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Portable Surface Plasmon Resonance Immunosensor for the Detection of Fluoroquinolone Antibiotic Residues in Milk

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ABSTRACT: An inexpensive and portable surface plasmon resonance (SPR) sensor, *SPReeta* Evaluation Kit SPR3, has been used to develop a biosensor for the determination of fluoroquinolone antibiotics (FQs) and to demonstrate its performance analyzing FQ residues in milk samples. The *SPReeta* three-channel gold chips were activated with a mixed self-assembled monolayer (m-SAM) and functionalized with a FQ haptenized protein. Binding of the antibody produced a concentration-dependent increase of the SPR signal as a result of the change in the refraction index. Similarly, the presence of the FQ produced a dose-dependent decrease of the response, which allowed a good limit of detection (LOD) to be obtained ($1.0 \pm 0.4\ \mu g\ L^{-1}$ for enrofloxacin in buffer). The response was reproducible in all three channels, on different injections and days, and also between chips. Milk samples could be analyzed after a simple sample treatment involving fat removal by centrifugation and dilution with water. Under these conditions calibration curves were obtained showing that FQ residues can be analyzed in milk samples with an IC_{50} value of $26.4 \pm 7.2\ \mu g\ L^{-1}$ and a LOD of $2.0 \pm 0.2\ \mu g\ L^{-1}$ (for enrofloxacin), far below the European Union regulations for this antibiotic family in this matrix. Finally, the paper also demonstrates that the biosensor is able to selectively detect the presence of FQs in milk samples, even in the presence of other antibiotics. Enrofloxacin, ciprofloxacin, and norfloxacin residues were detected in blind samples supplied by Nestlé Co.

KEYWORDS: surface plasmon resonance, biosensor, antibodies, fluoroquinolone antibiotics, milk, residue analysis, SAM

INTRODUCTION

Fluoroquinolone (FQ) antibiotics are widely used in veterinary medicine to treat and prevent animal diseases. Their extensive use and misuse may lead to the appearance of residues in edible tissues and foodstuffs.[1−3] For the dairy industry, these residues may affect fermentation processes and reduce the quality of the milk.[4,5] Moreover, the improper use of antibiotics in the veterinary field has been identified as one of the reasons driving the development of antimicrobial resistance phenomena and other adverse health effects.[1,6,7] To ensure public health protection, maximum residue levels (MRLs) have been established for different veterinary drugs on distinct foodstuffs of animal origin. Thus, for FQ antibiotics, MRLs are in the range between 30 and 1400 $\mu g\ kg^{-1}$ for different edible samples. In the case of milk, the maximum levels vary from 30 $\mu g\ kg^{-1}$, for danofloxacin, to 100 $\mu g\ kg^{-1}$, for enrofloxacin, including its metabolite, ciprofloxacin. The MRLs of other quinolones such as norfloxacin or ofloxacin have not been fixed.[8]

Nowadays, residue analysis is mainly made using chromatographic methods combined with fluorescence or mass spectrometry detection.[2,9−13] However, complex sample treatment processes are necessary, including in some cases concentration to reach the necessary detectability. This fact increases substantially the time and cost of each analysis.

Due to their great selectivity, immunochemical techniques offer shorter times of analysis and a significant decrease of the time invested in sample treatment/purification procedures. Thus, fluoroquinolones have been determined in milk by ELISA without any sample treatment, or only after a dilution or centrifugation step.[5,14−16] Moreover, the cost of the equipment needed is often also very low, because usually measurement can be made visually or with simple optical devices. In this context, during recent years several biosensor devices have appeared on the market. Although having a higher degree complexity, they keep the advantages of the bioreceptors, adding the possibility of automation and integration with data acquisition/handling systems.[17−19] Particularly, surface plasmon resonance (SPR) has become one of the most well-known biosensor principles. SPR biosensors are able to detect target analytes without the need of labels, allowing monitoring of biological interactions in real time. The plasmon generated on the surface of noble metals as a result of the incidence of light at a particular angle (critical angle) is used to detect biomolecular interactions, because this phenomenon is strongly affected by the refraction index of the adjacent media.

In recent years, the number of publications reporting applications of SPR biosensors in the clinical, environmental, and food safety fields has grown very quickly,[20−22] and a variety of devices can nowadays be found on the market.[23] Particularly, the *SPReeta* system (Texas Instruments Inc.) is a very affordable, small-size portable SPR device suitable for on-site analysis and has already been applied to the detection of small proteins[24] and low molecular weight analytes such as endocrine disruptors[25] and hormones,[26] but to our knowledge, nobody has yet reported their potential use to detect antibiotic residues in milk samples. In comparison with high-performance laboratory SPR systems, portable biosensors may present lower sensitivity due to their worse resolution ($\sim 2 \times 10^{-6}$ as compared to $\sim 2 \times 10^{-7}$ refractive index units, RIUs).[20,27−29] However, recently, new

