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Abstract

A novel dual electrochemical immunosensor was fabricated for the rapid and sensitive detection of enrofloxacin (EF) antibiotic in meat. Anti-quinolone antibody was immobilized onto screen-printed dual carbon electrodes *via* carbodiimide coupling. A new electrochemical probe was synthesized by conjugating difloxacin and aminoferrocene, whose oxidation was measured at +0.2 V *vs*. Ag/AgCl by differential pulse voltammetry. The detection principle was based on the competitive binding of this conjugate and free EF on immobilized antibodies. The proposed immunosensor allowed detection of EF at concentrations ranging from 0.005 $\mu g.mL^{-1}$ to 0.01 $\mu g.mL^{-1}$ with a detection limit of 0.003 $\mu g.mL^{-1}$. The immunosensor was stable for at least 1 month at 4°C and displayed a good specificity for other fluoroquinolones. The new dual electrode design offered an improved accuracy as one electrode was used as negative control. The efficiency of the sensor and the adequacy of the extraction process were finally validated by detecting EF in different meat samples.

Keywords

Immunosensor, Enrofloxacin (EF), Fluoroquinolones (FQs), Competitive assay, Screen-printed dual carbon electrodes (SPdCEs), Meat analysis

1. Introduction

Fluoroquinolones (FQs) are broad-spectrum synthetic antibiotics which selectively inhibit bacterial enzymes involved in DNA replication of Gram-negative and Gram-positive bacteria (Blondeau, 2004). These antibiotics, especially enrofloxacin (EF), were mainly used in large-scale breeding to prevent or treat animal diseases or to improve livestock growth and productivity (Cháfer-Pericás et al., 2010; Wolfson & Hooper, 1985). The use of these compounds as growth promoters is banned in European Union since 2006 (Brown et al., 2017; Diana et al., 2020) and they have been recently classified as restricted antibiotics (European Medicines Agency, 2019). Despite these limitations, Diana *et al*. recently reported that FQs are the most frequently used antimicrobials (Diana et al., 2020). Moreover, they are still considered as valuable polyvalent tools by a wide number of farmers to treat a variety of diseases. The overuse of FQs in animal husbandry or the disrespect of withdrawal periods settled by the manufacturers can result in the accumulation of antibiotic residues in animal-derived food, such as meat. Once consumed by humans, these residues present various health risks such as apparition of allergy and toxic reactions in liver, nervous system and skin (Aronson, 2016). This misuse can also lead to the prevalence of multidrug resistant pathogens, representing a serious public health concern (Alekshun & Levy, 2007; Tenover, 2006).
In order to avoid these problems and to protect consumers, the use of antibiotics in food animals has been regulated by establishing maximum residue limits for authorized antimicrobials for each species (MRLs, Commission Regulation (EU) No 37/2010). Regarding EF, MRLs are ranging from 100 $\mu g.kg^{-1}$ to 300 $\mu g.kg^{-1}$, depending on the animal species and the target tissue. Therefore, the sensitive and effective monitoring of this antibiotic in food samples is crucial.

Various analytical methods have been developed for the detection of EF in animal tissues, and they can be classified into two categories: screening and confirmatory. Screening methods allow the detection of EF at the concentration of interest but they usually correspond to qualitative or semi-quantitative tests. These methods include enzyme-linked immunosorbent assays (Hu et al., 2019; Z. Wang et al., 2014; Zhang et al., 2011), immunochromatographic strip tests (Chen et al.,

2012; Huang et al., 2013), microbiological inhibition tests (Mata et al., 2014; Tumini et al., 2019; Q. Wu et al., 2019) and fluorescent (Dolati et al., 2018; Ha et al., 2016) and colorimetric sensors (Rezende et al., 2019). On the contrary, confirmatory methods, such as capillary electrophoresis (L. Wang et al., 2005; Xu et al., 2015) or high throughput liquid chromatography coupled with mass spectrometry (Panzenhagen et al., 2016), fluorescence (Peris-Vicente et al., 2017) or UV detection (Dunnett et al., 2004) allow both identification and quantification of analytes. However, these methods require sophisticated material, experimented users, as well as complex extraction and clean-up processes.

Despite the progress made in recent years, there is still a strong demand for new strategies to improve sensitivity, simplicity and feasibility of FQs detection methods. In this regard, electrochemical immunosensors have gained special interest as they combine the high specificity of antigen-antibody interaction and the sensitivity, ease of use, and low cost of electrochemical devices. Thereby, many portable and affordable immunosensors have been designed for various applications in clinical diagnosis, environmental monitoring and food analysis (Felix & Angnes, 2018; Mollarasouli et al., 2019). Screen-printed carbon electrodes appear as attractive transducers for immunosensor development since they can be mass-produced at low cost, they are chemically stable and they can be used as disposable devices (Sharma et al., 2017; Taleat et al., 2014).

Electrochemical immunosensors detecting FQs such as ciprofloxacin (Garifallou et al., 2007; Giroud et al., 2009; Ionescu et al., 2007), ofloxacin (He et al., 2015; Zang et al., 2013), norfloxacin (Liu et al., 2018) and enrofloxacin (Wu et al., 2009) have already been described in literature. The detection methods involved in these immunosensors were mostly based on changes in impedance related to antigen-antibody binding event (Garifallou et al., 2007; Wu et al., 2009), or on voltamperometric detection of a redox molecule generated by an enzyme-labelled secondary antibody (Liu et al., 2018; Zang et al., 2013). However, electrochemical impedance spectroscopy procedures are complex, sensitive to non-specific binding and above all, they are not suitable for the sensitive detection of small molecules (Bahadir & Sezgintürk, 2016; Kivirand et al., 2019).

On the other hand, the use of reporter enzymes as antibody labels is expensive and the measurement protocol involves mandatory incubation and washing steps, resulting in increased measurement times (Liu et al., 2018; Zang et al., 2013). For these reasons, a promising strategy consists in the modification of the antigen with an electro-active probe like ferrocene. Ferrocene (dicyclopentadienyl iron) is a well-known redox molecule appreciated for its stability and low toxicity, which displays favorable electrochemical characteristics related to the reversible oxidation of its iron center (Štěpnička, 2008). These properties have been exploited for the development of several sensors where ferrocene derivatives were conjugated with peptides (Martić et al., 2011), proteins (Dechtrirat et al., 2014), oligonucleotides (Nakayama et al., 2002) or sugars (Casas-Solvas et al., 2009). This organometallic molecule was also used to improve antibacterial or antifungal properties of different antibiotics such as ampicillin (Skiba et al., 2012), β-lactams (Lewandowski et al., 2020), sulfonamides (Chohan, 2009) and FQs like ciprofloxacine (Khor et al., 2011).

