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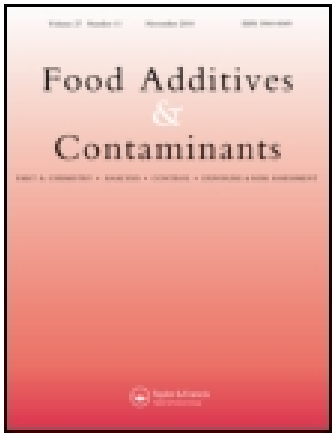

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Comparison of a fluoroquinolone surface plasmon resonance biosensor screening assay with established methods

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Comparison of a fluoroquinolone surface plasmon resonance biosensor screening assay with established methods

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The performance of a previously developed immunochemical biosensor screening method for fluoroquinolone (FQ) antibiotics in poultry muscle, fish and egg was compared with established methods. Blank sample material of the target matrices was individually spiked with the FQs at half maximum residue levels. Homogeneity of the test materials was confirmed by liquid chromatography–mass spectrometry (LC–MS/MS). Identical sets of spiked samples as well as incurred samples from a previous feeding experiment were sent to three independent laboratories and analysed by LC–MS/MS, a microbiological assay and the new biosensor assay. The new method correctly identified all contaminated samples and demonstrated advantages in sensitivity and analysis time compared to the microbiological screening assay.

Keywords: immunoassay; surface plasmon resonance; microbiological assay; LC–MS/MS; veterinary drug residues; poultry; fish; egg

Introduction

Fluoroquinolones (FQs) are synthetic antibacterial drugs used both in human and veterinary medicine. They act by inhibiting the enzymes DNA-gyrase and topoisomerase IV, which play an important role in the packing, replication and transcription of prokaryotic, but not eukaryotic DNA (Martinez et al. 2006). The first generation of quinolone antibiotics comprised non-fluorinated carboxylated quinolones or naphthyridone (e.g. nalidixic acid) and was mainly active against gram-negative bacteria. These drugs were clinically introduced in the 1960s, while their use in agri- and aquacultural practice started in the late 1970s to early 1980s. Later generations were rendered more effective against a broader range of bacteria (including gram-positive) and pharmacologically more active by the introduction of fluorine in the 6-position of the quinolone ring, as well as aryl or alkyl groups in the 1-position and a (substituted) piperazine ring in the 7-position. These FQs were introduced into veterinary use in the late 1980s. The general structure of (fluoro) quinolones, as well as the individual structures of the compounds included in the present study, are depicted in Figure 1.

The use of fluoroquinolone antibiotics in agriculture and aquaculture has been associated with a number of adverse effects. The main concern is the increase in human pathogenic strains which are resistant to this class of chemotherapeutics (FAO/WHO/OIE 2008). Therefore, it is desirable to reduce the presence of fluoroquinolone residues in food to a minimum. Maximum residue limits (MRL) have been set in the European Union for a number of fluoroquinolones in various matrices of selected species, while the use of other FQs is not permitted at all, including their use in laying hens (Council of the European Communities 1990). For the enforcement of official regulations, cost-efficient and reliable analytical tools are indispensable.

Various analytical methods have been developed for the determination of fluoroquinolone residues in food. These include thin layer chromatography, HPLC with UV, fluorescence and mass spectrometric detection, capillary electrophoresis, immunochemical methods and even gas chromatography after decarboxylation of FQs (Hernández-Arteseros et al. 2002; Andreu et al. 2007). Currently, the majority of analytical laboratories apply liquid chromatographic methods with (tandem) mass spectrometry detection (Schneider and Donoghue 2003; Hermo et al. 2008). However, this technique requires sophisticated instrumentation and experienced staff. For scenarios in which no quantitative results are necessary and high throughput is mandatory, e.g. monitoring of large

