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Surface Plasmon Resonance imaging-based sensing for anti-bovine immunoglobulins detection in human milk and serum

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ABSTRACT

Only few papers deal with Surface Plasmon Resonance imaging (SPRi) direct detection on complex matrices, limiting the biosensor application to real analytical problems. In this work a SPRi biosensor for anti-bovine IgG detection in untreated human bodily fluids, i.e. diluted human serum and milk, was developed. Enhanced levels of cow's milk antibodies in children's serum are suspected for their possible correlation with Type 1 diabetes during childhood and their detection in real samples was up to now performed by classical immunoassays based on indirect detection. The biosensor was optimised in standard samples and then in untreated human milk for anti-bovine IgG direct detection. The key novelty of the work is the evaluation of matrix effect by applying to real samples an experimental and *ex ante* method previously developed for SPRi signal sampling in standard solutions, called "Data Analyzer"; it punctually visualises and analyses the behaviour of receptor spots of the array, to select only spot areas with the best specific vs. unspecific signal values. In this way, benefits provide by SPRi image analysis are exploited here to quantify and minimise drawbacks due to the matrix effect, allowing to by-pass every matrix pre-treatment except dilution.

1. Introduction

Strong interest has been recently devoted to the latest advancement of SPR technology: SPR imaging (SPRi) transduction. It exploits spatial capabilities given by digital imaging analysis, allowing multi-analyte and simultaneous detection in array format. SPRi was reported for nucleic acids, protein/DNA, and protein–protein interactions studies [1]. Moreover, hyphenated techniques involving MS spectrometry [2–4], and electrophoresis [5] coupled with SPRi were described for protein detection, demonstrating its wide applicability. We reported how signal sampling and data management could lead to impressive improvement in terms of analytical performances [6], studying human IgG/anti-human IgG interaction in standard conditions as proof of principle of the method. Here we applied this method, "Data Analyzer", to one of the most relevant drawbacks in biosensors development, i.e. the matrix effect in real and complex samples. In fact, despite the numerous works on SPRi, only few of them deal with real matrices, usually performing heavy pre-treatments to minimise and/or eliminate the source of unspecific binding, but often affecting the analyte detection as well. This is the approach reported by Dong et al. [7] for human IgG detection by SPRi in hIgG-free diluted serum (1/10) obtained by adding 0.05% Tween 20 to polyethylene glycol (PEG)-treated serum. Although demonstrating the quantitative detection in hIgG-free spiked samples (LOD = 0.001 g L^{-1}), this procedure has difficult applicability for real hIgG analysis, since proteins removal from patient's samples will cause hIgG analyte depletion as well. Examples of SPRi immunosensing in sera samples are relative to semi-quantitative detection of anti-hepatitis virus antibody in undiluted human sera [8], and anti-citrullinated protein in diluted sera (1/50) for studies on auto-antibodies related to rheumatoid arthritis [9]. Other SPRi measurements on bodily fluids relevant for diagnostics are the cancer marker mucin quantified in spiked saliva [10], and human chorionic gonadotropin in diluted blood plasma (1/10) and activated leukocytes cell adhesion molecule, relevant in cancer diagnostic [11].

In this work we approached the quantitation of anti-bovine IgG (anti-bIgG) in human milk (HM) and serum (HS) by SPRi to go deeper into protein determination in human complex matrices of potential interest in diagnostics. Differently from the cited literature, here we performed an experimental and *ex ante* evaluation of matrix effects based on the application of "Data Analyzer" method to by-pass any sample pre-treatment except dilution. After preliminary calibration of the biosensor with anti-bIgG in buffer, its capability in detecting the analyte in untreated HS and HM was investigated. The key novelty of the work is the investigation of receptor spots behaviour for possible unspecific binding during a pre-analytical step. Each spot is mapped and analysed for its response to matrices, and thus "Data Analyzer" selects only areas

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performing the best specific vs. unspecific signal values. In this way, the contribution of unspecific binding can be automatically subtracted before starting measurements on real samples.