Despite the high selectivity and sensitivity of electrochemical immunosensors, inter-electrode variability is often observed due to the inherent differences in surface modification, as well as effect of storage over time. The measurement of a reference signal is therefore required to take into account these variations. Usually employed for the simultaneously detection of biomolecules (Escamilla-Gómez et al., 2009; Sánchez-Tirado et al., 2017) or pesticides (Shi et al., 2018), screen-printed dual carbon electrodes (SPdCEs) appear as suitable platforms to develop robust and reproducible electrochemical assays as they allow differential measurements by using one electrode as a blank electrode. It was recently demonstrated that such a dual device allows the reduction of both inter-electrode variations and total measurement time (Shi et al., 2018).

In this study, we describe a novel electrochemical immunosensor composed of two working electrodes for easy and rapid detection of fluoroquinolones in meat from different species. The detection of enrofloxacin (EF) was based on the competition between free EF and a newly synthesized conjugate, difloxacin-ferrocene, for their binding onto immobilized antibodies (Figure 1). To the best of our knowledge, the conjugation of enrofloxacin with ferrocene has never been

described in literature. Differential pulse voltammetry (DPV) was employed to reveal the binding of difloxacin-ferrocene on immobilized antibodies, and the presence of free EF signal induced a proportional decrease of output signal. Finally, the proposed immunosensor was applied to the detection of EF in different meat samples with reduced preliminary processing.

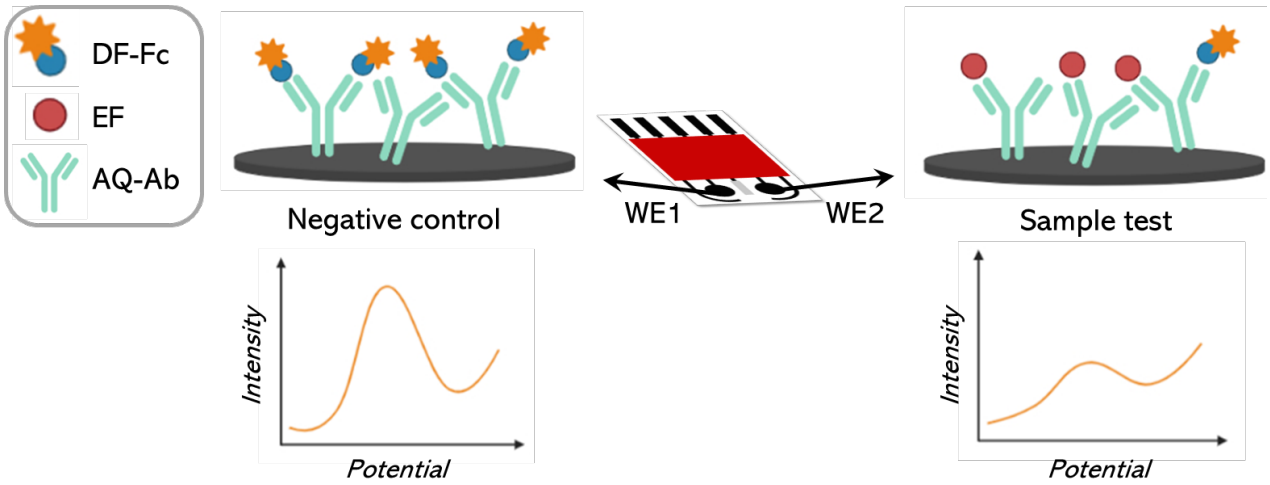


Figure 1. Detection principle of electrochemical dual immunosensor for EF detection. Working electrode 1 (WE1): In the absence of EF (negative control), the binding of DF-Fc onto anti-quinolone antibodies (AQ-Ab) leads to a high electrochemical signal; Working electrode 2 (WE2): In the presence of EF, competition takes place leading to a decrease in DF-Fc binding, which results in a lower electrochemical signal.

2. Material and Methods

2.1. Reagents and apparatus

4-aminobenzoïc acid, amoxicillin, ampicillin, bovine serum albumin (BSA), chloramphenicol, dichloromethane, disodium hydrogenophosphate (Na_2HPO_4), ethanolamine, anhydrous ethyl acetate 99,8%, hydrochloric acid 37%, kanamycin, magnesium sulfate ($MgSO_4$), methanol, 2-(N-morpholino)ethanesulfonic acid (MES), N-hydroxysuccinimide (NHS), potassium chloride (KCl), potassium dihydrogenophosphate (KH_2PO_4), sodium carbonate (Na_2CO_3), sodium chloride (NaCl), sodium nitrite ($NaNO_2$), spectinomycin, sulfuric acid 98%, tetracycline hydrochloride, and Tween® 20 were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier,

115 France). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), ciprofloxacin, difloxacin
116 hydrochloride, enoxacin, enrofloxacin, flumequine, norfloxacin, pefloxacin and pipemidic acid
117 were from Alfa Aesar (Kandel, Germany), benzotriazol-1-yloxytris(dimethylamino)phosphonium
118 hexafluorophosphate (BOP) and triethylamine from Merck (Saint Quentin Fallavier, France),
119 methanol and dichloromethane from VWR Chemicals and aminoferrocene from Tokyo Chemical
120 Industry (Zwijndrecht, Belgium). Polyclonal anti-quinolone antibodies from sheep were purchased
121 from Abcam (Cambridge, United Kingdom).
122 Electrochemical measurements were performed using an Autolab PGSTAT100
123 potentiostat/galvanostat from Metrohm Autolab BV (Utrecht, Netherlands) driven by Nova 1.11
124 software. Mass spectral analysis was performed using electrospray ionization mass spectrometry
125 (ESI-MS) (Thermo Scientific, France). Liquid chromatography coupled with mass spectrometry
126 (LC-MS) analyses were carried out using a Thermo Fisher Scientific Accela LC/MS device,
127 coupled to a LCQ Fleet equipped with an electrospray ionization source and a 3D ion-trap analyzer.
128 **2.2. Synthesis of difloxacin-ferrocene (DF-Fc) conjugate**
129 The difloxacin-ferrocene (DF-Fc) conjugate was easily produced by a one-step synthesis.
130 Difloxacin (43 mg, 0.1 mmol, 1 eq) was dissolved in dichloromethane (10 mL) under stirring.
131 Benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) (65 mg, 0.15
132 mmol, 1.5 eq) was added followed by a first moiety of triethylamine (20 µL, 0.15 mmol, 1.5 eq) in
133 order to facilitate the dissolution of difloxacin. After 2-3 min of stirring, aminoferrocene (20 mg,
134 0.1 mmol, 1 eq) was introduced followed by the second moiety of triethylamine (20 µL, 0.15 mmol,
135 1.5 eq). The mixture was left to react overnight and a dark brown color appeared. The organic layer
136 was diluted in ethyl acetate and washed with 1 M HCl (to eliminate unreacted ferrocene), and
137 Na_2CO_3 10% (w/v) (to eliminate the unreacted difloxacin) and lastly with saturated NaCl (to
138 eliminate traces of water). Finally, the product was dried over $MgSO_4$, filtered through Büchner
139 funnel, evaporated and lyophilized. A stock solution of DF-Fc was prepared by dilution of 1 mg of

