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Philippe Delahaut

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**Validation of an optical surface plasmon resonance biosensor assay for screening (fluoro)quinolones in egg, fish, and poultry meat**

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4 1 **Validation of an optical surface plasmon resonance biosensor**  
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6 2 **assay for screening (fluoro)quinolones in egg, fish, and poultry**  
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9 3 **meat**  
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13     **Abstract**

14     A surface plasmon resonance biosensor immunoassay has been developed for  
15     multi-residue determination of 13 (fluoro)quinolone antibiotics in poultry meat,  
16     egg, and fish. The following performance characteristics were determined  
17     according to the guidelines laid down for screening assay validation in the  
18     European Decision 2002/657/EC: detection capability, specificity/selectivity,  
19     decision limit, repeatability, ruggedness, and stability. The detection capability  
20     estimated for norfloxacin, the reference fluoroquinolone, was below 0.5, 1, and 1.5  
21     ng g<sup>-1</sup> for poultry meat, egg and fish respectively. The screening assay proved  
22     specific and showed satisfactory sensitivity, below the MRL levels even though  
23     flumequine and oxolinic acid had lower cross-reactivities. A wide range of non-  
24     MRL substances were also detected at concentrations below 10 ng g<sup>-1</sup>.  
25     Repeatability was good with both intra-assay and inter-assay coefficients of  
26     variation < 6 % and ruggedness was also demonstrated.

27  
28     **Keywords:** quinolones, biosensor, surface plasmon resonance, validation

## 29 Introduction

30 Over the last few decades, consumers, regulators, and industry have been very  
31 concerned about the quality and safety of food. Several crises, mainly of animal  
32 origin, have broken out in the last 20 years, causing a decline of consumption of  
33 certain foodstuffs. To prevent such crises in the future and to ensure that food is  
34 safe for consumers, effective monitoring is necessary. This concern has been  
35 integrated into a European project aiming to develop new technologies to screen  
36 for multiple chemical contaminants in foods (<http://www.biocop.org/>). Among  
37 these contaminants, fluoroquinolones (FQs) have been chosen as one of the  
38 priority residue groups.

39  
40 FQs constitute an entirely synthetic class of antibiotics that have found wide  
41 application in both human and veterinary clinical practice. The widespread use of  
42 FQs in agriculture has resulted in the potential presence of residues of these  
43 compounds in foodstuffs of animal origin. In parallel to exposure to low levels of  
44 these compounds, an upsetting increase in resistant human pathogens has been  
45 observed. This constitutes a public health hazard, primarily through the increased  
46 risk of treatment failure (Sarközy, 2001). The European Commission has thus  
47 established maximum residue levels (MRLs) for various FQs in different tissues  
48 (EMEA, 1998-2002). Rapid diagnostic methods for screening for the presence of  
49 all FQs in animal products are therefore needed for effective control, i.e. to ensure  
50 the correct use of these antibiotics.

51  
52 Recently, our team developed a generic immunoassay based on surface plasmon

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2  
3 53 resonance (SPR) biosensor technology for a set of 13 (fluoro)Qs in three matrixes  
4  
5 54 (Huet et al., 2008). The current paper describes the validation of this qualitative  
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7 55 screening assay according to the criteria specified in European Commission  
8  
9 56 Decision 2002/657/EC (EEC, 2002). The validation parameters  
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11 57 specificity/selectivity, decision limit ( $CC\alpha$ ), detection capability ( $CC\beta$ ),  
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13 58 repeatability, ruggedness, and stability are presented in this work. In the literature,  
14  
15 59 two main references have been reported which dealt with validation of a  
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17 60 multiresidual method by SPR biosensor (Traynor et al., 2003 and Gaudin et al.,  
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19 61 2007).  
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26

