Method validation

Instrument detection levels (IDLs) were determined as three times the standard deviation of the instrument response for a low-concentration standard analysed 10 times. If the estimated IDLs were less than half the standard concentration they were checked by analysing a lower-concentration standard in triplicate. IDLs for all analytes were determined to be 0.5 ng/ml or less. Method detection limits (MDLs) were calculated by extracting 7–8 replicate samples spiked with low levels of all analytes and determining the standard deviation of concentrations found. These standard deviations of the recovery-corrected concentrations were multiplied by the Student *t*-test value ($t=2.896$ at the 99.0% confidence level for number of replicates $n=8$, $t=2.998$ for $n=7$) to give estimates for the MDLs of 1–3 μg/kg. Limits of quantitation of approximately three times the MDLs were set at 10 μg/kg for ciprofloxacin and 5 μg/kg for the remaining analytes.

Instrument sensitivity and repeatability were investigated using a series of six standards over the

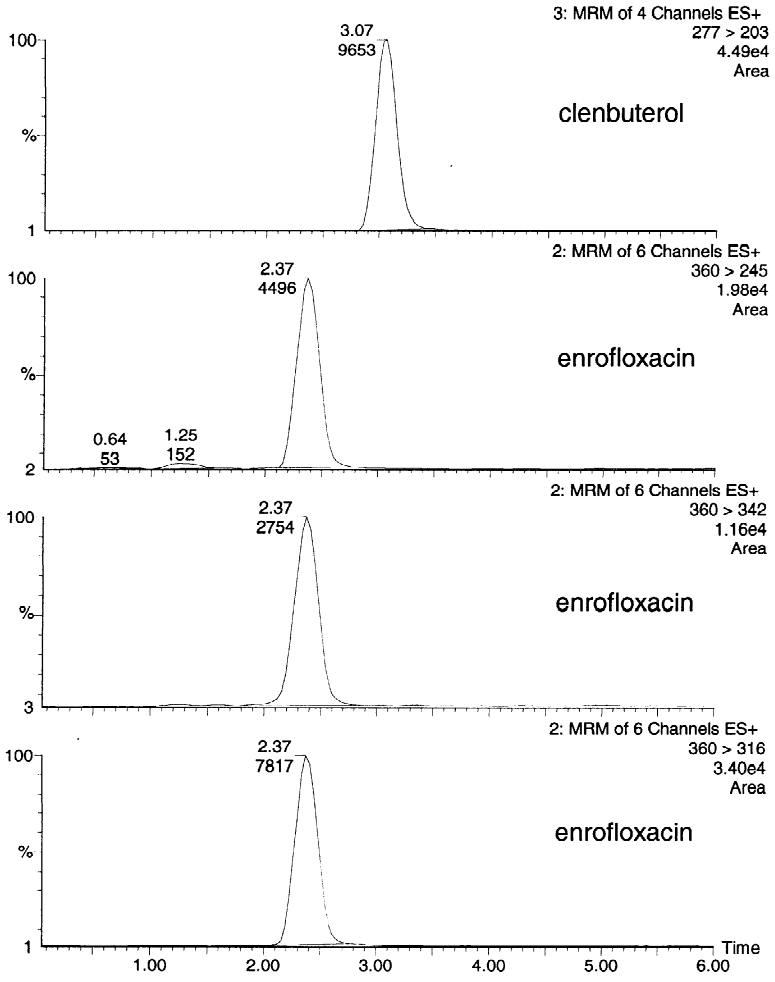

Fig. 4. LC–MS–MS chromatograms (Zorbax Extend C_{18} column) of extract of trout spiked at 200 μg/kg using the confirmation method for enrofloxacin. Time scale in minutes.

concentration range 2 to 200 ng/ml. Each standard was analysed seven times and quadratic calibration curves gave the best fit for the data. As examples, the curves for oxolinic acid and enrofloxacin are shown in Fig. 5, with their equations and associated coefficients of determination. A 10 ng/ml standard was analysed 22 times, and RSDs of the instrument response (relative to the I.S.) were 2–4% for the

