Simultaneous Determination of the Residues of Fourteen Quinolones and Fluoroquinolones in Fish Samples using Liquid Chromatography with Photometric and Fluorescence Detection

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Abstract

Cañada-Cañada F., Espinosa-Mansilla A., Jiménez Girón A., Muñoz de la Peña A. (2012): **Simultaneous determination of the residues of fourteen quinolones and fluoroquinolones in fish samples using liquid chromatography with photometric and fluorescence detection**. Czech J. Food Sci., **30**: 74–82.

A chromatographic method is described for assaying fourteen quinolones and fluoroquinolones (pipemidic acid, marbofloxacin, norfloxacin, ciprofloxacin, danofloxacin, lomefloxacin, enrofloxacin, sarafloxacin, difloxacin, oxolinic acid, nalidixic acid, flumequine, and pyromidic acid) in fish samples. The samples were extracted with *m*-phosphoric acid/acetonitrile mixture (75:25, v/v), purified, and preconcentrated on ENV + Isolute cartridges. The determination was achieved by liquid chromatography using C_{18} analytical column. A mobile phase composed of mixtures of methanol-acetonitrile-10mM citrate buffer at pH 4.5, delivered under optimum gradient program, at a flow rate of 1.5 ml/min, allows accomplishing the chromatographic separation in 26 minutes. For the detection were used serial UV-visible diode-array at 280 nm and 254 nm and fluorescence detection at excitation wavelength/emission wavelength: 280/450, 280/495, and 280/405 nm. The detection and quantification limits were between 0.2–9.5 and 0.7–32 μg/kg, respectively. The procedure was applied to the analysis of spiked salmon samples at two different concentration levels (50 μg/k and 100 μg/kg). Mean recoveries of fluoroquinolones from the salmon samples ranged from 50% to 102%, depending on the analyte.

Keywords: chromatographic method; UV detectecton; salmon

Quinolones and fluoroquinolones are an important family of synthetic antibacterials used in both human and veterinary medicine. In the veterinary field, they are used for the prophylaxis and treatment of veterinary diseases in most types of farm animals, and in aquaculture (Carlucci 1998). These drugs act by inhibiting the bacterial DNA-gyrase. They form a group with different chemical structures and spectra of activity (Gigosos *et al.* 2000). The general structure consists of a 1-substituted-1,4-dihydro-4-oxopyridine-3-carboxylic moiety, combined with an aromatic or heteroaromatic ring. The introduction of the fluorinated quinolones represents important therapeutic advantages, because this antibiotic group shows higher antibacterial activities than the parent compounds (Jackson

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et al. 1998). Their extensive administration to fish, destined for human consumption, has become a serious problem because their residues can persist in edible animal tissues. Quinolones may be directly toxic or be a source of resistant human pathogens, representing a possible risk to human health (Juan-García *et al.* 2006).

The use of antibiotics in food producing animals has generated a considerable interest. The accumulated scientific evidence is that certain uses of antibiotics in food producing animals can lead to antibiotic resistance in intestinal bacteria, and this resistance can then be transmitted to the general population, causing treatment-resistant illness. These uses of antibiotics can also create antibiotic resistance in non-pathogenic bacteria. The resistance genes can be transferred to disease-causing bacteria, resulting in antibiotic-resistant infections for humans. Dissemination of resistant micro-organisms may occur both in hospitals and communities. It is recognised that a major route of transmission of resistant microorganisms from animals to humans is through the food chain (Hernández-Serrano 2005). To protect human health, the European Union and other regulatory agencies around the world, such as the U.S. Food and drug administration (FDA), have established safe maximum residue limits (MRLs) for these drugs and other veterinary substances, for the use as veterinary drugs in animal products entering the human food chain. The use of veterinary drugs is regulated through EU Council Regulation 2377/90/EC (1990), which describes the procedure for establishing MRLs for veterinary medicinal products in foodstuffs of animal origin. In the EU Council Regulation 2377/90, eight fluoroquinolones; danofloxacin (DANO), flumequine (FLUME), marbofloxacin (MARBO), oxolinic acid (OXO), difloxacin (DIFLO), sarafloxacin (SARA), and sum of enrofloxacin (ENRO) and ciprofloxacin (CIPRO), have been included to set up MRLs.