lyophilized powder in 1 mL methanol. Difloxacin, aminoferrocene and DF-Fc were analyzed by LC-MS by injecting 1 $mg.L^{-1}$ of each solution prepared in methanol.

2.3. Fabrication of screen-printed dual carbon electrodes (SPdCEs)

Screen-printed dual carbon electrodes (SPdCEs) were fabricated using a DEK248 (Weymouth, United Kingdom) screen-printing system according to a new dual-electrode design (Figure 2A): two working electrodes (4 mm diameter disk) made of carbon paste (Electrodag 423 SS, Scheemda, Netherlands) with two carbon graphite counter electrode (16 mm × 0.8 mm, curved paths), shared a unique pseudo-reference electrode (5 mm × 1.5 mm straight path) made of Ag/AgCl paste (Acheson Electrodag PF-407C, Scheemda, Netherlands). An insulating paint was used to isolate the connectors from the measurement area.

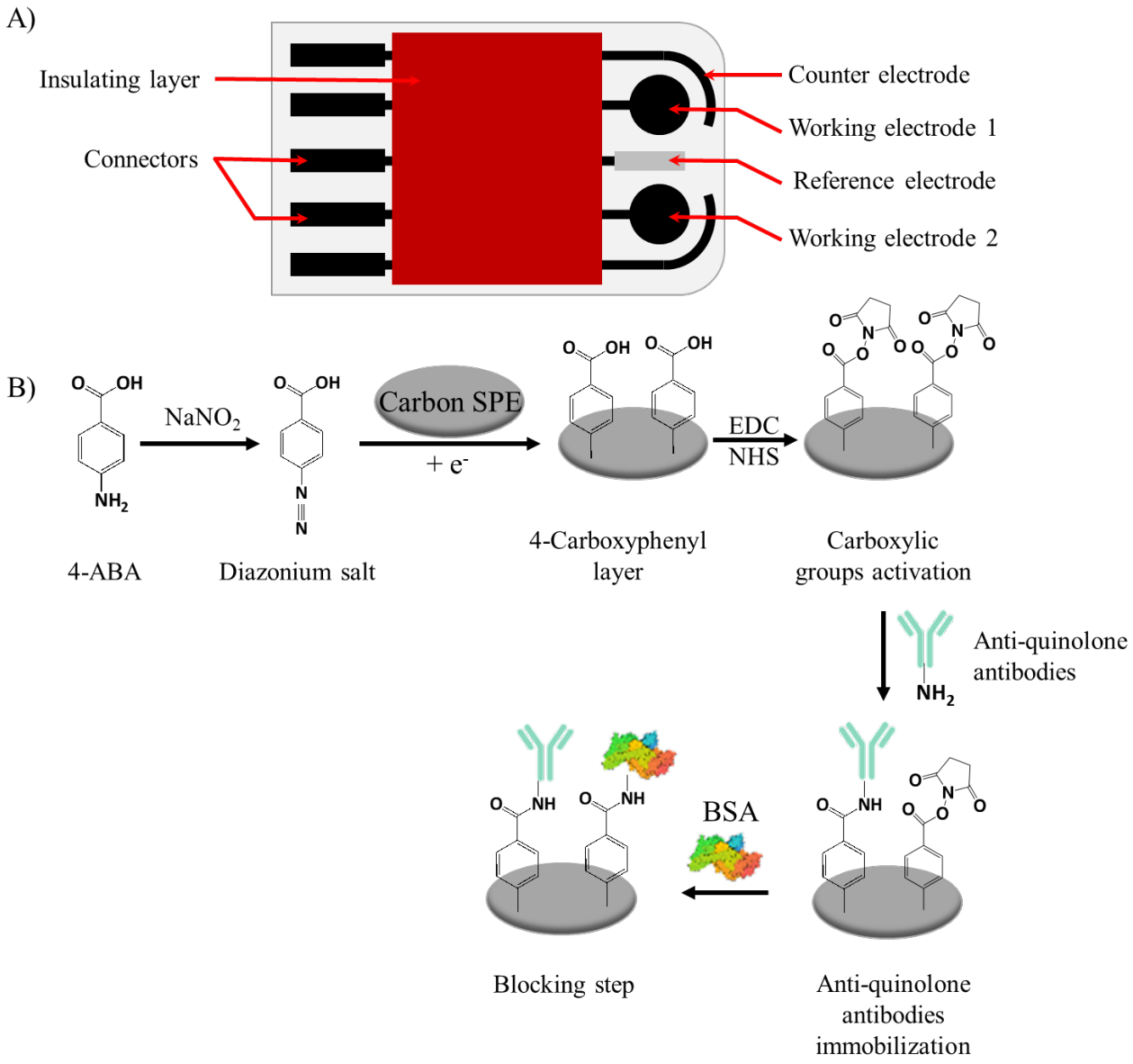


Figure 2. A) Design of the new screen-printed dual working-electrodes device. B) Steps of electrochemical immunosensor elaboration.