Attention to bovine IgG detection in HS and HM has significantly grown, since strong interest is devoted to abnormal levels of human anti-bovine antibodies in children's serum; it has been evidenced as a possible relationship between the time of introduction of formula containing bovine proteins in infant feeding could lead to seroconversion to positivity for diabetes-associated auto-antibodies and progression to clinical Type 1 diabetes (T1D) [12–14]. Moreover, it was reported that also the mother's diet during lactation has a crucial role in autoimmune processes leading to T1D, by the possible direct transmission of cow milk proteins [15–17] and/or anti-bovine immunoglobulin present into breast milk [18].

Detection of immune response toward allergenic bovine proteins (β-lactoglobulin, casein, and bovine serum albumin) are classically performed by Enzyme Linked Immunosorbent Assay (ELISA) immunoassays by indirect detection [19] or Radio Immuno-Assay (RIA) as in the case of anti-BSA antibodies detection [20]. However, SPR-based biosensors have shown to be also very competitive with ELISA assays [9,21], representing a possible new detection technology suitable for this emerging issue.

2. Materials and methods

2.1. Materials

11-Mercaptoundecanoic acid (MUA), 11-mercaptoundecanol, 1-mercaptohexanol, *N*-hydroxysuccinimide (NHS), sodium acetate trihydrate, ethanolamine hydrochloride (EA), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), Tween 20, and human serum were from Sigma–Aldrich (Italy). Anti-bovine IgG and anti-human IgG antibodies (mouse monoclonal), bovine and human IgG serum (certified material) were all purchased from Sigma–Aldrich (Milan, Italy). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) was from Calbiochem (USA). Glycine was from Merck KGaA (Germany). Absolute ethanol from Carlo Erba Reagenti (Italy). 184 Silicone Elastomer kit from Dow Corning Corporation (USA). Receptor immobilisation solution: 10 mM sodium acetate pH 5.0. Binding buffer was HEPES 10 mM, Tween20 0.1%, pH 7.4. All buffers and solutions were filtered (0.45 μm, Millipore) before use. Human milk was kindly provided by voluntary women during lactation.

2.2. SPR imaging instrumentation and measurements

Measurements were carried out with SPRi-Lab$^+$ instrument and SF-10 gold chips (Genoptics-Horiba Scientific, France) recording reflectivity variation % ($\Delta\%R$) at fixed angle and 635 nm. A 6-μL flow cell is connected to a Rheodyne 7125 valve (IDEX Health & Science Group, Wertheim-Mondfeld, Germany) for sample injection (175 μL per injection), and a peristaltic pump (Gilson, Middleton, USA) set on 20 μL min^{-1} flow rate. Measuring cycle: surface was equilibrated with binding buffer, and sample injected through the valve loop; after 10 min of sample/surface interaction, the surface was rinsed with binding buffer and then the bound analyte was removed by injecting 10 mM glycine solution, pH 1.9 for 30 s. SPRi signals are displayed both as sensorgrams and as difference images in real time by the software SPRi-View L 3.1.0. (Genoptics-Horiba Scientific).

2.3. Receptors immobilisation

Gold chips were washed with absolute ethanol and milliQ water then dried under nitrogen. Bovine IgG (bIgG) array was built starting from the gold layer of the chip by following a previously developed method based on the use of PDMS masks [6]. The immobilisation chemistry was based on the formation of a MUA (1 mM) self-assembled monolayer, followed by its activation with 50 mM NHS and 200 mM EDAC aqueous solution (20 min) to covalently bind proteins (150 ppm in 10 mM sodium acetate pH 5.0) through the amino-coupling reaction. Two spots were used as control surfaces (no receptor, only immobilisation solution). Finally, the biochip was treated for 20 min with EA solution (1 mM water solution, pH 8.0) to block unreacted sites.

