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# Validation of a liquid chromatography–tandem mass spectrometry method for the simultaneous quantification of 11 (fluoro)quinolone antibiotics in swine kidney

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**Abstract**

A LC–MS–MS method has been validated for the simultaneous quantification of 11 (fluoro)quinolone antibiotics at the maximum residue level (MRL) in swine kidney. The studied compounds were danofloxacine, cinoxacine, ciprofloxacine, enoxacine, enrofloxacine, flumequine, marbofloxacine, nalidixic acid, norfloxacine, ofloxacine and oxolinic acid. The method involves solid-phase extraction of these compounds followed by LC–MS–MS analysis using an electrospray ionisation interface. Limits of quantification ≤50 μg/kg could be obtained in swine kidney, much lower than every MRL. The validation is discussed. This work was carried out in order to support the European Union policy on consumer health protection.



*Keywords:* Validation; Fluoroquinolones; Quinolones; Antibiotics

## 1. Introduction

The 4-quinolones represent a recent and highly potent group of antibiotics used in human and in veterinary medicine. Their antibacterial activity is based on a selective inhibition of bacterial DNA synthesis [1]. The 6-fluorinated piperazinyl derivatives (fluoroquinolones) are even more active against Gram-negative bacteria and also moderately against Gram-positive bacteria [2–4]. These compounds were initially applied in the treatment of urine tract infections but are now broadly used in the treatment of respiratory diseases and enteric bacterial infections in humans and in food-producing animals such as cattle, swine, turkey, chicken [5,6] as well as for diseases in aquacultured fish [7].

The widespread use of 4-quinolones and other antibiotics in agriculture has resulted in the potential presence of these compounds residues in foodstuffs from animal origin. In parallel to the exposure to low levels of these compounds, an upsetting increase of resistant human pathogens has been observed [8–10] constituting a public health hazard, primarily through the increased risk of treatment failures [11–14].

In the frame of its policy on consumer health protection, the European Union (EU) established maximum residue levels (MRLs) for various classes of antibiotics among which (fluoro)quinolones, in different animal tissues (Council Regulation 2377/

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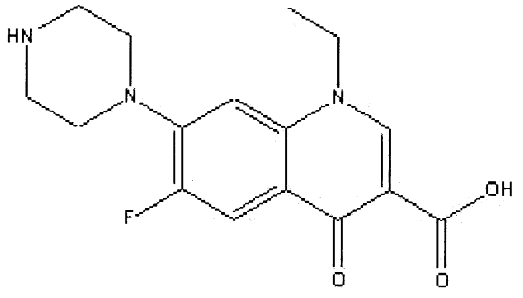
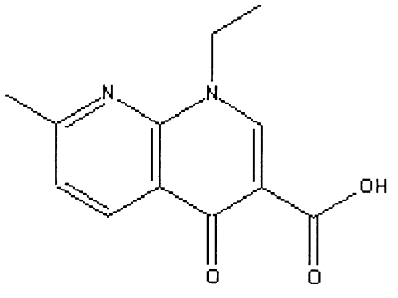
90/EEC [15]). State laboratories of the EU have to monitor the residues possibly present in samples coming from the slaughterhouses (Council Directive 96/23/EC [16]). The great chemical variety of 4-quinolones and the possibility of trace level residues made it necessary to develop sensitive multiresidue screening methods. Several procedures have been described in literature for the determination of 4-quinolones in various biological matrices, the majority being based on liquid chromatography using UV detection [17–19], fluorimetric detection [20–24] or mass spectrometric detection [25–31] but 4-quinolones have also been studied by thin-layer chromatography [32] and capillary electrophoresis [33]. Although most of these methods can achieve the quantification of 4-quinolones at very low concentration levels, and particularly under the MRLs, they are usually limited to the determination of a few 4-quinolones at a time (maximum seven [26]) [34]. This is often due to coelution problems for compounds belonging to the same chemical family and also due to p$K_a$ differences that hamper the sample preparation step [24,26]. However, there is a high demand for reference screening methods targeting high number of antibiotics and consuming reduced amount of solvents and for method validation [34]. With this goal a liquid chromatography–tandem mass spectrometry (LC–MS–MS) method has been previously designed in our laboratory for the identification of eleven 4-quinolones in swine kidney [35]. The present work was thus intended to validate the method for the simultaneous quantification of the eleven compounds at (and under) MRLs. The studied (fluoro)quinolones were danofloxacine, cinoxacine, ciprofloxacine, enoxacine, enrofloxacine, flumequine, marbofloxacine, nalidixic acid, norfloxacine, ofloxacine and oxolinic acid (Fig. 1) which MRLs are presented in Table 1. As no MRL has been defined yet for a few (fluoro)quinolones, 150 ppb was selected as an average limit for these compounds.




Norfloxacine




Nalidixic acid

Fig. 1. Chemical structures of two (fluoro)quinolones.