2.4. Activation of electrode surface and antibodies immobilization

Figure 2B describes each step of the electrochemical immunosensor fabrication. Before surface modification, the SPdCEs were electrochemically pre-treated by applying five voltammetric scans from +1.0 to −1.5 V *vs*. Ag/AgCl in a sulfuric acid solution (0.5 M H_2SO_4) containing 0.1 M KCl (Dekanski et al., 2001). As previously described, the surface was activated though electrografting of a diazonium salt. 4 mL of reagent was prepared by mixing 1 M $NaNO_2$ with 2 mM of 4-aminobenzoic acid in 0.5 M HCl (Paniel et al., 2017). After 5 min of reaction at room temperature, surface 4-carboxyphenyl groups were generated by performing a linear sweep

voltammetry from +0.4 to −0.6 V *vs.* Ag/AgCl (Paniel et al., 2017). This electro-grafting was followed by activation of carboxylic groups using EDC/NHS: 30 µL of 100 mM MES buffer pH 5.5 containing 100 mM EDC and 25 mM NHS were incubated onto each working electrode during 2 h at room temperature. After washing the electrode with deionized water, 20 µL of anti-quinolone antibodies prepared in 0.1 M PBS pH 7.4 were deposited onto the activated surface and allowed to react for 2 h at room temperature. A non-specific adsorption control was prepared by using 1 M ethanolamine instead of antibodies. After the drop removal, modified SPdCEs were finally incubated with 30 µL of 1% (w/v) BSA overnight at 4°C to block unreacted succinimide groups. The electrochemical immunosensor was then ready for enrofloxacin determination.

2.5. Immunosensor electrochemical characterization

Characterization of SPdCEs was performed in the presence of 2.5-100 $\mu g.mL^{-1}$ DF-Fc prepared in 0.1 M PBS pH 7.4 containing 10% (v/v) methanol. Differential pulse voltamogramms (DPV) were recorded by scanning the potential between -0.6 and +0.5 V *vs*. Ag/AgCl (step 0.001 V, modulation amplitude 0.2 V, modulation time 0.02 s, modulation time 0.1 s, scan rate 0.0168 V/s).

For immunosensor optimization, one working electrode was used as negative control (in the absence of EF) and the other for the detection of EF, each condition was tested in triplicate. For this purpose, 30 µL of DF-Fc (negative control) or 30 µL of a mix of DF-Fc and EF (positive control) were deposited onto the corresponding working electrodes and incubated for 45 min. After washing the electrodes with PBS buffer containing 0.05% (v/v) Tween-20, 300 µL of 0.1 M PBS pH 7.4 were deposited on the whole dual-electrode surface to perform DPV measurements. Different DF-Fc concentrations (5 to 100 $\mu g.mL^{-1}$), anti-quinolone antibodies concentrations (3.65 to 3650 $ng.mL^{-1}$) and incubation times (5 to 60 min) were tested to determine optimal experimental conditions for the detection of EF at 0.1 $\mu g.mL^{-1}$. For each optimization step, the ratio I_{WE2}/I_{WE1} was calculated, where I_{WE1} is the current intensity measured in the absence of EF (negative control) and I_{WE2} is the current intensity measured in the presence of EF. Each intensity value was corrected by

187 the signal measured in the absence of antibodies, corresponding to the non-specific adsorption of
188 DF-Fc. Optimal conditions corresponded to those leading to the lower ratio. Finally, a calibration
189 curve was established using EF solutions at concentrations ranging from 0.001 to 0.1 $\mu g.mL^{-1}$. This
190 dose-response curve was fitted by non-linear regression using the logistic equation (**Eq.1**) by Origin
191 8.6 program:

192 **Equation 1.** Logistic equation for the description of the dose-response curve.

193 $$I_{WE2}/I_{WE1} = \frac{B - A}{1 + \left(\frac{[EF]}{EC_{50}}\right)^{P}} + A \quad (\mathbf{1})$$

194 where A is the y-value at the bottom of the plateau of the curve, B is the y-value at the top plateau
195 of the curve, EC_{50} is the EF concentration required to halve the I_{WE2}/I_{WE1} ratio, and P is the
196 calculated logistical power.

197 **2.6. Meat samples preparation**

198 Incurred meat samples were obtained from the sample bank built by Serrano et al. (2020)
199 from pigs treated with enrofloxacin (Baytriluno, Bayer, Leverkusen, Germany). Muscle samples
200 from loins, sirloins, forequarters and hindquarters were kept frozen at -20ºC and analyzed by
201 HPLC-FLD according to an accredited method (International Organization for Standardization
202 (ISO) 17025:2017). The concentrations described ranged between 18 ± 3 and 1044 ± 44 $\mu g.kg^{-1}$.

203 In order to perform immunosensor analysis, a previous step of extraction of the serum was
204 needed. Meat serum was obtained using an extraction protocol optimized for the analysis of
205 antimicrobials in meat (Mata et al., 2014). Briefly, a piece of 3-4 g of muscle was placed into a 50-
206 mL Falcon tube and microwaved in defrost position for 3 min in a water bath. The meat serum
207 produced during this step was collected and centrifuged 3 min at 4000 g. The supernatant was
208 analyzed with the electrochemical immunosensor without any other treatment. A certified free-
209 antibiotic meat sample from this bank was used as negative control.

210

211

2.7. Detection of fluoroquinolones in meat sample with the electrochemical immunosensor

The matrix effect of meat serum on the immunosensor response was analyzed by determining the ratio I_{WE2}/I_{WE1} in buffer solution containing 20-80% (v/v) meat serum certified free of antibiotics.

The incurred meat serums were diluted 1:5 in the detection solution (50 $\mu g.mL^{-1}$ DF-Fc in PBS buffer pH 7,4 containing 10% (v/v) methanol). A negative control was also prepared with the free-antibiotic certified meat. These solutions were incubated onto modified SPdCEs: the negative control was incubated onto WE1 and incurred meat extract was analyzed on the WE2. The ratio I_{WE2}/I_{WE1} was calculated for each incurred meat. Fluoroquinolone concentrations were then determined using the calibration curve (**Eq.1**).

3. Results and Discussion

3.1. Synthesis of difloxacin-ferrocene conjugate

The working principle of the immunosensor is based on the competition between free EF and a newly synthesized conjugate, difloxacin-ferrocene (DF-Fc) for their binding to immobilized antibodies. Difloxacin is another fluoroquinolone that was selected due to its lower affinity for anti-quinolone antibodies (18% relative binding, according to Abcam product datasheet), which allows promoting the binding of EF during competition step. The synthesis of DF-Fc conjugate was based on the formation of an amide bond between difloxacin **(1)** and the redox probe aminoferrocene **(2)** (Figure 3A). This one-step synthesis was mediated by a coupling agent, benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP), allowing the activation of difloxacin hydroxyl group. The purity of the resulting DF-Fc conjugate **(3)** was controlled by liquid chromatography coupled with mass spectrometry (LC-MS), showing a unique molecular ion at 583.04 g/mol that corresponds exactly to the protonated DF-Fc molar mass (Supplementary data, Figure S1).

