Lab-on-a-chip for multiplexed biosensing of residual antibiotics in milk†

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A multiplexed immunoassay-based antibiotic sensing device integrated in a lab-on-a-chip format is described. The approach is multidisciplinary and involves the convergent development of a multi-antibiotic competitive immunoassay based on sensitive wavelength interrogated optical sensor (WIOS) technology and a polymer-based self-contained microfluidic cartridge. Immunoassay solutions are pressure-driven through external and concerted actuation of a single syringe pump and multiposition valve. Moreover, the use of a novel photosensitive material in a 'one step' fabrication process allowed the rapid fabrication of microfluidic components and interconnection port simultaneously. Pre-filled microfluidic cartridges were used as binary response rapid tests for the simultaneous detection of three antibiotic families – sulfonamides, fluoroquinolones and tetracyclines – in raw milk. For test interpretation, any signal lower than the threshold value obtained for the corresponding Maximum Residue Limit (MRL) concentration (100 μg L^{-1}) was considered negative for a given antibiotic. The reliability of the multiplexed detection system was assessed by way of a validation test carried out on a series of six blind milk samples. A test accuracy of 95% was calculated from this experiment. The whole immunoassay procedure is fast (less than 10 minutes) and easy to handle (automated actuation).

1. Introduction

The concern for 'food safety' surely emerged very early in human history, contributing to the settlement of certain rules and customs. Nowadays in technologically developed countries, food safety is subject to strict legislations that regulate the presence of undesired substances in food. Focusing on the milk industry, levels of residues of veterinary medicinal products, of which antibiotics represent a significant part, are regulated by European Council (EC) Regulation no. 2377/90. More precisely, a series of four antibiotic families are found to be of particular interest due to their routine use for treatment in bacterial infection and/or prophylactic purposes: fluoroquinolones, sulfonamides, β-lactams and tetracyclines. The Maximum Residue Limits (MRLs) determined by the EC concerning those antibiotics are 100 μg L^{-1} for fluoroquinolones, sulfonamides, tetracyclines and 4 μg L^{-1} for β-lactams. Their excessive use in dairy cow diet for the last decades gave rise to stronger bacterial resistance which consequently represents a serious problem in the efficiency of classic anti-bacterial treatment in humans.[1]

To face this situation, numerous antibiotics detection protocols have been developed based on conventional chromatographic techniques such as high performance liquid chromatography coupled with UV spectroscopy (HPLC-UV)[2] or mass spectrometry (HPLC-MS).[3] Despite their efficiency and high screening capability, those techniques usually require sample preparation and have to be run by skilled technicians in a laboratory environment. Further standard test methods used for antibiotic detection in milk at pasteurizing plants are based on microbial inhibition assay. Delvotest® is one of those tests commercially available that offer broad spectrum screening covering all major groups of antibiotic families.[4] Similarly, immunoassay tests for the detection of multiple antibiotics have been developed based on enzyme-linked immunosorbent assay (ELISA)[5] and parallel affinity sensor array (PASA).[6] In spite of their reliability and high screening capability, those tests have to be run in a laboratory environment and require at least a few hours for the assay time.

In this context, one of the major challenges of the dairy industry for detecting antibiotic residues in milk is the ability to integrate the quality control analysis in the milk intake process at the field or farm. In response to the milk industry demand for rapid and cheap detection protocols, a few tests based on lateral flow immunochromatography recently appeared on the market. The key advantage of those strip tests, also known as 'dipsticks', is the easy-to-use protocol where the sample mixed with immuno-reagents (labeled with colloidal gold or carbon) is absorption-driven by lateral flow through a nitrocellulose strip. Typically, those binary response tests are fast (less than 10 min), easy to perform, cheap, sensitive and specific. A few years ago, a rapid test in a dipstick format was commercialized for the simultaneous detection of two families of antibiotics (β-lactams and tetracyclines).[7] However, as far as our knowledge goes, no

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† Electronic supplementary information (ESI) available: Experimental details, microfluidic cartridge, HBP gasket and whole multianalyte detection system images, and schematic of assay protocol. See DOI: 10.1039/b819688e