It is the first time that an analytical method is proposed to determine all these compounds in a single-run analysis. The method implies the solid-phase extraction of the samples followed by the LC–MS–MS analysis of the analytes using an electrospray (ESI) interface. The compounds are detected in the multiple reaction monitoring (MRM) mode which provides a high level of selectivity for targetting the analytes in a complex biological matrix. Deuterated norfloxacine was especially synthesised and selected as an internal standard having chemical and physical properties similar to those of the analytes. A complete ''one-laboratory'' validation was performed following the very good SFSTP (Société Française des Sciences et Techniques Pharmaceutiques) guide on validation of bioanalytical methods [36,37]. The Washington Conference report [38] which is being used as a basis for bioanalytical method validation gives indeed the minimum requirements but does not provide any validation strategy. Consequently, a dedicated SFSTP Commission was created in 1995 to define both experimental and statistical strategies that allow the analyst to perform the most profitable experimental work possible. The strategy presented in the SFSTP report is principally designed for chromatographic methods in

Table 1
MRLs defined by the EU in swine kidney [15] for the studied (fluoro)quinolones: estimated LODs and LOQs determined by two different methods (see text)

| | MRL (μg/kg) | LOQ (μg/kg), $S/N=10$ | LOQ (μg/kg), LCL≥80% UCL≤120% | LOQ (μg/kg) =0.3×LOQ |
|---|---|---|---|---|
| Cinoxacine | Not defined | 12 | Not determined | |
| Ciprofloxacine | –[a] | 0.1 | <36 | <11 |
| Danofloxacine | 200 | 4 | 50 | 15 |
| Enoxacine | Not defined | 13 | <36 | <11 |
| Enrofloxacine | –[a] | 0.1 | 47 | 14 |
| Flumequine | 1500 | 19 | Not determined | |
| Marbofloxacine | 150 | 4 | <38 | <11 |
| Nalidixic acid | Not defined | 10 | Not determined | |
| Norfloxacine | Not defined | 5 | <36 | <11 |
| Ofloxacine | Not defined | 2 | 50 | 15 |
| Oxolinic acid | 150 | 13 | Not defined | |

[a] MRL for ciprofloxacine + enrofloxacine = 300 μg/kg.

pharmaceutical applications but it can be reasonably applied to other techniques and other application fields with similar specifications. In this paper, using the same strategy, maximum information could be driven from pre-validation experiments reducing the usually time-consuming validation step. Selectivity, linearity, accuracy and precision results are discussed.

## 2. Experimental

### 2.1. Instrumentation and methods

Here is given an overview of the experimental conditions used. If needed, more detailed information is presented in our previous work [35].

The solid-phase extraction (SPE) of the samples was performed using SDB-RPS disposable extraction disk cartridges (10 mm, 6 ml) from 3M Empore (St. Paul, MN, USA) containing mixed $C_8$ and cation-exchange phases. A 1-g amount of swine kidney was sliced and spiked with 150 ng of internal standard. (Fluoro)quinolones were also added to the samples at concentrations corresponding to MRL/4, MRL/3, MRL/2, MRL and MRL×2 for the preparation of calibration curves. The fortified samples were disrupted and homogenised in acetonitrile before centrifugation [35]. The supernatants were acidified with acetic acid 96% and the SPE was performed using acetonitrile–96% acetic acid (95:5, v/v) as a conditioning mixture. The (fluoro)quinolones were eluted by a mixture of methanol–1 *M* ammonia (75:25, v/v). The eluates were evaporated to dryness and the residues were redissolved in 300 μl of diluted formic acid (pH 2.5) before injection into the LC system.

The LC equipment was a Waters Alliance 2690 quarternary solvent delivery system (Waters, Milford, MA, USA). The chromatographic separation of the (fluoro)quinolones was performed using a Nucleosil 100-5 $C_{18}$ (70×4 mm; 5 μm particle diameter) reversed-phase column from Macherey-Nagel (Düren, Germany). A linear elution gradient from 2 to 70% of B in 5 min (70% of B for 1 min) was employed, where A was diluted formic acid (pH 2.5) and B was acetonitrile. The flow-rate of the mobile phase was 1.0 ml/min. A Valco zero dead volume T-piece splitter (9/1) from Micromass was used between the LC column and the ESI interface in order to reduce the flow-rate of the chromatographic effluent down to 100 μl/min. Injection volumes of 50 μl were used in LC.

The ESI–MS–MS detection of the (fluoro)-quinolones was achieved using a Quattro LC triple stage quadrupole instrument from Micromass (Manchester, UK). The positive ionisation mode was used and the ions were monitored in the MRM mode. The capillary voltage was 3.2 kV. The source block and desolvation temperatures were set at 130 and 400 °C, respectively, while the desolvation and nebuliser gas ($N_2$) flow-rates were 650 and 75 l/h, respectively.

Argon was used as a collision gas at $2.5 \cdot 10^{-3}$ mbar. Cone voltage and collision energy were optimised for each (fluoro)quinolones separately [35].

