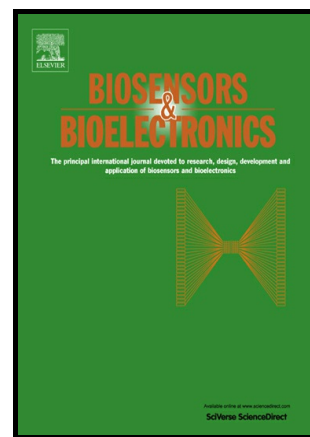# Author's Accepted Manuscript

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ACCEPTED MANUSCRIPT

An SPR based sensor for allergens detection

J. Ashley[1], M. Piekarska[1], C. Segers[1], L. Trinh[2], T. Rodgers[2], R. Willey[3], I.E. Tothill[1]*

[1]Cranfield University, Advanced Diagnostics and Sensors Group, Cranfield, Bedford, MK43 0AL, UK.

[2]University of Manchester, School of Chemical Engineering and Analytical Science, Oxford Road, Manchester, M13 9PL, UK.

[3]Safety and Environmental Assurance Centre, Colworth Science Park, Unilever plc, Sharnbrook, Bedford, MK44 1LQ, UK.

*Corresponding authors

I.E. Tothill

Tel: +44 (0) 7500766487,

E-mail: i.tothill@cranfield.ac.uk

Abstract

A simple, sensitive and label-free optical sensor method was developed for allergens analysis using α-casein as the biomarker for cow's milk detection, to be used directly in final rinse samples of cleaning in place systems (CIP) of food manufacturers. A Surface Plasmon Resonance (SPR) sensor chip consisting of four sensing arrays enabling the measurement of samples and control binding events simultaneously on the sensor surface was employed in this work. SPR offers several advantages in terms of label free detection, real time measurements and superior sensitivity when compared to ELISA based techniques. The gold sensor chip was used to immobilise α-casein-polyclonal antibody using EDC/NHS coupling

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procedure. The performance of the assay and the sensor was first optimised and characterised in pure buffer conditions giving a detection limit of 58 ng mL^{-1} as a direct binding assay. The assay sensitivity can be further improved by using sandwich assay format and amplified with nanoparticles. However, at this stage this is not required as the detection limit achieved exceeded the required allergens detection levels of 2 µg mL^{-1} for α-S1-casein. The sensor demonstrated good selectivity towards the α-casein as the target analyte and adequate recoveries from CIP final rinse wash samples. The sensor would be useful tool for monitoring allergen levels after cleaning procedures, providing additional data that may better inform upon wider food allergen risk management decision(s) that are made by food manufacturer. In particular, this sensor could potentially help validate or optimise cleaning practices for a given food manufacturing process.

1. Introduction

Cow's milk is one of the most common sources of food allergens globally and is considered a public health issue. The condition is caused by an adverse inflammatory response of the immune system which can be triggered by several milk proteins. There are at least 25 proteins that are known to be present in cows' milk and these are present in two distinct fractions, casein and whey. Some of the major allergens found in milk that are known to cause an allergic reaction are bovine serum albumin (BSA), γ-globulin, casein and β–lactoglobulin (BLG), (Lifschitz and Szajewska, 2015). The main fraction of milk proteins consist of casein, this represents 80 % of the proteins in milk. The remaining 20 % is made up of the serum proteins or whey proteins of which BLG is one such protein (de Jong et al.,

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1993; Eigenmann, 2007). It is estimated that 0.6-2.5 % of children and 1 % of adults have some form of allergy towards milk (Jo et al., 2014; Taylor et al., 2002). It is known that relatively low levels of milk allergen can cause reactions in allergic individuals; this is indicated by the Voluntary Incidental Trace Allergen Labelling (VITAL) framework. In particular, the VITAL framework proposes that a reference dose of at least 0.1 mg of milk protein per portion of product is a sufficient basis for food manufacturers to apply precautionary allergen labelling for allergic consumers (Allen et al., 2014; Taylor et al., 2014). EU regulation 1169/2011 currently requires that food manufacturers correctly label food products which either contain allergen containing ingredients or which have come from a factory where allergen containing ingredients have been used in other products (Council of the European Union, 2008). However, it is also important to emphasize that errors can occur during food manufacturing processes which can result in the unintentional presence of these allergen in finished products. As food allergy is considered a public health issue, food manufacturers have an increasing need for a robust analytical tools that support effective food allergen risk management, hence, the use of precautionary labelling on food packaging.

