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A label-free and portable multichannel surface plasmon resonance immunosensor for *on site* analysis of antibiotics in milk samples

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ABSTRACT

Techniques for immunosensing like surface plasmon resonance (SPR) may respond to the need for rapid screening methods to improve food safety. This paper describes the development of a novel portable six channel SPR biosensor based on the plasmon of gold diffraction grating surface for simultaneous multianalyte antibiotic detection in milk samples. Representative congeners from three important antibiotic families (FQs: fluoroquinolones, SAs: sulfonamides and CAP: phenicols) were chosen for this study. The chips are covalently biofunctionalized with haptenized proteins by means of a previously formed mixed self assembled monolayer (m-SAM) prepared using two types of mercapto alkyl reagents containing polyethyleneglycol (PEG) units. The samples or standards are mixed with specific polyclonal antibodies and injected into the sensor device. The detectability accomplished is very good (i.e. in buffer, enrofloxacin, 0.30 $\mu g\,L^{-1}$; sulfapyridine, 0.29 $\mu g\,L^{-1}$; and chloramphenicol, 0.26 $\mu g\,L^{-1}$) and whole milk samples can be analyzed directly without clean-up steps, by just diluting the sample five times with water to remove non-specific interferences caused by the matrix. Although the detectability of CAP regarding the MRPL (minimum required performance limit) is slightly compromised by the dilution, the detectability accomplished by FQs and SAs was far below the maximum residue levels (MRLs) established by the European Union.

1. Introduction

The use of pharmaceuticals on farm animals generates the risk of chemical poisoning or allergic reactions among others due to the transfer of these residues into the food chain. Furthermore, the overuse of antibiotics can promote the development of bacterial resistance, leading to a decrease in their efficiency of treating human or animal diseases (Ferguson et al., 2005). In the case of milk, besides health of consumers, the presence of antibiotics can also affect the fermentation process, making this matrix one of the most heavily regulated food (Neubert, 2006; Van Coillie, 2004). Fluoroquinolones (FQs), sulfonamides (SAs), β-lactames (βLs) and tetracyclines (TCs) are antibiotics whose use is allowed to prevent and treat animal diseases in veterinarian medicine. In order to protect public health, maximum residue levels (MRLs) have been established for most of these substances in several tissue samples. Thus, MRLs of 100 $\mu g\,kg^{-1}$ have been settled for the most important FQs and SAs congeners in milk samples (ECC/2377/90, 1990). In contrast, the use of chloramphenicol has been prohibited in food-producing animals because of its serious secondary effects and any residue confirmed in a sample is considered an infringement of the UE Legislation (2377/90, 1999). A minimum required performance limit (MRPL) of 0.3 $\mu g\,kg^{-1}$ in milk or meat (2003/181/EC, 2003) has been assigned for the analytical methods used for monitoring this substance. These requirements generate the need to develop reliable analytical methods like screening techniques with a broad spectrum detection range, sufficient detectability and high sample throughput (HTS) capabilities. Moreover, there is a demand for easy-to-use devices suitable for *on site* analysis in order to detect presence of antibiotic residues in milk samples in the farm, before loading them into the truck.

Sample treatments, including extraction and clean-up/preconcentration procedures, are often the bottle neck of residue laboratory analysis. The use of methods based on the use of selective receptors often allows avoiding some of these steps due to their selectivity to interact with the target analyte even

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in complex samples. This fact explains the extraordinary growth of the biochemical and immunochemical analytical methods on food safety, environmental monitoring and clinical laboratories (Adrian et al., 2010; Salvador et al., 2007). Additionally to this, there is the rapid advance of the knowledge and fabrication procedures of micro(nano)technologies and their application to the analytical sciences field. The combination of bioreceptors with this latest technological advances has given rise to a new generation of devices with excellent capabilities and enormous potential in detectability, selectivity, reliability, automation and HTS possibilities. Thus, biosensors are contemplated today as the future of the analytical laboratories in all fields, and particularly in the food sector (see recent reviews on this topic, Mi et al., 2009; Pandey et al., 2008; Sozer and Kokini, 2009; Yogeswaran and Chen, 2008). In this context, surface plasmon resonance (SPR) allows for qualitative and quantitative measurements of biomolecular interactions in real-time without requiring a labeling procedure. In the framework of food safety, SPR has been used for the detection of antibiotics in honey (Ashwin et al., 2005; Ferguson et al., 2005; Yuan et al., 2009), shrimps (Dumont et al., 2006), milk (Ashwin et al., 2005; Ferguson et al., 2005; Gaudin and Maris, 2001; Laurentie and Gaudin, 2009; Mellgren and Sternesjo, 1998; Sternesjo et al., 1995), porcine muscle (McGrath et al., 2005), eggs, fish, poultry meat (Ferguson et al., 2005; Huet et al., 2008), prawns (Ferguson et al., 2005), kidney (Ashwin et al., 2005) or broiler (Haasnoot et al., 2007), achieving good detectabilities. Specifically for milk samples, detectabilities reaching sub and low $\mu g\,L^{-1}$ range for CAP (Ashwin et al., 2005; Ferguson et al., 2005; Gaudin and Maris, 2001), fluoroquinolones (Mellgren and Sternesjo, 1998) and sulfonamides (Sternesjo et al., 1995), have been reported using laboratory equipment devices such as Biacore.

Nowadays, last SPR developments are towards the design of compact, low-cost, and sensitive biosensors, thanks to the advances in micro-fabrication technology that have made available integrable opto-electronic components (Dostálek and Homola, 2008; Homola, 2008; Piliarik et al., 2009; Shankaran et al., 2007). Recently, Piliarik et al. (2009) have reported a high performance SPR sensor based on a novel approach to spectroscopy of surface plasmons. This approach employs a special diffraction grating structure which simultaneously couples light into a surface plasmon and disperses the diffracted light for spectral readout of SPR signal (SPR Coupler and Disperser, SPRCD). Combined with a set of microfluidic channels parallel to the plane of diffraction, the sensor can be used for multiple measurements. This approach has allowed the construction of a compact and portable device with a six channel configuration suitable for field use and showing excellent features regarding sensitivity in respect of variations of the refractive index on the surface of the sensor. Combined with appropriate bioreceptors Adrian et al. (2009a) able to selectively recognize a wide range of antibiotics of the most important families used in the veterinary field, this device may have a great impact in the food safety field. In this context, the purpose of the work presented here has been to demonstrate the potential of this novel device as screening tool to detect the presence of antibiotic residues in milk samples.