## 27 **Materials and methods**

### 28 *Apparatus and reagents*

29  
30 64 The Optical SPR Biosensor System Biacore Q and HBS-EP buffer were obtained  
31  
32 65 from Biacore AB (Uppsala, Sweden).  
33  
34 66 Sarafloxacin, ofloxacin, flumequine, norfloxacin, ciprofloxacin hydrochloride,  
35  
36 67 oxolinic acid, lomefloxacin, enoxacin, and nalidixic acid were provided by Sigma  
37  
38 68 (St Louis, MO, USA). Enrofloxacin was obtained from Bayer (Leverkusen,  
39  
40 69 Germany), difloxacin hydrochloride from Chemos GmbH (Regenstauf, Germany),  
41  
42 70 pefloxacin from Rhone Poulenc (Vitry sur Seine, France), danofloxacin from  
43  
44 71 Pfizer Inc (New York, USA), and marbofloxacin was kindly provided by UBE  
45  
46 72 Europe (Lure, France). The related compounds nicotinic acid, nicotinamide, and  
47  
48 73 ethoxyquin were purchased from Sigma-Aldrich (St Louis, MO, USA).  
49  
50 74 Acetonitrile was provided by Biosolve (Valkenswaard, the Netherlands); hexane  
51  
52 75 from Merck (Haar, Germany); sodium chloride, sodium hydroxide, di-sodium  
53  
54 76 hydrogen phosphate, and potassium dihydrogen phosphate from VWR  
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3 78 International (Leuven, Belgium); HPLC-grade water from Acros Organics (Geel,  
4  
5 79 Belgium).

7  
8 80 *Antibodies and core-FQ sensor chip preparation*

9  
10 81 The polyclonal antibodies raised against norfloxacin hapten-flumequine-BSA  
11  
12 82 were produced in our animal facilities. They were purified by affinity  
13  
14 83 chromatography on a Protein A-Sepharose column (Amersham Biosciences,  
15  
16 84 Belgique) and then concentrated with a centricon (with a molecular weight cut-off  
17  
18 85 of 30,000) purchased from Millipore (Brussels, Belgium). Antibody concentration  
19  
20 86 was measured by the Bio-Rad protein assay (Bio-Rad Laboratories, Hemel  
21  
22 87 Hempstead, UK). Antibody stock solution was diluted 1/4 (v/v) with HBS-EP  
23  
24 88 buffer just before use.

25  
26 89  
27  
28 90 The core-FQ derivative was immobilised by a standard amine-coupling procedure,  
29  
30 91 it was synthesized from starting product 6-fluoro-4-oxo-1,4-dihydro-3-  
31  
32 92 quinolinecarboxylic acid (Apollo Scientific Limited, Bredbury, UK) on which a 3-  
33  
34 93 aminopropyl was added at position 1. Between analyses the chips were stored over  
35  
36 94 desiccant at 4°C in a sealed container.

37  
38 95  
39  
40 96 The full antibody production and FQ derivative immobilisation protocols have  
41  
42 97 been described in a previous publication (Huet et al., 2008).

43  
44 98

45  
46 99 *Preparation of standard curves in matrixes*

47  
48 100 A stock solution of norfloxacin at 100 µg mL<sup>-1</sup> was prepared in methanol. From  
49  
50 101 this solution, spiking solutions were prepared in HBS-EP buffer. A set of five  
51  
52 102 matrix-matched standards at 0.1, 0.5, 1, 2.5 and 10 ng g<sup>-1</sup> (poultry meat and egg)

1  
2  
3 103 or 0.1, 1, 2.5, 5 and 100 ng g<sup>-1</sup> (fish) were prepared by fortifying blank extracts.

4  
5 104 Duplicate injections at each concentration were performed and the mean response  
6  
7  
8 105 was used to construct a calibration curve.  
9

### 10 106 *Extraction of samples*

11  
12  
13 107 *Egg and poultry meat:* 2g homogenised muscle or whole egg were extracted with

14  
15 108 8 mL acetonitrile. Each tube was immediately vortexed before vigorous head-

16  
17  
18 109 over-head shaking for 10 min. After centrifugation, all of the supernatant was

19  
20 110 evaporated to dryness. The residue was reconstituted in 1 mL PBS pH 7.4 and

21  
22 111 mixed. Then 1 mL hexane was added and the tubes were vortexed. The sample

23  
24  
25 112 was centrifuged and the hexane layer and any traces of emulsion at the interface

26  
27 113 were removed. The washing step was repeated once again and the reconstituted

28  
29  
30 114 sample was quickly vortexed and centrifuged just before application to the 96-well

31  
32 115 plate.

33  
34 116 *Fish:* as above except that only a 2-mL aliquot of the supernatant was transferred

35  
36  
37 117 into a new tube and evaporated to dryness.  
38

### 39 118 *Sample analysis*

40  
41  
42 119 Antiserum freshly diluted in HBS-EP buffer was mixed 25% : 75% with sample

43  
44 120 extract and injected for 210 seconds over the sensor chip surface at a flow rate of

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47 121 25µl min<sup>-1</sup>. The chip surface was regenerated between cycles with a 30-second

48  
49 122 pulse of 10mM NaOH prepared from 1M NaOH with HPLC grade water at a flow

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51  
52 123 rate of 10 µl min<sup>-1</sup>.  
53

### 54 124 *Method validation*

55  
56  
57 125 Validation of our SPR biosensor method was conducted in accordance with table 9

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59  
60 126 of the document of European Commission Decision, 20002/657/EC (EEC, 2002).