As a result, food manufacturers are looking to develop cheap, sensitive and reliable tests for the detection of food allergens during routine manufacturing processes which includes cleaning in place (CIP) of food processing equipment. In 2004, Stephan et al. conducted a validation study of CIP cleaning by measuring the nut and celery allergen levels in pre rinse solutions as a positive control, and after cleaning had taken place (Stephan et al., 2004). Protein measurements were taken using, both Bradford assay, peanut specific enzyme-linked immunosorbant assay ELISA and PCR. This work indicated that methods for the quantification of food allergens in the rinse water collected from CIP procedures could be applicable to food allergy risk management.

Immunochemical tests such as ELISA and lateral flow assays are generally the most popular and widely used analytical techniques for milk protein analysis. ELISA is based on the detection of an antigen using antibodies directed against milk protein allergens and assays can be based on competitive or sandwich based formats (Johnson et al., 2014; Monaci et al., 2011). The procedure can be quick and easy to carry out and sensitive down to low ppm levels. However recent studies on the comparison of performance of commercially available kits has resulted in large variations in both peanut and milk allergen detection between kits (Jayasena et al., 2015; Poms et al., 2007). Lateral-flow immuno-chromatographic assays (LFA) offer a simplified cheap offline format for the qualitative detection of milk protein

allergens (Schubert-Ullrich et al., 2009). Currently lateral flow based methods are limited to qualitative analysis. Rocket immune electrophoresis has also been used as a technique for measuring casein protein in a large number of dairy samples (Yman et al., 1994). However, the technique suffers from poor sensitivity and is largely obsolete due to the advent of automated methods. Multiplex analysis is an immunoassay- based technique which has allowed for the simultaneous detection of multiple milk protein allergens. A recent paper demonstrated the technique on the detection of 14 different food allergens, reporting a sensitivity of about 5 ng mL^{-1} (Cho et al., 2015).

LC-MS has proven to be a bench mark technique for the sensitive quantitative analysis of milk protein allergen in a different food matrices and offers high accuracy and reproducibility (Monaci et al., 2016; Parker et al., 2015; Tolin et al., 2012). Although mass spectrometry based methods offer superior sensitivity compared to other analytical methods available for analysis of milk allergens, analysis still needs to be carried out in a dedicated lab by trained personnel.

Biosensors are an attractive alternative to traditional immunoassay methods offering comparable sensitivities and selectivity while allowing for on-site detection (Turner, 2013, Tothill, 2011). A number of papers have reported the use of biosensors based quartz crystal microbalance (QCM), surface plasmon resonance (SPR) and electrochemical for the detection of milk protein allergens (Rebe Raz et al., 2010; Yman et al., 2006). Surface plasmon resonance allows for the sensitive, on-line and label-free detection of milk proteins. Several studies have been done on the detection of milk allergens using SPR based sensors. Haasnoot et al. developed a sensor for the detection of κ-casein with a detection limit of 0.1 % or 100 μg mL^{-1} (Haasnoot et al., 2004). Indyk & Filonzi (2005) and Muller-Renaud et al. (2005), designed biosensors against lactoferrin (Limit of Detection [LOD]: 19.9 μg mL^{-1}) and α-s1-casein (LOD: 0.87 μg mL^{-1}) respectively (Indyk and Filonzi, 2005; Muller-Renaud et al., 2005). More recently, researchers have developed SPRi based affinity tests to diagnose hypersensitivity (Chardin et al., 2014).

In the current work, an immunoassay based SPR sensor was developed for the detection of α-casein in cleaning in place (CIP) final rinse water samples. This is because caseins are a significant fraction of milk protein and the detection of α-casein would be a useful marker for monitoring levels of milk during the CIP process in combination with other known allergen sampling methods such as surface swabbing. The sensor showed sub ppm

sensitivity, good selectivity and was able to detect spiked casein levels after acidic precipitation and buffer exchange using G-25 SPE column. To the best of our knowledge, a sensor for the detection of milk proteins in CIP wash samples has not previously been reported.