2.4. Signal sampling method and data management

SPRi signals were recorded on areas selected as Regions of Interests (ROIs) on biochip, and visualised as sensorgrams and difference images during measurements. A number of suitable ROIs (140 μm diameter) were selected on spots using "Data Analyzer" software [6]. The software was developed for SPRi data sampling and management procedures, and showed to improve the analytical performances of the system. Here it was exploited to evaluate matrix effects of untreated, except dilution, HS and HM on anti-bIgG quantitative detection. An array was designed as reported in Fig. 1A, and a grid of 61 ROIs/spot was defined with "Data Analyzer" (Fig. 1B and b (one spot zoomed in)). This fine grid allows the punctual evaluation of the SPRi response of each ROI. To this aim, a pre-analysis consisting of injections (three replicates, $n = 3$) of HS and HM alone and, subsequently, the same matrices spiked with 3 mg L^{-1} anti-bIgG, was performed. Moreover, injections of 3 mg L^{-1} anti-bIgG in buffer were also carried out (Fig. 1C). All SPRi data obtained from pre-analysis were thus aimed to evaluate the matrix effect, both for HS and for HM, and how possible unspecific binding can affect the quantification of anti-bIgG present in the same matrices. "Data Analyzer" was conceived to collect all SPRi data from pre-analysis (61 SPRi signals/spot, i.e. the overall number of ROIs/spot, for each sample injection) and manage them to obtain a final ranking of the ROIs. At the top of the rank "Data Analyzer" shows only ROIs displaying specific/unspecific values above a fixed threshold previously set in the software. At the end of the procedure, 3 ROIs/spot with the best performance are selected (Fig. 1D) and used for subsequent measures. Remaining ROIs are automatically discarded. Thus this method allows the preliminary and experimental evaluation of the biochip response, carried out on a large number of ROIs (61 ROIs/spot, i.e. 976 ROIs), followed by a rational selection of a limited number (3 ROIs/spot, i.e. 48 ROIs), which answers specific analytical requirements, i.e. a high specificity vs. matrix effect in this case.

For each injection, 48 SPRi signals were collected and averaged. The reproducibility of each measurement was thus expressed as coefficient of variation percentage (CV%) on the 48 SPRi signals relative to each sample injected. Reproducibility among injections was evaluated by comparing CV% values on three replicates ($n = 3$). In this case, the relative coefficient of variation percentage (CV%) is expressed as averaged CV% (CV_{av}%).

3. Results and discussion

3.1. Sample dilution

For sera and milk samples, a dilution step was performed as pre-treatment to partially reduce the matrix effect during measurements. The proper dilution factor was considered as the best compromise between sensitivity toward anti-bIgG, and minimisation of the matrix effect. Here we applied the spike method, which quantitatively assesses matrix effects by comparing the response of the matrix alone (HS and HM) to the response of the same matrix spiked with the analyte. For both matrices, dilutions of 1/10,

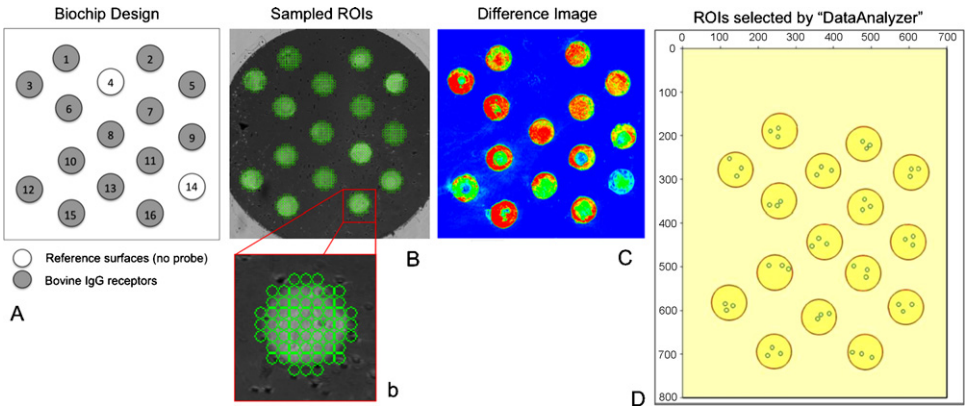

Fig. 1. Steps of "Data Analyzer" application: (A) biochip design; (B) grid of ROIs (green circles, 61/spot) utilised during pre-analysis for fine mapping of each spot of the array; (b) zoom-in image of one receptor spot sampled with 61 ROIs; (C) experimental difference image after 3 mg L^{-1} anti-bIgG injection in HS; (D) "Data Analyzer" output of selected ROIs to be used for analysis. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