### 2.2. Standards

Norfloxacine, ofloxacine, cinoxacine, oxolinic acid, nalidixic acid and flumequine were obtained from Sigma–Aldrich (St. Louis, MO, USA). Enoxacine, ciprofloxacine and enrofloxacine were provided by Bayer (Leverkusen, Germany). Danofloxacine was from Pfizer (Groton, CT, USA) and marbofloxacine from Vetoquinol (Aartselaar, Belgium). Deuterated norfloxacine was synthesised by the ''Centre de Recherche du Cyclotron'' (University of Liège, Belgium).

Swine kidney samples were provided by the ''Institut d'expertise vétérinaire'' (IEV, Ministry of Health, Bruxelles, Belgium). These samples gave a negative reaction to the official Belgium renal test published in the ''Moniteur Belge 28/07/1995 20368-20371'' indicating the absence of agents inhibiting the bacterial growth in the samples.

### 2.3. Standard solutions

Three different stock solutions (100 mg/l) were prepared for each (fluoro)quinolone in methanol–1 *M* NaOH (99/1, v/v) in order to perform three calibration curves (series) in swine kidney. Three stock solutions of the internal standard, deuterated norfloxacine (100 mg/l) were also prepared in methanol. Secondary standard solutions were then obtained by dilution in methanol, one for the internal standard (150 μg/l), one for flumequine (6000 μg/l) and one for the mixture of all the other (fluoro)-quinolones (600 μg/l). Ternary solutions containing 100 μl of the mix solution, 100 μl of flumequine solution and 100 μl of the internal standard solution were finally prepared in order to provide five concentration levels: a concentration at a low level (MRL/4), a concentration two-fold higher (MRL/2), a concentration at the maximum expected level (MRL×2) and intermediate concentrations (MRL/3 and MRL). These solutions were used to spike the swine kidney samples for the preparation of both biological and non-biological calibration curves. The use of three different kidney sources would have been an ideal but only one source was available in this case.

## 3. Results and discussion

The experiments were divided in two parts: pre-validation and validation. Because of the large amount of data resulting of the analysis of eleven (fluoro)quinolones, only figures related to norfloxacine analysis will be illustrated in this paper while the results obtained for the ten other compounds will be summarised into tables.

### 3.1. Pre-validation step

During the pre-validation step:

(i) the response function of the calibration curve obtained for each (fluoro)quinolone in swine kidney was identified;
(ii) the limits of detection (LOD) and quantification (LOQ) were estimated;
(iii) the range of calibration levels was defined;
(iv) the absolute recovery was determined; and
(v) the selectivity was evaluated.

#### 3.1.1. Response function

Three calibration curves (series) were successively analysed in swine kidney using triplicates at each concentration level. The responses were expressed as:

$$\text{response} = \text{area} \cdot \frac{\text{I.S.}_{\text{concentration}}}{\text{I.S.}_{\text{area}}}$$

where I.S. is the internal standard.

Responses obtained for the three series were plotted as a function of the concentration. A determination coefficient ($r^2$) of 0.9963 could be obtained for norfloxacine in the calibration range MRL/4-MRL×2.

The homogeneity of the variances (homoscedasticity) over the calibration range was then tested in order to decide for a least squares regression model or for a transformation method. Plotting the variances as a function of the concentrations, a linear increase of the variances could be observed when increasing the concentrations as it is often reported with LC–MS–MS methods. However, the Bartlett

Table 2
Pre-validation tests for norfloxacine in swine kidney

| | Results | Test interpretation |
|---|---|---|
| Calibration curve | $Y = 1.8827X - 3.4813$ | |
| Coefficient of determination | 0.9971 | |
| Curve type | Linear | |
| Origin | Exclude | |
| Weighting | $1/X$ | |
| Homoscedasticity: | | |
| Bartlett test: $\chi^2$ table (0.95; $k-1$)=9.49 | 5.96$<\chi^2$ table | No significant |
| Cochran test: $g$ table (0.95; $k$; $n-1$)=0.68 | 0.63$<g$ table | difference between |
| Hartley test: $r$ table (0.95; $k$; $n-1$)=202.00 | 28.59$<r$ table | variances |
| where $k$=number of groups; | | |
| $n$=number of replicates | | |
| Lack-of-fit test: | | |
| Slope: Snedecor's $F$ (0.95; 1; $n-2$)=4.67 | $F_1$=3535.94$>F$ table | The slope is significantly different |
| Linearity: Snedecor's $F$ (0.95; $k-2$; $n-k$)=3.71 | $F_2$=0.49$<F$ table | from zero. The model fits with the data |
| Absolute recovery (%) and RSD (%) at: | | |
| MRL/4 | 102.9±9.9 | |
| MRL/3 | 103.8±0.9 | |
| MRL/2 | 95.7±7.5 | |
| MRL | 97.8±5.7 | |
| MRL×2 | 101.7±3.5 | |

test, the Cochran test, and the Hartley test gave no significant difference between variances at the different concentration levels (Table 2). Nevertheless, a weighted regression model using a weighting factor ($W_j$) of $1/X$ was applied instead of an ordinary least squares regression model in order to obtain a constant relation between variances and concentrations. The calibration curve obtained using this model was not forced through zero as this can introduce a bias in the method. By this way, a determination coefficient ($r^2$) of 0.9971 could be obtained for norfloxacine in swine kidney (Table 2).