2. Materials and Methods

2.1. Materials

Acetic acid, α-casein, 11-mercaptoundecanoic acid 95 %, nonfat-dried milk, phosphate buffered saline (PBS) (one tablet dissolved in 200 mL of deionized water yields 0.01 M phosphate buffer, pH 7.4, at 25 °C), sodium acetate and sulphuric acid 95.0-98.0 % , BCA protein assay were bought from Sigma Aldrich (Saint-Louis, USA). Casein rabbit polyclonal antibody and BLG antibody was purchased from Abcam (Cambridge, UK). Mouse IgG antibody was obtained from Jackson Immunoresearch laboratories (West Grove, USA). Sodium hydroxide was obtained from Fisher Scientific (Loughborough, United Kingdom). Ethanolamine and ethanol were supplied from Fluka analytical (Buchs, Switzerland). Hydrogenperoxide 35 % wt was obtained from Acros Organics (Geel, Belgium). Thermo Scientific (Waltham, USA) supplied N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC). All buffers were prepared using deionised water from a Millipore Direct-Q® 3 UV (Merck Millipore, Darmstadt, Germany) water dispenser and were filtered and degassed under vacuum. G-25 MiniTrap columns were purchased from GE healthcare.

2.2 Sensor surface functionalization

All SPR based assays were performed on a Sierra Sensor SPR 2/4 machine and the SPR affinity sensor chips from Sierra Sensors (Hamburg, Germany). Bare gold sensor chips were prepared by forming a self-assembled monolayer (SAM) (Uludag and Tothill, 2012). The sensor chips were submerged in a 5 mM solution of 11-MUA dissolved in ethanol (100 mL) overnight. The resultant sensor chips were then rinsed with water followed by ethanol to quench the reaction and quickly dried with nitrogen. The sensors were stored at 4 °C until use.

The activation of the sensor surface was accomplished using the Sierra Sensors SPR 2/4 instrument. The sensor chip was docked in the machine the flow cell was degassed with 10 mM PBS pH 7.4 as the flow buffer. The machine was then primed three times with flow buffer prior to antibody immobilization. For the surface immobilisation, the flow rate was set to 10 µL min^{-1}. For the immobilisation protocol, 50 µL of a mixture of 0.1 M NHS /0.4 M EDC were injected over the sensor surface on two spot channels. The antibodies were then attached to the sensor surface via an amine coupling reaction. 50 µg mL^{-1} of each antibody was dissolved in 10 mM sodium acetate buffer pH 4.0 and 50 µL of the α-casein antibody was injected onto spot 1 and 50 µL of the control antibody IgG was injected onto spot two. The sensor surface was then blocked by injecting 50 µL of 30 µg mL^{-1} BSA onto both spots followed by 50 µL of 1 M ethanolamine pH 8.5. The change in refractive index was recorded after each injections and the signal reaching a steady state.

2.3 Binding assays on the SPR chip

α-Casein standards were prepared daily by dissolving the solid protein in 10 mM sodium borate buffer pH 9.0. Concentrations of each stock were determined using BCA protein assay. Standards were further diluted into the working range by serial dilution in 10 mM PBS pH 7.4.

All binding assays were conducted by firstly priming the sensor surface three times with 10 mM PBS buffer pH 7.4. Each injection cycle consisted of a 75 µL injection of α-casein standard (0.195 - 12.5 ppm) injected over two spots at a flow rate of 25 µL min^{-1}. Responses were measured after the end of the dissociation phase (3 minutes) with the control response being subtracted from the analyte response for each injection and plotted against protein concentration. Standard assays were performed in duplicate. The sensor surface was then regenerated by a 75 µL injection of 50 mM NaOH. The specificity of the sensor was determined by injection of BLG and k-casein. Each protein was injected onto the sensor surface at the same concentration range as for the α-casein standards using the same assay conditions.

2.4 CIP final rinse sample analysis

CIP final rinse samples were collected from Unilever plc (Colworth, Bedford, UK). Spiked α-casein samples were prepared by acid precipitation followed by gel filtration. α-Casein standard was spiked into CIP final rinse samples (0.5 – 12 ppm). A 4 mL of 10 mM sodium acetate buffer pH 4.0 was added to each sample and centrifuged for 5 minutes at 1000 g^{-1}. The solute was then taken off and the procedure repeated 2 further times. On the last wash, the sodium acetate residue was removed and 0.5 mL of 10 mM sodium borate buffer, pH 9.0 was added to dissolve the precipitate. G-25 spin trap columns were equilibrated with 8 mL of 10 mM PBS pH 7.4. Each sample was then added to the gel filtration column and spun at 1000 g^{-1} for 2 minutes. The eluted sample was then collected and directly injected on the sensor surface as the sample. Responses for the spiked samples were measured using the same conditions as described for α- casein standards.