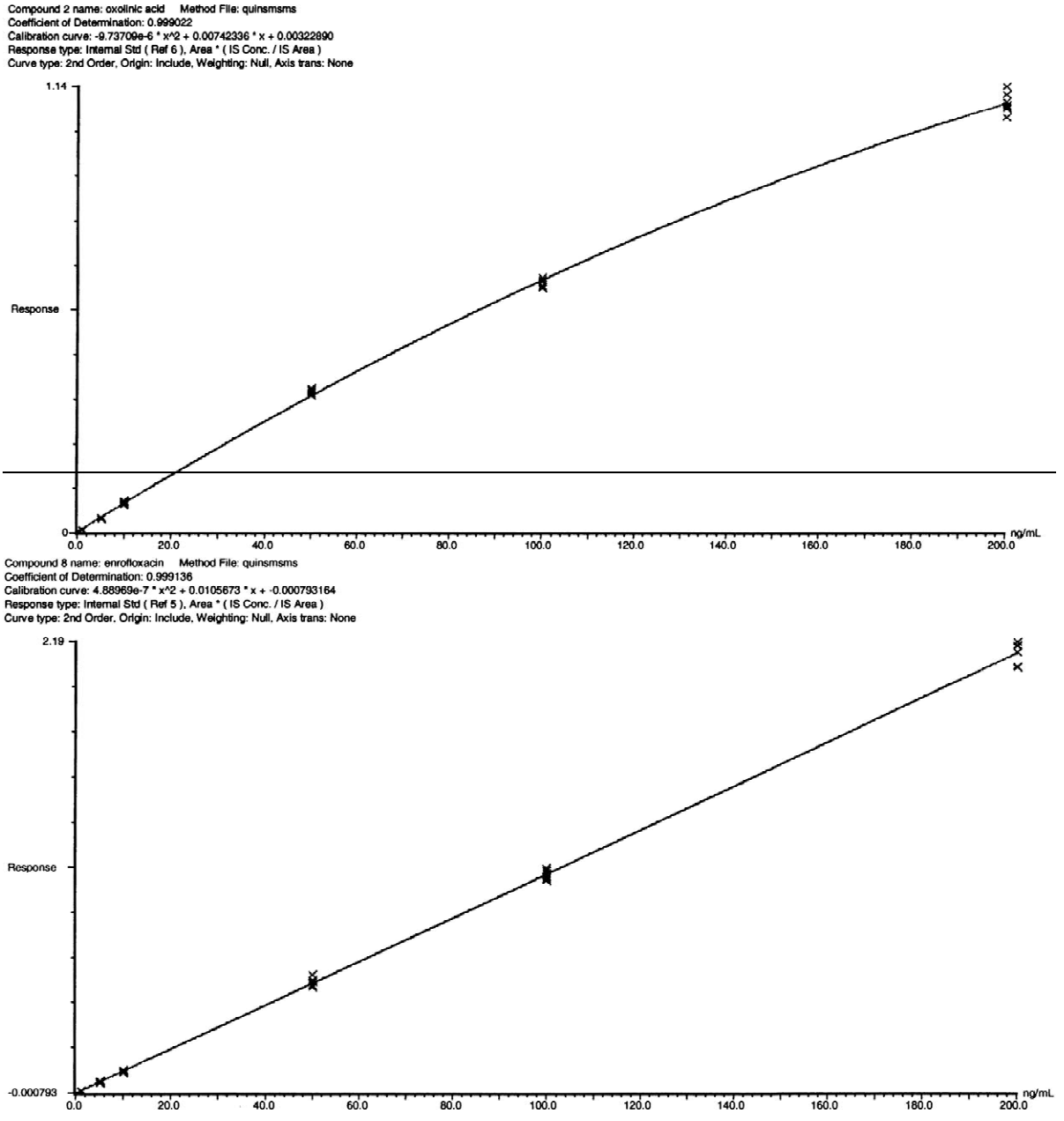

Fig. 5. Calibration curves constructed for oxolinic acid and enrofloxacin from standard solutions at six concentration levels in the range 2 to 200 ng/ml.

quinolones and 5–9% for the fluoroquinolones. Method linearity over the range 5 to 400 μg/kg was established by extraction as a single batch of 3–4 replicate trout samples spiked at each of six levels (20 samples). Plotting response against spiking concentration gave linear curves with coefficients of determination greater than 0.995 for all analytes. The RSD of the recoveries at all levels was less than 11% for all analytes except danofloxacin, 15%.

Method recoveries and repeatabilities were determined for each analyte by extracting 6–8 replicate samples spiked with all the analytes at each of five levels, including the level used to determine the MDLs as discussed above. The extractions of trout samples were performed in several batches by two different operators to demonstrate intralaboratory reproducibility. Over a range of spiking concentrations of 10–400 μg/kg, recoveries for oxolinic acid, flumequine and piromidic acid were 60–80%, ciprofloxacin recoveries were 35–60% and recoveries for the remaining fluoroquinolones were 50–80%. RSDs are generally good (with ciprofloxacin recoveries being the most variable), indicating that results of analyses could be corrected for recovery if desired. Recoveries of the nalidixic acid surrogate from 26 samples extracted in two different batches averaged 75%, with an RSD of 6%.