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rapid detection system has yet been described that is able to detect in a single operation more than two antibiotics. In this context, the present work proposes a fully integrated and automated multi-antibiotic detection system based on the wavelength interrogated optical sensor (WIOS) technology[8] and that is able to identify the presence in raw milk of sulfonamides, fluoroquinolones, and tetracyclines simultaneously. The whole immunoassay protocol is carried out automatically in the microfluidic cartridge which is operated by external pump and valve concerted actuation. Reservoirs for reagent solutions and sensor chip for WIOS detection are integrated in the microfluidic cartridge. Unlike conventional techniques such as HPLC, microbial inhibition assay, ELISA and PASA, the present system offers a fast, low-cost and easy-to-use test. Moreover, in contrast to the dipstick format assays, the developed system performs the detection of at least three antibiotics in a single test.

2. Experimental

2.1. Optical transduction principle

The optical detection is based on the recently developed wavelength interrogated optical sensing (WIOS) approach, the principle and instrument details of which have been carefully reported in previous work.[8]

The WIOS sensing principle is based on using a collimated light beam from a vertical cavity surface emitting laser (VCSEL) as wavelength-tunable source (763 nm ± 2 nm tuning range) for exciting waveguide modes in a dielectric waveguide by means of an input grating coupler (Fig. 1(1)). If the resonance condition is fulfilled, light is transmitted *via* an output grating coupler to a photodetector (Fig. 1(2)). Due to the continuous wavelength scanning, the output signal of the photodetector represents the grating coupler resonance peak whose position indicates the quantity (surface mass density) of analyte molecules adsorbed on the sensor chip's surface. The sensing layer can be composed of recognition molecules like binding proteins, antibodies, DNA strands or chemical receptors (Fig. 1(3)).The resonant coupling occurring at the first grating is governed by the grating equation:

$$\lambda_r(t) = \Lambda(n_e(t) - \sin\theta) \quad (1)$$

where λ_r is the resonance wavelength for which coupling occurs, Λ the grating period, θ the incident angle, and $n_e(t)$ the effective index of the waveguide mode.

For a given optical configuration (θ, Λ fixed), monitoring λ_r will give access to effective refractive index variations of the waveguide mode due to the molecular binding occurring at the waveguide–bulk interface, as shown in Fig. 1(3).

The grating waveguides have a period of 360 nm and a depth of 12 nm. The input and output grating coupler have a thickness of 150 nm and 300 nm, respectively. They are fabricated by a dry etching technique on the AF45 glass substrate[8] and then coated with a high refractive index layer (Ta_2O_5 dielectric film). Each