Liquid chromatography (LC) is the analytical method most widely applied for the determination of these compounds, and both UV and fluorescence detections are usually employed. The high fluorescence quantum yield exhibited by several quinolones and fluoroquinolones allow for highly sensitivite analytical methods.

Numerous LC methods have been reported describing the analysis of quinolones and fluoroquinolones in different matrices such as biological fluids (Hernández-Arteseros *et al.* 2002; Samanidou *et al.* 2005; Espinosa-Mansilla *et al.* 2006; Cañada-Cañada *et al.* 2007), environmental samples (Ferding *et al.* 2004; Turiel *et al.* 2005; Cañada-Cañada *et al.* 2009a), and pharmaceutical formulations (Hernández-Arteseros *et al.* 2002; Samanidou *et al.* 2005; Santero *et al.* 2006). In the food area, a huge number of multiresidue methods have been described for the analysis of these compounds in animal tissues or animal feeds. Pecorelli *et al.* (2003) proposed a multi-residue LC method for 13 quinolones in feeds using photodiode-array and fluorescence detection. The separation on a C_5 LUNA column took less than 27 min (Pecorelli *et al.* 2003). Hermo *et al.* (2005) have developed a LC method to determine nine quinolones in pig muscle on XDB-C_8 column and photometry detection. Samanidou *et al.* (2005) have proposed a method for direct determination of five fluoroquinolones in chicken whole blood on Inertsil C_8 (250 × 4 mm) column, using UV detection at 275 nm. Regarding the chromatographic methods for determination of these antibiotics in fish samples, Jonhston *et al.* (2002) have determined eight quinolones in fish tissue and seafood by LC with electrospray ionisation tamdem mass spectrometric detection. Recently, multiresidue determination of seven quinolone antibiotics in gilthead seabream (Dufresne *et al.* 2007) and quinolones and fluoroquinolones in fish and shrimp (Samanidou *et al.* 2008), using LC tandem mass spectrometry, have been reported. Capillary electrophoresis methods for analysing fluoroquinolones in fish matrices have also been reported (Lu *et al.* 2005; Juan-García *et al.* 2006). Recently, two reviews on the determination of antibiotic residues in fish have also been reported (Samanidou *et al.* 2007; Cañada-Cañada *et al.* 2009b).

In general, these methods employ liquid-liquid extraction (LLE) and/or liquid-solid extraction (LSE), in combination with a clean up by solid phase extraction (SPE) procedure. For multi-residue analysis, sample pre-treatments are necessary to extract drugs with different physical/chemical properties. A specific combination of LLE and SPE can be very selective for a specific class of veterinary drugs.

The aim of this study was the development of a chromatographic method for the simultaneous determination of 14 quinolones; pipemidic acid (PIPE), MARBO, enoxacin (ENO), norfloxacin (NOR), CIPRO, DANO, lomefloxacin (LOME),

ENRO, SARA, DIFLO, OXO, nalidixic acid (NALI), FLUME, and pyromidic acid (PYRO) in salmon samples with both diode-array and fluorescence detections. The proposed method determines a wide range of quinolones and fluoroquinolones simultaneously, and would be very useful for routine determination of these drugs in food samples.

MATERIAL AND METHODS

Reagents and materials. Marbofloxacin was supplied from Molekula (Gillingham, UK). Ciprofloxacin, enrofloxacin, and citric acid were purchased from Fluka (Madrid, Spain). Norfloxacin, pipemidic acid, enoxacin, danofloxacin, lomefloxacin, sarafloxacin, difloxacin, oxolinic acid, nalidixic acid, flumequine, and piromidic acid were obtained from Sigma-Aldrich (Madrid, Spain). Acetonitrile and methanol (HPLC-grade) and acetic acid and trifluoroacetic acid (TFA) were supplied from Merck (Barcelona, Spain). Formic acid came from Probus (Badalona, Spain). *m*-Phosphoric acid was purchased from Panreac (Córdoba, Spain). Sodium hydroxide was supplied by Scharlau (Barcelona, Spain). Demineralised ultrapure water, obtained from a MIlli-Q purification system, was used throughout the study.