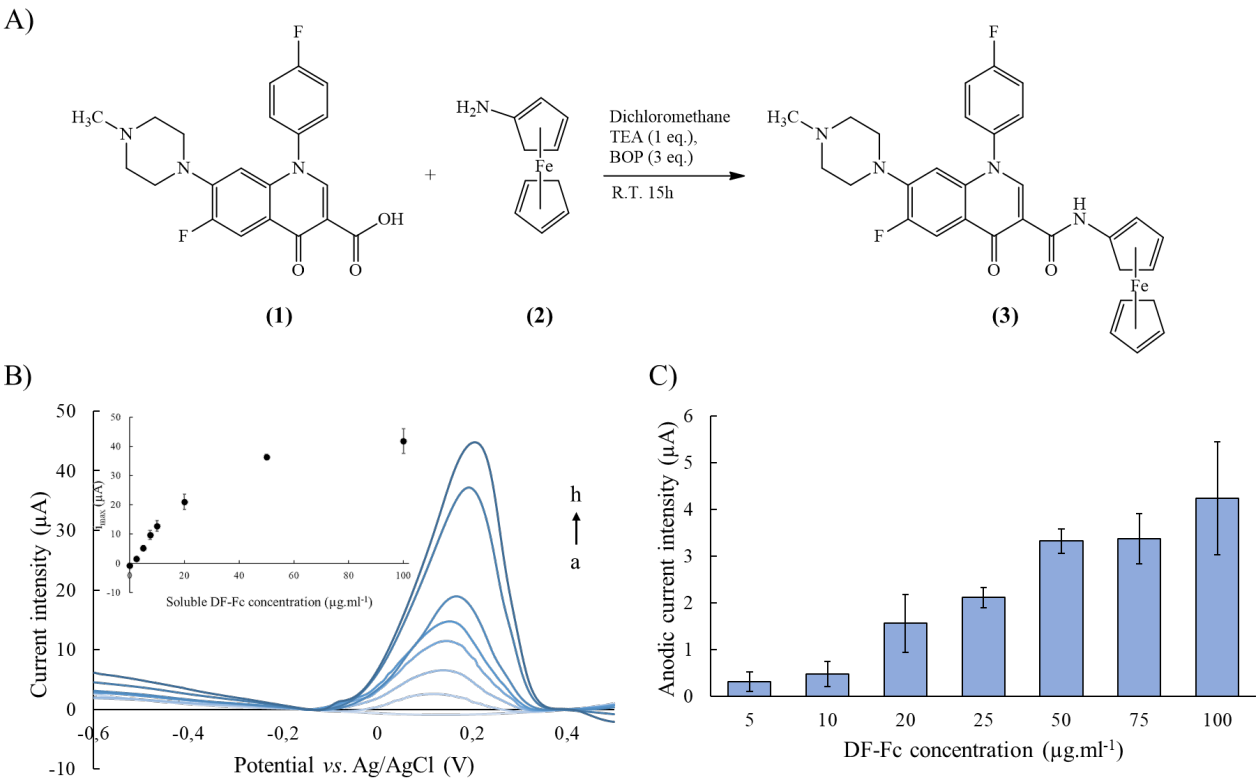


Figure 3. A) Synthesis of difloxacin-ferrocene conjugate **(3)** by the formation of an amide bond between aminoferrocene **(1)** and difloxacin **(2)**. B) DPV scans (from -0.6 to +0.5 V; step 0.001 V, modulation amplitude 0.2 V, modulation time 0.02 s, modulation time 0.1 s, scan rate 0.0168 $V.s^{-1}$) in the presence of 0 (a), 2.5 (b), 5 (c), 7.5 (d), 10 (e), 20 (f), 50 (g) and 100 (h) $\mu g.mL^{-1}$ DF-Fc (in 0.1 M PBS pH 7.4 containing 10% (v/v) methanol). The inset shows maximal anodic current intensity for each DF-Fc concentration. C) Anodic current intensity measured with the immunosensor after incubation of 5-100 $\mu g.mL^{-1}$ DF-Fc (antibody theoretical loading: 1.46 ng).

3.2. Electrochemical characterization of difloxacin-ferrocene conjugate

Firstly, the newly synthesized difloxacin-ferrocene conjugate (DF-Fc) was characterized by carrying out differential pulsed voltammetry (DPV) experiments between -0.6 and +0.5 V *vs.* Ag/AgCl in 0.1 M PBS pH 7.4 containing 10% (v/v) methanol to improve the solubility of the conjugate (Figure 3B). Oxidation of conjugate-linked Fc was observed at around +0.2 V *vs.* Ag/AgCl, and the obtained anodic current allowed establishing a calibration curve for DF-Fc concentrations ranging from 2.5 to 100 $\mu g.mL^{-1}$ (Figure 3B inset). The resulting curve follows a hyperbolic shape, with a linear range between 2.5 and 10 $\mu g.mL^{-1}$, and a sensitivity of 1202 $\mu A.mg^{-1}.mL$. It was calculated that variability of the signal measured with 10 different electrodes in

presence of 50 µg.mL^{-1} DF-Fc was 2%, showing that home-made screen-printed electrodes are suitable transducers for DF-Fc detection.

In a second step, the affinity of DF-Fc for anti-quinolone antibodies was evaluated by incubating the designed immunosensor with DF-Fc at concentrations ranging from 5 to 100 µg.mL^{-1} (Figure 3C). For each concentration, current intensities were corrected by the signal measured in the absence of antibodies, corresponding to the non-specific adsorption of DF-Fc. It was shown that the corrected current increases linearly with DF-Fc concentration and reaches a plateau at 3.3 µA in the presence of 50 µg.mL^{-1} DF-Fc, corresponding to the saturation of immobilized antibodies (Figure 3C). In these conditions, a satisfying variability was measured with a relative standard deviation of 4.3%, measured using three different electrodes.