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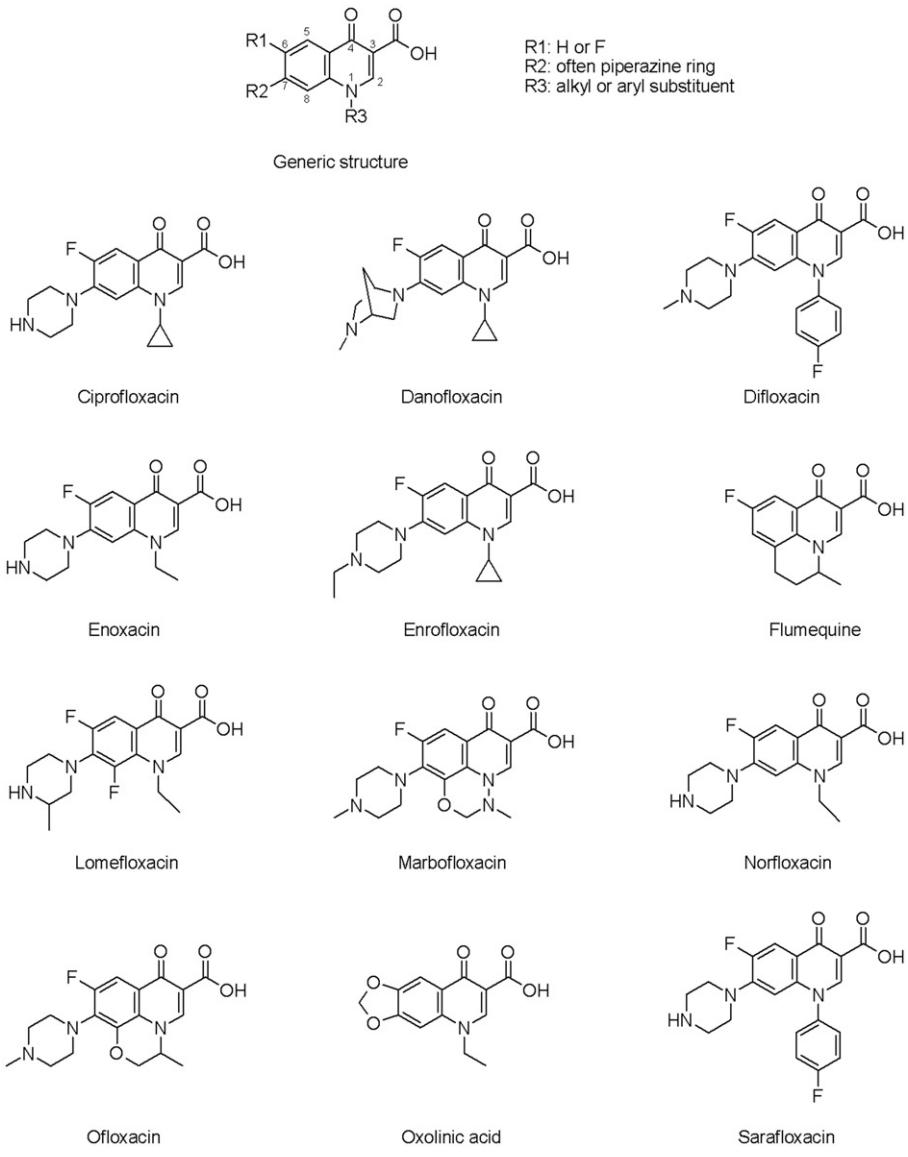

Figure 1. Generic and individual chemical structures of the investigated (fluoro)quinolones.

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sample series, the costs and turn-around times associated with confirmatory LC–MS methods do not make them the method of choice. In this case, screening methods are better suited. To date, the available screening assays are either microbiological assays (Korsrud et al. 1998) or immunochemical methods in an ELISA format (Huet et al. 2006; Wang et al. 2007). Commonly used microbial growth inhibition tests, such as the EU Four-Plate test (Bogaerts and Wolf 1980) and the Premi®Test (DSM, Geleen, The Netherlands) fail to detect the presence of (fluoro)quinolones and require extension with a more specific test bacterium. This can be achieved by including *Escherichia coli* ATCC 11303 (Ellerbroek 1991; Okerman et al. 2001), but *Yersinia ruckeri* NCIMB 13282 might be preferable due to better sensitivity towards flumequine (Pikkemaat et al. 2007). The majority of ELISA assays has a higher sensitivity than microbiological assays and meets or exceeds the regulatory requirements. However, most of these assays exhibit cross-reactivity profiles which do not satisfactorily cover all of the relevant fluoroquinolones. Biosensor assays have gained increasing interest in the screening of food for residues of veterinary drugs (Ricci et al. 2007; Homola 2008). In particular, surface plasmon resonance (SPR)-based optical biosensors have already been applied to a variety of antibiotics and growth promoters (Haughey and Baxter 2006), e.g. chloramphenicol (Ferguson et al. 2005), ß-lactam antibiotics (Gustavsson et al. 2002), ß-agonists (Traynor et al. 2003). Most of these assays use antibodies as recognition molecules in a competitive format. In contrast to other immunoassay techniques, e.g. ELISA, SPR assays do not require incubation and are handled in a micro-fluidic flow-through set-up. Detection is achieved via mass changes upon binding

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of free antibodies on the surface of the sensor chip, which carries immobilised derivatives of the target analytes. Thus, no labelled components have to be used. To date, only few SPR assays for fluoroquinolones have been reported. These only cover single compounds, but not the entire class of FQs (Mellgren and Sternesjö 1998; Haasnoot et al. 2007).

The need for a cost-efficient rapid high-throughput screening assay for the entire class of fluoroquinolones was approached within the EC-funded project "New Technologies to Screen Multiple Chemical Contaminants in Foods" (acronym: BioCop, www.biocop.org) by the development of a SPR biosensor method for the screening of 13 different fluoroquinolone antimicrobial chemotherapeutics in poultry muscle, fish and egg (Huet et al. 2008). Before a new (screening) method can be applied in routine and statutory analysis it should be demonstrated that the assay is fit for the intended purpose. Within the project, a tiered approach was taken for this purpose. First, a comprehensive in-house validation of the method, according to the European validation guideline for veterinary drugs (Commission of the European Communities 2002), was carried out. This was followed by a critical comparison with existing methods and was complemented by an interlaboratory method performance study. In the present study, a comparison of the new method with established screening and confirmatory methods for the determination of fluoroquinolone residues in the target matrices is described. The methods chosen for comparison were a fluoroquinolone-specific microbiological screening assay and a LC–MS/MS confirmatory method. A set of samples individually spiked with fluoroquinolones at 0.5 MRL level in poultry, fish and egg was prepared and shipped to the laboratories participating in the comparison study. The performance of the different methods was compared, not only in terms of quality of the results, but also in cost-efficiency and speed of analysis. It was shown that each of the compared methods has its specific benefits in different analytical scenarios. The present study is the first direct comparison of different analytical methods, both screening and confirmatory, for fluoroquinolones, applied on identical sets of naturally incurred and spiked samples.