3. Results and Discussion

3.1 Sensor surface functionalisation

Casein- antibodies were immobilised on one spot and anti-IgG on spot 2 as a control. Before covalent immobilisation was conducted pH scouting was carried out to select the optimal buffer pH for antibodies immobilisation. This was done using the sensor chip without surface activation with EDC/NHS. The best pH was found to be pH 4.0 (supplementary information, Fig. S1 & Fig. S2). Therefore, pH 4.0 was used to immobilise the antibody in further experiments. After immobilisation, the sensor surface on both spots was blocked with BSA (30 μg mL^{-1}) followed by ethanolamine (1 M). NaOH (50 mM) was implemented after each casein injection in order to regenerate the sensor surface as the use of a low pH regeneration buffer would result in a protein precipitation on the sensor surface. 10 mM PBS pH 7.4 was used as the running buffer. The average response for each level of antibody binding on the sensor chip was >2000 response units (RU) and a typical sensorgram is shown in Fig. 1. The pH and concentration of the capture antibody was optimised to give the highest degree of immobilisation respectively. 50 μg ml^{-1} of anti α-casein mixed in 10 mM sodium acetate (pH 4.0) gave the highest extent of immobilisation and is shown in the supplementary information (Fig. S3). It is worth noting that the level of protein adsorption with the unactivated sensor does not always translate into the same level of immobilisation on an EDC/NHS activated surface due to the change in surface charge upon activation. However in this case there was little variance between the two types of surface and optimised immobilisation buffer. The effect of blocking the sensor was also investigated and is shown

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in the supplementary information (Fig. S4). Injections of α-casein at different concentrations on a sensor blocked with BSA (30 µg mL^{-1}) and ethanolamine (1 M) was found to be optimal for reducing non-specific binding (Fig. S4) and these conditions were used in all subsequent binding experiments.
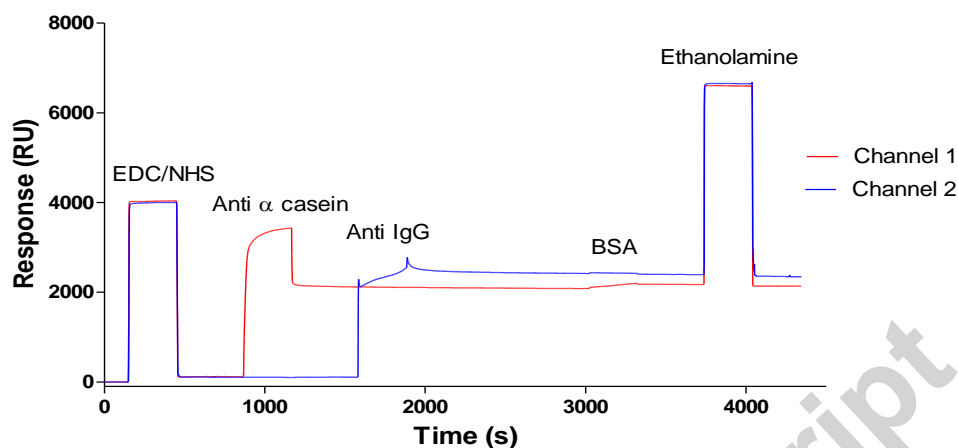

Fig. 1. SPR sensorgram of anti-α casein and anti-IgG control onto a 11-MUA SAM sensor surface. Functionalisation consisted of a 50 µL injection of EDC/NHS followed by a 50 µL injection of each antibody. The surface was blocked by injection of BSA followed by ethanolamine.

3.2   Binding assays on the SPR sensor

Binding assays were performed in duplicate using the optimised assay conditions. Different concentrations of casein (0.78 -50 ppm) were injected on both the active and control spots with a regeneration in between each injection (50 mM NaOH). By plotting the response measured after each casein concentration reached a saturation level and then dissociation phase taken place with washing buffer (3 minutes) in the sensorgram (Fig. 2), against different concentrations of α-casein standard, a linear correlation was achieved followed by saturation at the higher casein concentrations (Fig. 3A).