2. Experimental

2.1. Chemicals and immunochemicals

The preparation of the haptenized proteins (10b-BSA, SA2-BSA) and the antibodies (As171 and As155) for fluoroquinolones and sulfonamides has been described before (Adrian et al., 2009a; Font et al., 2008; Pinacho et al., submitted for publication). For chloramphenicol, the antibodies (As-antiCAP, ref 61611) were produced at the Center of Rural Economy (CER, Marloie, Belgium, CER, 2009) and kindly given as gift from Dr. Benoit Granier (Unisensor S.A., Liege, Belgium) and the preparation of the coating antigen (CAP1–BSA) will be described elsewhere. The analytes used as standard were of high purity and were supplied by Biochemika Fluka (Buchs, Switzerland) or Sigma–Aldrich (St. Louis, MO). The alkylthiol reagents (HS-C11-$(EG)_4$-OH and HS-C11-$(EG)_6$-COOH) used for gold functionalization consist of polyethyleneglycol (PEG) units with hydroxyl (–OH) and carboxylic acid (–COOH) ending groups and were supplied by Prochimia (Gdansk, Poland). All the other chemicals and reagents were obtained from Sigma–Aldrich.

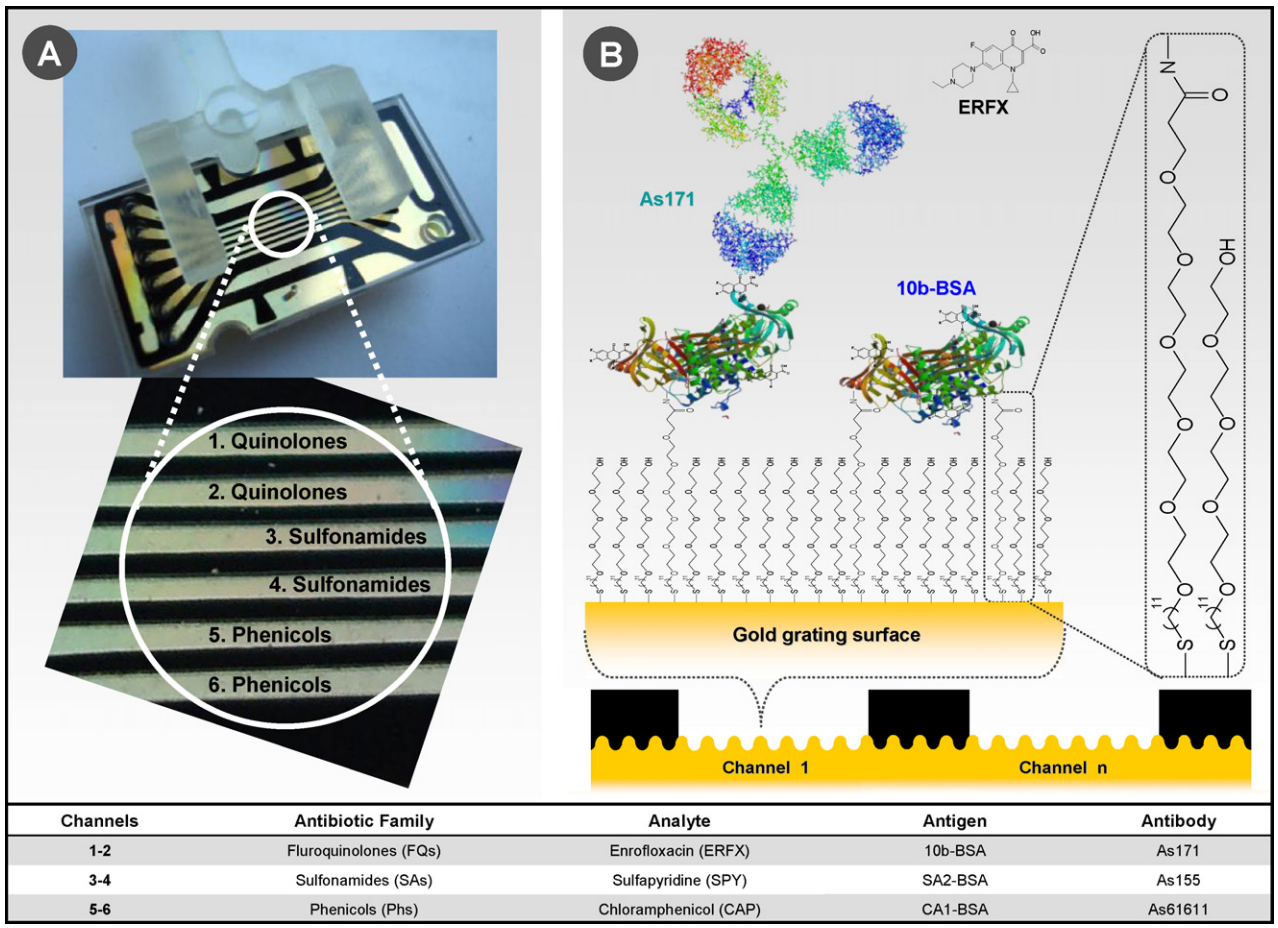
Buffers and solutions. Phosphate-buffered saline (PBS) is a 10 mM phosphate buffer in 0.8% saline solution, pH 7.4. PBSCa is PBS with 1 mM $CaCl_2$. SA buffer is 10 mM in sodium acetate, pH 5 and pH 4. CB buffer is 58.5 mM in citric acid and 115.5 mM in NaOH, pH 3. Aqueous solutions of NHS (N-hydrosuccinimide) 75 $mg\,mL^{-1}$, EDC (1-ethyl 3-(3-dimethylaminopropyl)-carbodiimide hydrochloride) 11.5 $mg\,mL^{-1}$, are from an amine coupling kit from Biacore (Uppsala, Sweden).