Materials and methods

Preparation of samples

The selection of the concentration levels for the spiked samples followed the rationale that the assay should be capable of detecting contaminated samples at least at half the current MRL. In cases in which a MRL existed for one matrix but not another, the existing MRL was extrapolated to this latter matrix. In the case of a specific fluoroquinolone not being approved (and thus not having a MRL), the lowest MRL of the fluoroquinolones approved for the respective matrix was targeted.

Incurred chicken muscle and egg sample materials were obtained from controlled feeding experiments (Pikkemaat et al. 2007). Incurred and blank trout samples were produced by controlled exposure experiments. The animal experiments were approved by the Institutional Animal Experiment Commission under permission no. 2005054.

Incurred fish muscle and skin samples were obtained by dosing two groups of rainbow trout (live weight 300–400 g, obtained from a commercial breeder) with flumequine (Flumequine, 50% water-soluble powder; Dopharma, Raamsdonksveer, The Netherlands). A third group remained untreated as blank reference. Medication was administered for 24 h via the basin water after a 16-day acclimation period in non-medicated water in a flow-through system. One group was exposed to 25 mg l^{-1} basin water, the second group to 10 mg l^{-1} basin water. At the end of the medication period, the fish were killed by electrocution, collected and rinsed with clean water. The fish were scaled and filleted. Filet samples were homogenized using a household chopper (Moulinette, Moulinex) and individually packed and stored at −20°C. The drug residue concentration in each filet sample was determined using the microbiological assay. Samples containing drug concentrations around the MRL were obtained through a cryogenic grinding and mixing procedure. Frozen chopped samples were blended to a fine powder under liquid nitrogen using a Grindomix GM200 (Retsch, Haan, Germany), after which the material was sieved through a 1.25-mm sieve. Batches of 1.5 kg containing flumequine at approximately 0.5, 1, and 1.5 times the MRL were prepared by combining the proper amount of incurred and blank reference material. After collecting the sieved material, it was homogenized by additional stirring for 10 min under liquid nitrogen. Homogeneity of the trout fillet samples was established by the procedure described by Fearn and Thompson (2001): 10 random samples from a batch were analysed in duplicate by the same LC–MS/MS method as applied for the poultry and egg samples (Pikkemaat et al. 2007).

Spiked poultry and fish samples were prepared by adding 5 ml of antibiotic standard solution to 295 g (poultry) or 245 g (fish) roughly chopped blank reference material. The blank reference material was obtained from the same controlled animal feeding experiments as the incurred samples. The spiked materials were roughly homogenized using a moulinette food chopper (Moulinex) after which they were frozen (−20°C). The frozen material was partially crushed and transferred into a cutting mill (UMC 5 Electronic; Stephan Machinery, Hameln,

Germany) where liquid nitrogen was added. The meat was first roughly ground, then smaller portions were blended to a fine powder using a Grindomix GM200 (Retsch GmbH, Haan, Germany), after which the material was sieved through a 1.25-mm sieve. This product was finally homogenized by additional stirring for 10 min under liquid nitrogen. Spiked egg samples were prepared by adding 5 ml of antibiotic standard solution to 245 g of blank reference material which was previously homogenized using an Ultra-Turrax, and mixed thoroughly.

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Determination of homogeneity

The spiked sample material was analysed for homogeneity by means of a confirmatory LC–MS/MS method (Pikkemaat et al. 2007). After addition of water and internal standards, the sample material was homogenised and centrifuged. The supernatant was filtered through a 0.45 µm filter and the filtrate was further purified by ultrafiltration (30 kDa filter unit at 3500 *g*). Finally, the ultrafiltrate was analysed by LC–MS/MS in electrospray ionisation mode. The limit of detection was below 10 µg kg^{-1} for all the target analytes in the investigated matrices. The homogeneity of the materials was tested according to The International Harmonized Protocol for Proficiency Testing of Analytical Laboratories (Thompson and Wood 1993) and ISO 13528 on statistical methods for use in proficiency testing by inter-laboratory comparison (ISO 2005), taking into account the insights discussed by Thompson (2000) regarding the Horwitz equation. With this procedure, the between-sample standard deviation (s_s) is compared with the target standard deviation derived from the Horwitz equation (σ_H). The materials are considered adequately homogeneous if $S_S \leq 0.3\sigma_H$.