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3 127 Specificity means the ability of a method to distinguish between the analyte being  
4  
5 128 measured and other substances. In keeping with the EU Decision requirements,  
6  
7 129 two approaches were used to test this aspect. Firstly, 20 blank samples from  
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10 130 different origins of each of the three studied matrixes (egg, poultry meat, and fish)  
11  
12 131 were analysed by the biosensor procedure to detect the presence of possible  
13  
14 132 interferences from endogenous substances and matrix constituents. Secondly,  
15  
16 133 cross-reactivity (CR) profiles were determined for a range of potentially  
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18 134 interfering substances by spiking blank samples at relevant concentration (1000 ng  
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22 135 g<sup>-1</sup>) with ethoxyquin, nicotinamide, and nicotinic acid considered as FQ analogues.  
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25 136 The CR profile of the polyclonal antibody was also determined for a range of  
26  
27 137 common (fluoro)Qs. Calibration curves were prepared in each matrix using the  
28  
29 138 compounds of interest in the range 0-1000 ng g<sup>-1</sup> (0, 0.25, 1, 2.5, 10, 25, 100, and  
30  
31 139 1000). The CR was calculated from the relevant calibration curves as IC<sub>50</sub>  
32  
33 140 (norfloxacin)/CR<sub>50</sub> (competitor) x 100, where IC<sub>50</sub> (inhibitory concentration) is the  
34  
35 141 concentration at midpoint of the standard curve and CR<sub>50</sub> is the concentration of  
36  
37 142 competitor required to cause the same decrease in signal as obtained with  
38  
39 143 norfloxacin used at its IC<sub>50</sub> concentration. The calculations were done with the  
40  
41 144 BIAevaluation software provided by Biacore AB (Uppsala, Sweden).  
42  
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46 145  
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48 146 The detection capability CC<sub>β</sub> was determined for the reference molecule  
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51 147 norfloxacin. According to the Commission Decision, in the case of substances for  
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53 148 which no permitted limit has been established, where no quantitative results are  
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55 149 available (i.e. qualitative screening), the CC<sub>β</sub> can be determined by investigation  
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57 150 of fortified blank material at and above the decision limit (CC<sub>α</sub>). In this case the  
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59 151 concentration level at which only ≤ 5% false compliant results remain equals the  
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3 152 CC $\beta$ . In practice, the CC $\beta$  was chosen as the lowest tested fortification level  
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6 153 giving no negative result in an analysis of 20 spiked blanks. This decision should  
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8 154 avoid the problem of false negatives. At least twenty negative samples of fish,  
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10 155 egg, and chicken muscle were fortified with norfloxacin at 1.5, 1, and 0.5 ng g<sup>-1</sup>  
11  
12 156 respectively, and then assayed in the biosensor assay.

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17 158 For the repeatability step, a standard curve and six samples fortified with  
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19 159 norfloxacin at the corresponding CC $\beta$  value were prepared in each studied matrix,  
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21 160 and then samples were extracted and injected. This experiment was realized three  
22  
23 161 times in three days, giving intra- and interday variability. The detected  
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25 162 concentration of each spiked sample was calculated as were the mean  
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27 163 concentrations, the standard deviations (STDEVPA), and the coefficients of  
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29 164 variation (CVs).

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34 166 To prove the ruggedness of the developed SPR-based FQ assay, the same  
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36 167 experiment was performed three times by changing one factor per experiment,  
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38 168 namely (i) the pH of the buffer (phosphate-buffered saline, PBS) in which dried  
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40 169 residues were reconstituted: pH 6.4 instead of PBS pH 7.4; (ii) the operator; (iii)  
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42 170 the time of evaporation (it was doubled). Each experiment was conducted as  
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44 171 follows: one calibration curve of norfloxacin was extracted simultaneously with  
45  
46 172 five blank samples of different origins and five blank samples fortified with  
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48 173 norfloxacin at its CC $\beta$  value in the matrix used. The detected concentration of  
49  
50 174 each spiked sample and each known negative sample was calculated for the egg,  
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52 175 fish, and chicken muscle matrix.