The Limit of detection (LOD) was determined from the linear proportion of the saturation graph by calculating 3 x standard deviation (s.d.) of the blank response and extrapolating the response in the linear calibration plot to convert the value to concentration. The limit of detection was determined to be 57.80 ng mL^{-1} with a linear range from 0.195 ppm to 12.5 ppm as detailed in Fig. 3B. These results show that the SPR based assay was more sensitive towards α-casein than the commercially available ELISA kit which reported a LOD of 2.5 ppm (Johnson et al., 2014).
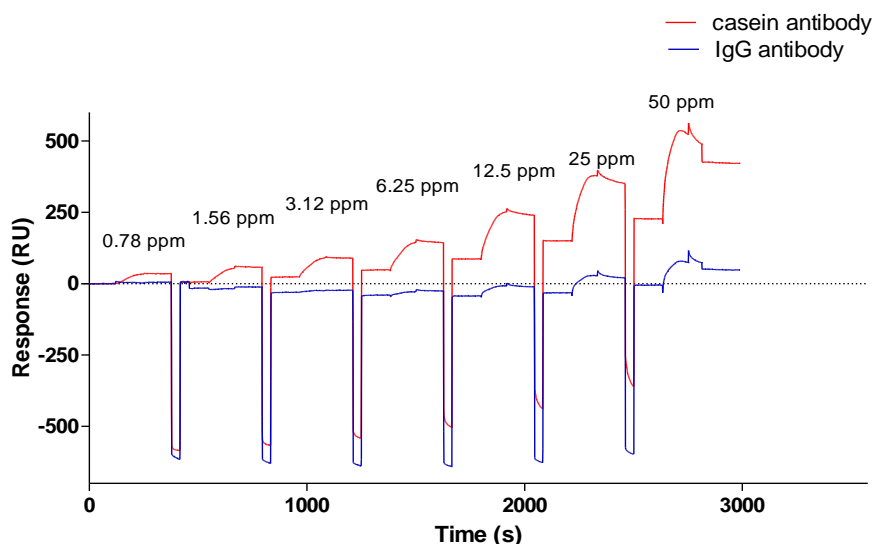
Fig. 2. Real time sensorgram of casein binding (0.78 -50 µg mL^{-1}) to the casein-antibody as the active sample and IgG antibody as the control, with NaOH (50 mM) regeneration. 10 mM PBS pH 7.4 was used as a flow buffer at a flow rate of 25 µL min^{-1}.

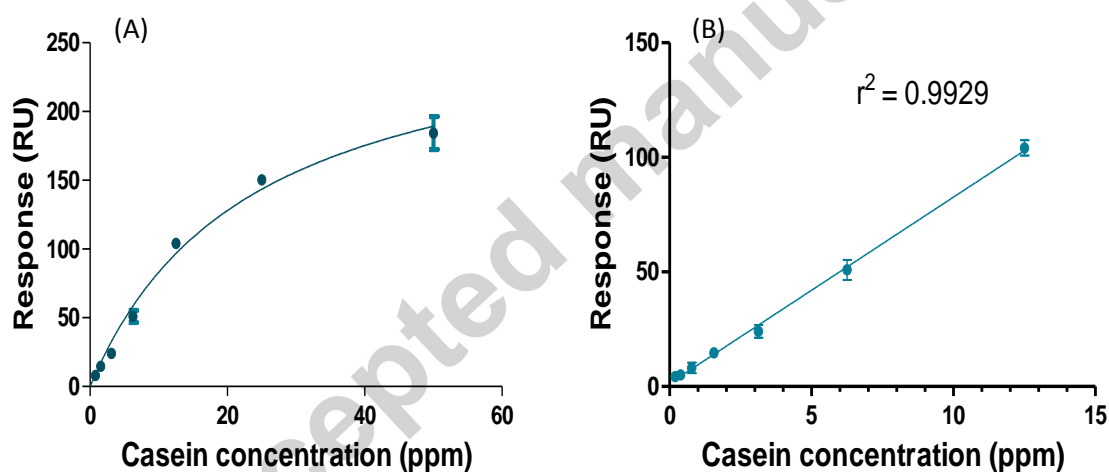
Fig. 3. (A) Nonlinear calibration plot and (B) linear calibration plot of the α-casein sensor response versus protein concentration (linear range = 0.195 ppm to 12.5 ppm). Assays were performed in duplicate and LOD was determined to be 57.80 ng mL^{-1}.

The selectivity of the sensor to detect α-casein was determined by comparing the response of BLG and k-casein to that given for α-casein. Fig 4, shows the relative responses of different protein injections over a range of concentrations. The antibody shows very little cross reactivity towards BLG while the k-casein shows some binding suggesting that the antibody has some cross reactivity towards different isoforms of casein. The polyclonal