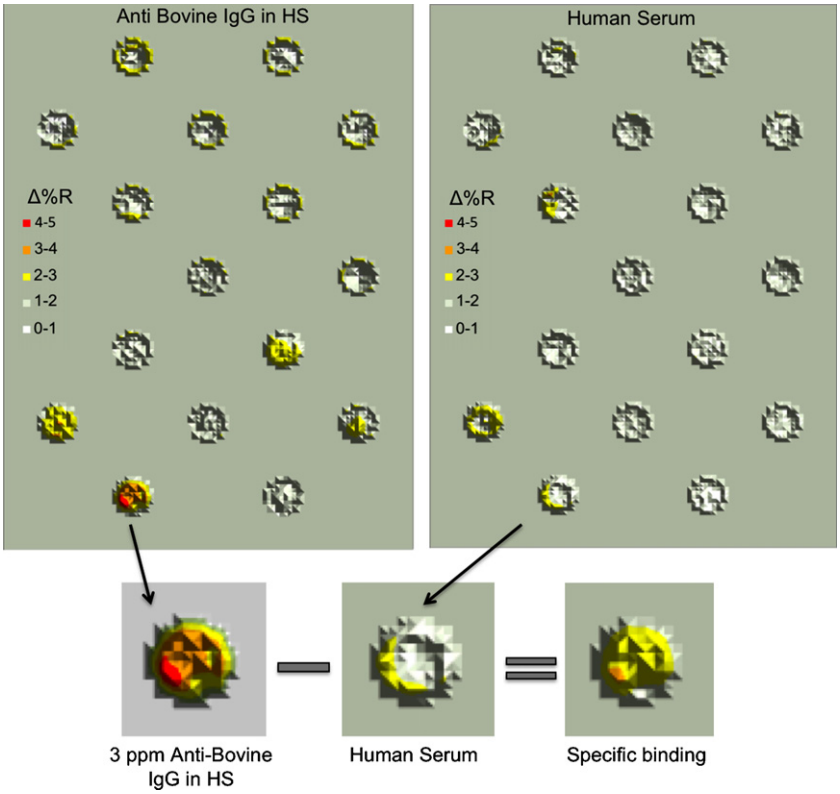

Fig. 2. 3D Charts elaborated by "Data Analyzer" during pre-analysis to evaluate the behaviour of HS on the array. Left chart: biochip response to 3 mg L^{-1} anti bovine IgG in diluted HS; right chart: biochip response to diluted serum alone. Below, one spot is zoomed in to better visualise how matrix contribution can be subtracted obtaining anti-bovine IgG specific signal.

1/20, 1/40, 1/60, 1/80 and 1/100 in binding buffer were analysed, recording signals due to the corresponding unspecific binding alone. The results were then compared with those from HS and HM spiked with anti-bIgG (3 mg L^{-1} for HS and 10 mg L^{-1}) and then diluted following the same factors. Best results were obtained for 1/40 dilution factor for HS and 1/100 for HM, respectively (data not shown). HM showed higher unspecific binding on biochip and, consequently, a worse sensitivity toward anti-bIgG.

3.2. Evaluation of the matrix effect with "Data Analyzer"

As often happens when dealing with complex matrices, despite the pre-analytical dilution step, SPR signal due to unspecific binding still remains an important limiting factor during measurements. In fact, dilution is useful as long as instrumental sensitivity remains adequate. For this reason here we approached this issue by an experimental and *in silico* method called "Data Analyzer". The first step of the method involves a pre-analysis step by testing the biochip response after injections of HS an HM. During this stage, each spot was sampled by 61 ROIs and the relative binding data were visualised by "Data Analyzer" as 3D charts displaying the biochip behaviour (each pixel of the chart corresponds to an experimental SPRi signal).