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## 177 **Results and discussion**

178 As explained by Spinks in 2000, the current need is to develop assays for more  
179 than one analyte per test and to increase sample throughput. Progress in  
180 immunoassay development is constrained by the availability of antibodies with  
181 appropriate affinity and specificity characteristics. It was a real challenge to  
182 develop a single efficient biosensor immunoassay for the entire class of FQ  
183 antibiotics. Four immunogens and one engineered antibody were synthesised, and  
184 three different FQ derivatives were immobilised on the sensor chip surface. Huet  
185 et al. (2008) described all the tested approaches in detail, revealing for the first  
186 time the new concept of the bi-active antibody. The most difficult aspect of  
187 development was taking into account the fact that some substances have different  
188 MRLs, others have no MRLs, and the use of some is prohibited in animals from  
189 which eggs are produced for human consumption.

190  
191 All samples were determined free of FQ residues by LC-MS/MS before being  
192 used in the biosensor procedure. One norfloxacin calibration curve was prepared  
193 and extracted for each experiment of the validation study (see experimental  
194 section). The midpoints of all these standard curves were evaluated with the  
195 BIAevaluation software and compared with the expected values, midpoints at  
196 about 1, 1.5, and 3 ng g<sup>-1</sup> having been previously determined for poultry muscle,  
197 egg, and fish, respectively. All IC<sub>50</sub> values obtained in the validation study were in  
198 keeping with the values just mentioned since they ranged from 0.8 to 1.2 ng g<sup>-1</sup> for  
199 poultry, from 0.9 to 2 ng g<sup>-1</sup> for egg and from 2.6 to 4.1 ng g<sup>-1</sup> for fish.

200  
201 *Specificity/selectivity*

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3 202 The specificity of the entire procedure was investigated against the following  
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5 203 quinolones: norfloxacin, flumequine, enrofloxacin, ciprofloxacin, sarafloxacin,  
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7 204 pefloxacin, enoxacin, difloxacin, lomefloxacin, danofloxacin, ofloxacin,  
8  
9  
10 205 marbofloxacin, and oxolinic acid. The calibrants selected for the test were added  
11  
12 206 at six levels concentration. The CR of the binding protein used in the assay is  
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14 207 shown in table 1. The specificity of the antibody towards norfloxacin is the 100%  
15  
16 208 reference. It was confirmed that the selected antibody could detect 11  
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18 209 fluoroquinolones with high cross-reactivities ranging from 26 to 183 %. Lower  
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20 210 cross-reactions (between 1 and 14 %) were observed for flumequine and oxolinic  
21  
22 211 acid. Although low, these cross-reactivities remain acceptable, as the European  
23  
24 212 Union has established a maximum residue limit (MRL) at 100 ng g<sup>-1</sup> for oxolinic  
25  
26 213 acid in fish and chicken muscle, at 600 for flumequine in fish, and at 400 ng g<sup>-1</sup> for  
27  
28 214 flumequine in poultry muscle. The cross-reactivity profile was already presented  
29  
30 215 in the previous article (Huet et al., 2008), the results were compared and some  
31  
32 216 differences appeared. The assay's sensitivity was better with lower IC<sub>50</sub> values in  
33  
34 217 this work. The two CR profiles for oxolinic acid were quite different, much lower  
35  
36 218 here. These differences are explained by the use of an antibody purified on a  
37  
38 219 protein A-Sepharose column in this publication whereas a crude antibody was  
39  
40 220 working last year. On the other hand, there was no measurable cross-reactivity  
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42 221 with the common FQ analogues ethoxyquin, nicotinamide, and nicotinic acid at  
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44 222 high concentration.  
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55 224 The specificity of the SPR assay was also demonstrated after analysis of 20 known  
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57 225 negative poultry meat, egg, and fish samples. These showed no interference, when  
58  
59 226 analyzed against a corresponding matrix curve. Determination of the CC<sub>α</sub> is not  
60

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3 227 required by document 2002/657/EC but can easily be calculated from the data  
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5 228 obtained so far. The decision limit  $CC\alpha$ , determined as the concentration  
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7  
8 229 corresponding to the mean response for negative samples minus three times the  
9  
10 230 standard deviation, was 0.13 (poultry meat), 0.29 (egg), and 0.31  $\text{ng g}^{-1}$  (fish).  
11