Before taking a definitive decision about the fit of the $1/X$ weighted regression model, the residuals ($Y_{\text{observed}} - Y_{\text{estimated}}$) from the three calibration curves were calculated and plotted as a function of the concentrations. The graph showed that the residuals were slightly decreasing when increasing the concentrations indicating that the upper concentrations were better represented by the model than the lowest concentrations. Nevertheless, the distribution of residuals around the fitted calibration curve was satisfactory. Finally, the regression model was evaluated using an analysis of variance (ANOVA) lack-of-fit test that determines the ratio between the residual error and the pure experimental error [36,39,40]. As expressed in Table 2, the regression model fitted with the data in the range MRL/4 to MRL×2.

### *3.1.2. Evaluation of LOD and LOQ and selection of the calibration range*

Once the weighted regression model was selected, LOQs were evaluated for each (fluoro)quinolone using two different methods. The fist method is based on the calculation of the lowest and the highest quantifiable values (LQV and HQV) between which the analyte recoveries and their confidence intervals are included in 80–120%. By this way, LQV and HQV define the calibration range where the accuracy of the method is verified. The second method is the common determination of LOQs and LODs corresponding to the concentrations giving signal-to-noise ratios of 10 and 3, respectively.

In order to calculate LQV and HQV, the concentrations were first back calculated from the analytical response using the regression equation of each calibration curve. The concentrations obtained for the three calibration curves were then pooled by concentration level and a mean recovery was determined from calculated and theoretical concentrations. Lower and upper confidence limits (LCL

and UCL) at the 95% level could be attributed to these recoveries using the following formulas [37]:

$$\mathrm{LCL}\left(R_j^{\%}\right) = \frac{\left(\bar{u}_j - t\left(0.1, \sum_k^p n_{jk} - p\right) s_{\mathrm{IP}}\right)}{X_j} \cdot 100$$

$$\mathrm{UCL}\left(R_j^{\%}\right) = \frac{\left(\bar{u}_j + t\left(0.1, \sum_k^p n_{jk} - p\right) s_{\mathrm{IP}}\right)}{X_j} \cdot 100$$

where 0.1 = 10% significance level ($\alpha = 10\%$) in the table; $\bar{u}_j$ is the mean estimated concentration; $R_j^{\%}$ the mean recovery (%); $s_{j(\mathrm{IP})}$ the standard deviation for intermediate precision; $X_j$ the introduced concentration; LCL($R_j^{\%}$) the lower confidence limit at the 95% level of the mean recovery (%); UCL($R_j^{\%}$) the upper confidence limit at the 95% level of the mean recovery (%).

Example is given in Fig. 2 for norfloxacine in swine kidney. Lower and upper confidence limits of the mean percentage recovery at concentrations from MRL/4 to MRL×2 were included in the acceptance interval of 80–120%. If a confidence limit (lower or upper or both) was out of the acceptance interval, the concentration level should be excluded and the calibration range reduced to the next concentration level giving acceptable confidence limits. The graph also shows that in the case of norfloxacine the LOQ is probably inferior to MRL/4 (<36 μg/kg). The LOQs obtained for each (fluoro)quinolone in swine kidney using the same approach are presented in Table 1. They were equal or lower than 50 μg/kg.

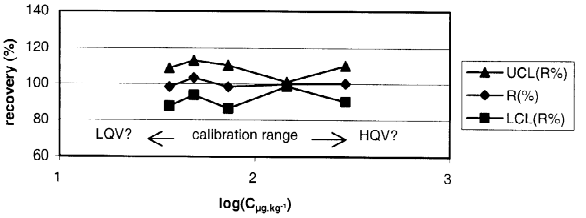



Fig. 2. Accuracy profile and estimation of the limits of quantification for norfloxacine in swine kidney. Log($C_{\mu g/kg}$) is the logarithmic transformation of the concentration (μg/kg). UCL(R%) is the upper confidence limit at the 95% level of the mean recovery (%); LCL(R%) is the lower confidence limit (%); R is the mean recovery (%). LQV is the lowest quantifiable value; HQV is the highest quantifiable value. UCL(R%) and LCL(R%) are included in the acceptance interval of 80–120% over the whole calibration range selected.

On the other hand, using a linear regression model, LODs could be estimated from the LOQs by the relation LOD = 0.3×LOQ (Table 1).

In addition, Table 1 shows that the LOQs obtained for the (fluoro)quinolones in LC–MS–MS are much lower than the MRLs. However the LOQs of four (fluoro)quinolones could not be determined using LCL and UCL because of higher $s_{j(\mathrm{IP})}$ values generating confidence limits out of range. In this case, as an alternative, LODs and LOQs could be estimated by the second method involving the measurement of the signal-to-noise ratio ($S/N$). In order to allow a comparison between analytes, the LOQs of the other (fluoro)quinolones were also estimated by this method (Table 1). Cinoxacine, enoxacine, flumequine, nalidixic acid and oxolinic acid gave higher LOQs than the other (fluoro)quinolones. On the other hand, all LOQs were lower using the $S/N$. It is important to note the difference between LOQs obtained by both methods. If the use of LCL and UCL acceptance interval is much stricter, it takes into account the regression model and the measurements accuracy whereas the signal-to-noise ratio can be considered as more informative because no accuracy is guaranteed at $S/N = 10$.