Ten randomly taken samples (~5 g) from each batch were analysed by LC–MS/MS in duplicate for the 12 fluoroquinolones to establish the concentration and homogeneity of the materials.

Analytical procedures for method comparison

Identical sets of homogeneity-checked test materials were sent frozen to the participating laboratories. They were analysed by the new SPR assay, as well as by established microbiological and LC–MS/MS methods.

LC–MS/MS

A 50 µl aliquot of internal standard solution (norfloxacin-D5 from Witega, Germany, 1 µg ml^{-1} in H_2O/MeCN/HFA, 9:1:0.1, v/v), 2 ml water and 4 ml acetonitrile were added to the ground samples (1 g). After homogenisation (Ultra-Turrax) and centrifugation (2300 *g*, 10 min) the supernatant was decanted, filtered (0.45 µm) and diluted five-fold in HPLC eluent. The chromatographic separation was carried out by gradient elution (A: 10% MeCN in water, 0.1% HFA; B: 100% MeCN, 0.1% HFA; 0% B to 100% B in 10 min, flow rate 0.4 ml min^{-1}) on an Agilent 1100 system with a HyPurity C_{18} (150 × 3 mm, 5 µm) column. The HPLC was coupled to an Applied Biosystems API 4000 mass spectrometer with a TurboIonSpray® source. The substances were measured in a negative ion mode and quantified with two MRM transitions. The limit of detection was 10 µg kg^{-1} or below for all 12 investigated FQs.

Microbiological assay

Approximately 20 g of the blended sample material (fish and poultry) was heated in a centrifuge tube for 20 min at 64°C and the sample was then centrifuged for 10 min at 27 000 *g*. The supernatant (meat fluid) was applied directly onto the test plate in punch holes with a diameter of 14 mm. Egg samples were directly applied to the test plates after homogenisation. The agar test plates for the analysis of poultry and egg were prepared and seeded with *Yersinia ruckeri* NCIMB 13282, as described by Pikkemaat et al. (2007). *Yersinia* is not affected by natural inhibitors present in egg, such as lysozyme and avidin. The analysis of fish was performed on Plate Count Agar (Difco) adjusted to pH 8.0 and seeded with 10^5 cfu ml^{-1} *E. coli* ATCC 11303. A sample volume of 250 µl was applied and plates were incubated overnight at 30°C.

SPR biosensor assay

Sample extracts were prepared by extraction of the blended samples (2 g) with acetonitrile (8 ml) by vortexing (30 s) and head-over shaking for 10 min. After centrifugation at 2500 *g* and 4°C, the supernatant was transferred to a new tube and evaporated to dryness under a stream of nitrogen at 50°C. In the case of fish, only 2 ml of the supernatant were transferred. The residue was reconstituted in 1 ml phosphate-buffered saline (PBS) at pH 7.4. Residual fat was removed by duplicate washing with *n*-hexane. The SPR analysis was run on a Biacore Q biosensor instrument. The carboxymethylated CM5 sensor chip surface was functionalised by immobilisation of a specific fluoroquinolone derivative. A tailored bi-functional polyclonal antibody was used for recognition of the target molecules. The overall procedure is described in detail by Huet et al. (2008). The method achieves detection capabilities cc ß, as determined in accordance with Commission Decision 2002/657/EC (Commission of the European Communities 2002), of 0.5 µg kg^{-1} (chicken muscle), 1.5 µg kg^{-1} (fish),

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and 1 μg kg^{-1} (egg) for the calibration compound norfloxacin. Detection capabilities for further fluoroquinolones are determined by differences in cross-reactivity and extraction efficiency, and are below 4 μg kg^{-1} for most analytes in the three matrices, except for oxolinic acid (7–14 μg kg^{-1}), and flumequine (52–68 μg kg^{-1}).

Results and discussion

Homogeneity of samples

The homogeneity of the incurred sample materials has been demonstrated previously (Pikkemaat et al. 2007). The spiked sample materials, which were prepared for this method comparison study, were homogeneity tested prior to their distribution for analysis. In the case of chicken, all samples were shown to be homogeneous ($S_S \leq 0.3\sigma_H$). Only for the sample material P-FQ-8 spiked with sarafloxacin at 5 μg kg^{-1} (=half the MRL for skin and fat), the homogeneity could not be confirmed because the level in the sample was below the quantification limit of the method. In the case of fish, all samples were shown to be homogeneous. For the egg materials, homogeneity was proven for all samples except for those spiked with enoxacin (E-FQ-9) and lomefloxacin (E-FQ-10). The measured concentrations (4 and 1 μg kg^{-1}, respectively) were below the quantification limit of the LC–MS/MS method. Furthermore, they were well below the scheduled spiking level (15 μg kg^{-1}). It remains to be elucidated whether this discrepancy is due to a mistake in the spiking procedure or due to other effects. However, for the other matrices, no such effect was observed.