Standards. Stock solutions of each analyte were prepared at 10 mM and stored at 4 °C. Sulfapyridine (SPY) and chloramphenicol (CAP) were dissolved in DMSO (dimethyl sulfoxide) and enrofloxacin (ERFX) was dissolved in NaOH 50 mM aqueous solution.

Milk samples. Certified bovine milk samples, testing negative for the antibiotics under study, were obtained from the Spanish Agency of Food Security and Nutrition (AESAN, 2009). Prior measurement samples were diluted with milliQ water and filtered through PVDF (polyvinylidene fluoride) filter (0.5 μm).

2.2. SPRCD instrument

A compact SPR sensor system developed at the Institute of Photonics and Electronics, Prague (Czech Republic) was used in this work. This sensor is based on a novel approach to spectroscopy of surface plasmons (Telezhnikova and Homola, 2006) which utilizes special diffraction grating (referred to as surface plasmon coupler and disperser – SPRCD) for coupling light into the surface plasmon via the second order of diffraction and simultaneous dispersion of the light diffracted into the first diffraction order over a position-sensitive detector. The SPRCD sensor system consists of SPRCD reader and SPRCD cartridge. In the SPRCD reader a light emitting diode (central wavelength – 850 nm) is used as a source of light illuminating the SPRCD cartridge. The light diffracted by the SPRCD is imaged by means of a toroidal mirror on a CMOS detector (complementary metal oxide semiconductor). The integrated electronics acquires and preprocess images from the CMOS detector and transfers them to a computer. The device is connected to the computer through a USB port and does not need a source of external current supply. Six individual spectra corresponding to six sensing channels are extracted from each image and used to calculate the resonant wavelength (Piliarik et al., 2009). Acquisition and processing of data is managed by a special software (SPRCoDe) developed at the Institute of Photonics and Electronics. The SPRCD diffraction gratings were produced by fabrication of a master grating by holographic method and subsequent replication of the master to polycarbonate substrates (dimensions: 30 mm × 15 mm) by means of soft-lithography and hot-embossing techniques. The substrates with replicated gratings were cleaned using high-frequency plasma cleaner (Femto, Diener Electronics, Germany) and coated with 5 nm thick adhesion-promoting layer of titanium followed by 150 nm thick SPR-active gold layer by thermal evaporation in vacuum using Pfeiffer PLS 570 thin film deposition facility. The substrate with SPRCD was interfaced with a transparent cover slide via a gasket



Channels	Antibiotic Family	Analyte	Antigen	Antibody
1-2	Fluroquinolones (FQs)	Enrofloxacin (ERFX)	10b-BSA	As171
3-4	Sulfonamides (SAs)	Sulfapyridine (SPY)	SA2-BSA	As155
5-6	Phenicols (Phs)	Chloramphenicol (CAP)	CA1-BSA	As61611

Fig. 1. (a) Gold chip assembled with the flow cell. Channels 1 and 2 will be used for quinolones screening, channels 2 and 3 for sulfonamides screening and channels 5 and 6 for phenicols screening. (b) Scheme of the immunosensor format in the case of fluoroquinolones detection. The table shows the employed elements in the assays of the different channels.

made of 100 μm thick self-adhesive plastic foil. Six 300 μm wide flow channels defined by the gasket were formed on the sensor surface. Input and output ports were machined into the cover slide to interface with the sample delivery tubing in the SPRCD reader. The cartridge was kept together using a special clip to form an enclosed standalone and disposable unit (see Fig. 1a). The SPRCD sensor was connected to a sample management system consisting of a pair of four channel peristaltic pumps Reglo Digital from Ismatec (Switzerland). Peristaltic tubing (i.d. 0.25 mm) made up of tygon was used throughout the experiments.

2.3. Biofunctionalization of the SPRCD chip for multiple antibiotic residue analysis

Before functionalization, the gold SPRCD chip was cleaned by washing with absolute ethanol, drying with nitrogen stream and by cleaning in an UV ozone cleaner for 10 min to remove organic contaminants. Afterwards, the chip was washed with deionised water followed by absolute ethanol and dried with a stream of pure nitrogen. Subsequently, the chip was activated through the formation of a mixed self assembled monolayer (m-SAM) and biofunctionalized with the haptenized proteins according to the following protocol:

(1) *Activation of the gold chip*. The gold chips were incubated overnight in 80% EtOH solution containing in HS-C11-$(EG)_4$-OH (140 μM) and HS-C11-$(EG)_6$-COOH (60 μM). This m-SAM provides the necessary chemical functional groups for covalent immobilization of biomolecules while providing an appropriate biocompatible environment favoring biomolecular recognition reactions and reducing non-specific adsorptions.

(2) *Biofunctionalization of the chips*. Two channels were used for each antibiotic family (FQs: channels 1 and 2; SAs: channels 3 and 4; and CAP: channels 5 and 6; see Fig. 1a). The carboxylic groups of the m-SAM layer were activated by flowing (15 μL min^{-1}) through the six channels a 1:1 mixture (volume ratio) of aqueous solutions of NHS and ECD for 4.5 min. Solutions of the haptenized proteins (10b-BSA at 5 μg mL^{-1} in SA buffer at pH 5; SA2-BSA at 5 μg mL^{-1} in SA buffer at pH 4 and CAP1–BSA at 10 μg mL^{-1} in CB buffer at pH 3) were flowed (30 μL min^{-1}) through the different channels for 30 min. The bioconjugate concentrations and the pH of the solutions were adjusted to reach the same signal (0.10 and 0.15 nm) in all six channels. Finally, the remaining active groups were capped by passing a 1 M ethanolamine aqueous solution for 5 min at the same flow. Fig. 1b shows a scheme of the format of the SPR sensor.

2.4. SPR protocol for multiple antibiotic residue analysis

(1) *Binding step*. The samples were mixed 1:1 with the antibody solutions (FQS, As171, SAs, As155 and CA As61611 diluted 1/2000, 1/500 and 1/1000, respectively in PBSCa or in 20 mM PBS when measuring milk samples) and pre-incubated for 10 min in polypropylene tubes. Then, the mixtures were flowed

Table 1
Features of the calibration curves of the SPR assay.