### *3.1.3. Absolute recovery*

Calibration curves were performed for each (fluoro)quinolone in aqueous solution and in spiked swine kidney samples. The absolute recoveries of the analytes from swine kidney were evaluated by the ratio of the mean response obtained at each concentration level in swine kidney to the mean corresponding response in aqueous solution. Results obtained at each concentration level for norfloxacine are detailed in Table 2. In general, mean absolute recoveries between 97.9 and 100.8% could be obtained for all (fluoro)quinolones (Table 3). Absolute recoveries very close to 100% indicate that the behaviour of the internal standard during the sample preparation step is very similar to that of the analytes as expected. In addition, mean relative standard deviations (RSDs) between 5.0 and 6.2% were determined for most of the compounds, indicating a very good reproducibility of the extraction procedure throughout the whole calibration range (Table 3).

Table 3
Mean absolute recoveries (%) and mean RSDs (%) obtained for each (fluoro)quinolone over the calibration range

| | Mean absolute recovery (%) | Mean RSD (%) |
|---|---|---|
| Danofloxacine | 100.7 | 4.8 |
| Cinoxacine | 98.8 | 12.8 |
| Ciprofloxacine | 99.9 | 6.0 |
| Enoxacine | 100.8 | 5.6 |
| Enrofloxacine | 99.9 | 6.2 |
| Flumequine | 97.9 | 12.1 |
| Marbofloxacine | 100.5 | 5.8 |
| Nalidixic acid | 98.4 | 11.1 |
| Norfloxacine | 100.4 | 5.5 |
| Ofloxacine | 100.1 | 6.1 |
| Oxolinic acid | 98.11 | 13.22 |

Cinoxacine, flumequine, oxolinic acid and nalidixic acid showed higher RSDs between 11.1 and 13.2% but still inferior to the 15% commonly accepted in biological samples.

### *3.1.4. Selectivity*

The selectivity of the method could be demonstrated by the SPE–LC–MS–MS analysis of swine kidney samples considered as negative in terms of (fluoro)quinolones MRLs by the ''Institut d'expertise vétérinaire''. No interference was observed with the monitored MS reactions. Fig. 3 illustrates the selective monitoring of the eleven (fluoro)quinolones at the concentration MRL/4 in a swine kidney sample. On the other hand, flumequine residue could be detected in ''negative'' samples at an estimated concentration of 24 μg/kg, much lower than MRL/4 (Fig. 4).

## *3.2. Validation step*

The validation step provided three different informations:

(i) the confirmation of the weighted regression model selected during the pre-validation;
(ii) the accuracy of the method; and
(iii) the precision of the method including repeatability and intermediate precision.

The validation step was performed for the eleven (fluoro)quinolones simultaneously in swine kidney during three different days. The five concentration levels (MRL/4, MRL/3, MRL/2, MRL and MRL× 2) defined in the pre-validation were investigated. Three replicates were analysed at each concentration level for the determination of the calibration curves whereas six replicates were used to calculate the accuracy and the precision of the method at three concentration levels. The validation results obtained for the 11 (fluoro)quinolones are presented in Table 4.

### *3.2.1. Confirmation of the regression model*

Using the weighted regression model, coefficients of regression superior to 0.99 could be obtained for seven (fluoro)quinolones over the 3 days of validation. However lower coefficients of regression were obtained for cinoxacine, flumequine, oxolinic acid and nalidixic acid.

### *3.2.2. Accuracy*

Satisfactory accuracy results were achieved for the three calibration curves of danofloxacine, ciprofloxacine, enoxacine, enrofloxacine, marbofloxacine, norfloxacine and ofloxacine with recoveries and confidence intervals (CIs, %) together included in the interval 80–120% at MRL/4, MRL/3 and MRL. Whereas, cinoxacine, flumequine, oxolinic acid and nalidixic acid showed recoveries from 79.1 to 132.1% and confidence intervals from 10.4 to 83.3% at the same concentration levels. In Table 4, because of the large amount of data, only pooled recoveries and confidence intervals obtained for the three concentration levels are presented.

### *3.2.3. Precision*

The precision of the method was evaluated at MRL/4, MRL/3 and MRL by repeatability and reproducibility coefficients (%). Repeatability and reproducibility coefficients much lower than 15% at MRL/3 and MRL and much lower than 20% at LOQ (MRL/4) could be obtained for the seven (fluoro)-quinolones giving satisfactory accuracy results. Mean repeatability and reproducibility coefficients were 7.1 and 13.0%, respectively. However, repeatability and reproducibility coefficients of 33.2 and 37.3%, respectively, were obtained for cinoxacine, flumequine, oxolinic acid and nalidixic acid.