Apparatus. Agilent Model 1100 LC instrument (Agilent Technologies, Santa Clara, USA) was used equipped with degasser, quaternary pump, manual six-way injection valve, containing a 20 µl loop, UV-visible diode-array detector, rapid scan fluorescence spectrophotometer detector, and the Chemstation software package to control the instrument, data acquisition, and data analysis. LC solvents and the extracts, before their injection into the chromatographic system, were filtered through a 0.22 µm nylon filter membranes (Millipore, Miford, USA). The flow rate was 1.5 ml/minute. UV detection was performed at 280 nm for all quinolones, while the fluorescence excitation/emission wavelengths were 280/450 nm for pipemidic acid, norfloxacin, ciprofloxacin, lomefloxacin, danofloxacin, enrofloxacin, sarafloxacin, difloxacin, and oxolinic acid, 280/495 nm for marbofloxacin, and 280/405 nm for enoxacin, respectively. Oxolinic acid, flumequine, and nalidixic acid were recorded at 254 nm. Analytical column was a Zorbax Eclipse XDB-C_{18}, 150 mm × 4.6 mm, 5 µm particle size (Agilent Technologies, Palo Alto, USA). The SPE cartridge used in this study was ENV + Isolute (3 cm^3/200 mg) obtained from Symta (Madrid, Spain). The pH was measured with a CRISON MicropH 501 meter (CRISON Instruments, Barcelona, Spain). A centrifuge, SELECTA a Mixtasel (Barcelona, Spain), was used to separate the extracts.

Table 1. Gradient program used for the separation of 14 quinolones

Time (min)	Buffer[a] (%)	Acetonitrile (%)	Methanol (%)
0	82	8	10
12	82	8	10
29	40	50	10
30	82	8	10

[a]citric acid/citrate 10 mmol/l at pH 4.5

Solutions. The mobile phase, consisting of citric acid/citrate buffer 10 mmol/l of pH 4.5, acetonitrile and methanol, was mixed by the pump in the gradient mode described in Table 1. The individual stock solutions of each compound were prepared at the concentration of 100 µg/ml. Pipemidic acid, marbofloxacin, enoxacin, norfloxacin, ciprofloxacin, lomefloxacin, danofloxacin, enrofloxacin, sarafloxacin, and difloxacin were prepared by dissolving the exact quantity in 50mM acetic acid aqueous solution. Stock solutions of nalidixic acid, flumequine, oxolinic acid, and piromidic acid were prepared by dissolving the compound in acetonitrile. These solutions were stored at 4°C and were stable for a month at least. The intermediate working solution was prepared by mixing the individual solutions and diluting them to the concentrations of 5 µg/ml with acetonitrile.

All the statistical calculations; regression lines, figures of merit, and analytical quality parameters, were calculated by the ACOC software, a Matlab routine developed by our research group (Espinosa-Mansilla *et al.* 2005).

Tissue sample fortification. A fortified salmon muscle sample was prepared by spiking 5 g of minced blank of salmon, in the range of 50–200 µg/kg, by the addition of an appropriate volume of the working solution of quinolones (50, 100, and 200 µl). After the addition, the spiked salmon samples were allowed to stand 20 min at room temperature, to allow the total interaction between the antibiotics and the salmon sample prior to the extraction.

In order to evaluate the recoveries, calibration graphs were constructed using as signal the peak area versus analyte concentration. For the cali-

brations, samples of 5 g of minced blank salmon were fortified after the SPE procedure, that was considered the 100% extracted, at five concentration levels (20, 30, 50, 100, 200 µg/kg). All samples were analysed in triplicate.