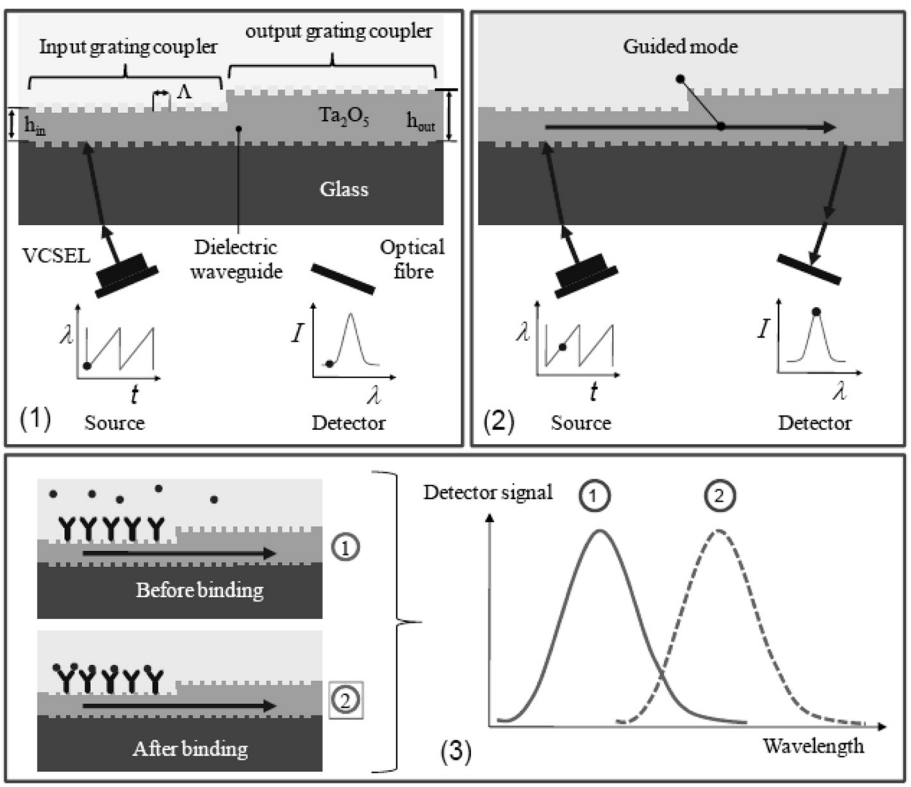

Fig. 1 Sensing principles of wavelength interrogated optical sensor (WIOS) technology based on grating waveguide resonant coupling. Characteristics of the WIOS system used are given by the following parameters: dielectric film thickness: h_{in} (150 nm), h_{out} (300 nm); grating period: Λ (360 nm), grating groove depth 12 nm. At resonance wavelength a fraction of the incident light is transmitted into the waveguide and coupled out through the output pad. Output light is collected by plastic optical fibres and analyzed by a photodiode detector. Biofunctionalization of the sensing layer converts the WIOS into a biosensing device where the shift in the resonance wavelength reflects the quantity of bio-analyte bound.

sensing chip (17.5 mm × 17.5 mm) is constituted of eight individual sensing regions (grating pads) allowing simultaneous and real-time monitoring.

2.2. Immunoassay formats

Individual and multiplexed competitive immunoassay formats have been developed using the ELISA approach[5] and then implemented on WIOS technology[9] in previous work. The three assays were performed on indirect formats where specific haptens for sulfonamides and fluoroquinolones as well as neutravidin were coated onto different sensing regions (gratings) of the chip. The chip biofunctionalization was carried out using a polysaccharide-based photolinker polymer (OptoDex™, Arrayon Biotechnology) thin-film coating followed by a nanoplotting technique. The whole immunoassay sequence is composed of three consecutive steps: milk sample introduction; washing step and signal amplification using secondary antibody (anti-mouse and anti-rabbit). Prior to introduction into the fluidic system the milk to be analyzed was mixed (1 : 5 dilution) with a 'cocktail' solution containing the different antiserum receptors that specifically react with their corresponding antibiotics, if any are present in the sample. In contact with the sensing surface, the excess of antibodies (not bound to antibiotics) attached to the hapten coated on a specific sensing region. After a washing step the attached antibodies were revealed by reacting with a secondary antibody. Typically, as for any competitive assay, the higher the concentration of analyte (a given antibiotic), the lower the signal obtained, reflecting a low amount of free antibody able to attach to the sensing surface. The list of reagents used for immunoassay is provided in the ESI.†

2.3. Microfluidic cartridge fabrication

The microfluidic cartridge consists of two parts: the reservoir plate and the sensor chip. The reservoir plate is made of the widely used poly(methyl methacrylate) (PMMA)[10–16] where reservoirs and delivery channels are shaped by micromilling and sealed by thermal bonding. External dimensions of the cartridge are 100 × 40 × 7 (in millimeters) and the reservoirs exhibit a volume capacity of around 100 μL. Channels have a rectangular-shaped section with dimensions of 0.9 mm × 0.5 mm. The sensing chamber is made of a UV-curable hyperbranched polymer (HBP). The HBP used in this work is an acrylated, third generation polyether core HBP. It combines a high Young's modulus, a hydrophilic character and low internal stresses resulting in high dimensional accuracy.[17–19] Alternatively to a previously described UV molding process for microfluidic devices,[20–23] a novel fabrication technique combining UV micromolding and photolithography allowed the fabrication of the microfluidic components and interconnection port simultaneously, as depicted in Fig. 2. Firstly, a 100 μm thick SU-8 layer was structured by a lithography process on a silicon substrate. The liquid HBP monomer mixed with a photoinitiator was dispensed on the SU-8 master and exposed to UV light for 20 seconds (50 mW cm^{-2}) through a Cr photomask which exhibits patterns of the interconnection port. The control of the monomer layer thickness was achieved by using spacers between the SU-8 master and the photomask. Thereafter, the photomask was removed and the uncured part of the HBP was developed using an organic solvent solution. The sensing chamber was generated on the biofunctionalized sensing chip by bonding the HBP gasket using UV adhesive. Finally, the sensor chip was assembled in the reservoir plate and the reservoirs filled with the different solutions required for the immunoassay. Pictures of the microfluidic cartridge and HBP gasket are available in the ESI† (Fig. S1) as well as a list of reagents.