Fig. 2 reports, as example, data sampling for human serum. Upper left chart shows biochip behaviour after injection of 3 mg L^{-1} anti-bIgG in 1/40 HS: all spots show high signals due to the high contribution of potential interferents from serum. A second injection with HS alone, on the previously regenerated surface, is carried

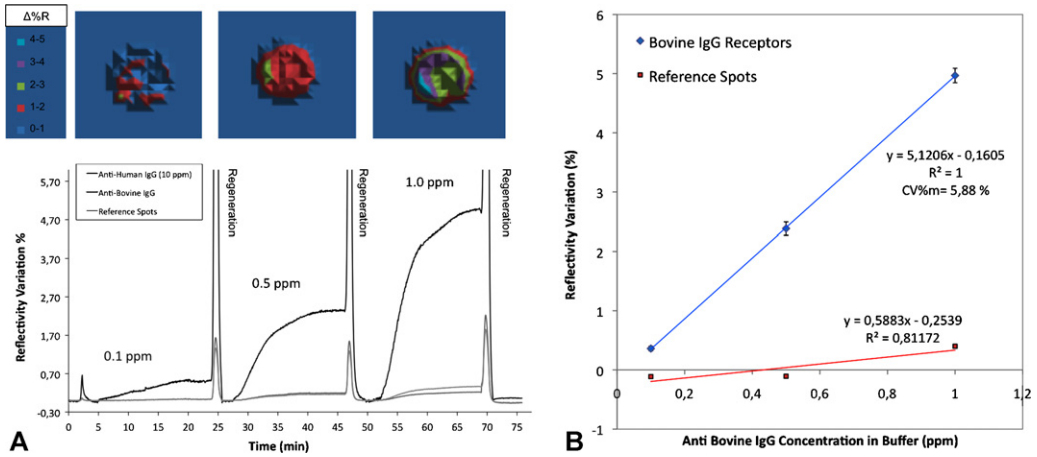

Fig. 3. (A) Sensorgrams of anti-bovine IgG concentrations relative to calibration curve and, above, the response of one bovine IgG spot reported as "Data Analyzer" output. In the sensorgram, the specific responses are reported together with three replicates of 10 mg L^{-1} anti-human IgG tested as negative control. (B) Anti-bovine IgG calibration obtained in binding buffer.

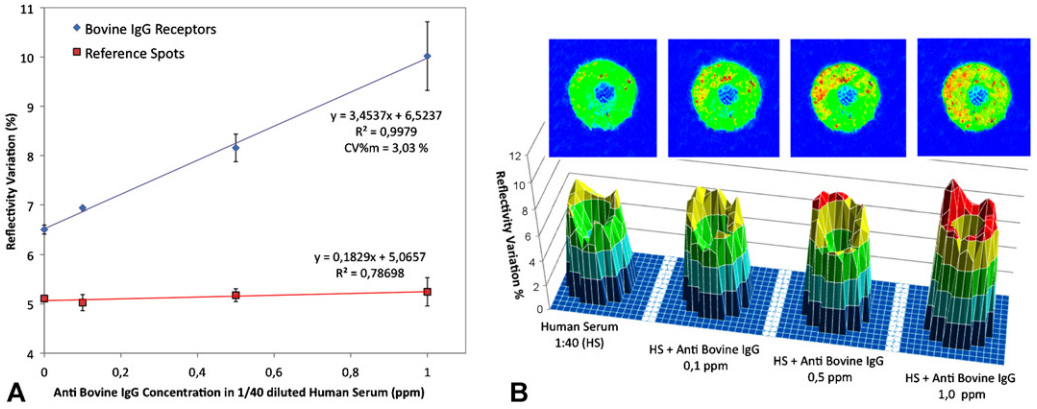

Fig. 4. (A) Anti-bovine IgG calibration in 1/40-diluted human serum. (B) Difference images of one bovine IgG spot of the array after the binding event with anti-bIgG at different concentrations and, lower, 3D elaboration of the same binding data obtained by "Data Analyzer".

out and the result displayed on the right 3D chart. Thus contribution of the matrix can be simply identified.

Furthermore, some important considerations can be advanced. We can observe an important "crown" or "edging" effect (Fig. 2) when the injected sample interacts with the spots. This inconvenience, which could significantly affect the analytical performances of the biosensor, was overcome by performing a targeted and rational selection of the ROIs. To this aim, "Data Analyzer" subtracts each SPRi signal relative to the matrix alone (HS in this case) from the signal obtained when the same matrix is spiked with the analyte (HS + anti-bIgG). The result is visualised both as 3D plot and as $\Delta\%R$ data. Fig. 2 (bottom) shows one spot zoomed-in, in which we can observe that ROIs in the inner part of the spot display the best specificity. Finally "Data Analyzer" automatically sorts the