Extraction and purification procedures. The extraction and purification procedures of the quinolones from the salmon samples were performed as reported by Hermo *et al.* (2005). Briefly, five grams of the salmon samples were extracted using two sequential volumes (25 ml and 10 ml) of m-phosphoric acid 0.3%:acetonitrile (75:25, v/v). After the extraction step, the mixtures were centrifuged at 3000 rpm (10 min) at room temperature and the whole liquid extract was filtered through a 22-µm nylon filter. Purification and concentration of the samples was achieved with ENV + Isolute cartridges. The SPE cartridges were conditioned with 2 ml of methanol, 2 ml of water, and 2 ml 50mM H_3PO_4 at pH 3. After the samples had passed, the cartridges were flushed with 2 ml of water and 7.5 ml of hexane in order to defat the extracts. The analytes were eluted with 5 ml of 2%TFA in water and acetonitrile (25:75, v/v). The elutes were evaporated to dryness under a nitrogen stream at 50°C. The residue was dissolved in 500 µl of the mobile phase. The resulting solution was injected into the chromatographic system.

RESULTS AND DISCUSSION

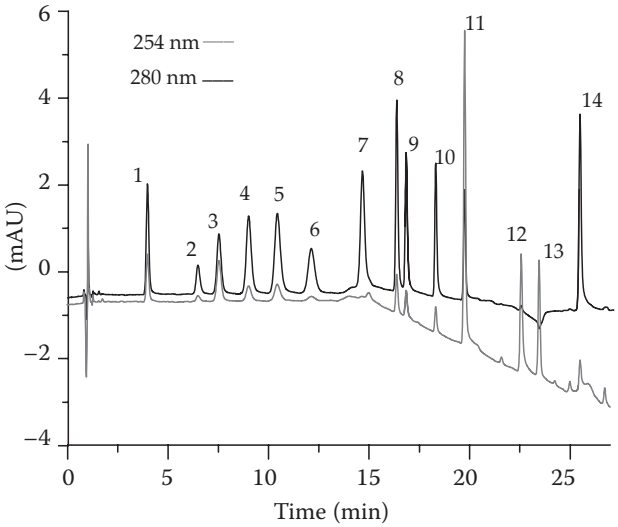
Method development

Several mobile phases were investigated during the chromatographic method development. Different organic solvents such as acetonitrile and/or methanol in combination with different aqueous buffers (formic acid of pH 3, acetic acid of pH 4.5, and citric acid of pH 4.5) were tested. The best separation performance was achieved by using a mobile phase consisting of methanol-acetonitrile-citric acid 10 mmol/l, pH 4.5, with a gradient mode, according to Table 1. The flow rate was maintained at 1.5 ml/minute.

To detect the fourteen analytes simultaneously, the conditions of the detection were specifically optimised. The fluorescence response varied considerably among the quinolones. Excitation/emission wavelengths were 280/450 nm for PIPE, NOR, CIPRO, LOME, DANO, ENRO, SARA, and DIFLO; 280/495 nm for MARBO; 280/405 nm for ENO. DANO exhibited higher fluorescence than most of the other compounds. OXO, FLUME, and NALI were measured at 254 nm. ENO and PIRO were preferably quantified by UV absorbance at 280 nm.

The chromatogram of the mixed standard solution of fourteen quinolones shows a satisfactory separation with good resolution and peak shapes (Figure 1). The separation was achieved in 26 minutes. Under the assay conditions described above, the fluoroquinolones examined were well resolved with the retention times of 3.9 min for PIPE, 6.4 min for MARBO, 7.4 min for ENO, 8.9 min for NOR, 10.3 min for CIPRO, 11.9 min for LOME, 14.6 min for DANO, 16.3 min for ENRO, 16.8 min for SARA, 18.3 min for DIFLO, 19.7 min for OXO, 22.6 min for NALI, 23.5 min for FLUME, and 25.5 min for PIRO. The resolution factors ranged from 2.1 to 11.3, indicating a satisfactory separation as shown in the chromatograms of a standard mixture with photometry detection in Figure 1, and with fluorescence detection in Figure 2.