Obviously, the method is perfectly accurate and precise for danofloxacine, ciprofloxacine, enoxacine, enrofloxacine, marbofloxacine, norfloxacine and ofl-

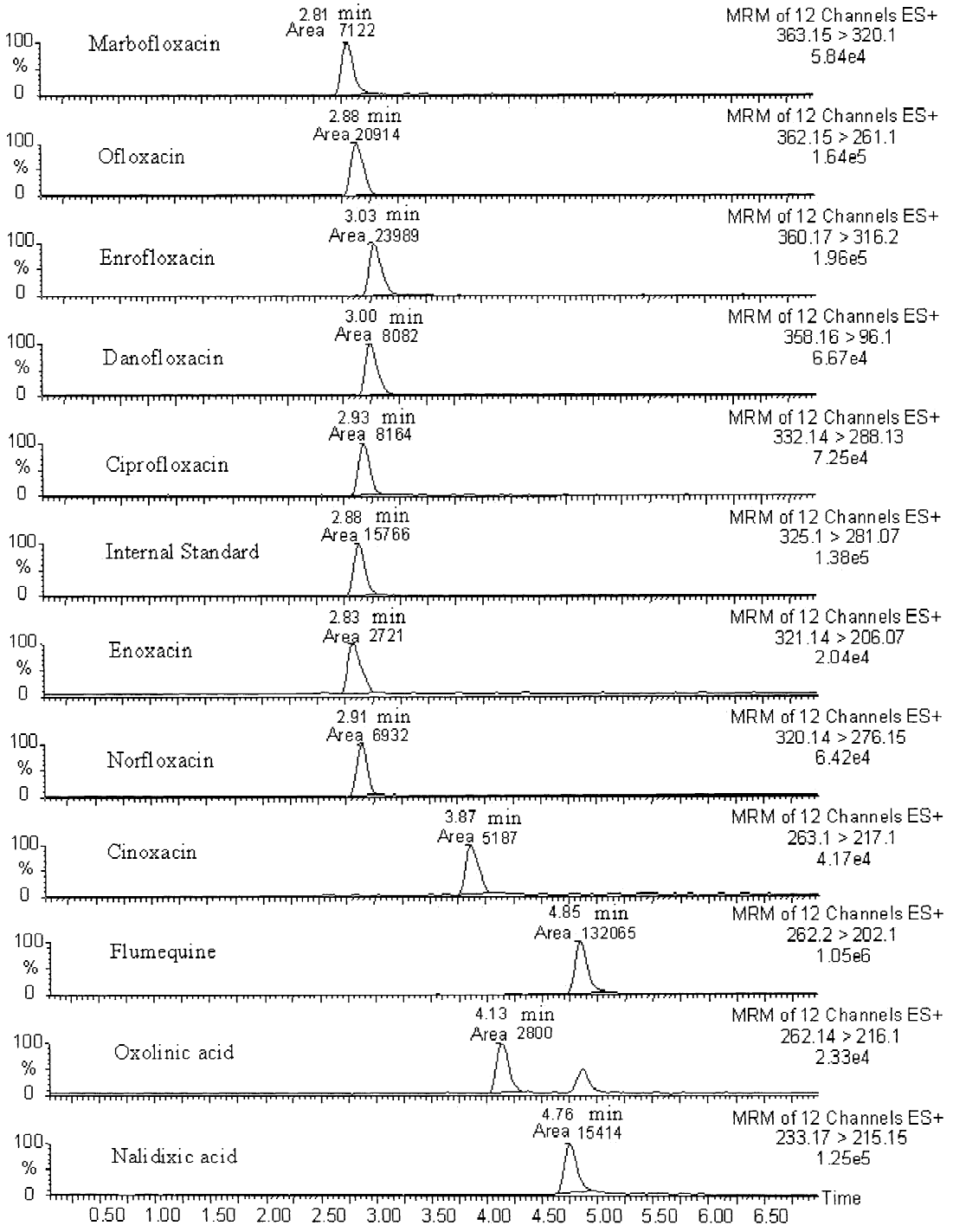



Fig. 3. Reconstituted ion chromatogram obtained after the SPE–LC–MS–MS analysis of a swine kidney sample spiked with 11 (fluoro)quinolones and one internal standard at MRL/4.

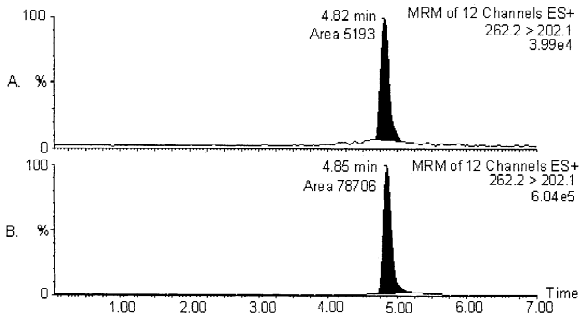



Fig. 4. Comparison of the reconstituted ion chromatograms obtained after the SPE–LC–MS–MS analysis of a ''negative'' swine kidney from the ''Institut d'expertise vétérinaire'' (A) and a swine kidney sample spiked with flumequine at MRL/4 (B). An approximate amount of 24 μg/kg flumequine has been detected in the ''negative'' swine kidney sample by monitoring the flumequine ions transition $m/z$ 262.2>$m/z$ 202.1 (MRM mode).

oxacine whereas cinoxacine, flumequine, oxolinic acid and nalidixic acid are more susceptible to quantitative variations using the SPE–LC–MS–MS method.