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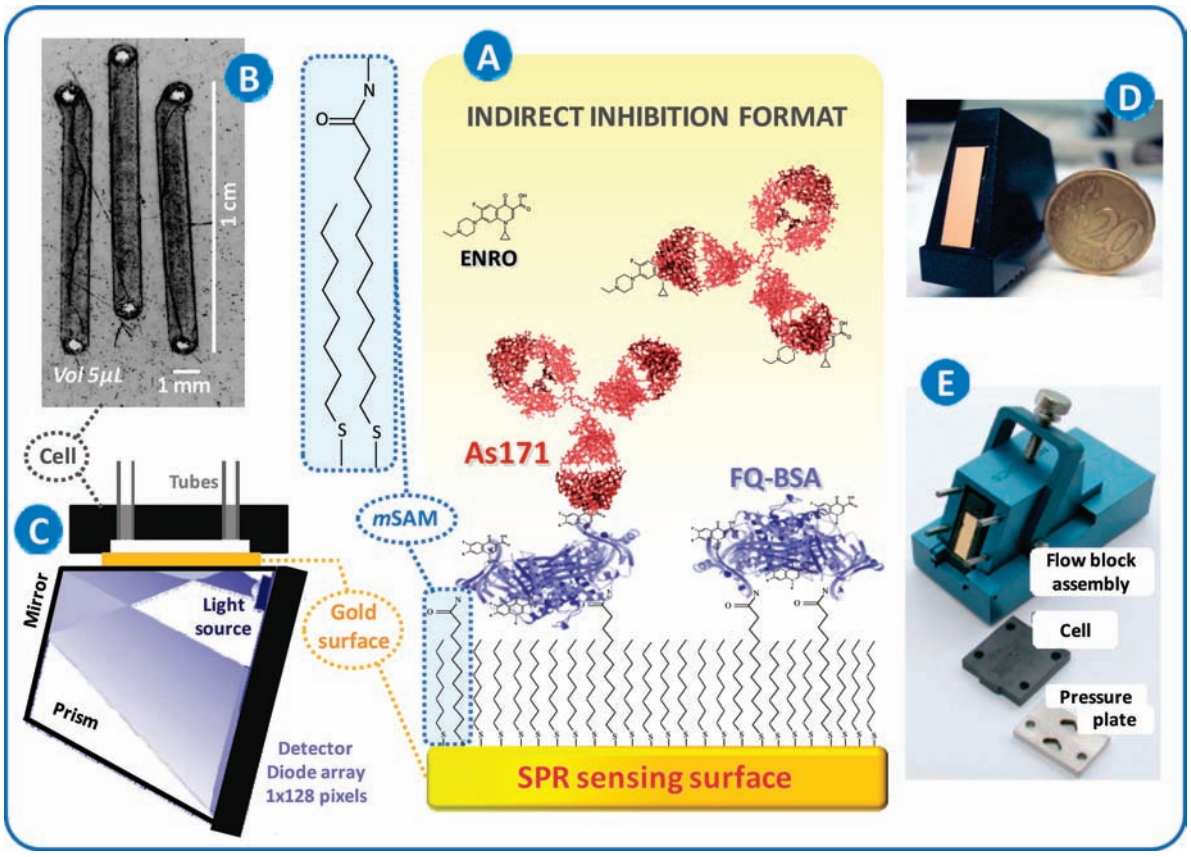

Figure 1. (A) Illustration of SPR working format for detection of FQs; (B) picture of the three channels in polypropylene cell; (C) scheme of the components of the *SPReeta* chip (model TSPR1K23) coupled to the cell; (D) real view of the chip; (E) chip in the flow block assembly.

portable SPR biosensors have been reported to be able to reach resolutions of $(3-5) \times 10^{-7}$[30,31] and have been demonstrated to be good enough for FQ-residue analysis in the food safety field.[32] Here we report the use of an even simpler SPR system such as the *SPReeta* Evaluation KIT SPR3 to develop an immunosensor for fluoroquinolone antibiotic residue analysis with sufficient detectability with regard to EU legislation.[8] Three samples or controls can be simultaneously analyzed by using a three-channel flow cell over the gold chip biofunctionalized with FQ haptenized protein. The FQ *SPReeta* immunosensor has been used to analyze milk samples after very simple treatment. Although the analysis of fluoroquinolones in milk samples using SPR devices has already been reported,[3,32] this is the first time that the possibility of performing these analyses employing a simple, portable, and inexpensive SPR device such as the *SPReeta* system is reported. The results shown here demonstrate that the *SPReeta* FQ immunosensor is able to selectively detect contamination of milk samples by FQ, even in the presence of other antibiotics.