12 231

### 15 232 *Detection capabilities*

17  
18 233 To calculate the detection capability ( $CC\beta$ ), 20 known negative samples were  
19  
20 234 spiked with the reference molecule norfloxacin, chosen because the polyclonal  
21  
22 235 antibody used as binding protein was raised against a norfloxacin hapten.  
23  
24  
25 236 Norfloxacin is also commonly used for treatment of animals in many countries  
26  
27 237 (Webber and Piddock, 2001) and very often mentioned in the literature. The  
28  
29  
30 238 negative samples of fish, egg, and chicken muscle were fortified with norfloxacin  
31  
32 239 at 1.5, 1 and 0.5  $\text{ng g}^{-1}$  respectively and then assayed in the biosensor assay. All  
33  
34 240 fortified samples were declared positive (above their respective cut-off values).  
35  
36  
37 241 The  $CC\beta$  of the assay thus proved inferior to the tested level of fortification. To  
38  
39 242 ascertain if a sample was negative or not, the cut-off level for each matrix was  
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41  
42 243 determined from the graphs presented in figure 1, which combine the calculated  
43  
44 244 mean norfloxacin concentrations for blank samples and for samples spiked at the  
45  
46 245 appropriate  $CC\beta$  level. The means calculated for blank and fortified samples were  
47  
48  
49 246 found to be different and the variability of known negative samples was much  
50  
51 247 lower than that of samples containing norfloxacin. To limit the number of pseudo-  
52  
53 248 false positives, all cut-off levels were set near the mean of the spiked samples, as  
54  
55  
56 249 in the case of MRL-regulated compound/matrix combinations, a considerable rate  
57  
58 250 of pseudo-false positive results can be generated; for instance samples containing  
59  
60 251 FQs, but below the MRL. In fact, this high sensitivity of the assay is essential to

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2  
3 252 detect flumequine and oxolinic acid at satisfactory levels even if it implies the  
4  
5 253 acceptance of a certain pseudo-false positives rate. This aspect is well-known in  
6  
7  
8 254 the field of immunochemical screening assays using a generic antibody  
9  
10 255 (Scortichini et al., 2009). The cut-off values were set at 1.5, 1, and 0.5 ng g<sup>-1</sup> for  
11  
12 256 fish, egg, and poultry meat respectively. More precisely, when the calculated  
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14  
15 257 concentration of an unknown sample was below the corresponding cut-off level,  
16  
17 258 this sample was considered negative, but when it was above or equal to the cut-off  
18  
19 259 level, the sample was considered suspect and to require analysis by a confirmatory  
20  
21  
22 260 method for identifying and quantifying (fluoro)Qs.  
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261

262 The determination of cross-reactivities with other (fluoro)Qs (specificity/  
263 selectivity section) then enabled us to calculate the detection capabilities of all  
264 investigated compounds. This was done for each matrix according to the following  
265 formula:  $CC\beta \text{ compound} = (CC\beta \text{ norfloxacin} / \text{corresponding CR}) \times 100$ . The  
266 obtained values are presented in table 2. They range from 0.9 to 3.7 ng g<sup>-1</sup> for the  
267 fish matrix, from 0.5 to 2.2 ng g<sup>-1</sup> for the egg matrix, and from 0.4 to 1.9 ng g<sup>-1</sup> for  
268 chicken muscle, except for flumequine and oxolinic acid which show higher  
269 values due to their lower specificity. The assay could nevertheless detect both  
270 residues at or below their half-MRL levels. These results are satisfactory, since the  
271  $CC\beta$  values for all (fluoro)Qs were well below the established MRL levels set by  
272 EMEA for specified analyte/matrix combinations (table 1). Moreover, the assay  
273 proved sensitive enough to detect also the non-MRL substances at levels below 10  
274 ng g<sup>-1</sup>.

275

276 *Repeatability*

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3 277 As explained in the experimental section, six replicate samples were spiked with  
4  
5 278 norfloxacin at the appropriate  $CC\beta$  level and then extracted simultaneously with  
6  
7  
8 279 one calibration curve. The experiment was performed three different times for  
9  
10 280 each matrix of interest. The intra- ( $n = 6$ ) and interday variations ( $n = 3$ ) calculated  
11  
12 281 are listed in tables 3 and 4, respectively. CVs varied from 2.8 to 5.8 % under  
13  
14 282 intraday repeatability-testing conditions and from 1.9 to 5.7 % under interday  
15  
16 283 repeatability-testing conditions. Since a CV of 15% was set for the desired  
17  
18 284 repeatability, these repeatability results are satisfactory.  
19  
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285