The detection was optimised for the simultaneous determination of the 14 quinolones and fluoroquinolones of interest. It allowed their quantification in the range of µg/kg. Except for OXO, NALI, FLUME, and PIRO that were detected photometrically, all the quality parameters were calculated by



Peaks: (1) pipemidic acid; (2) marbofloxacin; (3) enoxacin; (4) norfloxacin; (5) ciprofloxacin; (6) lomefloxacin; (7) danofloxacin; (8) enrofloxacin; (9) sarafloxacin; (10) difloxacin; (11) oxolinic acid; (12) nalidixic acid; (13) flumequine; (14) piromidic acid

Figure 1. Chromatograms of a mixture of 14 quinolones (500 ng/ml) with UV detection at 254 nm (grey line) and 280 nm (black line)

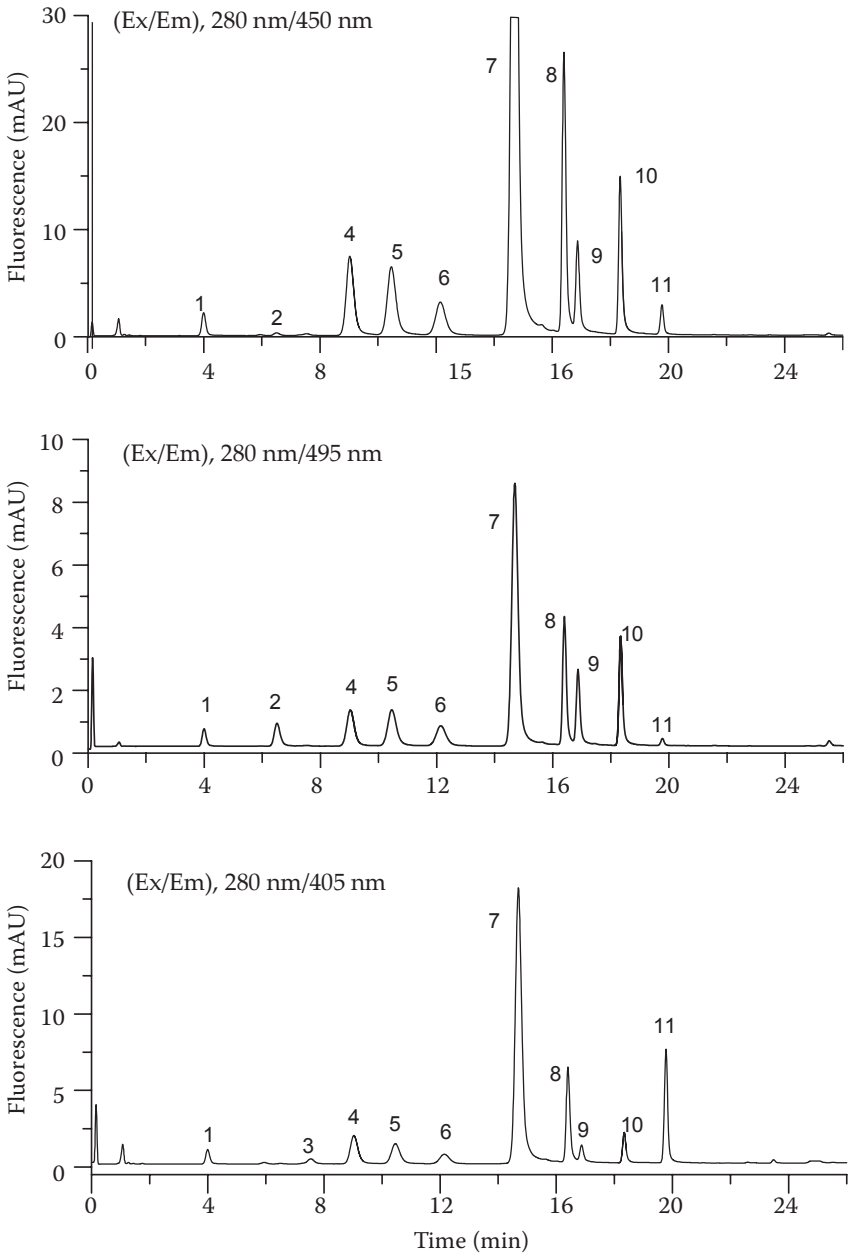


Figure 2. Chromatograms of a mixture of 14 quinolones (500 ng/ml) with fluorimetric detection at λ_{exc} = 280 nm, and λ_{em} = 405, 450, and 495 nm. All peaks as in Figure 1