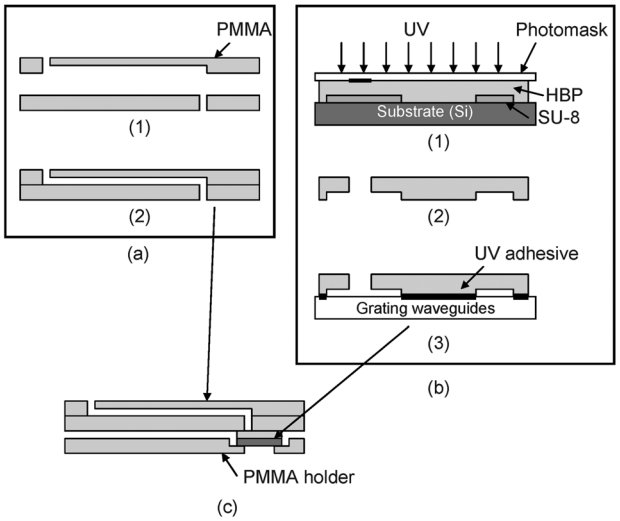

Fig. 2 Fabrication process of the LOC cartridge: (a) reservoir plate: (1) micromilling of PMMA plate for reservoirs and microchannels; (2) thermal bonding for the sealing of reservoirs and microchannels; (b) sensor chip with sensing chamber: (1) UV curing of HBP on SU-8 through photomask for the sensing chamber; (2) development of uncured HBP; (3) bonding of sensing chamber on the grating waveguide chip using UV adhesive; (c) assembly of the reservoir plate and sensor chip.

2.4. Fluidic setup

Fluidics motion in microfluidic systems has been widely studied and several strategies are well established in the lab-on-a-chip field. One of the most elegant approaches is the on-chip electro-osmotic pump,[24,25] particularly convenient for low flow rate devices (sub μL min^{-1}). However, electro-osmotic pumps generally require a high voltage source (up to kV) and the fabrication process tends to increase the cost of the microfluidic chip. Alternatively, pressure-driven fluidic setups allow easy control of the flow rate and do not require active channels on the chip. This approach has been reported regarding a microarray system actuated by eight syringe pumps for automated analysis of multiple antibiotics in milk.[6] In this work, fluids motion is driven by a single syringe pump (Cavro™ XCalibur) working in aspiration mode in combination with a multiposition valve (Cavro™ Smart Valve). Both pump and valve are controlled through PumpLink32 software (Cavro™) that allows programming a sequence of coordinated operations. A picture of the whole multianalyte detection system is available in the ESI† (Fig S2).

All along the multiplexed immunoassay, the different solutions pre-contained in the microfluidic cartridge reservoirs have to be sequentially delivered on the sensing chamber. In the meantime, the air plug between reservoirs has to be kept away from the sensing chamber. This is achieved using the coordinated