286 *Ruggedness*

287 A series of key parameters (the pH of reconstitution buffer (PBS), the operator,  
288 and the duration of evaporation) were investigated. Usually, reconstitution of  
289 dried residues after evaporation is carried out in 1 ml PBS adjusted to pH 7.4. In  
290 this experiment 16 samples per matrix, corresponding to 6 calibration points and  
291 10 known negative samples of which 5 were fortified at the corresponding  $CC\beta$   
292 level were reconstituted in 1 ml PBS at pH 6.4. All blank samples were declared  
293 negative because the results were lower than the respective cut-off levels (0.5, 1,  
294 and 1.5  $ng\ g^{-1}$  respectively in poultry meat, egg, and fish). The result for one  
295 sample of chicken muscle, however, was very near to the cut-off value for this  
296 matrix. This suggests that reconstitution in PBS with a small change in pH could  
297 generate a false positive in this matrix. All spiked samples were declared positive,  
298 giving values slightly higher than with the unchanged protocol.  
299

300 A different operator carried out all the steps described under the instructions for  
301 use of the prototype kit: preparation of calibrant solutions and spiking solutions,

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3 302 fortification, extraction, preparation of solutions for the run, running assay. The  
4  
5 303 change of operator did not affect the performance of the method. The third  
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7  
8 304 parameter, the drying time, being quite variable in the routine execution of the  
9  
10 305 assay, we multiplied it by two. No variation was observed except that fortified  
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12 306 samples gave values slightly higher than with the unchanged protocol.  
13  
14 307  
15  
16  
17 308 The midpoints of the calibration curves obtained in this ruggedness experiment are  
18  
19 309 summarized in the table 5. The sensitivity proved similar to that of the usual  
20  
21 310 method except less sensitivity for the fish and egg matrixes reconstituted in PBS at  
22  
23 311 lower pH.  
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### 313 *Stability*

314 No stability study was performed since this SPR assay is a qualitative screening  
315 method and could mean to unreliable observations. As described in a recent  
316 publication (2007), the team of Gaudin et al. showed that their biosensor method  
317 was not suitable for conducting stability studies due to the variability of  
318 quantitative results. For instance, they observed an increase of residue  
319 concentration in spiked samples stored at -20°C for 20 weeks; no scientific  
320 explanation was found for this kind of phenomenon.  
321

322 The literature does provide, however, some stability data worth mentioning here.  
323 The stability of some FQs in solution has been demonstrated by Okerman et al.,  
324 2007. In this study, frozen stock solutions of flumequine, enrofloxacin, and  
325 marbofloxacin were evaluated in an agar diffusion test. Diameters of inhibition  
326 zones were measured at monthly intervals for 6 months, and the decline in active



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3 327 substance was calculated. Stock solutions of  $1 \text{ mg ml}^{-1}$  in  $0.1 \text{ N NaOH}$  were kept  
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5 328 at  $-20^{\circ}\text{C}$  until needed and were never exposed to direct strong light. The loss of  
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7  
8 329 activity was less than 10% after 6 months of storage at  $-20^{\circ}\text{C}$ . The studied FQs are  
9  
10 330 thus relatively stable.

11 331

12  
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14  
15 332 The stability of FQs in chicken muscle has been demonstrated by Bailac et al.,  
16  
17 333 2006 and Shu-Chu et al., 2003, but we have found no information in the literature  
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19  
20 334 for the two other matrixes. The stability of spiked chicken samples at low ( $25 \text{ ng}$   
21  
22 335  $\text{g}^{-1}$ ) and high concentration ( $250 \text{ ng g}^{-1}$ ) was evaluated by liquid chromatography-  
23  
24 336 ultraviolet (LC-UV). In Bailac et al., 2006, the matrix was fortified with 7  
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26  
27 337 different quinolones: ciprofloxacin, danofloxacin, enrofloxacin, sarafloxacin,  
28  
29 338 difloxacin, oxolinic acid, and flumequine. Then these samples were stored at -  
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31 339  $20^{\circ}\text{C}$  for 3 months. Afterwards, they were analyzed and compared with fresh  
32  
33  
34 340 chicken samples fortified at the same levels. The results proved the long-term  
35  
36 341 stability of most of FQs in chicken muscle, showing no significant differences in  
37  
38 342 concentration. In Shu-Chu et al., 2003, the stability of incurred chicken muscle  
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41 343 samples was evaluated by HPLC. The authors studied the stability of residual  
42  
43 344 enrofloxacin after refrigeration in wu ku chicken muscle. Incurred samples were  
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45 345 stored at  $4^{\circ}\text{C}$  for 1, 4, and 7 days to test the stability of enrofloxacin. The level of  
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47  
48 346 residual enrofloxacin in samples was found not to have decreased after 7 days at  
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50 347  $4^{\circ}\text{C}$ .