the fluorimetric signal. In addition to the higher sensitivity, fewer interfering compounds were observed in the chromatogram. The chromatograms of a representative blank and spiked salmon sample (at 50 μg/kg level) with photometric and fluorimetric signals are shown in Figures 3 and 4, respectively. It can be observed that the fluorimetric blank salmon chromatogram is purer than the photometric one, but both chromatograms presented interference at the same retention time as enoxacin, and because of this, this compound was not quantified in the application.

On the other hand, it is important to note that in the blank salmon (without fortifying) some peaks were present at the same retention times as the studied analytes peaks, such as (4) NOR and (7) DANO (Figure 4; Ex/Em, 280/450 nm). In addition, in Figure 3 (254 nm and 280 nm) an unknown peak (17.9 min) appeared close to DIFLO. This unknown peak did not appear in the fluorimetric signal. To confirm the presence of these fluoroquinolones, a MS detector would be required. Nevertheless, the amounts of those analytes present in the blank salmon samples would be below MRLs.

Quality parameters of the method

The calibration graphs were constructed in the presence of the salmon background by means of

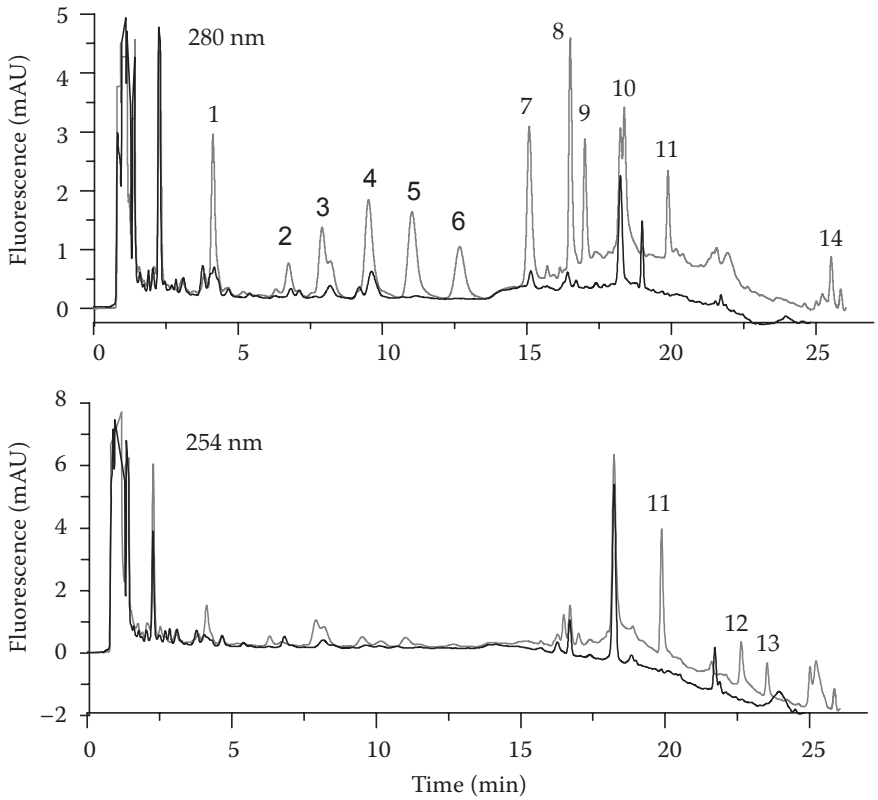


Figure 3. Chromatograms of a salmon blank (black line) and spiked salmon at 50 µg/kg (grey line) with UV detection at 254 and 280 nm. All peaks as in Figure 1

fortifying salmon blank sample, after the SPE procedure, at five concentration levels (20, 30, 50, 100, 200 µg/kg). The calibration plots were linear in the concentration range studied for all compounds, with correlation coefficients ranging between 0.9941 and 0.9987 as can be seen in Table 2. The detection (LOD) and quantification (LOQ) limits were calculated on the basis of the results obtained with five replicates of salmon sample spiked at the 50 µg/kg level. The LOD and LOQ were obtained as 3 and