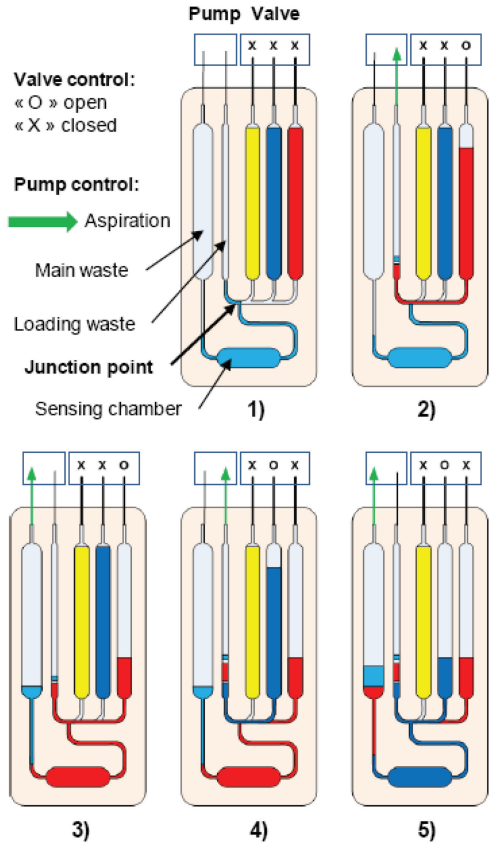

Fig. 3 Schematic representation of fluidic setup for sequential liquid delivery from pre-filled reservoirs to the sensing chamber. The configuration of the microfluidic cartridge during storage is depicted in (1) with the sensing chamber filled with buffer solution. When the assay begins the first reservoir starts emptying into loading waste to ensure liquid–liquid continuity in the junction point (2). Then flow is deviated towards the sensing chamber (3). The same operation is repeated for liquid contained in further reservoirs (4) and (5). Through this setup the whole sequence is controlled by external and concerted actuation of one multiposition valve connected to atmospheric pressure and one syringe pump (3 ports).

actuation of pump and valve, together with a loading waste on the cartridge as described in the schematic representation of Fig. 3. Waste reservoirs ensure that no residual liquid is going out of the cartridge, limiting the risk of contamination. The description of the 'holder interface' that ensures cartridge positioning on the WIOS instrument and connection to the fluidic functions is detailed in the ESI† (Fig. S3).

Under laminar flow conditions, the reaction rate depends principally on the analyte concentration leading to higher stability and reproducibility of the measurement. Regarding the present system, the hydrodynamic flow generated by negative pressure actuation through the microfluidic channels and incubation chamber is laminar (flow rate set at 25 μL min^{-1}), as indicated by the very low Reynolds number, $R_e = 0.85$, calculated from the following equation:

$$R_e = \frac{4Q}{\pi d_h \nu} \quad (2)$$

where Q represents the volumetric flow; and d_h and ν are the hydrolic diameter and the dynamic viscosity, respectively.

3. Results and discussion

Prior to immunoassay-based multidetection, the fluidic setup that includes the microfluidic cartridge and external pump and valve was successfully evaluated. As shown in Fig. 4, liquid delivery from the cartridge reservoirs to the sensing chamber is achieved by controlling the position of the valve which is connected to the air. Moreover, the residual air plug that prevents solutions mixing between reservoirs during storage is efficiently maintained out of the sensing chamber during the whole assay sequence *via* the so-called loading waste that acts as a flow diversion path (Fig. 4B). This system design also ensures the continuous delivery of the sample volume through the sensing chamber with no air plug between adjacent solutions.

The full assay protocol using the present system consists of a few simple handling operations: (1) insert the pre-filled cartridge on the instrument, (2) introduce the milk sample into the vial, (3) plug the vial onto the instrument, and (4) turn-on the laser source and run the program for pump/valve control (Fig. S4 in the ESI†).