51 348

### 52 349 *Method comparison*

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55 350 Besides the in-house validation of the method, the new method was compared  
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58 351 with established methods for the determination of FQ residues in the target  
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3 352 matrixes. The methods chosen were an FQ-specific microbiological screening  
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5 353 assay and a liquid chromatography-mass spectrometric (LC-MS) confirmatory  
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7  
8 354 method. This method comparison study demonstrated the performance of our  
9  
10 355 developed SPR biosensor screening assay, since all spiked and incurred samples  
11  
12 356 containing one of the investigated FQ antibiotics, sometimes even at very low  
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14  
15 357 concentration, were identified as suspicious by the biosensor method. Further  
16  
17 358 practical details and complete results are presented in a publication (Weigel et al.,  
18  
19 359 2009).  
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22 360

## 23 24 361 **Conclusions**

25  
26  
27 362 Multi-residue identification of 13 fluoroquinolones in fish, egg, and poultry  
28  
29 363 muscle by means of our SPR-based biosensor assay has been successfully  
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31 364 validated. Satisfactory results were obtained with respect to selectivity/specificity,  
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33 365 detection capability, repeatability, and ruggedness of analysis in each matrix. The  
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35 366 pH of the reconstitution buffer might, however, influence the chicken matrix and  
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37  
38 367 lead to false positives. Stability data from a bibliographical study are included in  
39  
40  
41 368 the paper.  
42  
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44 369

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47  
48  
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50  
51 372 "New technologies to screen multiple chemical contaminants in foods". BioCop,  
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418 **Tables**419 Table 1. IC<sub>50</sub> values and CR profile of FQ binding protein with a range of

420 (fluoro)quinolones in the matrixes

	fish		egg <sup>b</sup>		chicken muscle	
	IC <sub>50</sub> (ng g <sup>-1</sup> )	CR (%)	IC <sub>50</sub> (ng g <sup>-1</sup> )	CR (%)	IC <sub>50</sub> (ng g <sup>-1</sup> )	CR (%)
norfloxacin	2.4	100	0.9	100	0.6	100
ciprofloxacin	2.3 (50) <sup>a</sup>	106	1.1	82	0.7 (50) <sup>a</sup>	82
danofloxacin	3.4 (100)	71	0.7	124	1.1 (200)	50
difloxacin	4.3 (300)	57	0.8	106	1.2 (300)	45
enoxacin	5.7	43	1.6	56	2.1	26
enrofloxacin	1.5 (50) <sup>a</sup>	163	0.5	183	0.5 (50) <sup>a</sup>	118
flumequine	106 (600)	2	46	2	75 (400)	1
lomefloxacin	5.1	48	2.0	45	1.3	43
marbofloxacin	3.6	67	0.7	122	0.7	80
ofloxacin	2.7	91	0.8	117	0.9	65
oxolinic acid	23 (100)	11	6	14	12 (100)	5
pefloxacin	2.0	125	0.5	180	0.5	116
sarafloxacin	6.0 (30)	41	1.9	47	1.9	30

421 The corresponding MRL value is indicated as ( )

422 <sup>a</sup> Sum of ciprofloxacin and enrofloxacin = 100 ng g<sup>-1</sup>423 <sup>b</sup> Not for use in animals for which eggs are produced for human consumption424  
425 Table 2. Calculated CC<sub>β</sub>, expressed in ng g<sup>-1</sup>, for all studied (fluoro)Qs, as

426 determined by antibody cross-reactivity

	fish	egg	chicken muscle
norfloxacin	1.5	1.0	0.5
ciprofloxacin	1.4	1.2	0.6
danofloxacin	2.1	0.8	1.0
difloxacin	2.6	0.9	1.1
enoxacin	3.5	1.8	1.9
enrofloxacin	0.9	0.5	0.4
flumequine	65	52	68
lomefloxacin	3.1	2.2	1.2
marbofloxacin	2.2	0.8	0.6
ofloxacin	1.6	0.9	0.8
oxolinic acid	14	7.0	11
pefloxacin	1.2	0.6	0.4
sarafloxacin	3.7	2.1	1.7

427

428 Table 3. Intra-assay parameters determined for the FQs SPR-assay (n=6)

spiking concentration	fish	egg	chicken
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norfloxacin, ng g <sup>-1</sup>	1.5			1			0.5		
mean, ng g <sup>-1</sup>	1.8	2.0	2.0	1.3	1.3	1.1	0.7	0.7	0.6
standard deviation, ng g <sup>-1</sup>	0.08	0.12	0.10	0.06	0.05	0.04	0.02	0.02	0.02
coefficient variation, %	4.2	5.8	4.9	4.4	4.0	3.7	3.5	2.8	3.6