MATERIALS AND METHODS

SPR Sensor Setup. The employed system was a *SPReeta* Evaluation Kit SPR3 (Nomadics, Inc., Stillwater, OK) composed by SPR gold chips with an integrated detector (model TSPR1K23, Texas Instruments, Inc., Dallas, TX), a flow cell, a flow block assembly, and a control box. The SPR gold chips consist of a borosilicate glass surface coated with a 50 nm layer of gold placed over the plastic prism used for coupling of the light to the surface plasmon. The light source is a LED emitting near-infrared light (840 nm) and is polarized to enhance SPR (see Figure 1). The light is reflected in a mirror after the interaction with the surface and collected onto a linear array of silicon photodiodes detector (1 × 128). The minimum resolvable refractive index is about $(3-5) \times 10^{-6}$ RIU. The sensor also contains a memory chip for recording identification and calibration information. The flow cell is made of polypropylene and provides three independent flow channels (1 mm × 1 cm) with a volume of about 5 μL each (see Figure 1B). This cell is assembled to Teflon microfluidic tubes (i.d. = 0.76 mm, Tygon 2765-175) for its attachment to the tubes of a peristaltic pump (ISM 404B, ISMATEC, Glattbrugg, Switzerland). The flow block assembly is used to secure the flow cell to the face of the *SPReeta* by means of a stainless steel pressure plate (see Figure 1E) and to interconnect the *SPReeta* sensor module with an electronic control box. The control box connects the sensor with a computer provided with data handling and processing software (Multiple Channel *SPReeta* program, version 21.15, from Texas Instrument, Inc., Dallas, TX and GraphPad Prism, version 4.0, from GraphPad Sofware Inc., San Diego, CA).

Chemicals and Immunochemicals. Unless otherwise indicated, chemicals and biochemicals were supplied by Sigma-Aldrich (St. Louis, MO). Enrofloxacin, ciprofloxacin, and norfloxacin (ENRO, CIP, and NOR, respectively) were supplied by Biochemika, Fluka (Milan, Italy), in a high-purity degree. The preparation of immunoreagents, haptenized protein FQ—BSA and polyclonal antiserum As171, is described elsewhere.[33]

Buffers and Solutions. Phosphate-buffered saline (PBS) is 10 mM phosphate buffer on an 0.8% saline solution, pH 7.5. Assay buffer, PBSCa, is PBS with 1 mM in $CaCl_2$. PBS20 is PBS 20 mM. McIlvaine's buffer is 80 mM in Na_2HPO_4 and 60 mM in citric acid, pH 4.2. On the FQ *SPReeta* immunosensor, the running buffer used to measure samples or standards in buffer was PBS, whereas the running

buffer used to measure milk samples or controls was a 1:1 mixture of 20 mM PBS and blank treated milk (see procedure below). The solution used to form a self-assembled monolayer (SAM solution) on the gold chip was a freshly prepared mixture of mercaptoundecanoic acid (MUA, 2.5 mM) and octanethiol (OT, 7.5 mM) in EtOH. The carboxylic acids were activated using a NHS/EDC solution (5 mg mL^{-1} each) containing *N*-hydrosuccinimide (NHS) and 1-ethyl 3-(3-dimethylaminopropyl)carbodiimide hydrochloride (ECD) in PBS and prepared just prior use. A solution of the FQ haptenized protein (FQ−BSA) at 10 μg mL^{-1} in PBS was prepared to biofunctionalize the gold chips. A 0.3 M NaOH aqueous solution was employed for regeneration steps, and a 1% Triton X-100/0.1 M NaOH aqueous solution was employed for cleaning the gold surface. All of the buffers used for the SPR system were filtered and degassed prior to use.

Biofunctionalization of the SPR chips was performed under flow conditions at 33 μL min^{-1}. First, the gold chips were cleaned by passing through a Triton X-100/NaOH solution (20 min) followed by Milli-Q water (10 min) and EtOH (10 min). Activation of the gold surfaces was accomplished through the formation of a mixed self-assembled monolayer (m-SAM) by flowing the SAM solution (3 h). Subsequently, the carboxylic acids of the MUA were activated by passing through the NHS/EDC solution (30 min). The chips were then washed with PBS (5 min), and immediately the FQ−BSA solution was flowed (30 min). After the chip had been washed with PBS (5 min), a solution of BSA was passed (5 mg mL^{-1} in PBS, 5 min) to block the remaining surface and, finally, PBS again (5 min). The biofunctionalized chips could be store at 4 °C on a dry chamber when not in used for more than 1 month. See Figure 1A for details of the functionalized surface. Similar cleaning procedures[24,34] and methods of covalent functionalization[28,35−37] can be found in other SPR works.

SPR Protocol for Fluoroquinolone Analysis. A protocol to work under inhibition indirect format conditions was developed (see Figure 1A). The flow rate used during the whole procedure was 33 μL min^{-1}. Prior to analysis, the standards prepared in the assay buffer (or in treated milk samples) were mixed with the antibody solution (As171 diluted 1/500 in assay buffer for standards and As171 diluted 1/250 in PBS20 for milk samples) in a 1:1 volume ratio and incubated for 10 min at room temperature. Afterward, the mixture was flowed into the sensor for 15 min. The chip was then washed with running buffer (5 min). The change in RIU between the baseline before passing the sample and after the washing step was used to quantify the FQ concentration. Finally, the chip was regenerated by passing through 0.3 M NaOH (3 min) followed by running buffer (7 min) to recover the baseline level. The calibration of the FQ SPR immunosensor was performed by preparing standard curves of ENRO (3500−0 μg L^{-1} in eight calibration points) in assay buffer (or treated milk samples) and measuring them with the *SPReeta* FQ immunosensor as described above. The alkaline regeneration in SPR sensors has been reported before,[38,39] as well as binding processes in phosphate buffer.[28,35,38,40]

The three channels were used to get a mean value for every calibration point (n = 3 channels). The data recorded were adjusted with a four-parameter logistic equation, $Y = [(A - B)/(1 - (C/x)^{\wedge}D)] + B$, where A is the maximal signal, B is the minimum signal, C is the concentration producing 50% of the maximal signal (IC_{50}), and D is the slope at the inflection point of the curve.[41] Reproducibility of the SPR assay was studied by assessing the variation of calibration curve parameters on three different days (n' = 3 days). The IC_{90} (the concentration producing 90% of the maximal signal) value, frequently employed to estimate the limit of detection (LOD) of immunochemical assays,[28,32,35,41,42] was used to assess the detectability of the SPR method.