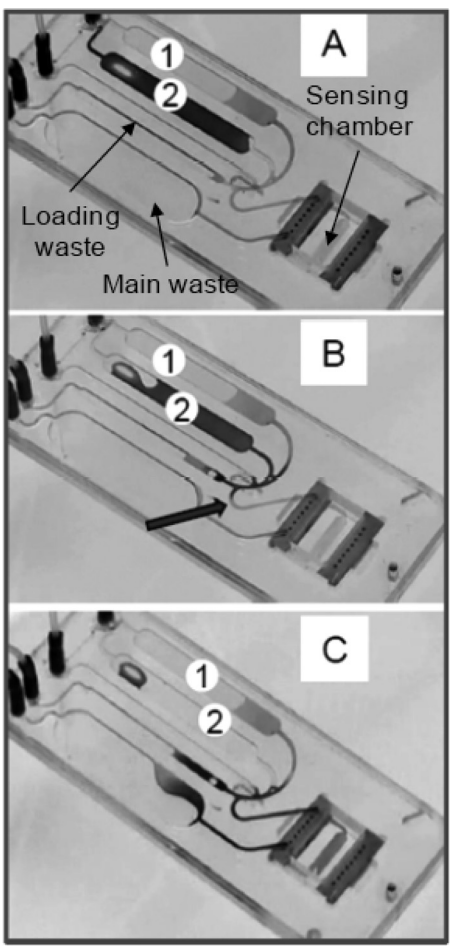

Fig. 4 Photograph sequence showing liquid delivery from reservoirs 1 and 2 to the sensing chamber. (A) Reservoir 1 connected to air: solution successively delivered to loading waste and sensing chamber. (B) Reservoir 2 connected to air: solution 2 driven to loading waste. No residual air between solutions 1 and 2. (C) Solution 2 driven to sensing chamber. The arrow in (B) shows the liquid–liquid junction with no residual air going to the sensing chamber.

Consequently, the automated multiplexed immunoassay takes place in the sensing chamber and its progress is monitored by the parallel WIOS signals measured for each antibiotic family. In order to simplify the typical WIOS response obtained during multiplexed measurement (one test curve and reference per antibiotic family) the graph of Fig. 5 represents the WIOS response curve obtained for a single antibiotic. The immunoassay sequence comprises a series of consecutive steps: (1) baseline stabilization (buffer), (2) milk sample introduction, (3) washing and (4) introduction of the secondary antibody for signal amplification. The differential signal value between the test curve and reference is measured after 10 minutes from the assay start. Typically for a binary response test the value obtained at the maximum residue level (MRL) concentration defines two qualitative domains: for any value inferior or equal to the value at MRL the test is considered 'positive', the remaining domain being 'negative'.

On this basis, calibration was carried out for multiplexed measurement of sulfapyridine (sulfonamide), ciprofloxacin (fluoroquinolone) and oxytetracycline (tetracycline) antibiotics in raw milk. The differential WIOS response corresponding to the amplification step of the assay and obtained for the multiplexed detection of three antibiotics at MRL concentrations – that is 100 μg mL^{-1} for each antibiotic – is depicted in Fig. 6. Accordingly to the competitive format of the immunoassays, those values measured after a 10 minute assay time are significantly lower than the ones obtained with a milk sample free of antibiotic, as shown in Fig. 7. In the case of ciprofloxacin the positive domain clearly covers a larger response range than the negative one. This result suggests that the half maximal effective concentration, EC_{50}, which corresponds to the part of the sigmoidal calibration curve with maximal sensitivity is lower than the MRL concentration. However, this occurrence did not represent a limitation for the sensitive detection of fluoroquinolone in the present binary test approach. Finally, a validation test was carried out to evaluate the reliability of the multiplexed test by analyzing six blind milk samples provided by Nestlé. Prior to the test, the reservoirs of six microfluidic cartridges were pre-filled with

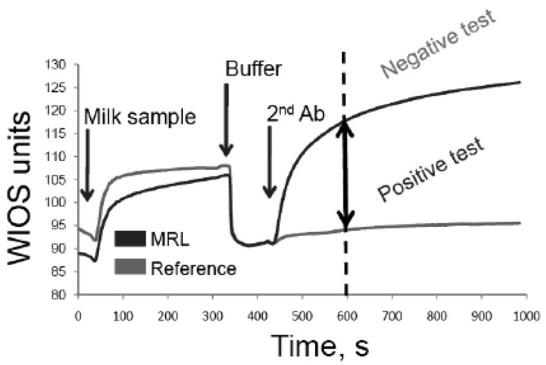

Fig. 5 Typical WIOS response curve obtained with lab-on-a-chip immunoassay test where threshold curve obtained at maximum residue level (MRL) concentration defines positive and negative domains.