	Enrofloxacin (ERFX)		Sulfapyridin (SPY)		Chloramphenicol (CAP)	
	PBSCa[a]	Milk 1/5[b]	PBSCa[a]	Milk 1/5[b]	PBSCa[a]	Milk 1/5[b]
S_{max} (nm)	0.150 ± 0.018	0.120 ± 0.023	0.129 ± 0.005	0.116 ± 0.015	0.117 ± 0.037	0.103 ± 0.027
S_{min} (nm)	0.012 ± 0.001	0.010 ± 0.004	0.019 ± 0.007	0.014 ± 0.004	0.021 ± 0.09	0.013 ± 0.09
Slope	−0.82 ± 0.02	−0.82 ± 0.02	−0.95 ± 0.06	−0.89 ± 0.25	−0.86 ± 0.16	−0.85 ± 0.10
IC50	3.21 ± 0.52	2.90 ± 0.51	2.55 ± 1.32	2.03 ± 0.41	3.64 ± 0.29	2.92 ± 0.69
($\mu g\,L^{-1}$)	3.15 ± 0.09[c]	2.55 ± 0.07[c]	2.93 ± 0.38[c]	1.63 ± 0.21[c]	3.18 ± 0.19[c]	4.05 ± 0.32[c]
LOD ($\mu g\,L^{-1}$	0.30 ± 0.09	0.34 ± 0.02	0.29 ± 0.10	0.43 ± 0.25	0.26 ± 0.03	0.22 ± 0.07
R^2	0.994 ± 0.000	0.996 ± 0.002	0.995 ± 0.005	0.995 ± 0.004	0.992 ± 0.006	0.996 ± 0.004

Data extracted (mean ± standard deviation) from the four-parameter logistic equation used to fit standard curves.
[a] $n = 2$ channels and $n' = 2$ days.
[b] $n = 3$ days.
[c] IC50 value in presence of high concentrations the other two antibiotics (300 nM, each).

($30\,\mu L\,min^{-1}$) for 15 min in duplicate through the corresponding channels (1–2, 3–4 and 5–6, see Fig. 1). When measuring milk samples, one of the two channels (2, 4 and 6) used for each family was used as reference passing only diluted milk (no antibody) to record the unspecific signal. (2) *Washing step*. A buffer solution (PBSCa for samples diluted in buffer or PBS for milk samples) was flowed ($30\,\mu L\,min^{-1}$) for 5 min through the six channels. (3) *Regeneration step*. A 40 mM HCl aqueous solution was passed ($30\,\mu L\,min^{-1}$) across the flow cell for 15 min. Before starting a new cycle, PBSCa was flowed ($30\,\mu L\,min^{-1}$) for 5 min. The change in λ produced by the binding of the Ab was determined by subtracting the initial background signal on each cycle to the signal remaining after the washing step. For milk samples, the signal recorded on the reference channels (2, 4 and 6, no antibody) was subtracted from the signal of the binding channels (1, 3 and 5, respectively). The data recorded from measuring the antibiotic calibration curves were used to build standard curves that were fitted to a four-parameters logistic equation ($Y = [(A - B)/(1 - (C/x)^D)] + B$, where A is the maximal signal, B is the minimum signal, C is the concentration producing 50% of the maximal signal, and D is the slope at the inflection point of the curve). The data for sigmoidal calibration curves were treated with a GraphPad Prism (GraphPad Sofware Inc., version 4.0, San Diego, CA).

2.5. SPRCD calibration for antibiotic determination

Standard calibration curves. Stock solutions were used to prepare the calibration points (8 different concentrations within the range of 720–0 $\mu g\,L^{-1}$ prepared using 1/5 serial dilutions in PBSCa or diluted milk).

Specificity studies. Calibration curves were also prepared for each analyte in the presence of high concentrations of the other antibiotics (100 $\mu g\,L^{-1}$ each) and the response compared to that obtained when the analyte was alone.

2.6. Evaluation of the SPR assay

Repeatability intrachannel and interchannel was investigated by running 5 cycles in duplicate (2 channels) and recording the signal produced by the binding of the antibodies at zero concentration of the analyte. The coefficient of variation, CV (%CV = 100 × X/SD, where X is the average value and SD is the standard deviation) was calculated for all data ($n = 10$). Reproducibility was investigated comparing the achieved signal at the same conditions in $n = 4$ days (Table 1).

The parameters of the calibration curve (A, B, slope, IC50 and R^2) and the limit of detection, LOD (concentration producing 90% of the maximum signal), were used to assess the variability of the antibiotic detection method. The data shown are the average and standard deviation of the parameters from standard curves run in 2 different days and 2 channels each day. For milk samples, the data shown is the result of calibration curves analyzed in 3 different days.

Table 2
Antibiotic content of spiked samples in whole bovine milk ($\mu g\,L^{-1}$).

	Enrofloxacin (ERFX)	Sulfapyridine (SPY)	Chloramphenicol (CAP)
Sample 1	100 (1 × MRL)	0	0
Sample 2	0	100 (1 × MRL)	0
Sample 3	0	0	5 (17 × MRL)
Sample 4	50 (0.5 × MRL)	160 (1.6 × MRL)	13 (43 × MRL)
Control max	0	0	0

MRL: maximum residue level, MPRL: minimum performance residue limit.

2.7. Analysis of milk samples

Matrix effect studies. Non-specific matrix effects were evaluated by preparing different dilutions of milk samples using milliQ water and recording the change in λ produced by the zero concentration in comparison to that provided in PBSCa buffer in absence of the analyte.

Blind milk samples measurements. Whole milk samples were spiked with at ERFX, SPY and CAP at different levels (see Table 2). Prior the analysis the samples were diluted at least 5 times with milliQ water and the measurements made as described above. For FQs and SAS, further dilutions (20 times) were usually necessary in order to place the concentration values within the dynamic range of the sensor. The signal of the samples was compared with that provided by controls prepared in buffer or milk at zero concentration of analytes.