3.3. Performance of the electrochemical immunosensors for the detection of enrofloxacin

The proposed electrochemical immunosensor was used to detect EF based on a competition between EF and DF-Fc for their binding to immobilized antibodies (Figure 1). Due to the higher affinity of antibodies for EF than for DF-Fc, the preferential binding of EF led to a decrease of electrochemical signal. The immunosensor performances were optimized in order to reach the desired threshold of 0.1 µg.mL^{-1} EF. The new dual working electrode design allowed the simultaneous measurement of negative control on WE1 (in the absence of EF) and positive control on WE2 (for various EF concentrations). The measured currents were corrected from the error due to non-specific binding of DF-Fc, which was calculated from triplicate measurements prior to each measurement series. The ratios I_{WE2}/I_{WE1} were calculated for different DF-Fc concentrations, using various antibody loadings and different incubation times. It was shown that the detection of EF was optimal using an antibody loading of 1.46 ng in presence of 50 µg.mL^{-1} DF-Fc and using an incubation time of 45 min. Under these optimal conditions, a calibration curve was established with EF at concentrations ranging from 0.001 to 0.1 µg.mL^{-1} (Figure 4A). The I_{WE2}/I_{WE1} ratio decreases with the increase of EF concentration, showing a typical behavior of a competitive assay. According the logistic model (**Eq.1**) used to fit this dose-response curve, the EF concentration necessary to

halve the signal (EC_{50}) was 0.010 µg.mL^{-1} and the limit of detection calculated for 90% response ratio was 0.003 µg.mL^{-1}. These results show that the performance of the developed sensor is suitable for the determination of EF in meat samples.

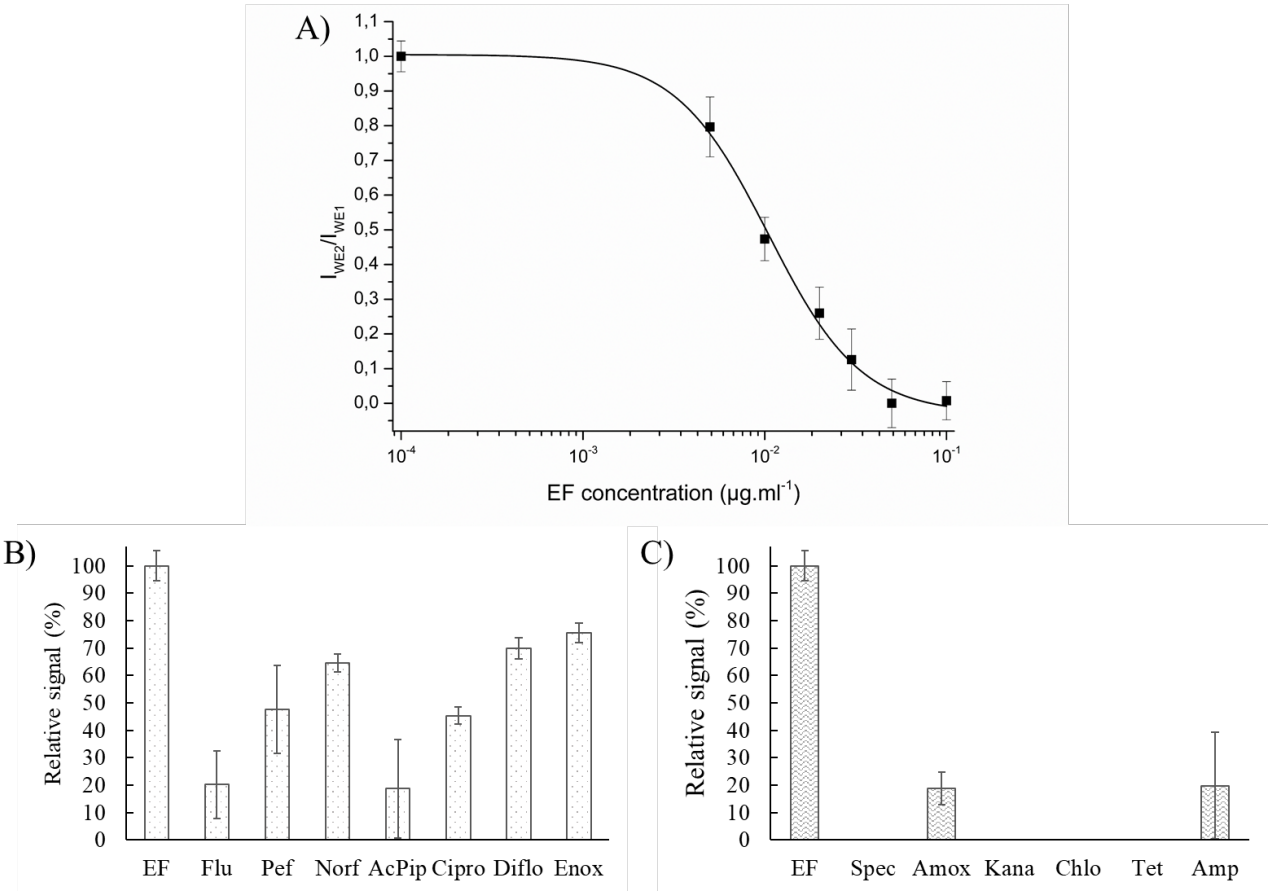


Figure 4. A) Dose response curve measured in the presence of 0.001 to 0.1 µg.mL^{-1} EF and 50 µg.mL^{-1} DF-Fc (in PBS pH 7.4, incubation time 45 min, antibody 1.46 ng). Curve fitting using a logistical model (black line) exhibiting the following equation: $I_{WE2}/I_{WE1} = (1/(1+([EF]/0.010)^{1.84}))$ ($R^2 = 0.989$). B) Relative signal (expressed in percentage of EF signal) measured in the presence of 0.1 µg.mL^{-1} of spectinomycin (Spec), amoxicillin (Amox), kanamycin (Kana), chloramphenicol (Chlo), tetracycline (Tet) and ampicillin (Amp). C) Relative signal (expressed in percentage of EF signal) measured in the presence of 0.1 µg.mL^{-1} quinolones: flumequine (Flu), pefloxacin (Pef), norfloxacin (Norf), pipemidic acid (AcPip), ciprofloxacin (Cipro), difloxacin (Diflo) and enoxacin (Enox).

3.4. Selectivity towards other antibiotics

The selectivity of the electrochemical immunosensor was evaluated by incubating modified SPdCEs with 0.1 µg.mL^{-1} of various antibiotics from different classes: spectinomycin and kanamycin (aminoglycosides class), chloramphenicol (phenicols class), tetracycline (cyclines class)

296 and amoxicillin and ampicillin (β-lactams class). According to Figure 4B, these antibiotics do not
297 significantly interfere with EF detection.
298 The immunosensor was then tested using different quinolones including flumequine, pefloxacin,
299 norfloxacin, pipemidic acid, ciprofloxacin, difloxacin and enoxacin (Figure 4C). Due to the non-
300 specificity of antibodies for one fluoroquinolone, an inhibition was detected for all the
301 fluoroquinolones tested. It must be stressed that flumequine and pipemidic acid presented lower
302 signals, probably due to the absence of a piperazine ring in flumequine and of a fluor atom in
303 pipemidic acid (Supplementary data, Figure S2). These two elements thus seem to be mandatory for
304 antibodies specific recognition.