Comparative measurements

The results of the analysis of the sample sets for poultry, fish and egg by LC–MS/MS, microbiological assay and SPR assay are summarised in Tables 1–3, and compared to the spiking levels and the respective MRLs. For interpretation of the results, it is important to note that numerical results from the microbiological and the SPR assay cannot be compared directly to the concentrations obtained by LC–MS. In the case of the microbiological assay, the diameter of the inhibition zone not only depends on the concentration of the inhibiting compound (fluoroquinolone), but also of the susceptibility of the test strain for this specific compound. Similarly, in the case of the SPR immunoassay, the cross-reactivities of the respective fluoroquinolones largely influenced the calculated concentration (which is related to the calibration compound norfloxacin). Nevertheless, these figures help to understand the performance of the assay. Even a compound like flumequine gives a clear positive result despite its low cross-reactivity (1–2%).

LC–MS/MS

LC–MS/MS can currently be regarded as the gold standard for the analysis of fluoroquinolones in food. Thus, this method was included in the comparison study as a reference. As a confirmatory method, it delivers reliable quantitative results. With a view to current legal requirements (MRLs in the range 30–600 μg kg^{-1}) and a tendency among food industry for a reporting limit of 10 μg kg^{-1} in the case of non-regulated substances, most LC–MS/MS methods have not been optimised for concentrations below 10 μg kg^{-1}. As a result, all samples with a content of 10 μg kg^{-1} or more were reliably identified and quantified by the LC–MS/MS methods (both the one used for the determination of homogeneity and that used in the method comparison by a second laboratory). Only the samples with concentrations below this value could not be quantified reliably. This affected samples P-FQ-8 (sarafloxacin in chicken, target level 5 μg kg^{-1}), E-FQ-9 (enoxacin in egg, target level 15 μg kg^{-1}, semi-quantitative result 4 μg kg^{-1}), and E-FQ-10 (lomefloxacin in egg, target level 15 μg kg^{-1}, semi-quantitative result 1 μg kg^{-1}).

Microbiological assay

The microbiological method generally performed well for those analyte/matrix combinations with a set MRL of 100 μg kg^{-1} or above. In poultry, all FQs with a MRL of ≥100 μg kg^{-1} (ciprofloxacin, danofloxacin, difloxacin, enrofloxacin, flumequine, marbofloxacin, oxolinic acid) were correctly identified as suspect positives at MRL/2 level in the spiked samples. For sarafloxacin, MRLs in chicken are only set for skin/fat (10 μg kg^{-1}) and for liver (100 μg kg^{-1}), but not for muscle. The assay detected contaminated samples at 50 μg kg^{-1} (1/2 MRL liver), but not at 5 μg kg^{-1} (1/2 MRL skin/fat). For all the FQs with no authorisation for use in chicken (norfloxacin, enoxacin, lomefloxacin, ofloxacin), the method was not able to detect them at the targeted low concentrations (15 μg kg^{-1}). Norfloxacin was not even detected at 50 μg kg^{-1}. All incurred samples (flumequine, difloxacin, enrofloxacin) were correctly classified as suspect positives at or below MRL/2 level.

A similar picture was obtained for fish. Most FQs with a MRL of ≥100 μg kg^{-1} (ciprofloxacin, danofloxacin, difloxacin, enrofloxacin, flumequine, marbofloxacin) were correctly identified as suspect positives at MRL/2 level in the spiked samples. Only oxolinic acid was not detected at MRL/2 level (50 μg kg^{-1}). Sarafloxacin, which has a lower MRL of 30 μg kg^{-1} in *Salmonidae*, was detected at

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Table 1. Results of the method comparison in poultry samples.

Sample ID	Description	MRL [µg kg^{-1}]	Spiking level [µg kg^{-1}]	LC–MS/MS concentration [µg kg^{-1}][a]	Microbiological assay		SPR	
					Inhibition zone[b] [mm]	Interpretation	Conc.[c] [µg kg^{-1}]	Interpretation (cut-off 0.5 µg kg^{-1})
P-Blk-1	Pooled blank		0	<LOQ	–	Negative	<0.1	Negative
Spiked samples								
P-FQ-1	Ciprofloxacin	100	50	40	27	Positive	9.1	Positive
P-FQ-2	Danofloxacin	200	100	110	26	Positive	5.6	Positive
P-FQ-3	Difloxacin	300	150	120	30	Positive	6.5	Positive
P-FQ-4	Enrofloxacin	100	50	55	29	Positive	>10	Positive
P-FQ-5	Flumequine	400	200	230	23	Positive	1.4	Positive
P-FQ-6	Marbofloxacin	150	75	70	31	Positive	8.6	Positive
P-FQ-7	Norfloxacin	no	15	15	–	Negative	>10	Positive
P-FQ-7b	Norfloxacin	no	50	50	–	Negative	>10	Positive
P-FQ-8	Sarafloxacin	10[d]/100[e]	5	<LOQ	–	Negative	1.3	Positive
P-FQ-8b	Sarafloxacin	10[d]/100[e]	50	75	17	Positive	3.0	Positive
P-FQ-9	Enoxacin	no	15	20	–	Negative	2.0	Positive
P-FQ-10	Lomefloxacin	no	15	10	–	Negative	4.2	Positive
P-FQ-11	Ofloxacin	no	15	15	–	Negative	5.8	Positive
P-FQ-12	Oxolinic acid	100	50	60	17	Positive	1.9	Positive
Incurred samples								
P-IC-1	Flumequine	400		90	17	Positive	1.5	Positive
P-IC-2	Flumequine	400		430	29	Positive	2.0	Positive
P-IC-3	Flumequine	400		680	34	Positive	2.7	Positive
P-IC-4	Difloxacin	300		260	34	Positive	9.0	Positive
P-IC-5	Difloxacin	300		180	32	Positive	7.9	Positive
P-IC-6	Difloxacin	300		390	37	Positive	>10	Positive
P-IC-7	Enrofloxacin	100		55	31	Positive	>10	Positive
P-IC-8	Enrofloxacin	100		110	35	Positive	>10	Positive
P-IC-9	Enrofloxacin	100		270	39	Positive	>10	Positive