3. Results and discussion

3.1. SPR assay features

In order to characterize the precision of the SPRCD sensor (see immunosensor scheme in Fig. 1), the variation of the signal produced upon binding of the specific antibodies (in absence of the analyte) to the sensor surface was investigated to know repeatability (intra-day variation) and reproducibility (inter-day variation) behavior. The repeatability on the consecutive cycles was found to be very good with coefficients of variation (%CV) below 5% for the three antibiotic families (FQs, 3.2%; SAs, 4.1%; and CAP, 4.4%; $n = 5$ cycles, 2 channels) demonstrating that regeneration of the chip surface did not affect consecutive cycles. Similarly, an excellent reproducibility was observed between measurements recorded in different days (FQs, 7.1%; SAs, 6.2%; and CAP, 12.4%; $n = 4$ days), demonstrating the robustness and the low variability of the sensor.

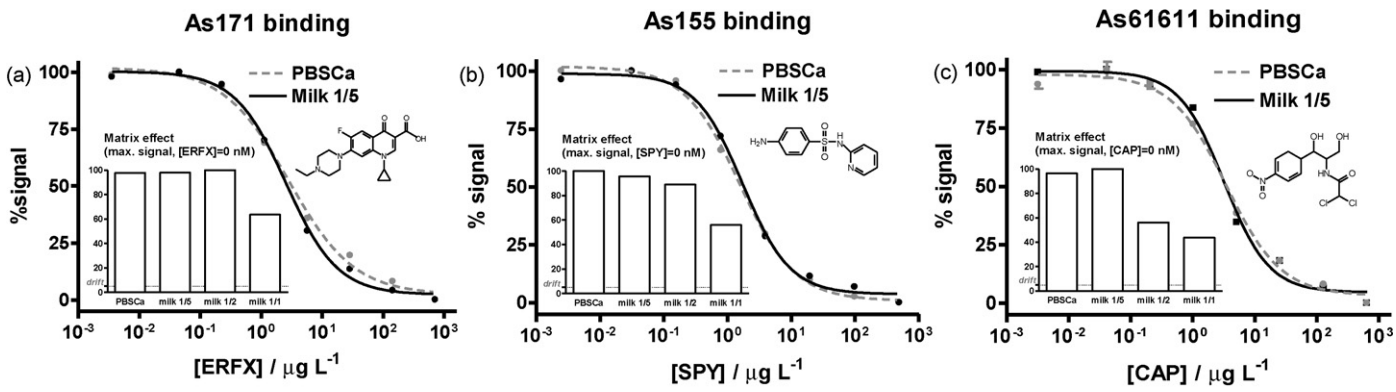


Fig. 2. Calibration curves obtained for each analyte in buffer (dotted line) and in milk 1/5 (black line). (a) Detection of ERFX, channels 1–2. (b) Detection of SPY, channels 3–4. (c) Detection of CAP, channels 5–6. In the case of buffer, the calibration points correspond to a mean value ($n = 2$ channels) of change in λ (nm) after the As binding. In the case of milk, the calibration points correspond to the λ change (nm) in one channel after the As binding and after the correction with the reference channel (no As). The inserted bar graphs indicate the matrix effect in maximal signal with different dilutions of milk. The parameters of calibration curves are reflected in Table 1.

As expected, the presence of the analyte in the sample produced a decrease of the sensor signal due to the competitive assay conditions, inhibiting the binding of the antibody to the surface on a proportional manner as a function of the analyte concentration. Calibration of the biosensor was made for the three analytes under study: ERFX, SPY and CAP (see calibration curves in Fig. 2, dotted line). The parameters of the calibration curves run in buffer are summarized in Table 1 and correspond to the average and standard deviation of experiments performed on 2 different days. The LODs (concentration producing 90% of the maximum signal) accomplished were much far below of the required values according to the EC legislation for the case of the FQs and SAs (0.30 $\mu g\,L^{-1}$ for ERFX and 0.29 $\mu g\,L^{-1}$ for SPY) and just at the MPRL for CAP (0.26 $\mu g\,L^{-1}$). The slopes were around −1 and the regression coefficient was in all cases very good ($R^2 \geq 0.992$). Moreover, when the calibration curves were prepared in presence of high concentrations of the other two antibiotics (100 $\mu g\,L^{-1}$ each), no significant variations were observed. Thus, the parameters of the fitting equation were very similar (see IC50 value in Table 1) demonstrating the specificity of the immunological reaction and indicating that quantification of the target was not affected by the presence of other analytes in the same sample.

3.2. Evaluation of SPRCD sensor for antibiotic determination in milk samples

Matrix effects studies showed that whole milk samples could be directly measured without previous clean-up steps. Calibration could be performed in buffer if desired by just diluting the milk samples 5 times in PBS (see Fig. 2 and Table 1). Under these conditions the calibration curves prepared in buffer or in milk were identical. Fig. 3 shows the sensograms obtained for the calibration curve prepared in milk (channels 1, 3 and 5) in comparison to the signal recorded in the reference channels (2, 4 and 6) through where only milk was passed.

Under these conditions (milk diluted 5 times) the LOD values accomplished were 1.7 $\mu g\,L^{-1}$ (1.7 $\mu g\,kg^{-1}$ of milk) for ERFX, 2.1 $\mu g\,L^{-1}$ (2.1 $\mu g\,kg^{-1}$ of milk) for SPY, 50 times lower than MRLs (100 $\mu g\,kg^{-1}$). However, the LOD accomplished for CAP was 1.1 $\mu g\,L^{-1}$ (1.1 $\mu g\,kg^{-1}$of milk), slightly superior to the MPRL (0.3 $\mu g\,kg^{-1}$). Using lower dilution factors would improve the detectability of the biosensor for CAP. However, although no significant unspecific interferences were recorded in the FQs and SAs channels and milk samples could be directly measured after just two times dilution with buffer, the matrix effect in the CAP channel was greater (see insert bar-graphs in Fig. 2). Thus, in order to reach the necessary detectability for CPA, measurements would have to be done without any dilution. In fact, as mentioned above good calibration curves are obtained when directly measuring milk samples without any dilution, however, for CAP the signal decreased about 40–60% (see insert graphs in Fig. 2) which prevented from using the buffer calibration. Thus, to reach the necessary detectability for CAP, calibration of the SPRCD biosensor has to be performed with standards diluted in reference whole milk. In these conditions, the maximum signal recorded is around 0.06 nm, still at least 10 times bigger than the variation of the background (noise, drift) in the same time of acquisition.