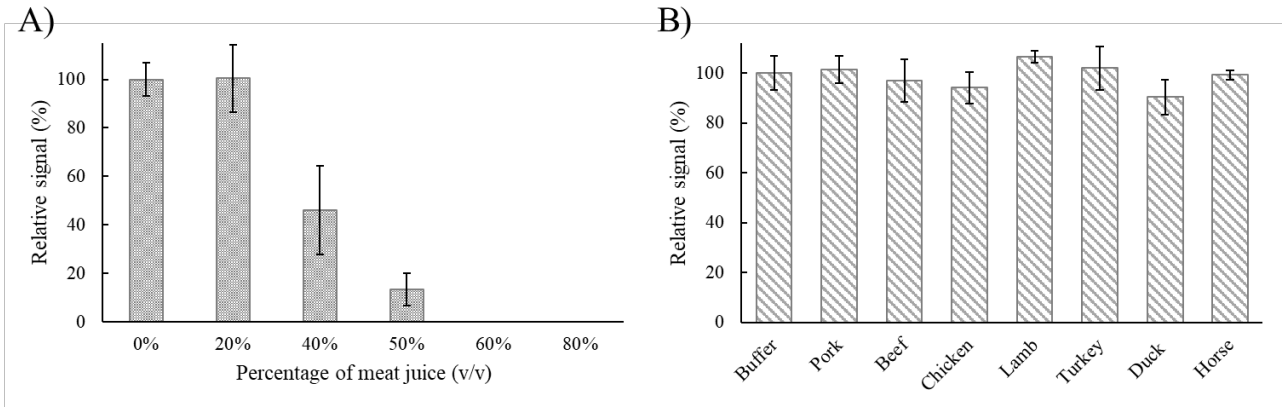
305 **3.5. Reproducibility and storage stability of the immunosensor**

306 The immunosensor reproducibility was evaluated by testing the response of five sensors,
307 prepared independently under the same conditions, in the presence of 0.1 $\mu g.mL^{-1}$ enrofloxacin. The
308 corresponding I_{WE2}/I_{WE1} ratios were calculated and a relative standard deviation of 5.5% was
309 obtained, indicating that the electrochemical immunosensor shows a satisfactory reproducibility.
310 Long-term stability was also evaluated using 6 immunosensors prepared in the same optimal
311 conditions and stored during one month at 4°C. The results showed that 94% of current intensity
312 was conserved after one month and that a comparable I_{WE2}/I_{WE1} ratio was obtained in the presence
313 of 0.1 $\mu g.mL^{-1}$ EF, showing an appropriate stability of this electrochemical immunosensor. These
314 storage conditions could allow the mass production of the proposed immunosensor but need further
315 optimization for potential commercialization.

316 **3.6. Detection of fluoroquinolones in meat samples**

317 The proposed immunosensor was used to detect EF from meat samples. Firstly, serum from
318 antibiotic-free meat samples was extracted and tested using the immunosensor to evaluate its
319 potential matrix effect. The electrochemical response (I_{WE1}) was recorded in the presence of 20-
320 80% (v/v) of meat serum in the incubation solution. The current intensity (I_{WE2}) was measured in
321 parallel with 20-80% (v/v) of the serum spiked with 0.1 $\mu g.mL^{-1}$ EF, and the response ratios

322 I_{WE2}/I_{WE1} were determined for each serum percentage. The achieved results were reported as
323 relative percentages, in comparison with the ratio measured without serum (Figure 5A). It was
324 shown that meat serum does not affect the immunosensor response when used at a volume ratio of
325 20%, while higher amounts dramatically decrease its response. The same ratio of 20% was thus
326 tested using muscle meat serums from other animals (chicken, beef, lamb, duck, horse and turkey),
327 which were extracted and analyzed using the same protocol. As shown in Figure 5B, the addition of
328 0.1 $\mu g.mL^{-1}$ EF led to very similar relative signals, showing that the optimized protocol is suitable
329 for the detection of fluoroquinolones in a wide range of meats.



330

331 **Figure 5.** A) Immunosensor relative signal (in comparison with the I_{WE2}/I_{WE1} ratio obtained in the absence of meat
332 serum) measured in the presence of 0.1 $\mu g.mL^{-1}$ EF and 20-80% (v/v) of meat serum. B) Immunosensor relative
333 signal (in comparison with the I_{WE2}/I_{WE1} ratio obtained in buffer) measured in the presence of 20% (v/v) meat
334 serums from pork, beef, chicken, lamb, turkey, duck and horse meat, spiked with 0.1 $\mu g.mL^{-1}$ EF.
335
336 Incurred samples obtained from pigs treated with different doses of fluoroquinolones were analyzed
337 using the developed immunosensor. For each piece of meat, the I_{WE2}/I_{WE1} ratio was determined
338 using the previously described strategy, allowing the determination of a fluoroquinolone
339 concentration expressed in $\mu g.L^{-1}$. Figure 6 presents the correlation between the fluoroquinolone
340 concentration found by HPLC-FLD (Serrano et al., 2020) and data obtained from the analysis
341 performed with the new immunosensor. A satisfying correlation coefficient ($R^2 = 0.975$) was found
342 between the results achieved using both technologies, showing that the electrochemical

immunosensor could be a very useful tool for the detection of fluoroquinolone antibiotics in meat samples. Moreover, it was shown that the simplicity of the extraction process was sufficient to detect these antibiotics with accuracy.

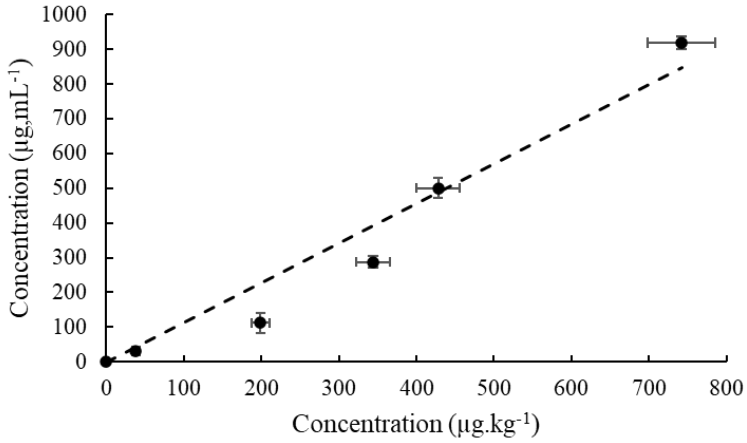


Figure 6. Correlation between the fluoroquinolone concentration determined by the proposed electrochemical immunosensor (expressed in $\mu g.L^{-1}$) and determined by HPLC-FLD (expressed in $\mu g.kg^{-1}$) for each incurred meat sample tested. The dotted line exhibited the following equation: $y=1.1408x$ ($R^2=0.975$).