[a]Rounded in intervals of 5 µg kg^{-1}; [b]Including the 14-mm diameter of the sample hole; [c]Calibration to norfloxacin; [d]Skin and fat; [e]Liver.

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Table 2. Results of the method comparison in fish samples.

Sample ID	Description	MRL] [μg kg^{-1}]	Spiking level [μg kg^{-1}]	LC–MS/MS concentration [μg kg^{-1}][a]	Microbiological assay		SPR	
					Inhibition zone[b] [mm]	Interpretation	Conc.[c] [μg kg^{-1}]	Interpretation (cut-off 1.5 μg kg^{-1})
F-Blk-1	Pooled blank		0	<LOQ	–	Negative	0.3	Negative
Spiked samples								
F-FQ-1	Ciprofloxacin	100	50	55	38	Positive	18	Positive
F-FQ-2	Danofloxacin	100	50	40	29	Positive	9.0	Positive
F-FQ-3	Difloxacin	300	150	110	23	Positive	13	Positive
F-FQ-4	Enrofloxacin	100	50	40	30	Positive	>100	Positive
F-FQ-5	Flumequine	600	300	350	16	Positive	4.1	Positive
F-FQ-6	Marbofloxacin	150	75	70	30	Positive	22	Positive
F-FQ-7	Norfloxacin	no	15	20	–	Negative	16	Positive
F-FQ-7b	Norfloxacin	no	50	50	21	Positive	>100	Positive
F-FQ-8	Sarafloxacin	30	15	15	–	Negative	4.1	Positive
F-FQ-8b	Sarafloxacin	30	50	50	22	Positive	6.7	Positive
F-FQ-9	Enoxacin	no	15	15	–	Negative	4.3	Positive
F-FQ-10	Lomefloxacin	no	15	10	–	Negative	5.6	Positive
F-FQ-11	Ofloxacin	no	15	10	18	Positive	11	Positive
F-FQ-12	Oxolinic acid	100	50	70	–	Negative	4.6	Positive
Incurred samples								
F-IC-1	Flumequine	600		350	16	Positive	4.0	Positive
F-IC-2	Flumequine	600		800	21	Positive	5.2	Positive
F-IC-3	Flumequine	600		930	26	Positive	6.7	Positive

[a]Rounded in intervals of 5 μg kg^{-1}; [b]Including the 14-mm diameter of the sample hole; [c]Calibration to norfloxacin.

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Table 3. Results of the method comparison in egg samples.

Sample ID	Description	MRL [μg kg^{-1}]	Spiking level [μg kg^{-1}]	LC–MS/MS concentration [μg kg^{-1}][a]	Microbiological assay		SPR	
					Inhibition zone[b] [mm]	Interpretation	Conc.[c] [μg kg^{-1}]	Interpretation (cut-off 1 μg kg^{-1})
E-Blk-1	Pooled blank		0	<LOQ	–	Negative	<0.1	Negative
Spiked samples								
E-FQ-1	Ciprofloxacin	no	50	40	29	Positive	>10	Positive
E-FQ-2	Danofloxacin	no	50	40	25	Positive	>10	Positive
E-FQ-3	Difloxacin	no	150	120	31	Positive	>10	Positive
E-FQ-4	Enrofloxacin	no	50	35	25	Positive	>10	Positive
E-FQ-5	Flumequine	no	100	90	–	Negative	2.6	Positive
E-FQ-6	Marbofloxacin	no	75	60	27	Positive	>10	Positive
E-FQ-7	Norfloxacin	no	15	15	–	Negative	>10	Positive
E-FQ-7b	Norfloxacin	no	50	50	20	Positive	>10	Positive
E-FQ-8	Sarafloxacin	no	15	10	–	Negative	5.5	Positive
E-FQ-8b	Sarafloxacin	no	50	40	22	Positive	7.9	Positive
E-FQ-9	Enoxacin	no	15	<LOQ (4)[d]	–	Negative	3.0	Positive
E-FQ-10	Lomefloxacin	no	15	<LOQ (1)[d]	–	Negative	9.4	Positive
E-FQ-11	Ofloxacin	no	15	10	–	Negative	>10	Positive
E-FQ-12	Oxolinic acid	no	50	50	–	Negative	5.8	Positive
Incurred samples								
E-IC-1	Flumequine	no		70	–	Negative	2.6	Positive
E-IC-2	Flumequine	no		140	23	Positive	3.5	Positive
E-IC-3	Flumequine	no		330	29	Positive	5.0	Positive
E-IC-4	Oxolinic acid	no		15	–	Negative	3.8	Positive
E-IC-5	Oxolinic acid	no		40	18	Positive	6.0	Positive
E-IC-6	Oxolinic acid	no		90	28	Positive	8.8	Positive

[a]Rounded in intervals of 5 μg kg^{-1}; [b]Including the 14-mm diameter of the sample hole; [c]Calibration to norfloxacin; [d]Estimated concentration below LOQ, mistake in spiking.