Recovery and repeatability data for abalone and prawn meat were similar to that for trout. Over a range of spiking concentrations of 10–400 μg/kg, recoveries from abalone for oxolinic acid, flumequine and orbifloxacin were 60–80%, ciprofloxacin recoveries were 29–43% from abalone and 47–57% from prawn meat (over the concentration range 50–400 μg/kg) and recoveries for piromidic acid and the remaining fluoroquinolones were 46–78%. Recoveries for quinolones were generally lower at the limit of quantitation (LOQ) and recoveries for fluoroquinolones from abalone were slightly higher at the LOQ. Recoveries of the nalidixic acid surrogate averaged 69% (RSD 6%) for all abalone samples and 68% (RSD 9%) for all prawn samples.

Recovery and repeatability data for trout tissue are given in Table 4.

Method ruggedness was evaluated using a Plackett–Burman experimental design and fortified trout samples. The seven method parameters investigated were the duration of disruption with the sonic probe, duration of rotation, the temperature of the water bath during concentration of the extracts, the final volume of the combined extracts after evaporation, the necessity of mixing the combined extracts after evaporation, the reuse of the polymer SPE cartridges and the concentration of aqueous sodium hydroxide. The two levels chosen for the first four of these factors differed from each other by ~20%. The remaining parameters had two possible states—either the extracts were vortexed or they were not, and the SPE cartridges were either new or washed, dried and reused. The sodium hydroxide solution for dilution was either prepared freshly or left standing open to the air for several weeks, after which time the pH was noticeably lower. The only factor that was found to have a significant effect on recovery was the concentration of aqueous sodium hydroxide used to dilute the loading solution.

3.5. Method uncertainty

The ISO/IEC 17025:1999 standard requires chemical testing laboratories to estimate the uncer-

Table 4
Recovery and repeatability for quinolones and fluoroquinolones extracted from trout

Spike level (μg/kg)	Analyte	OXO	FLU	PIRO	CIPRO	ENRO	DANO	SARA	ORBI
10	Recovery	78	82	72	59	60	79	68	66
($n=8$)	RSD (%)	5	4	3	10	7	6	7	7
50	Recovery (%)	80	78	68	41	56	57	58	63
($n=8$)	RSD (%)	8	4	3	12	10	9	5	7
200	Recovery (%)	68	73	62	36	56	55	51	55
($n=7$)	RSD (%)	5	6	5	18	20	11	4	9
400	Recovery (%)	69	73	65	50	79	68	64	65
($n=8$)	RSD (%)	5	6	5	8	4	4	7	4

tainty associated with their measurements. The uncertainty budgets for the quinolone and fluoroquinolone analyses were prepared from the data obtained during the method validation process using the procedures described in the Eurachem/CITAC guide [24].

The equation for the concentration (C) of an analyte in tissue, incorporating terms for all sources of uncertainty quantified, was as follows:

$$C = C_{\text{extract}}(\text{calibration}) \times S \times V(\text{extract})/[w(\text{samp}) \times R] \quad (1)$$

where C_{extract}(calibration) is the concentration of analyte in the sample extract as determined from the instrument response using the calibration curve; S is a factor to allow incorporation of uncertainty in the concentration of the calibration standards, and has an assigned value of 1; V(extract) is the final volume of the extract; w(samp) is the weight of sample extracted; R is the average method recovery for that analyte and matrix.

Sources of bias for which no data were available, and which were therefore beyond the scope of this uncertainty budget, include possible heterogeneity in incurred samples, and variation in sample recovery or instrument response (matrix effects) due to differences in composition between samples of different origin.

Relative uncertainty for a typical sample was around 8%. The largest contribution to the uncertainty was the variability in method recovery, accounting for ~6%. The next largest contributor, at 1.3% for a typical sample, was the uncertainty associated with determining the analyte concentration in the extract using the instrument response and the calibration curves. As these calibration curves are non-linear, simple statistical methods for the determination of associated uncertainties are not available. Instead, the uncertainty was estimated empirically as the typical RSD of the response for seven replicate injections of standards at each of six concentrations.

4. Summary

This paper describes the development and validation of an LC–MS–MS method for the quantification and confirmation of eight quinolones and fluoroquinolones in trout, abalone and prawns. A solid-phase extraction procedure was developed that extracts all eight analytes in a single procedure with satisfactory recoveries, in spite of the different chemical natures of the analytes. This procedure was automated to increase throughput and minimise operator-induced variability. An LC–MS–MS analysis procedure was established that allows highly selective identification of residues and quantification down to 5 μg/kg (10 μg/kg for ciprofloxacin). Confirmation to the degree of confidence specified in EU guidelines can be achieved with a second LC–MS–MS analysis of the same extract.

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