These results are in agreement with previous studies in which most of these immunoreagents (antibodies and haptenized proteins) were also employed to analyze milk samples by ELISA or evanescent waveguide optical biosensors (Adrian et al., 2008, 2009a,b,c; Font et al., 2008; Pinacho et al., submitted for publication). In these works, excellent detection limits were reached in addition of demonstrated that the broad recognition pattern of the immunoreagents used. Thus, since the selectivity of these immunoreagents is addressed to recognize a high number of sulfonamide and fluoroquinolones congeners (Adrian et al., 2008, 2009a), the combination of the immunoreagents in the present SPRCD biosensor could allow detecting up to 20 antibiotics below their MRLs.

3.3. Analysis of blind milk samples

As preliminary experiments to assess performance of the SPRCD biosensor, blind milk samples spiked with antibiotics around their MRL values were measured using the signal of the control zero and the MRL as reference. The SPR signal was completely inhibited under these conditions for the samples that contained FQs and SAs. In order to quantify the samples it was necessary to place them within the working range of the biosensor, for which reason it was necessary to dilute them about 20 times. Fig. 4 shows a graph bar how the samples that contained antibiotics below, above or at the MRL could be detected. The decrease in the signal was an indication of the presence of the antibiotic. Thus, sample 1 was predicted to be positive for FQs and negative for SAs and CAP. Sample 2 did only show inhibition in channel 3, indicating that it was contaminated by SAs. Sample 3 underwent inhibition in channel 5, indicating the presence of CAP residues. Finally, sample 4 produced a decrease in the signal of channels 1, 3 and 5, pointing to a contamination with the three antibiotic families. The concentrations of ERFX, SPY and CAP in whole milk are summarized in Table 2. As mentioned above, using these

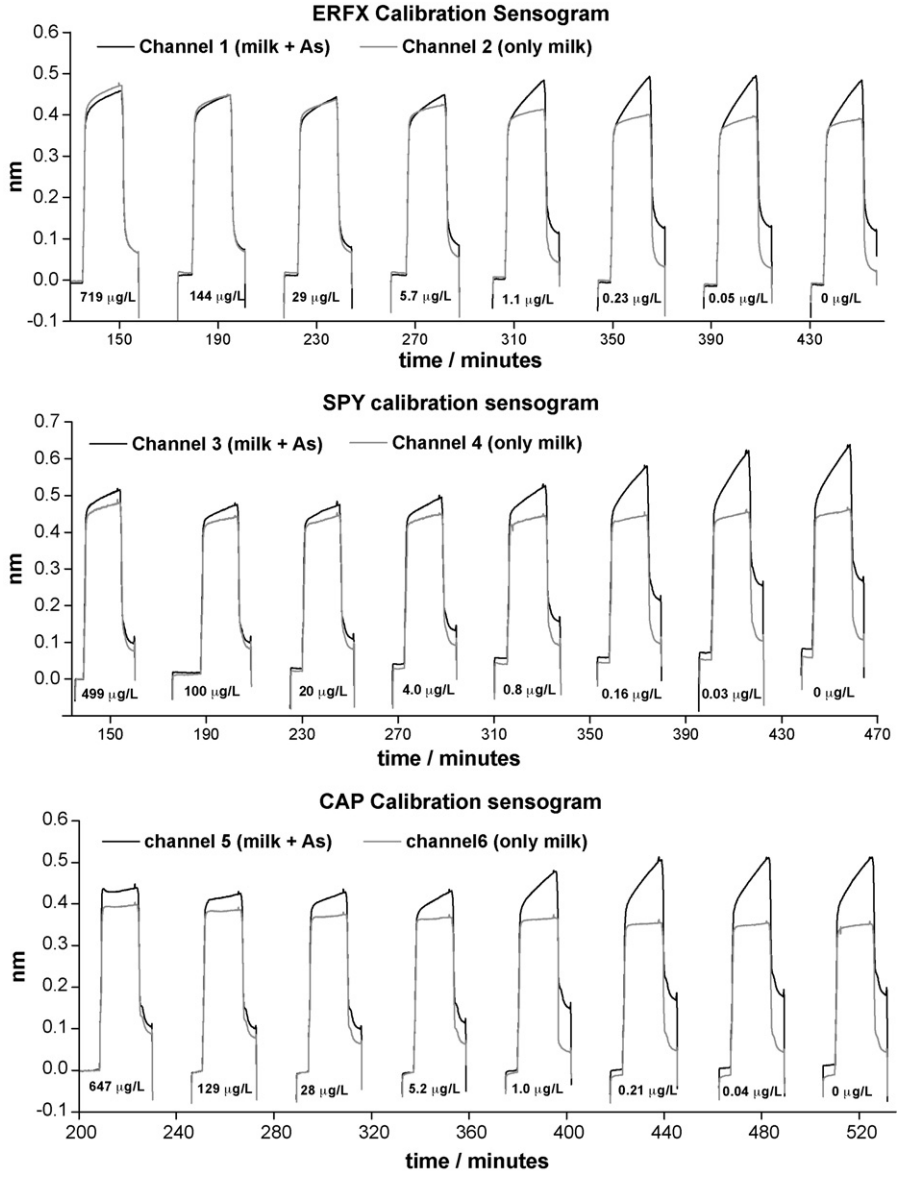


Fig. 3. Sensograms obtained when measuring different concentrations of analytes in milk. Each sensogram shows the response of the reference (2, 4, 6) and the measuring channels (1, 3, 5). The concentrations of ERFX (A), SPY (B) and CAP (C) decreased in each consecutive cycle.