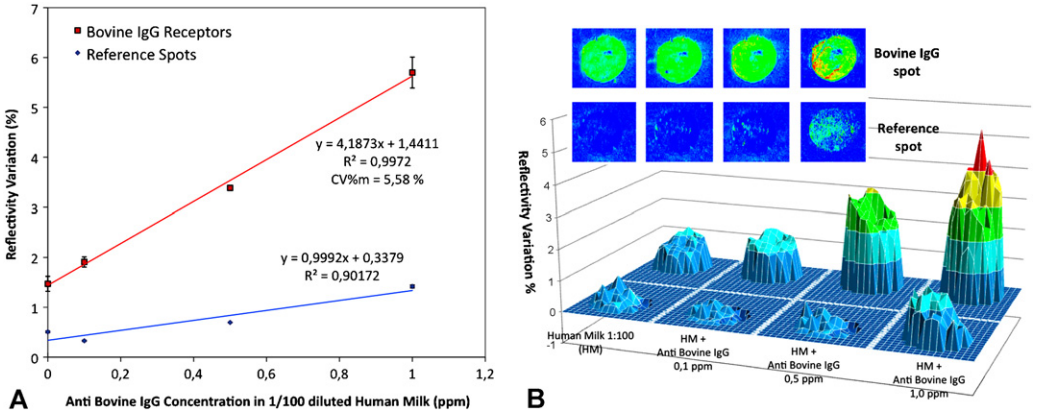

Fig. 5. (A) Anti-bovine IgG calibration in 1/100-diluted human milk. (B) Difference images of one bovine IgG spot of the array after the binding event with anti-bIgG at different concentrations and, lower, 3D elaboration of the same binding data obtained by "Data Analyzer". In this case one probe receptor is compared with a reference spot on which the matrix contribution can be observed.

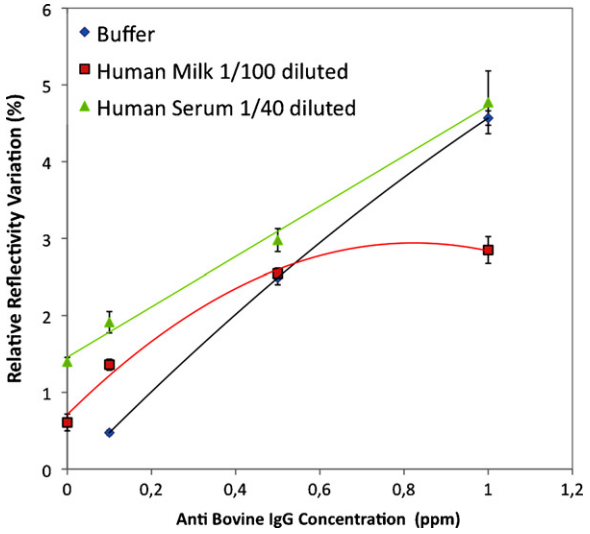

Fig. 6. Plot comparing the three calibration curves obtained in buffer, human serum, and human milk. The comparison was carried out by subtracting relative unspecific signals on reference spots for each tested matrix.

most performing ROIs for each spot, confirming that the most specific ROIs for anti-bIgG in HS are located in the inner part of the spot (see the final grid generated by "Data Analyzer" and displayed in Fig. 1D, in which the selected three ROIs on each spots are located). The same procedure was performed also in case of HM (1/100).