### *3.3. Particular behaviour of cinoxacine, flumequine, oxolinic acid and nalidixic acid*

In order to explain the different results obtained for cinoxacine, flumequine, oxolinic acid and nalidixic acid compared to the other (fluoro)-quinolones, the different steps of the analytical method were investigated. First, all phases of the sample preparation involving handling and physical separation processes (filtration, centrifugation) could be excluded from the possible sources of variation as an internal standard was added to the samples at the early step of the method. The solubilisation of the standards could also not be suspected in the given conditions.

On the other hand, processes involving the different chemical properties of the compounds could explain the different results obtained and not corrected by the internal standard. For instance, the variable retention of the analytes on the solid-phase extraction cartridge could generate a lack of accuracy and reproducibility for a few of them. However, the good solid-phase extraction recoveries obtained for acidic (fluoro)quinolones at pH 2.5 in swine kidney [35] demonstrate the efficiency of the selected mixed cation-exchange and reversed-phase stationary phase.

The ionisation process achieved in MS detection also highly depends on the chemical properties of the analytes and could be responsible for not reproducible responses. Because of the relatively large number of compounds simultaneously analysed in LC–MS–MS, a competition between analytes to be ionised in the MS source could even be suspected. Compounds undergoing preferential ionisation would give good repeatability results whereas a less efficient ionisation could also generate less reproducible response for some compounds.

In order to test this hypothesis, individual standard solutions of cinoxacine, flumequine, oxolinic acid and nalidixic acid at the concentration of MRL×2 and containing the internal standard were analysed by LC–MS–MS using eight replicates. The RSDs of the responses were compared to those obtained for norfloxacine and enoxacine in the same conditions. Then a mix solution of cinoxacine, flumequine, oxolinic acid, nalidixic acid, norfloxacine and enoxacine with internal standard was prepared at the concentration of MRL×2 (Sol A). A mix solution of the eleven (fluoro)quinolones with internal standard was prepared at the same concentration level (Sol B). Sol A and Sol B were analysed by the same method and the resulting RSDs are presented in Table 5.

The RSDs of the responses obtained for all compounds were lower in Sol B (11 compounds, mean RSD=5.5%) than in Sol A (six compounds, mean RSD=11.0%). Moreover, the C.V.s obtained in pure standard solutions (mean RSD=13.2%) were often higher than those obtained in Sol A (mean RSD=11.0%). Therefore it seems that the hypothesis of a competition between analytes for the ionisation in the MS source can be rejected. On the contrary, the ionisation process of the (fluoro)-quinolones seems to be better when the number of compounds simultaneously present in solution increases. Similar phenomena can sometimes be observed in biological samples compared to standard aqueous solutions as far as no particular ion suppression effect is generated by the biological matrix. These phenomena are related to the fact that the environment of the analytes in solution is very important for the ionisation process. In certain cases,

Table 4
Validation results of the studied (fluoro)quinolones in swine kidney

| | Norfloxacine | Ofloxacine | Cinoxacine | Flumequine | Enoxacine | Oxolinic acid | Nalidixic acid | Marbofloxacine | Enrofloxacine | Ciprofloxacine | Danofloxacine |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Calibration curve (5 points, $n=3$) | | | | | | | | | | | |
| Equation | $y=1.883x$ $-3.481$ | $y=4.034x$ $+35.386$ | $y=0.894x$ $+4.669$ | $y=1.986x$ $+299.512$ | $y=0.937x$ $-7.524$ | $y=0.531x$ $+0.928$ | $y=2.300x$ $+83.091$ | $y=1.257x$ $-0.66$ | $y=4.694$ $+60.870$ | $2.348x$ $-15.452$ | $1.605x$ $+19.795$ |
| Determination coefficient $r^2$ | 0.9971 | 0.9910 | 0.9910 | 0.9584 | 0.9963 | 0.9831 | 0.9782 | 0.9923 | 0.9926 | 0.9962 | 0.9950 |
| Mean accuracy ($n=6$) | | | | | | | | | | | |
| Recovery (%) | 100.9 | 102.7 | 106.6 | 97.3 | 100.9 | 111.3 | 95.7 | 103.8 | 102.5 | 101.5 | 99.8 |
| Confidence interval (%) | 5.3 | 9.4 | 25.7 | 41.3 | 6.4 | 29.3 | 30.8 | 8.5 | 8 | 4.8 | 8.2 |
| Precision (calculated from the recoveries) | | | | | | | | | | | |
| Mean repeatability ($n=6$; $k=3$) | 4.1 | 8.2 | 17.7 | 44.7 | 6.1 | 39.4 | 31.2 | 8.3 | 8.5 | 5.4 | 9.2 |
| Mean reproducibility ($n=18$, $k=3$) | 11.3 | 12.7 | 22.3 | 45.1 | 12.0 | 44.2 | 37.5 | 13.0 | 15.2 | 11.3 | 15.1 |

Confidence interval $=st/\sqrt{n}$, where $t=$Student's $t$ $(n-1)$ and $n=$number of replicates.