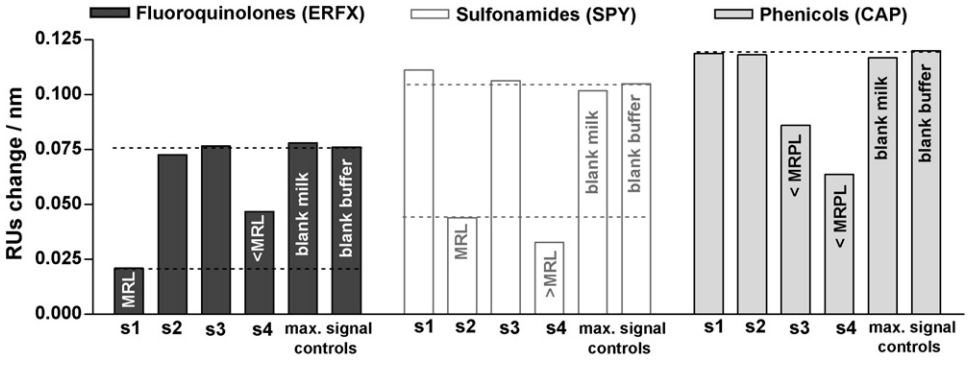


Fig. 4. Processed change (nm) for milk blind samples (s1: sample 1, s2: sample 2, s3: sample 3, s4: sample 4). The raw signal of antibody in milk (channels 1, 3, 5) was corrected with the signal of the reference channels (2, 4, 6). The two last bars indicate in each case the maximum signal (in milk and buffer) and are employed as a control sample. Corresponding concentrations of antibiotics are in Table 2.

dilution factors, standards can be prepared in buffer since both, the controls prepared in milk and in buffer (blanks) generated the same signal due to the removal of the matrix interferences by dilution. However, in order to increase detectability for CAP it is advisable to use reference milk samples to prepare standards and to inject the samples undiluted.

3.4. Comparison with other SPR systems

In comparison to other SPR immunosensors for detection of these antibiotics in milk, the immunosensor presented here shows several advantages like multiplexicity and portability while keeping the detectability of the high performance SPR laboratory systems. Moreover, there is no need of sample treatment or clean-up steps since matrix interferences can be removed by simple dilution. Other reported works in which antibiotic residue detection in milk samples was addressed using other SPR devices and immunoreagents, often need to pre-treat samples to eliminate the milk fat, using physical procedures (i.e. centrifugation) (Ferguson et al., 2005; Laurentie and Gaudin, 2009; Mellgren and Sternesjo, 1998; Sternesjo et al., 1995), or even to perform extraction/purification and preconcentration of the antibiotics prior injecting the milk sample into the sensor (Ashwin et al., 2005). In this way, it has been possible to detect CAP at lower concentration levels (<0.1 $\mu g\,kg^{-1}$) using Biacore Q system, at expenses of increasing analysis time and complexity of the whole procedure which reduces the possibilities to implement these procedures for *on site* analysis (Ashwin et al., 2005). The SPRCD sensor presented here has proven to reach detectability levels similar to those accomplished by commercial SPR devices such as Biacore for antibiotic residue analysis in milk. LODs of 1.5 $\mu g\,kg^{-1}$ for ERFX, 0.4 $\mu g\,kg^{-1}$ and close to 50 $\mu g\,kg^{-1}$ for sulfamethazine (SA antibiotic) have been reported using Biacore (Mellgren and Sternesjo, 1998; Sternesjo et al., 1995), and Biacore X instruments (Laurentie and Gaudin, 2009), respectively after the matrix fat removal. Higher detectability has been accomplished when using Biacore Q in combination with Qflex® kits. Thus, a 0.04 $\mu g\,kg^{-1}$ decision limit has been achieved for the analysis of CAP (Ferguson et al., 2005).

Although the use of different SPR configurations for multianalyte measurements has been described (Chinowsky et al., 2007; Dostálek et al., 2007; Homola et al., 2005; Kawazumi et al., 2005; Kim et al., 2007; Mauriz et al., 2007; Trevino et al., 2009; Yang and Kang, 2008), to our knowledge, this is the first time that a portable multiplexed SPR immunosensor has been reported for multiple antibiotic residue detection in milk samples. An SPR imaging has been reported to detect several antibiotic families have been detected. The system allows detection of most of them below the MRL (except phenicols) by just diluting the sample (Raz et al., 2009), however, the detectability accomplished is lower than that reported in this paper, and the device is not suitable for *on site* analysis of milk samples in farms before loading them into the truck.

4. Conclusions

An optical label-free multianalyte immunosensor has been developed for *on site* analysis of antibiotic residues from three different families in whole milk samples simultaneously. The transducer is based in the well-known SPR principle, although in this case a special diffraction grating (SPRCD, surface plasmon coupler and disperser) allows simultaneous excitation of the surface plasmons and dispersion of the diffracted light resulting in position-sensitive first order wavelength spectra, collected on a CMOS detector, which allows developing multiplexed biosensor devices. The device is a portable, small size and six channel device connected to the computer through a USB port and does not need an external power supply.

Fluoroquinolones, sulfonamides and phenicols, are detected in buffer with LODs of 0.30 $\mu g\,L^{-1}$ for ERFX, 0.29 $\mu g\,L^{-1}$ for SPY and 0.26 $\mu g\,L^{-1}$ for CAP, far below the MRLs and at the MPRL for the last one. Milk samples can be analyzed directly by applying a 1/5 dilution in water and identical calibration curves were built. Moreover, using this dilution, the matrix effect is removed and the same response is accomplished. The whole analytical procedure takes about 30 min for each sample. The chip can be totally regenerated and shows good repeatability (CV% = 3–4%) and reproducibility (CV% = 6–12%) on the binding process of different cycles.

The immunosensor is suitable for field use and due to the different channels which allows their use in different configurations such as increasing the number of replicates of the same sample, allowing measuring 6 samples for the same analyte, or as in this case three analytes in duplicates. Moreover, correction of the undesired non-specific signals caused by the matrix is possible, using a reference channel.