429

430 Table 4. Inter-assay parameters determined for the FQs SPR-assay (n=3)

spiking concentration norfloxacin, ng g <sup>-1</sup>	fish	egg	chicken
mean, ng g <sup>-1</sup>	1.9	1.2	0.7
standard deviation, ng g <sup>-1</sup>	0.11	0.07	0.01
coefficient variation, %	5.7	5.4	1.9

431

432 Table 5. Summary of midpoints of curve coming from the calibration curves of

433 norfloxacin realized during the ruggedness study and calculated thanks the

434 BIAevaluation software. These values were expressed in ng g<sup>-1</sup>

	fish	egg	chicken
standard method	3.0	1.5	1.0
small change in pH	4.0	2.5	1.2
operator	3.3	1.4	1.0
extended evaporation	3.3	1.5	1.0

435

436 **Figure**

437 Figure 1. Cut-off level determination for the analysis of egg (a), fish (b) and

438 chicken muscle (c). Each point represents the reference FQ, norfloxacin

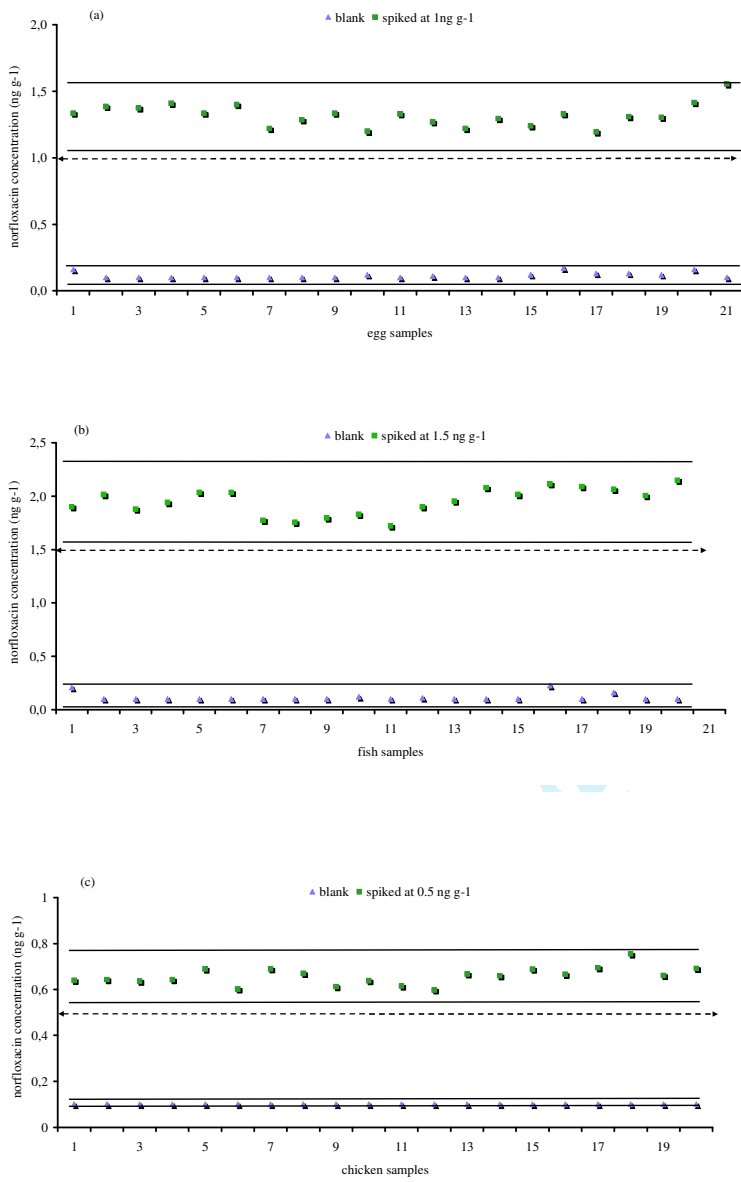
439 concentration obtained from the analyses of different samples (blank and spiked at

440 CC $\beta$  value). The black lines represent the corresponding standard deviations441 (mean  $\pm$  3 standard deviation SD). The cut-off values are represented on the graph

442 with dotted lines

443

Figure 1



Only