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antibody was used for this particular study due to the lack of availability of monoclonal antibodies.
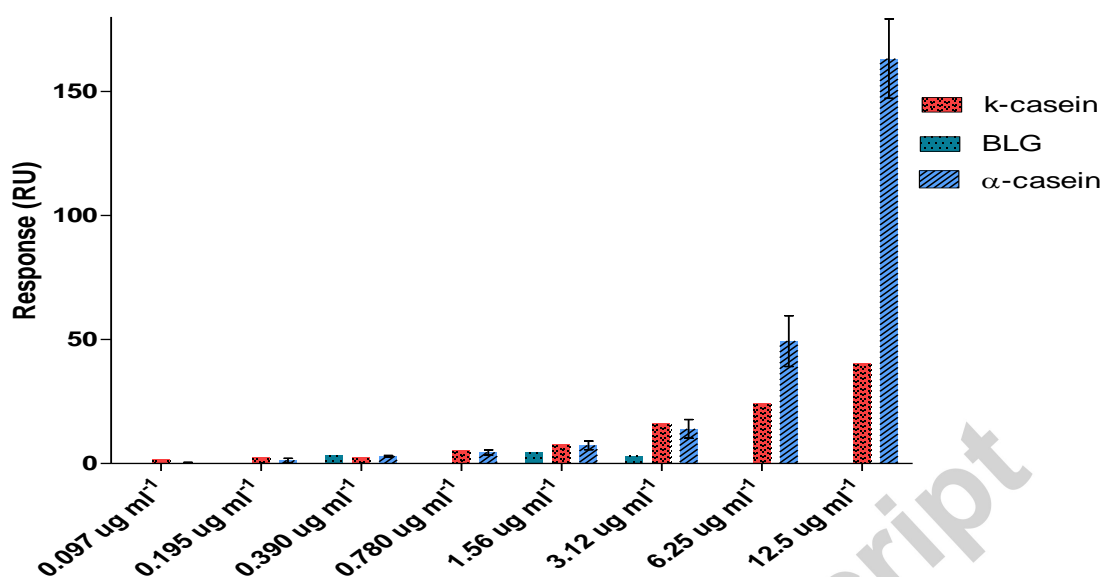

Fig. 4. Relative responses of α-casein, k-casein and BLG towards the sensor surface at different concentrations (0.097 – 12.5 μg mL^{-1}).

3.3   CIP final rinse sample analysis

To test the sensors ability to detect α-casein in CIP waste water, the binding assay was performed by injecting different concentrations of casein extracted from CIP final rinse water samples. Due to the unique properties of casein, the protein can be easily separated from other milk proteins using acidic conditions. Casein was extracted by adding 10 mM sodium acetate and centrifuging at 1000 g^{-1} for 5 minutes. The precipitate was washed several times with acetate buffer. The precipitate was reconstituted with 10 mM borate buffer at pH 9.0 and exchanged in a G-25 spin column equalibriated with PBS. The acid precipitation was used to remove other macromolecules such as other proteins, fats and carbohydrates while the gel column allowed for the removal of small molecules including detergents from the CIP samples. Table 1 shows the percentage recoveries for casein in CIP wash samples.

Table 1. Spiked sample percentage recoveries and RSDs of α-casein in final wash CIP samples after acid precipitation and gel filtration. Spikes were performed in duplicate.

Spiked concentration	Recovery %	%RSD

0.5 ppm	60.03	10.45
1.5 ppm	128.64	12.12
6 ppm	91.63	7.34
12 ppm	85.58	11.23

At low concentrations, the spiked recovery was below the accepted percentage recovery criteria of (± 20% recovery) with 0.5 ppm showing a percentage recovery of 60.03, ± 10.45 % and 1.5 ppm showing a recovery of 128.64, ± 12.12 %. As the spiked concentration increases, the percentage recoveries become more acceptable with 6 ppm and 12 ppm showing a percentage recovery 91.63 ± 7.34 % and 85.58 ± 11.23 % respectively, suggesting that the combination of acid precipitation and gel filtration can affect the recovery of the protein and the sensor's performance in measuring α-casein at low concentrations. At higher concentrations, the protein recovery does improve which further confirms that the sample extraction using gel filtration and acid precipitation is suitable for this application. It is also noted that the acid precipitation is a critical step in the sample preparation and that residues of sodium acetate can cause bulk effects in the sensorgram. This was a preliminary testing and further work is required to optimise the sample pre-treatment procedure and improve recoveries at the low protein concentrations. This proff of concept sensor is generic for α-casein detection and can be applied to other food samples for milk allergens detections, after taking in consideration sample handeling procedures.