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References

2003/181/EC, C.D., 2003. Official Journal of the European Union L71/17.
2377/90, R.n., 1999. Official Journal of the European Communities.
Adrian, J., Fernández, F., Muriano, A., Obregón, R., Ramón, J., Tort, N., Marco, M.-P., 2010. Immunochemical Analytical Methods for Monitoring the Aquatic Environment. In: Namieśni, J., Szefer, P. (Eds.), Analytical Measurements in Aquatic Environments. CRC Press/Taylor & Francis Group Ltd, pp. 139–187.
Adrian, J., Font, H., Diserens, J.M., Sanchez-Baeza, F., Marco, M.P., 2009a. Journal of Agricultural and Food Chemistry 57 (2), 385–394.
Adrian, J., Pasche, S., Diserens, J.-M., Sánchez-Baeza, F., Gao, H., Marco, M.P., Voirin, G., 2009b. Biosensors and Bioelectronics 24 (11), 3340–3346.
Adrian, J., Pasche, S., Pinacho, D.G., Font, H., Diserens, J.M., Sanchez-Baeza, F., Granier, B., Voirin, G., Marco, M.P., 2009c. Trends in Analytical Chemistry 28 (6), 769–777.
Adrian, J., Pinacho, D.G., Granier, B., Diserens, J.M., Sanchez-Baeza, F., Marco, M.P., 2008. Analytical and Bioanalytical Chemistry 391 (5), 1703–1712.
AESAN, 2009. http://www.aesan.msc.es/.
Ashwin, H.M., Stead, S.L., Taylor, J.C., Startin, J.R., Richmond, S.F., Homer, V., Bigwood, T., Sharman, M., 2005. Analytica Chimica Acta 529 (1–2), 103–108.
CER, 2009. http://www.cergroupe.be/.
Chinowsky, T.M., Soelberg, S.D., Baker, P., Swanson, N.R., Kauffman, P., Mactutis, A., Grow, M.S., Atmar, R., Yee, S.S., Furlong, C.E., 2007. Biosensors and Bioelectronics 22 (9–10), 2268–2275.
Dostálek, J., Homola, J., 2008. Sensors and Actuators B: Chemical 129 (1), 303–310.
Dostálek, J., Přibyl, J., Homola, J., Skládal, P., 2007. Analytical and Bioanalytical Chemistry 389, 1841–1847.
Dumont, V., Huet, A.C., Traynor, I., Elliott, C., Delahaut, P., 2006. Analytica Chimica Acta 567 (2), 179–183.
ECC/2377/90, 1990. ECC/2377/90. Official Journal of the European Union L224, 221–228.
Ferguson, J., Baxter, A., Young, P., Kennedy, G., Elliott, C., Weigel, S., Gatermann, R., Ashwind, H., Stead, S., Sharman, M., 2005. Analytica Chimica Acta 529 (1–2), 109–113.
Font, H., Adrian, J., Galve, R., Esevez, M.C., Castellari, M., Gratacos-Cubarsi, M., Sanchez-Baeza, F., Marcot, M.P., 2008. Journal of Agricultural and Food Chemistry 56 (3), 736–743.

Gaudin, V., Maris, P., 2001. Food and Agricultural Immunology 13 (2), 77–86.
Haasnoot, W., Gerçek, H., Cazemier, G., Nielen, M.W.F., 2007. Analytica Chimica Acta 586 (1–2), 312–318.
Homola, J., 2008. Chemical Reviews 108 (2), 462–493.
Homola, J., Vaisocherová, H., Dostálek, J., Piliarik, M., 2005. Methods 37 (1), 26–36.
Huet, A.C., Charlier, C., Singh, G., Godefroy, S.B., Leivo, J., Vehniäinen, M., Nielen, M.W.F., Weigel, S., Delahaut, P., 2008. Analytica Chimica Acta 623 (2), 195–203.
Kawazumi, H., Gobi, K.V., Ogino, K., Maeda, H., Miura, N., 2005. Sensors and Actuators B: Chemical 108 (1–2), 791–796.
Kim, S.J., Gobi, K.V., Iwasaka, H., Tanaka, H., Miura, N., 2007. Biosensors and Bioelectronics 23 (5), 701–707.
Laurentie, M., Gaudin, V., 2009. Journal of Chromatography B 877 (23), 2375–2379.
Mauriz, E., Calle, A., Manclus, J.J., Montoya, A., Lechuga, L.M., 2007. Analytical and Bioanalytical Chemistry 387 (4), 1449–1458.
McGrath, T., Baxter, A., Ferguson, J., Haughey, S., Bjurling, P., 2005. Analytica Chimica Acta 529 (1–2), 123–127.
Mellgren, C., Sternesjo, A., 1998. Journal of AOAC International 81 (2), 394–397.
Mi, L.J., Zhang, X., Yang, W.C., Wang, L.H., Huang, Q., Fan, C.H., Hu, J., 2009. Journal of Nanoscience and Nanotechnology 9 (4), 2247–2255.
Neubert, H.J., 2006. Analytical Chemistry 78 (23), 7908–17908.
Pandey, P., Datta, M., Malhotra, B.D., 2008. Analytical Letters 41 (2), 159–209.
Piliarik, M., Vala, M., Tichy, I., Homola, J., 2009. Biosensors and Bioelectronics 24 (12), 3430–3435.
Pinacho, D.G., Sánchez-Baeza, F., Marco, M.-P., submitted for publication, 2010. Analytical Chemistry.
Raz, S.R., Bremer, M., Haasnoot, W., Norde, W., 2009. Analytical Chemistry 81 (18), 7743–7749.
Salvador, J.P., Adrian, J., Galve, R., Pinacho, D.G., Kreuzer, M., Sanchez-Baeza, F., Marco, M.P., 2007. Comprehensive Analytical Chemistry, 279–334.
Shankaran, D.R., Gobi, K.V., Miura, N., 2007. Sensors and Actuators B: Chemical 121 (1), 158–177.
Sozer, N., Kokini, J.L., 2009. Trends in Biotechnology 27 (2), 82–89.
Sternesjo, A., Mellgren, C., Bjorck, L., 1995. Analytical Biochemistry 226 (1), 175–181.
Telezhnikova, O., Homola, J., 2006. Optics Letters 31 (22), 3339–3341.
Trevino, J., Calle, A., Rodriguez-Frade, J.M., Mellado, M., Lechuga, L.M., 2009. Analytica Chimica Acta 647 (2), 202–209.
Van Coillie, E.F., 2004. Journal of Agricultural and Food Chemistry 52 (16), 4975–4978.
Yang, G., Kang, S.W., 2008. Food Science and Biotechnology 17 (3), 547–552.
Yogeswaran, U., Chen, S.M., 2008. Sensors 8, 290–313.
Yuan, J., Addo, J., Aguilar, M.-I., Wu, Y., 2009. Analytical Biochemistry 390 (1), 97–99.