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50 μg kg^{-1}, but not at MRL/2 level (15 μg kg^{-1}). For all the FQs with no authorisation for use in fish (norfloxacin, enoxacin, lomefloxacin, ofloxacin), the method was not able to detect them at the targeted low concentrations (15 μg kg^{-1}). In contrast to chicken, norfloxacin was detected at 50 μg kg^{-1} (but not at 15 μg kg^{-1}) in fish. The incurred samples (flumequine) were correctly classified as suspect positives at or below MRL/2 level.

FQs are not permitted for use in laying hens. As a result, the absence of FQs in eggs is required and no MRLs have been set for this matrix. Nevertheless, spiking levels were chosen in the same range as for chicken muscle, as it is expected that MRLs will be extrapolated from licensed to non-licensed matrices in the future. Using this approach, most FQs with a pseudo-MRL of ≥100 μg kg^{-1} (ciprofloxacin, danofloxacin, difloxacin, enrofloxacin, marbofloxacin) were correctly identified as suspect positives. Only flumequine (100 μg kg^{-1}) and oxolinic acid (50 μg kg^{-1}) were not detected at the spiked concentrations. Sarafloxacin (pseudo-MRL at 30 μg kg^{-1}) was detected at 50 μg kg^{-1}, but not at 15 μg kg^{-1}. Among the FQs with no authorisation for use in any species (norfloxacin, enoxacin, lomefloxacin, ofloxacin), the method was not able to detect them at low concentrations (15 μg kg^{-1}). In contrast to chicken, norfloxacin was detected at the 50 μg kg^{-1} level (but not at 15 μg kg^{-1}) in egg. The incurred samples (flumequine, oxolinic acid) were correctly classified as suspect positives down to 151 μg kg^{-1} (flumequine) and 46 μg kg^{-1} (oxolinic acid), but not below (67 and 25 μg kg^{-1}, respectively).

SPR biosensor assay

The newly developed SPR biosensor assay identified all samples which contained FQs in all of the matrices as suspect positives. This also includes the non-authorised substances at low concentrations (15 μg kg^{-1}). Sarafloxacin was even detected at the 5 μg kg^{-1} level. Of the three compared methods, this assay was the most sensitive for the majority of analytes. When looking at the quantitative data obtained, some inconsistencies in comparison to the MS data became apparent. In the case of norfloxaxin in fish, the concentration determined by SPR (16 μg kg^{-1}) of sample F-FQ-7 was in line with that measured by LC–MS (20 μg kg^{-1}). For sample F-FQ-7b, there was a discrepancy (LC–MS: 50 μg kg^{-1}, SPR: >100 μg kg^{-1}). This effect may be explained by the fact that concentration determinations outside the quasi-linear range of the sigmoidal calibration curve of the immunoassay are not very precise. In other cases (e.g. F-FQ-8 and F-FQ-8b), this argument does not hold as both values fell in the quasi-linear range. The concentration measured for sarafloxacin by SPR must differ from that measured by LC–MS because sarafloxacin has a cross-reactivity which differs from that of the calibration compound norfloxacin. However, it is expected that the values obtained by SPR should have the same relation to each other as the corresponding MS data (i.e. the higher concentration should be 3.33 times that of the lower concentrated sample). This was not the case (a factor of 1.63 only). A possible explanation may be differences in extraction efficiencies at different concentration levels, although this is not very likely. As the method does not claim to be quantitative, these observations do not affect its usefulness for qualitative screening purposes. In the qualitative screening of routine samples, the new biosensor method well ensures that no sample containing FQs will falsely be declared as negative/compliant. A drawback of this high sensitivity is that, in the case of MRL regulated compound/matrix combinations, a considerable rate of pseudo-false positive results may be generated, i.e. samples containing FQs at levels below the MRL. This is a common problem of generic immunochemical screening assays. Due to different cross-reactivities (as well as different MRLs), it is nearly impossible to develop an assay which has both low false negative and low false positive rates (unless in the very unlikely case that the cross-reactivity profile exactly matches the different MRL levels). By the selection of the cut-off value, it will still be possible to optimise the rate of pseudo-false positives. The priority within the project was directed towards the development of a generic assay capable of detecting a broad range of FQ antibiotics (even unusual or unexpected ones) at low levels. As suspect samples are required to be analysed by confirmatory methods, the pseudo-false positives (concentrations below MRL) will easily be identified.