Milk Samples. Blank bovine whole milk samples (3.5% fat) were obtained from a grocery store and analyzed by HPLC-MS/MS by AESAN (Agencia Española de Seguridad Alimentaria y Nutrición, www.aesan.msc.es) to ensure the absence of antibiotic residues. These samples were used to prepare controls and also to evaluate the FQ *SPReeta* immunosensor as described below. Control (CTRL) milk samples were prepared at zero concentration (CTRL-zero, maximum SPR signal), at 100 μg L^{-1} of enrofloxacin (CTRL-ENRO, MRL 100 μg L^{-1}), at 100 μg L^{-1} of ciprofloxacin (CTRL-CIP, MRL 100 μg L^{-1}), and at 100 μg L^{-1} of norfloxacin (CTRL-NOR, no MRL has been established). Prior to the analysis, milk samples were centrifuged (20000*g* for 30 min) at 4 °C and diluted with Milli-Q water (five times). In some studies, the milk samples were treated with McIlvaine buffer in 1:1 volume ratio, shaken for 1 min, and centrifuged (20000*g*, 10 min) to remove the proteins. Further on, the samples were diluted with Milli-Q water (five times) prior to the analysis. Recovery studies were developed by comparing the concentration before and after the sample treatment ($\%R = 100 \times C_f/C_i$). For such a purpose, blank whole milk samples were spiked in duplicate at three different levels and the measurements of FQ content were performed by ELISA.

Evaluation of the *SPReeta* FQ Immunosensor. *Immunosensor Performance around the MRL.* Blank whole milk samples were spiked with ENRO at different concentrations, 200, 150, 75, and 50 μg L^{-1} (2×MRL, 1.5×MRL, 0.75×MRL, and 0.5×MRL), treated as described before, and measured with the *SPReeta* FQ immunosensor in triplicate (n = 3 channels). Blank samples (0×MRL) were used as zero-control.

Blind Samples. Milk samples (GF20, GF21, GF22, GF23, GF24, and GF25) spiked with several antibiotic families at different concentrations were supplied by the Nestlé Research Center (www.research.nestle.com) and analyzed with the FQ *SPReeta* immunosensor in our laboratory by comparing the responses of those samples with those of the controls to determine which samples were positive or negative according to EC regulations.

RESULTS AND DISCUSSION

The *SPReeta* integrated optical SPR sensor chips were biofunctionalized with the FQ haptenized protein (FQ−BSA) by covalent attachment to the m-SAM layer formed on top of the gold surface. Previously, a stable m-SAM providing carboxylic groups at a suitable density for immobilizing the immunoreagents[35] was achieved by flowing a solution of a 1:3 MUA/OT mixture for 3 h. The carboxylic groups were used to form amide bonds by reaction with the amino groups of the lysine residues of the FQ−BSA bioconjugate (see Figure 1A). The efficiency of this process can be corroborated by some examples in the literature employing a similar process of covalent functionalization.[28,35−37]

The concentration of FQ−BSA was selected from the saturation curve constructed by passing increasing concentrations over the activated SAM (see Figure 2a). The concentration chosen should provide enough coverage of the gold surface and an adequate signal when the antibody (As171) interacts with the functionalized surface in the subsequent binding step. As can be observed in Figure 2a, concentrations of 10 and 20 μg mL^{-1} of FQ−BSA provided 70 and 90% of saturation of the surface. However, the binding response for the same concentration of As171 was the same in both cases (data not shown). Therefore, 10 μg mL^{-1} was the concentration selected for the functionalization of the gold chips.

Once the concentration of antigen was fixed (10 μg mL^{-1}), the binding of the FQ antibody (As171) at increasing concentrations was assayed to select an antiserum dilution (1:1000) producing an adequate signal as a consequence of the variation produced in the refractive index of the media (see Figure 2b) without being significantly affected by baseline fluctuations, temperature changes, or the drift of the system (∼670 RUs).