4. Conclusion

An antibody based SPR sensor for α-casein was successfully fabricated and tested. The sensor showed good sensitivity as compared to other traditional methods such as ELISA showing a LOD of 57.8 ng mL^{-1}. The α-casein antibody was able to selectively bind to the analyte at over a range of concentations and that 50 mM NaOH was shown to adequetely regenerate the surface of the sensor. However, further optimisation is required to ensure full regeneration. It is also worth noting that batch to batch variation of antibodies can cause variation in the sensor performance. The sensor was found to give good recoveries at higher concentrations and the combination of gel filtration and acid precipitation could potentially be used as an extraction method for milk proteins from food samples.

It is important to emphasise that we have developed and shown the potential capability of an alpha-casein specific antibody SPR sensor. However, one possible next step

would be to utilise the same technology to generate antibody-based SPR towards other sources of food allergens that are concern to food manufacturing processes e.g. peanut, egg. It would also be of interest to test the sensor ability to detect the absence of residual protein in a CIP validation study as conducted by Stephan et al (Stephan et al., 2004). It is also clear that from the literature, there is a requirement for standardisation of milk protein standards.

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Figure Titles

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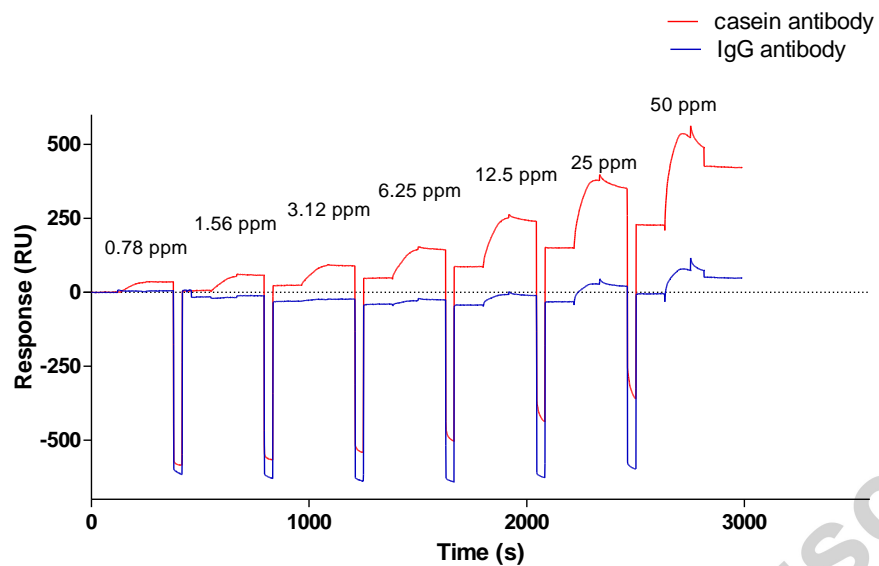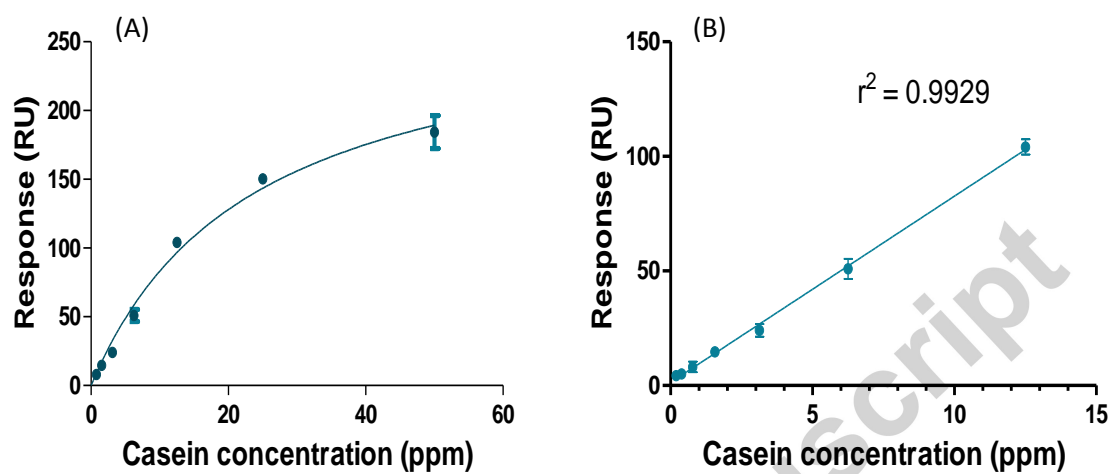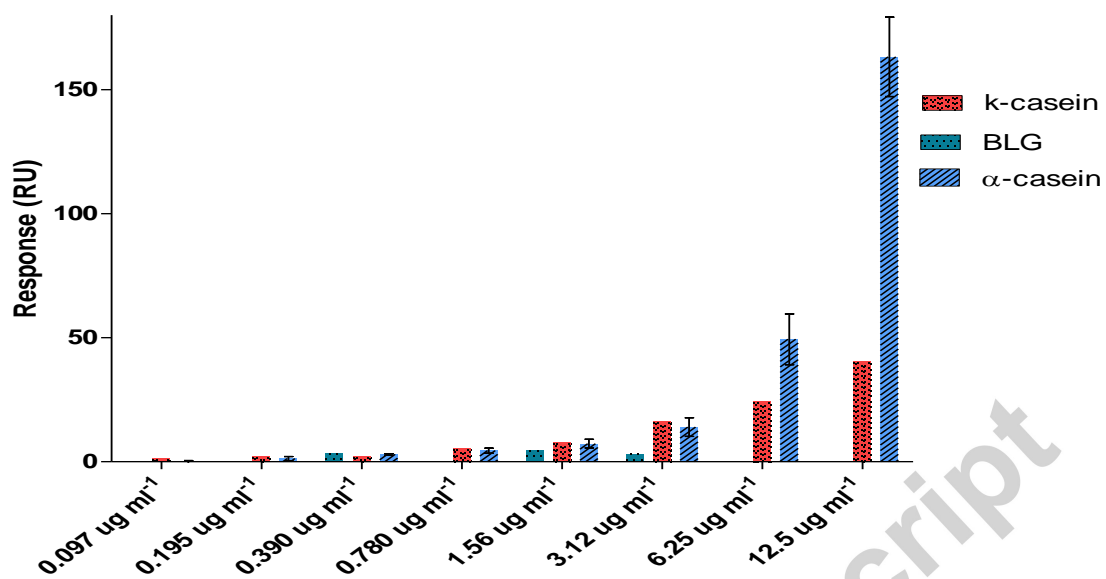
Fig. 1.
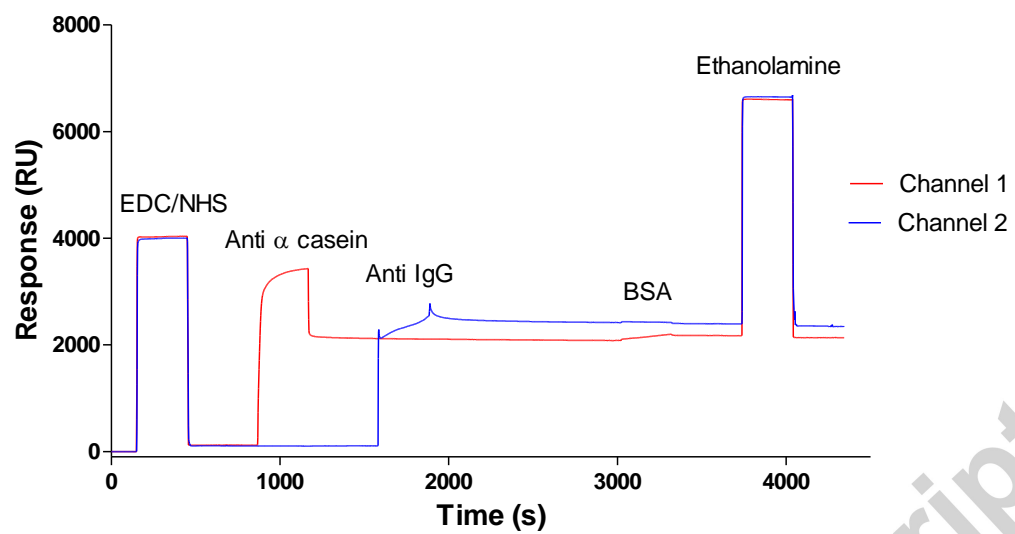
Fig. 2.

Fig. 3

Fig. 4

Table 1. Spiked sample percentage recoveries and RSDs of α-casein in final wash CIP samples after acid precipitation and gel filtration. Spikes were performed in duplicate.

Spiked concentration	Recovery %	%RSD
0.5 ppm	60.03	10.45
1.5 ppm	128.64	12.12
6 ppm	91.63	7.34
12 ppm	85.58	11.23

Highlights

- A Label-free SPR sensor was developed for milk allergens
- Direct immunoassay was constructed on the gold sensor chip.
- α-casein was used as the biomarker for cow's milk allergens detection
- Sample pre-treatment method was developed for CIP samples testing