Critical comparison

The method comparison study demonstrated the excellent performance of the newly developed SPR biosensor screening assay. All spiked and incurred samples which contained one of the investigated FQ antibiotics, sometimes even at very low concentrations, were identified as suspect positives by the new method in poultry muscle, fish and egg. The assay was even more sensitive than the confirmatory reference method for certain compounds. It can be assumed that the method will not miss any poultry, fish or egg sample containing FQs, even at low levels. Furthermore, it can be expected that the assay will show a comparable performance for other matrices. As a drawback of the high sensitivity, samples containing permitted FQs below the authorised MRL will also be detected as suspect positives. Thus, the method can be used to screen for samples which contain FQs at any level,

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even trace levels, but not to distinguish between samples which contain the target substances above or below the regulatory limit. However, from the perspective of food safety, it is more important not to miss any non-compliant sample, but with a view to economic implications (costs of confirmatory analysis), a balance has to be achieved between safety and cost-efficiency. This balance can be optimised by modification of the method (e.g. dilution of the final extract) and an appropriate selection of the cut-off value. If, for example, permitted fluoroquinolones are to be screened in poultry muscle at their respective MRLs, a five-fold dilution would still yield cc ß values below the MRL even for low cross-reactivity compounds, such as flumequine and oxolinic acid, while the number of positive findings for trace levels below the MRL would be reduced. Nevertheless, due to the generic nature of the assay and the given cross-reactivity profile, it will not be possible to avoid positives below MRLs completely. However, the main intention of a screening assay is to avoid false negatives and to sort out all samples which are clearly negative. This is perfectly achieved by the new assay.

The microbiological assay provided good identification of most contaminated samples at half MRL levels, but was less sensitive than the SPR method. Although single analytes (e.g. norfloxacin) will be missed at relevant concentrations, the assay provides a better balance between sensitivity and number of pseudo-false positives. In addition, it is easier to perform compared to the SPR biosensor assay and can be set up on-site. It provides good cost-efficiency.

To select an appropriate method for a given analytical purpose, it is important not only to compare the analytical performance data, but also further parameters. These include costs for instrumentation and consumables, labour needed for sample preparation and data analysis, turn-around times, required expertise of staff and type of available laboratory. In Table 4, some of these parameters are compiled for the compared methods. From the data, it becomes obvious that the two screening assays have quite distinct characteristics. The tested biosensor method is more labour intensive and requires more expensive instrumentation and consumables compared to the microbiological assay. These higher costs will be justified in situations where it is mandatory (i) to obtain the results on the same day and (ii) to screen at low levels ($10\,\mu g\,kg^{-1}$ or below), as in the case of unauthorised substances or matrices where there is an interest in sub-MRL levels (e.g. baby food). In contrast, the microbiological assay will be more appropriate in the screening for compliance with regulatory limits of authorised fluoroquinolones, e.g. in monitoring programs.

Conclusions

The three methods which were compared comprised an excellent suite of complementary analytical tools. LC–MS/MS is the standard confirmatory reference method for FQs. It is capable of reliably identifying and quantifying FQs down to levels of $10\,\mu g\,kg^{-1}$ and below. Among the two screening assays, the microbiological assay has clear advantages in front-line situations, where cost-efficiency, ease of use and robustness are mandatory. This holds even more when the analytes to be expected are known and regulated at MRL levels of $\geq 100\,\mu g\,kg^{-1}$. For those situations where safety is the main driving force and non-regulated fluoroquinolones at low concentrations

Table 4. Comparison of the methods in terms of performance and requirements.

	LC–MS/MS	SPR-biosensor	Microbiological assay
Method category	Confirmatory	Screening	Screening
Results	Quantitative	Qualitative (semi-quantitative)	Qualitative (semi-quantitative)
Sensitivity	$\sim 10\,\mu g\,kg^{-1}$	$1–50\,\mu g\,kg^{-1}$	$10–100\,\mu g\,kg^{-1}$
Sample preparation time	4 h	5 h	2 h[a]/0.5 h[b]
(batch 20 samples), thereof labour	3.75 h	4 h	1.5 h[a]/0.5 h[b]
Measurement time (batch 20 samples)	16 h	4.5 h	12–16 h[c]
Data evaluation (batch 20 samples)	2.5 h	0.25 h	0.5
Availability of results	Day 2	Day 1	Day 2
Instrumentation costs	200–250 k€	~150 k€	1–5 k€
Consumables costs	Low	Medium[d]	Low
Labour costs	High	Medium	Low
Required lab	Sophisticated analytical lab	Analytical lab or QA lab on site	Simple lab on-site
Market price per sample	100–150 €	30–50 €	5–15 €

[a]Poultry, fish; [b]Egg; [c]Incubàtion overnight; [d]For commercial test kits.

are expected, the SPR biosensor assay will be the screening tool of choice due to its high sensitivity. This is also true for the screening of products in which the complete absence of residues is regarded desirable (e.g. baby food) and for countries which practice a more restrictive veterinary use of fluoroquinolones. An major advantage of the SPR assay is that it is capable of delivering results on the same day, while the microbiological assay requires incubation overnight.

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