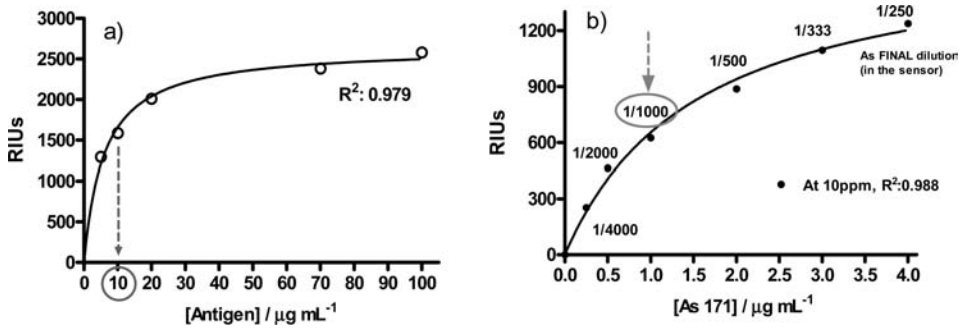

Figure 2. Graphs showing the variation of the SPR response as a function of the concentration of immunoreagents: (a) saturation of the response when increasing FQ−BSA concentrations are immobilized gold chip; (b) response recorded as a result of the specific binding of increasing concentrations of As171 to the biofunctionalized gold SPR chip (10 μg mL^{-1} FQ−BSA). In the *x*-axis is represented the theoretic concentration of specific antibody (1 mg mL^{-1} in undiluted As171).

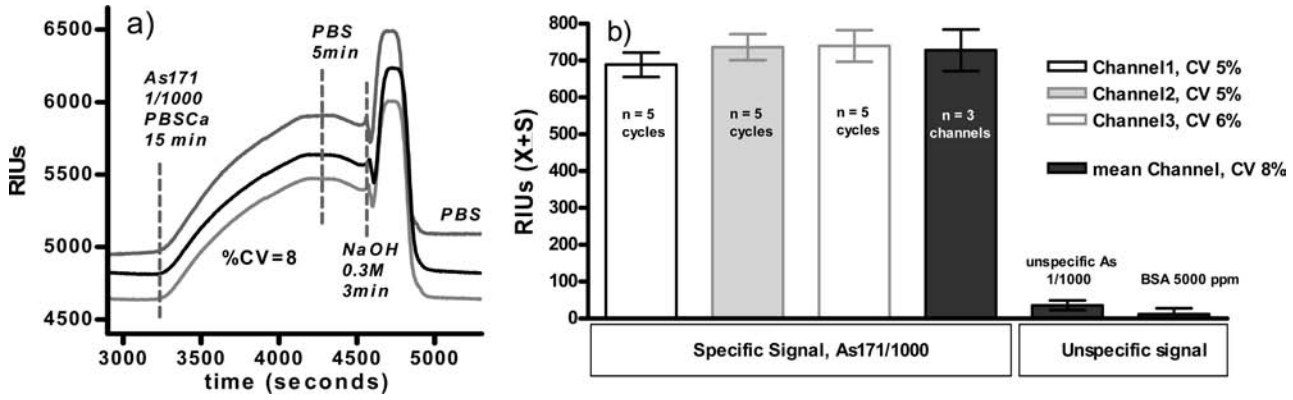

Figure 3. (a) Sensogram showing the repeatability observed between the three channels of the same SPR chip (measurements made in parallel). (b) Mean value ($X \pm S$) of achieved response in each channel for five cycles (dark bar indicates the value for the same cycle but for three channels). The mean response (n = 3 channels) provided by other nonspecific proteins (unspecific antiserum and BSA) is also represented.

Repeatability of the signal produced upon binding of the As171 between channels (n = 3) and between cycles (n = 5) showed coefficient of variations (%CV) of 8 and 6%, respectively. Moreover, the variability interdays (n = 3) and interchips (n = 3) was about 12 and 14%, respectively. Figure 3 shows the sensograms recorded measuring with the three channels in parallel for one binding cycle, including regeneration. The same chip could be reused. Thus, after 10 working days, in which a chip had been used for about 80 cycles, the signal recorded still was 80% of the value recorded in the first cycle. Similar behavior has been reported by other authors. Thus, as an example Gobi and Naimushin describe a drop of the signal to 93 and 80% after 30 and 60 cycles, respectively.[24,35] The causes of the signal decay are related to the access of the epitopes of the antigen. Changes in the structure and conformation of antigen can occur due to exposure to extreme pH (regeneration step) and variations between working (solution) and storage (air) conditions. Additionally, the surface can be partially blocked due to strong unspecific interactions or some irreversible antigen−antibody associations.[20,21,35,43] In this work, we have employed alkaline solution for the regeneration of the surface, but besides this method,[38,39] applications of acidic regeneration[28,35,40] or mixtures with organic solvent have also been reported.[6,44]

The specificity of the response of the FQ *SPReeta* immunosensor was assessed by comparing the signal (change in the refractive index) produced by the binding of As171 (a specific antiserum for FQs) with those produced by the interaction with an unspecific antibody and with BSA. These solutions were injected on a chip biofunctionalized with FQ−BSA, and as can be observed in Figure 3b, the signal provided by As171 was significantly higher than the signals provided by the other ones.

The ability of the FQ *SPReeta* immunosensor to detect FQ antibiotic residues was initially studied by building an ENRO calibration curve in assay buffer under inhibition indirect format conditions (see Figure 1A). The standard solutions were mixed with the antibody and, after a short preincubation time, flowed into the sensor chamber. The presence of the antibiotic inhibited the binding of the antibody to the sensor surface, as can be observed in Figure 4 (see dotted line). Table 1 summarizes the features of the immunosensor measuring FQ in buffer. In these conditions, a LOD = 1.04 ± 0.4 μg L^{-1} was achieved, which is 100 times lower than the established MRL.