Table 5
Repeatability study in aqueous solution: comparison of the RSD (%) obtained by the LC–MS–MS analysis (eight replicates) of (fluoro)quinolones in pure standard solution containing the internal standard, in Sol A (six analytes + internal standard) and in Sol B (11 analytes + internal standard)

| Repeatability RSD (%) ($n=8$) | Pure standard solution | Sol A | Sol B |
|---|---|---|---|
| Danofloxacine | | | 4.5 |
| Cinoxacine | 20.9 | 13.8 | 8 |
| Ciprofloxacine | | | 4.7 |
| Enoxacine | 5.2 | 5.9 | 4.4 |
| Enrofloxacine | | | 5.4 |
| Flumequine | 7.3 | 12.9 | 6.2 |
| Marbofloxacine | | | 3.8 |
| Nalidixic acid | 15.1 | 11.1 | 6.9 |
| Norfloxacine | 5.2 | 6.5 | 3.4 |
| Ofloxacine | | | 3.5 |
| Oxolinic acid | 25.4 | 15.6 | 9.6 |

RSD were calculated from the ratios of the analyte area to the internal standard area.

a mix solution is more favourable than a pure solution. In this case, it seems to improve the performances of the multi-residue analytical method compared to a single compound analysis.

Besides, in pure standard solution as well as in Sol A and Sol B, the repeatability observed for norfloxacine and enoxacine was higher than the one of cinoxacine, flumequine, oxolinic acid and nalidixic acid. The repeatability of the response obtained in LC–MS–MS in standard aqueous solution without any sample preparation step can thus vary significantly from a compound to another one. It could be related to an inherent less efficient and reproducible ionisation process. As an indication, the LOQs obtained from the $S/N$ of these compounds were slightly higher than those of the other (fluoro)-quinolones.

The differences in chemical structure between cinoxacine, flumequine, oxolinic acid or nalidixic acid and the other (fluoro)quinolones were taken into account. As mentioned by Yorke and Froc [23] and by Volmer et al. [26], cinoxacine, flumequine, oxolinic acid and nalidixic acid are acidic and show a $pK_a$ around 6 related to the carboxylic function. The other studied (fluoro)quinolones show an additional piperazinyl moiety. This basic moiety generates a second $pK_a$ value around 9 and makes these (fluoro)-quinolones amphoteric instead of acidic. At acidic pH, they exist mostly as cations whereas the acidic (fluoro)quinolones are neutral. In the given LC–MS conditions (pH 2.5), this could be responsible for a better ionisation of the amphoteric (fluoro)quinolones in the MS source compared to the acidic ones and could lead to a higher repeatability. Nevertheless, in terms of sensitivity, the expected weak protonation of acidic (fluoro)quinolones at acidic pH does not seem to be critical. Besides, Volmer et al. [26] noticed that the signal intensities (molar response) of the acidic (fluoro)quinolones was not significantly influenced by pH and proposed the positive ionisation mode at acidic pH for their MS detection.

## 4. Conclusion

This paper describes the validation procedure of a LC–MS–MS method for the simultaneous identification and quantification of eleven (fluoro)quinolones in swine kidney. The method involves the solid-phase extraction of all analytes in a single process followed by the multi-residue analysis of the extract using tandem mass spectrometry. The method is sensitive with limits of quantification $\leq 50$ μg/kg, much lower than the MRLs imposed by the EU. The validation procedure was divided in two parts: pre-validation and validation. During the pre-validation, the response function of the calibration curve was identified to a weighted regression model (weighting factor $1/X$) and a calibration range from MRL/4 to MRL$\times 2$ was defined. The absolute recovery and the selectivity of the method were also studied. The validation step was able to demonstrate the accuracy and the precision of the method for seven amphoteric (fluoro)quinolones, whereas four acidic (fluoro)-quinolones showed relatively poor accuracy and repeatability results. This could be related to the weak protonation of these compounds in MS and interesting correlations could be made with literature about the particular analytical behaviour of acidic (fluoro)quinolones compared to amphoteric (fluoro)-quinolones. Therefore, the method presented in this paper can be considered as a multi-residue method for the detection at the μg/kg level and the identification of eleven (fluoro)quinolones in swine kidney, including amphoteric and acidic species. Moreover, the method allows the simultaneous quantification of

amphoteric species. If acidic (fluoro)quinolones are identified, their concentration can already be estimated by this method and if their accurate quantification is required, it should then be performed separately. The method can probably be applied with slight modifications to other biological matrices.

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