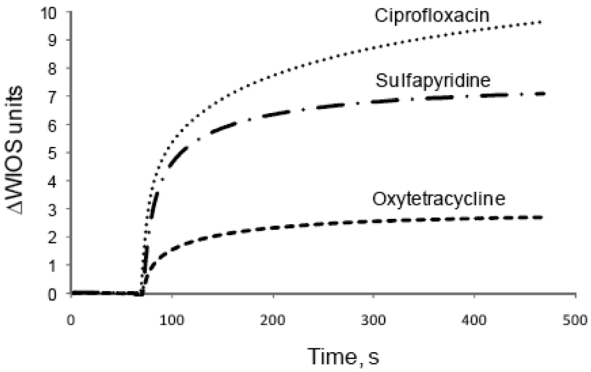

Fig. 6 WIOS differential response curves corresponding to the amplification step of the immunoassay obtained for multiplexed detection of three antibiotics: sulfapyridine (long dashed dot line), ciprofloxacin (dotted line), and oxytetracycline (dashed line) at MRL concentrations (100 μg mL^{-1}). Those threshold curves define positive and negative test domains for each antibiotic.

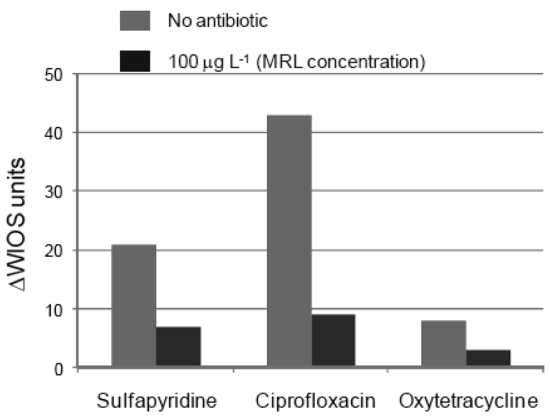

Fig. 7 Comparative representation of WIOS differential responses obtained from multiplexed measurements with a milk sample containing no antibiotic (light grey) and 100 μg L^{-1} (MRL concentration) of sulfapyridine, ciprofloxacin and oxytetracycline (dark grey).

Table 1 Test results obtained through the multiplexed blind analysis of six milk samples and the corresponding real antibiotic concentrations

	Sulfonamide		Fluoroquinolone		Tetracycline	
Sample no.	μg L^{-1}	Test	μg L^{-1}	Test	μg L^{-1}	Test
1	0	–	0	–	0	–
2	100	+	100	+	100	+
3	100	+	50	–	50	–
4	50	–	100	–	50	–
5	0	–	50	–	100	+
6	0	–	0	–	120	+

washing solution and mixture of secondary antibodies; meanwhile, competition reagents were introduced into sample vials. At this stage the assay protocol previously described was observed and repeated using one cartridge per sample analyzed. For each sample, the WIOS differential responses obtained for each antibiotic family were compared to the corresponding value at the MRL concentration and converted into a binary response: a positive or negative test. Afterwards, the test results for the panel of blind samples were analyzed from the sight of the sample concentration values revealed by Nestlé and summarized in Table 1. For those three antibiotic families analyzed – sulfonamides, fluoroquinolones and tetracyclines – the test accuracy reached 95% with only one false negative observed for 'sample 4' where the MRL concentration of fluoroquinolone was given as negative. Further experiments are currently being carried out to implement this multiplexed automated detection system to the β-lactam antibiotic family.

4. Conclusions

A fully automated lab-on-a-chip system for the simultaneous detection of multiple antibiotics in raw milk has been developed. The design and fabrication of a polymer-based microfluidic cartridge using a combination of micromachining and UV micromolding with an acrylated HBP make the test cheap, fast and easy to perform. The disposable and passive self-contained microfluidic cartridge is externally actuated *via* a simple fluidic setup involving a single pump/valve concerted operation. Solution mixing between reservoirs is prevented by air plugs that are deviated to loading waste during the assay, resulting in continuous liquid delivery on the sensor chip. The multiplexed automated immunoassay approach was successfully validated through the analysis of six blind milk samples containing sulfonamides, fluoroquinolones and tetracyclines. The test accuracy reached 95% for multiplexed measurements. The present a lab-on-a-chip-based detection system that is currently being implemented to β-lactam antibiotics opens a wide spectrum of applications in the fields of food analysis, environment monitoring and medical diagnosis.

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