Subsequently, the capability of the immunosensor to directly analyze FQ residues in milk samples was evaluated. Unfortunately, undiluted blank milk samples produced a significant unspecific response, which could not be removed just by dilution with Milli-Q water. Milk is a complex matrix and its components, mainly fat and proteins,[45,46] can interact unspecifically with the sensor surface by hydrophobic and electrostatic interactions.[47−49] For this reason, in many works, sample treatments such as fat removal[3,50−53] or protein precipitation[44] are necessary prior to SPR analysis. In our case, several sample treatment methods were assayed with the

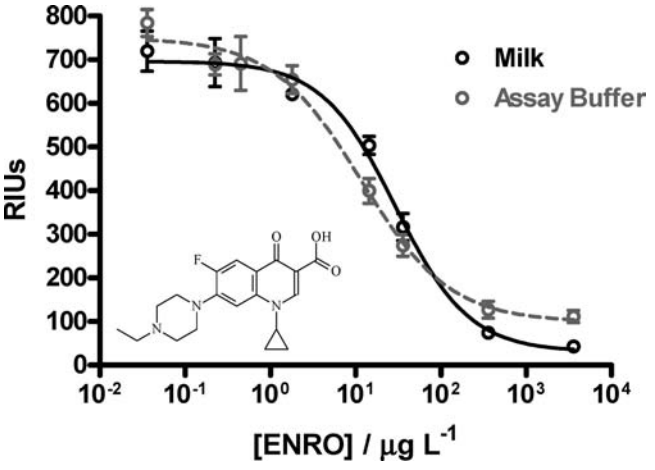

Figure 4. ENRO calibration curves recorded in the assay buffer (dotted line) and in milk. The SPR response of each point is the average and standard deviation of three measurements (three channels). Prior to the analysis, milk samples were centrifuged (20000*g*, 30 min) and diluted five times with water. The parameters ($X \pm S$) of these calibration curves are reflected in Table 1 for $n = 3$ days.

objective of removing matrix interferences. Precipitation of the proteins with the McIlvaine buffer, or removal of the fat by centrifugation, followed by a 1:5 dilution with Milli-Q water succeeded in reducing significantly the unspecific signal produced by the milk. On the other hand, the specific signal accomplished as result of the binding of the As171 to the gold chip was greater when milk samples that had been centrifuged were measured (~450 RIU) than those treated with the McIlvaine buffer (1:5 dilution, ~320 RIU). Therefore, centrifugation of the milk was selected as a sample treatment method prior to SPR analysis. The concentration of the antibody was adjusted to enhance the signal and to accomplish a maximum assay signal similar to that of the buffer (see Table 1), and the running buffer was a 1:1 mixture of 20 mM PBS and blank treated milk (centrifuged and diluted). By using this solution as running buffer, the remaining unspecific signal produced by the milk was corrected. Figure 4 shows the calibration in milk, and the features extracted from the four-parameter equation used to fit the curve are shown in Table 1. As can be observed, under these conditions the performance of the FQ *SPReeta* immunosensor is very good both in buffer and in milk. The LOD achieved is slightly higher than that accomplished in the assay buffer. Considering the sample dilution and the FQ recovery after centrifugation of the milk ($93 \pm 10\%$), the LOD value is 10.7 μg L^{-1} (or 10.7 μg kg^{-1}), still greatly below the MRL established for ENRO in this type of matrix (100 μg kg^{-1}). The criteria $AVG_{blank} - 3SD_{blank}$ provided equivalent values (data not shown). This LOD is within the range of those achieved using other immunochemical techniques such as ELISA (0.5−12.5 μg kg^{-1})[5,15,16] or chromatographic techniques such as HPLC (0.5−18 μg L^{-1})[9−12] to detect FQs in milk. Compared to other SPRs such as Biacore, a slightly better LOD has been reported (1.5 μg kg^{-1}) due to the higher resolution of that device.[3] However, our data suggest that for FQ antibiotic residue analysis, a portable and inexpensive system such as the one reported in this paper is sufficient. On the other hand, the sample treatment employed here (centrifugation and dilution) is much easier compared to the procedures employed prior to HPLC analysis[9−12] and some SPR measurements[39,44] where the treatment of the milk involved more steps.

Table 1. Features of the ENRO Calibration Curves[a]

	assay buffer	milk[b]
As171 final dilution	1/1000	1/500
S_{max}/RIUs	672 ± 78	671 ± 28
S_{min}/RIUs	104 ± 7	30 ± 20
slope	−1.0 ± 0.2	−1.0 ± 0.2
IC_{50}[c]	10.2 ± 2.7	26.4 ± 7.2
IC_{80} (LOQ)[c]	2.1 ± 0.9	5.4 ± 0.7
IC_{90} (LOD)[c]	1.0 ± 0.4	2.0 ± 0.2
R^2	0.981	0.986

[a] Results are extracted from the four-parameter logistic equation used to build the standard curves. Each concentration point of the standard curve was measured in triplicates (three channels) and on three different days. [b] Prior to the analysis, milk samples were centrifuged (20000*g*, 30 min) and diluted with water (five times). [c] Concentration values are expressed in μg L^{-1}. S_{max}, maximum signal; S_{min}, minimal signal; LOQ, limit of quantification; LOD, limit of detection.