4. Conclusions

This work describes for the first time the development of an electrochemical immunosensor for the detection of enrofloxacin (EF) antibiotic in meat samples. The device is based on the competitive binding of EF and a ferrocene-labelled conjugate (DF-Fc), which was synthesized and characterized by differential pulse voltammetry. Under optimal conditions, the proposed immunosensor was able to determine EF at concentrations ranging from 0.005 to 0.01 $\mu g.mL^{-1}$, with a detection limit of 0.003 $\mu g.mL^{-1}$. The use of a dual-working electrode design allowed taking into account the inter-electrode variability by using one electrode as negative control. This new method exhibited an acceptable stability, good reproducibility and adequate selectivity towards other antibiotics. Spiked EF was detected in pork, chicken, beef, lamb, horse, duck and turkey meats after a rapid and easy extraction method. Finally, real samples were also analyzed and led to an accurate EF quantification.

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Supplementary data

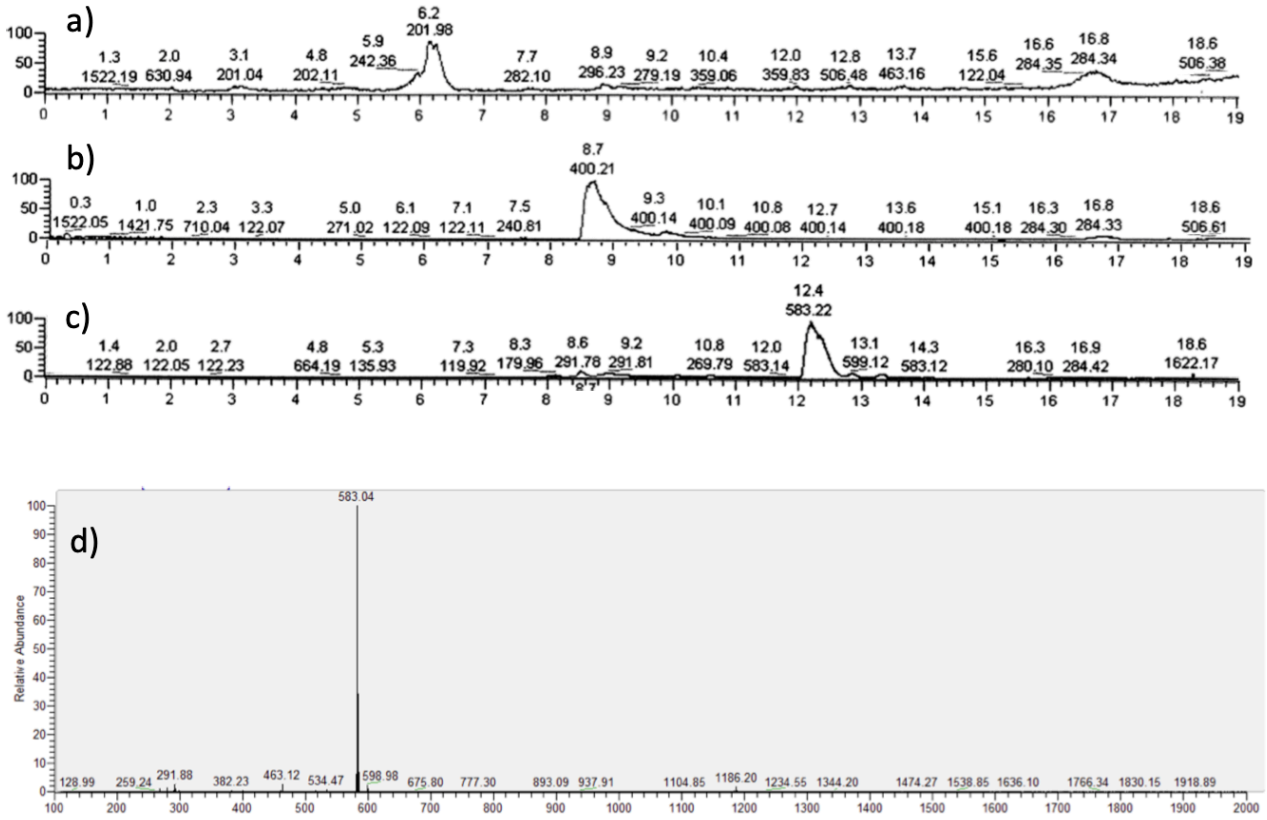

Figure S1. Chromatogram of aminoferrocene (A), difloxacin (B) and DF-Fc conjugate after synthesis, purification and lyophilization (C) (1 mg.l^{-1} prepared in methanol). Reagent peaks (difloxacin and aminoferrocene) were absent in this last spectrum, showing that they were totally consumed/eliminated. D) m/z spectrum of DF-Fc at 12.4 min retention time.

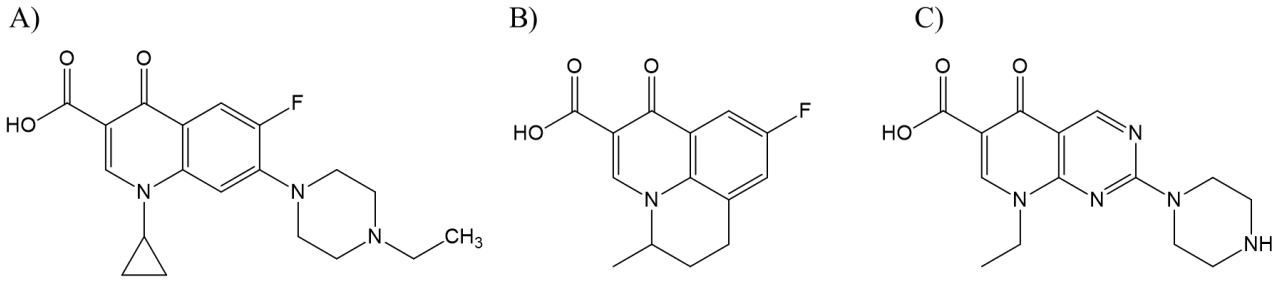

Figure S2. Structure of enrofloxacin (A), flumequine (B) and pipemidic acid (C).