Table 2. Linear regression data and limits of detection and quantification for the standard curves of the 13 analytes

Analyte	Detection signal (nm)	Slope ± σ	Intercept ± σ	Correlation coefficient (*r*)	LOD (µg/kg)	LOQ (µg/kg)
PIPE	280/450[a]	0.047 ± 0.001	2.3 ± 0.9	0.9974	1.8	5.7
MARBO	280/495[a]	0.023 ± 0.001	0.13 ± 0.34	0.9986	2.9	9.7
NOR	280/450[a]	0.281 ± 0.006	17 ± 6	0.9971	1.7	5.5
CIPRO	280/450[a]	0.273 ± 0.004	5.2 ± 4.7	0.9982	2.3	7.8
LOME	280/450[a]	0.154 ± 0.002	0.5 ± 0.2	0.9987	5.0	16.6
DANO	280/450[a]	1.657 ± 0.050	173 ± 51	0.9941	0.2	0.7
ENRO	280/450[a]	0.469 ± 0.001	19 ± 9	0.9973	0.5	1.7
SARA	280/450[a]	0.148 ± 0.002	2.1 ± 2.2	0.9986	1.8	5.7
DIFLO	280/450[a]	0.245 ± 0.003	3.6 ± 3.6	0.9987	1.0	0.33
OXO	254[b]	0.041 ± 0.001	–1.4 ± 0.8	0.9975	8.9	30.0
NALI	254[b]	0.049 ± 0.001	–2.6 ± 1.0	0.9972	9.2	31.0
FLUME	254[b]	0.032 ± 0.001	–0.8 ± 0.6	0.9979	9.5	32.0
PIRO	280[b]	0.069 ± 0.001	–3.8 ± 1.3	0.9976	8.7	29.0