The ratio of maximum and minimum signal (S_{max}/S_{min}) was about 22 in the case of calibration in milk (see Table 1), more than 3 times larger than in the case of calibration in buffer. This could be due to the blocking capacity of the matrix providing a lower minimum signal in the calibration curve.[54,55] Figure 5 shows the sensogram obtained on one of the channels after repetitive injections of milk samples containing decreasing concentrations of ENRO and, as expected, increasing signals were obtained. As can be observed, multiple measurements/regeneration cycles could be made without diminishing immunosensor performance even when complex matrices were analyzed.

To assess the capability of the FQ *SPReeta* immunosensor to discriminate between compliant and not compliant samples according to EC regulations, blank whole milk samples were spiked at different concentrations around the MRL value and later measured with the immunosensor. As can be observed in Figure 6a, milk samples containing ENRO around MRL values (2×MRL, 1.5×MRL, 0.75×MRL, and 0.5×MRL) produced an inhibition of the maximum signal (obtained at zero concentration) between 20 and 80%, which is very good because this wide interval of response ensures immunosensor reliability (linear range or working range). Thus, the linearity range has been estimated to be in the 30−250 μg kg^{-1} interval.

Finally, as a preliminary evaluation study, the FQ *SPReeta* sensor was used to analyze blind milk samples prepared at the Nestlé Research Center by spiking the samples with mixtures of antibiotics corresponding to some of the most important families used to treat cows, β-lactams (ampicillin and penicillin G), cephalosporins (cefazolin), fluoroquinolones (ciprofloxacin, enrofloxacin, and norfloxacin), and sulfonamides (sulfamethazine and sulfapyridine). As reported previously, the broad selectivity of the As171/FQ−BSA immunoreagents used in this study allows detection of least 10 different quinolones,[14,33,42] which supports the potential of this immunosensor for FQ residue analysis. In these experiments, the signal produced by the blind samples was compared to that of the controls (CTRL, right side bars). A signal below the CTRL-zero indicated the presence of FQs. Moreover, a signal equal to or below that of the CTRL-ENRO, CTRL-CIP, or CTRL-NOR, was an indication of a potentially noncompliant milk sample, which in a real case would require further confirmation with a well-established chromatographic method. On the other hand, if the signal was higher than

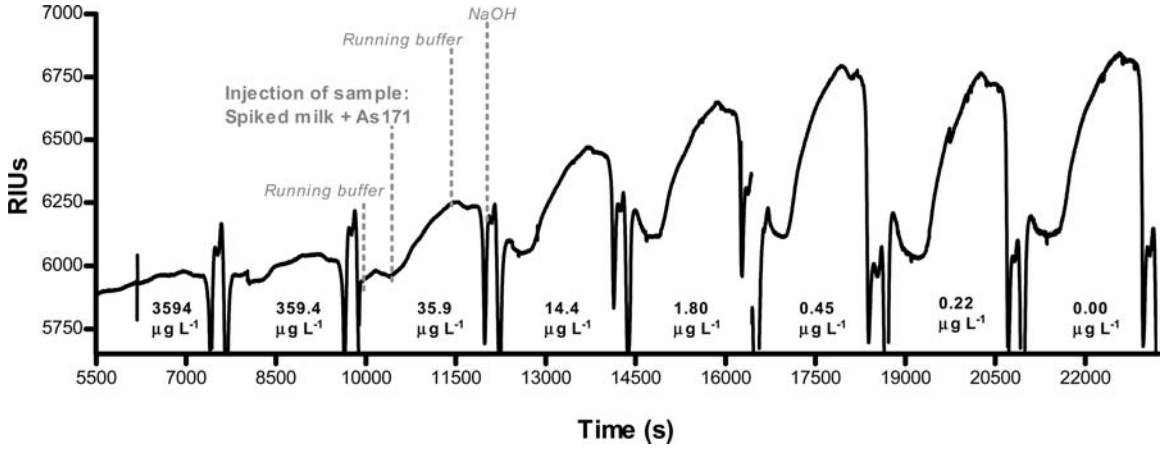

Figure 5. *SPReeta* sensogram used to build the calibration curve in milk. The sensogram is recorded on one of the sensor channels when subsequently injecting milk samples spiked with decreasing concentrations of enrofloxacin. The same sensograms were obtained when measurements were made in parallel on the three channels.