3.3. Anti-bovine IgG calibration in buffer

The biochip was exposed to anti-bIgG in binding buffer and the main analytical parameters of the array (i.e. selectivity, sensitivity, reproducibility, etc.) were evaluated. Fig. 3A (bottom) reports sensorgrams of anti-bIgG biorecognition on biochip, within the range 0.1–1 mg L^{-1}, and (top) the relative response of one bovine IgG spot displayed as 3D output by "Data Analyzer". In the sensorgram both specific responses on bovine IgG receptors and on reference spots are reported. Moreover, a control solution (10 mg L^{-1} anti-human IgG) was tested as negative reference, giving negligible unspecific signal on all bovine IgG receptors. Fig. 3B shows the linear range for anti-bIgG within 0.1–1.0 mg L^{-1} in binding buffer, together with the corresponding equation and R^2 coefficient of the curve. The detection limit (DL) was 0.04 mg L^{-1}, which was inferred through different experiments as the anti-bIgG concentration corresponding to the minimum reliable SPRi signal (0.06 $\Delta\%R$). This was calculated as three times the observed averaged standard deviation (SD) of the baseline, SD = ±0.02 $\Delta\%R$, The DL is in line with anti-bovine protein antibodies (i.e. anti-BSA IgG) amount reported for healthy blood donors in the literature [20]. The reproducibility among experiments, expressed as averaged coefficient of variation percentage, was $CV_{av}\% = 5.9$.

3.4. Detection of anti-bovine IgG in diluted human serum and milk

Standard additions of target analyte in 1/40 diluted HS were performed within 0.1–1.0 mg L^{-1}, showing a high linear response ($R^2 = 0.998$) with a $CV_{av}\% = 3$ (Fig. 4A). The DL estimated was of 0.07 mg L^{-1} and therefore the system is able to detect altered levels of anti-bIgG in undiluted HS presenting values higher than 2.8 mg L^{-1}, more than sufficient to detect altered sera samples. Clinical data [20] displayed values up to 258 mg L^{-1}, with median value of 19 mg L^{-1}. Even if matrix effect is relevant ($\Delta\%R = 5$), it is independent from analyte concentration allowing its reliable quantification. Fig. 4B displays experimental difference images of one representative bIgG spot after binding with anti-bIgG at concentrations tested. These images were obtained by SPRi-View L 3.1.0, provided with the instrument. In the lower part of Fig. 4B, the 3D chart obtained with "Data Analyzer" is also shown.

In the case of the second tested matrix (HM), anti-bIgG standard additions were carried out as for human serum calibration in diluted 1/100 HM within the range of 0.1–1.0 mg L^{-1}. Also in this case very good linearity ($R^2 = 0.997$) and reproducibility ($CV_{av}\% = 5.6$) were observed (Fig. 5A). DL was 0.11 mg L^{-1} in diluted matrix, corresponding to a DL of 11 mg L^{-1} in undiluted HM. In Fig. 5B, the 3D chart obtained for the target concentrations tested is reported. One probe receptor is compared with a reference spot displaying only the matrix effect. Some concentration-dependent effect on reference spots was observed above 0.5 mg L^{-1}, slightly affecting the quantitative detection of anti-bIgG. No recent data are available about anti-bovine IgG content in HM during lactation, but the existence of subgroups at risk for T1D via a mucosal immunity pathway [18] was recently suggested. In this sense, the detection of such type of allergens in breast-feeding is undoubtedly of interest in women with altered anti-bIgG levels.

Results on HS and HM were finally directly compared with those obtained in buffer by subtracting values obtained on reference spots (no receptor) from those obtained on bIgG spots. Fig. 6 shows that within the concentration range 0–0.5 mg L^{-1} both matrices display a positive deviation from buffer. This deviation fades nearly 0.5 mg L^{-1} and, in the case of HM, the quantification in matrix is quite coincident with that in buffer. For higher concentrations, HS displays a wider linear range, whereas HM is characterised by a negative deviation from linearity for concentrations above 0.5 mg L^{-1}.

4. Conclusions

We developed a SPRi biosensor for anti-bovine IgG detection in untreated human serum and milk, since high levels of these antibodies in children's serum are correlated with Type 1 diabetes. Bovine IgG microarrays were analysed and the unspecific adsorption evaluated by previously developed software, "Data Analyzer", applied here for the first time to real matrices. "Data Analyzer" is able to locally estimate matrix effects, selecting only spot areas with high specificity toward specific analyte. The evaluation is faced by an *ex ante* and standardised approach, and utilises image analysis to better exploit SPRi potentialities. By this approach, anti bovine IgG detection was selective and reproducible between 0.1 and 1.0 mg L^{-1}, recording reliable signals also in the case of untreated, except dilution, matrices (1/40 for serum and 1/100 for milk). These findings strongly encourage further studies on SPRimaging application to other complex matrices for fast, reproducible and quantitative detection of proteins.

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