Linear range for each one: 20–200 µg/kg (n = 5); [a]FLD: (Ex/Em) nm; [b]UV

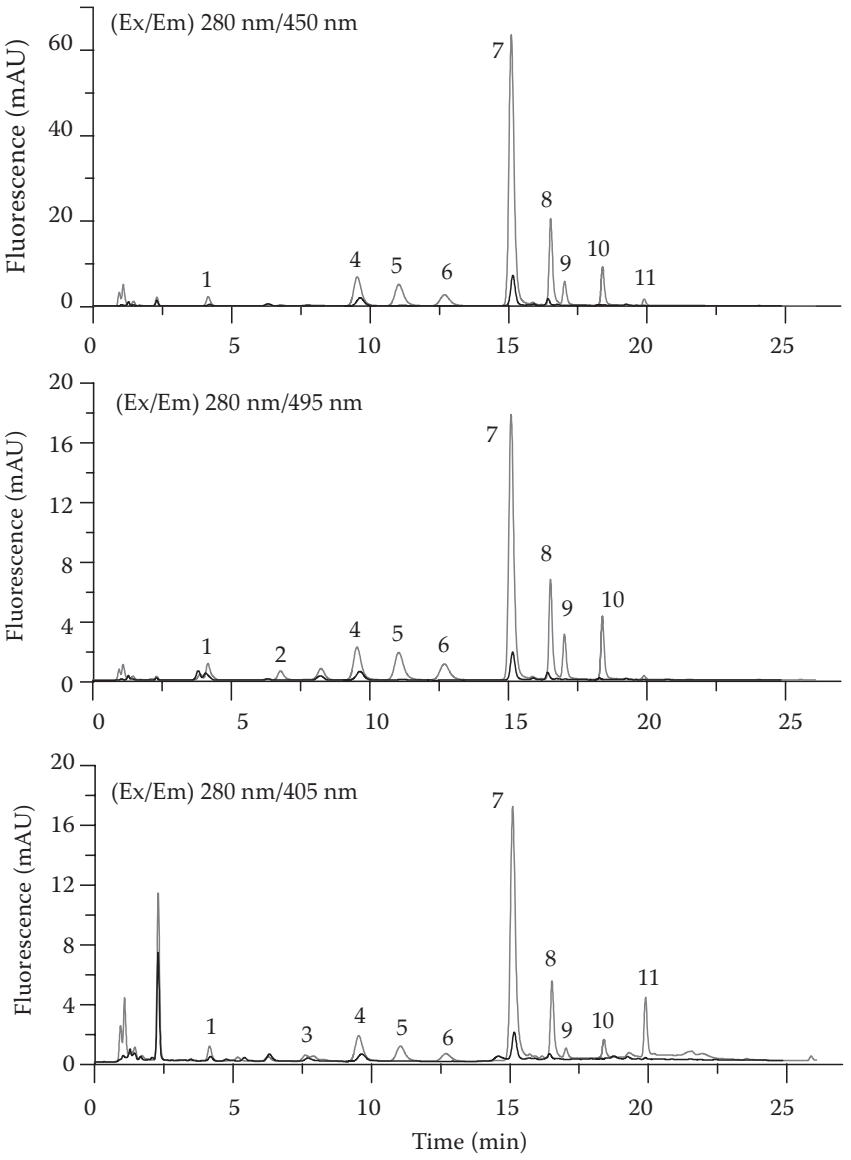


Figure 4. Chromatograms of a salmon blank (black line) and spiked salmon at 50 µg/kg (grey line) with fluorimetric detection at λ_{exc} = 280 nm and λ_{em} = 405, 450, and 495 nm. All peaks as in Figure 1

10 times, respectively, the signal-to-noise ratio. Thus, the average amplitude of the baseline of standard chromatograms, for the time intervals of around two minutes, was multiplied by 3 or 10, and the concentrations of the analytes corresponding to these signals were calculated using the respective calibration curves constructed by employing the peak height as the analytical signal. The detection and quantification limits found were between 0.2–9.5 µg/kg and 0.6–30 µg/kg, respectively.

Method validation

The developed method was applied to determine 13 quinolones and fluoroquinolones in salmon samples. To perform the determination, salmon blank samples were fortified with all the quinolones at two concentration levels (50 µg/kg and 100 µg/kg). Five samples were injected at each level. The recovery averages from salmon spiked samples were determined at each concentration level by comparing the peak area of each compound with the respective calibration curve. The validation data are listed in Table 3. The best recoveries were found for the minor level concentration and for the first seven quinolones. The worst results (R < 50%) were obtained for PIRO. Nevertheless, these recoveries are in accordance with those obtained by others authors (Pecorelli *et al.* 2003). Moreover, acceptable intraday precision (repeatability) was achieved, with relative standard deviation (RSD) values smaller than 8% (Table 3).

Table 3. Recoveries (%R) and repeatability at two levels of spiking

Analyte	Level (µg/kg)	
	50 (n = 5)	100 (n = 5)
	%R (RSD)	
PIPE	102 (5)	71 (5)
MARBO	78 (4)	64 (6)
NOR	86 (1)	72 (6)
CIPRO	80 (1)	69 (7)
LOME	83 (2)	67 (6)
DANO	87 (1)	78 (6)
ENRO	75 (2)	65 (6)
SARA	70 (1)	64 (7)
DIFLO	62 (1)	56 (7)
OXO	68 (4)	58 (5)
NALI	58 (2)	54 (7)
FLUME	53 (2)	50 (6)
PIRO	34 (7)	31 (3)

CONCLUSIONS

The proposed multiresidue SPE LC/DAD/FLD method can be applied for the simultaneous determination and quantification of 13 quinolones and fluoroquinolones from salmon samples. The limits of detections are lower than the MRLs established by the European Union for each antibiotic. Overall, the method is simple to apply to a routine analysis and particularly suitable for screening quinolones and fluoroquinolones residues in food products. We are currently working on improving the purification and extraction stages in order to achieve better recovery percentages for the compounds of interest, which will give rise to a more sensitive and competitive global method.

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