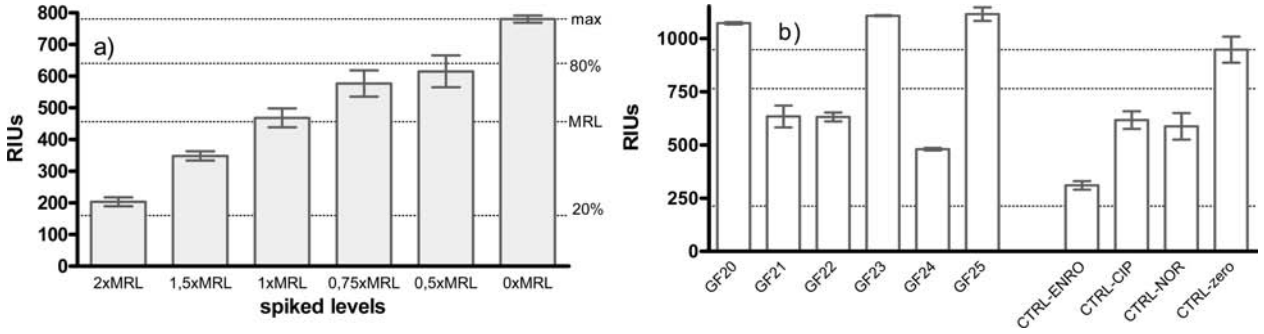

Figure 6. Results from the preliminary evaluation experiments to assess reliability of the SPR sensor to analyze FQ residues in milk samples: (a) response of milk samples spiked with ENRO around MRL; (b) response provided by blind samples compared with CTRL samples. Prior to the analysis, milk samples were centrifuged (20000*g*, 30 min) and diluted five times with water. Results correspond to the mean of the SPR response of $n = 3$ channels. The horizontal dotted lines represent the maximum SPR signal and the assay working range (80 and 20% of inhibition).

Table 2. Results from the Analysis of Blind Milk Samples with the FQ-SPR Immunosensor

		sample code					
		GF20	GF21	GF22	GF23	GF24	GF25
spiked values	FQ[b]	0	CIP (100)	NOR (100)	0	ENRO (100)	0
	other antibiotics[b]			SMZ (100) PENG (4)	SPY (100)	CEF (50)	AMP (4)
FQ *SPReeta* result		NEG	POS[a]	POS[a]	NEG	POS[a]	NEG

[a] The response was close to that given by the corresponding CTRL samples prepared in milk with CIP, ENRO, and NOR at 100 μg L^{-1} (see Figure 6b). [b] Antibiotic concentrations expressed in μg L^{-1}. AMP, ampicillin; CEF, cefazolin; CIP, ciprofloxacin; ENRO, enrofloxacin; NOR, norfloxacin; PENG, penicillin G; SMZ, sulfamethazine; SPY, sulfapyridine.

that of the controls, it would indicate FQ contamination, but below the MRL. Therefore, that sample would be considered as a compliant sample. The results of this study are shown in Figure 6b and are summarized in Table 2. As can be observed, in accordance with the antibiotics and spiked concentrations (see Table 2), samples GF20, GF25, and GF23 were predicted as compliant samples and samples GF21, GF22, and GF24 provided a signal around the MRL and therefore were classified as potentially noncompliant samples. Although different milk samples could provide distinct responses due to individual variability in the milk composition,[45,46] these results suggest that these differences are not so significant because the response of the controls was very similar to that of the blind samples. On the other hand, previous experiments performed by ELISA indicated that these differences are not so great as reported also for the immunochemical determination of sulfonamide antibiotics.[56]

The results of this preliminary study demonstrate the potential of the FQ *SPReeta* immunosensor developed as a screening tool and the fact that, despite the presence of other antibiotics, no false positives and no false negatives were obtained. Furthermore, these results agree with other studies in which these samples were also analyzed by ELISA[14] and by another optical biosensor.[42]

In summary, a portable SPR immunosensor with high potential as a screening tool has been developed to detect FQ residues in milk samples. The sensor showed a detectability that complies with EC requirements, because it is below the MRL values. Matrix interferences are minimized after a very simple sample treatment. Considering the broad specificity of the antibodies used in this study, the immunosensor shows great potential because three samples can be measured in <30 min. Moreover, the chip can be reused for several weeks with only a slight decrease in the maximum signal.

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ABBREVIATIONS USED

SPR, surface plasmon resonance; FQ, fluoroquinolone; m-SAM, mixed self-assembled monolayer; LOD, limit of detection; IC_{50}, 50% inhibition concentration; MRL, maximum residue level; ELISA, enzyme-linked immunosorbent assay; RIU, refractive index units; EU, European Union; LED, light-emitting diode; ENRO, enrofloxacin; CIP, ciprofloxacin; NOR, norfloxacin; FQ−BSA, FQ hapten−bovine serum albumin; PBS, phosphate-buffered saline; MUA, mercaptoundecanoic acid; OT, octanethiol; NHS, *N*-hydrosuccinimide; ECD, 1-ethyl 3-(3-dimethylaminopropyl)carbodiimide hydrochloride; HPLC-MS/MS, high-performance liquid chromatography−tandem masses spectrometry; CTRL, control; EC, European Commision; %CV, coefficient of variation; LOQ, limit of quantification; AVG, average; SD, standard deviation; AMP, ampicillin; CEF, cefazolin; PENG, penicillin G; SMZ, sulfamethazine; SPY, sulfapyridine.

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