



# An optical biosensor-based immunoassay for the determination of bovine serum albumin in milk and milk products



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## ABSTRACT

An automated biosensor immunoassay, exploiting surface plasmon resonance detection, is described for the quantitation of bovine serum albumin (BSA) in milk products. Antibody selection, assay conditions and potential non-specific binding interferences were defined. Analytical performance includes a working range of 10–1000 ng mL<sup>-1</sup>, a method detection limit of 0.02 mg g<sup>-1</sup> in milk, an instrument intermediate precision relative standard deviation (RSD<sub>IR</sub>) of 3.7%, an intermediate precision RSD<sub>IR</sub> of 8.9% for whey protein concentrate, and a single flow cell stable for at least 400 cycles. Accuracy was demonstrated by recovery, compliance with a certified reference material and comparison with a high performance liquid chromatographic method. The technique was applied to the estimation of BSA content of bovine milk, colostrum, whey protein fractions and infant formulae. The change in BSA expression during early bovine lactation and across a production season, and the thermal denaturation of BSA were also investigated.

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## 1. Introduction

Bovine serum albumin (BSA) is a carbohydrate-free, single-chain, 66.4 kDa globular protein with a pI of approximately 4.6, and is a component of the whey protein system in cows' milk, comprising approximately 1.5% of the total milk protein and 8% of the whey protein fraction. It consists of 583 amino acid residues of known sequence and includes 17 intramolecular disulphides and one free thiol group (Farrell et al., 2004). BSA is not synthesised in the mammary gland, but its presence in milk derives from passive diffusion from blood and it is therefore structurally and immunologically identical to blood serum albumin. The higher order structure of BSA facilitates its reversible binding to a wide range of low molecular mass ligands, and BSA has also been reported as possessing anticarcinogenic and antioxidant properties, although its biological function in milk remains obscure (Edwards & Jameson, 2014; Madureira, Pereira, Gomes, Pintado, & Malcata, 2007).

BSA has been implicated as a potential autoimmune trigger of insulin-dependent diabetes mellitus (IDDM), and the consumption

of bovine milk or milk-based paediatric formula during infancy has been linked with later onset IDDM, although the causal association remains a controversial topic (Persaud & Barranco-Mendoza, 2004). Hypersensitivity and allergy to bovine milk and milk-based paediatric formula amongst a small proportion of the human population has also implicated BSA as a significant allergenic protein (El-Agamy, 2007). The level of BSA in bovine milk has been used as a marker of the health of the mammary gland and of milk quality (Auldust, Walsh, & Thomson, 1998; Lieske, Jantz, & Finke, 2005) and the isolated protein has been extensively utilised in numerous biochemical test procedures as a blocking agent, hapten carrier, protein calibrant and surface immobilisation platform (Odunaga & Shazhko, 2013; Yang, He, Wang, Li, & Zhang, 2014).

Intact food allergen proteins are generally detected in food surveillance programmes by enzyme-linked immunosorbent assay (ELISA) or biosensor-based immunoassays, liquid chromatography (LC), mass spectrometry and DNA-based methods (Bremer, Smits, & Haasnoot, 2009; Cucu, Jacxsens, & De Meulenaer, 2013; Rebe Raz, Liu, Norde, & Bremer, 2010; Yman, Eriksson, Johansson, & Hellenäs, 2006). Methods reported specifically for the determination of BSA in milk also include colorimetry, radial immunodiffusion, ELISA, isoelectric focusing, SDS-PAGE and size exclusion chromatography, although high performance liquid chromatography (HPLC) procedures utilising a range of separation

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mechanisms have become the most commonly used platform for the simultaneous analysis of multiple whey proteins including BSA (do Amaral, Charles, & Brito, 1995; van Beresteijn & Meijer, 1996; Bobe, Beitz, Freeman, & Lindberg, 1998; Elgar et al., 2000; de Frutos, Cifuentes, Amigo, Ramos, & Diez-Masa, 1992; Lieske et al., 2005; Marincic, McCune, & Hendricks, 1999).

The surface plasmon resonance (SPR) optical biosensor platform has become a well-established real-time, label-free evanescent field technique for determining the concentration of both low and high molecular mass target analytes in complex food systems with minimal sample preparation. SPR detection is dependent on the change in refractive index at the sensor surface due to binding of the analyte to the immobilised ligand (Gauglitz, 2005; Narsaiah, Jha, Bhardwaj, Sharma, & Kumar, 2012). Although most commonly applied to single-analyte measurements, SPR biosensors have recently evolved to array formats for multiplex protein quantitation (Billakanti, Fee, Lane, Kash, & Fredericks, 2010; McGrath et al., 2013). Although SPR biosensor and quantum dot fluorescence techniques utilising molecularly imprinted polymers, silica or graphene oxide have recently been reported for the detection of BSA in simple model systems (Chiu & Huang, 2014; Wang & Wei, 2013; Yang et al., 2014), the use of a conventional SPR-based biosensor platform has not yet been reported for the determination of BSA content in milk. The aim of the present study was therefore to develop such an assay that would be applicable in the routine laboratory environment for the compliance testing of milk-based foods.

## 2. Materials and methods

### 2.1. Reagents

1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide-HCl (EDC, 0.4 M), N-hydroxysuccinimide (NHS, 0.1 M), ethanolamine-HCl (1 M, pH 8.5), sodium acetate buffer (10 mM, pH 4.5 and 5.0), glycine-HCl buffer pH 2.0, sensor chip CM5 and HBS-EP running buffer (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.005% surfactant P20, pH 7.4) were obtained from GE Healthcare. All water used was of >18 M $\Omega$  resistivity.

Affinity-purified sheep and rabbit polyclonal anti-bovine BSA antibodies (A10-113A and A10-127A respectively, 1 mg mL<sup>-1</sup>) were obtained from Bethyl Laboratories (Montgomery, TX, USA). Affinity-purified goat polyclonal anti-bovine BSA antibody (SC-50710, 0.2 mg mL<sup>-1</sup>) was obtained from Santa Cruz Biotechnology (Dallas, TX, USA) and lyophilised rabbit polyclonal anti-bovine BSA antibody (B7276) was sourced from Sigma-Aldrich (St. Louis, MO, USA). BSA from bovine serum (A4378) was obtained from Sigma-Aldrich. Antibody solutions were stable for 6 months during storage at 4 °C.

The bovine milk proteins  $\alpha$ -casein,  $\beta$ -casein,  $\kappa$ -casein,  $\alpha$ -lactalbumin,  $\beta$ -lactoglobulin, and immunoglobulin G (IgG) were obtained from Sigma-Aldrich and bovine lactoferrin was isolated from skim milk as described previously (Indyk, McGrail, Watene, & Filonzi, 2007).

### 2.2. Biosensor surface preparation

The four anti-bovine BSA antibodies were individually immobilised under instrument control (Biacore<sup>®</sup> Q optical biosensor, operating software v3.0.5, GE Healthcare, Uppsala, Sweden) on separately assigned flow cells of a CM5 sensor chip (GE Healthcare) by amine-coupling at 25 °C, following a protocol described previously (Indyk et al., 2007). Briefly, each surface was activated with a mixture of EDC and NHS (1:1, v/v, 10  $\mu$ L min<sup>-1</sup>, 7 min) followed by antibody (50  $\mu$ g mL<sup>-1</sup> in 10 mM sodium acetate, pH 5.0, 10  $\mu$ L min<sup>-1</sup>,

7 min). Similarly, BSA was immobilised (25  $\mu$ g mL<sup>-1</sup> in 10 mM sodium acetate, pH 4.5, 10  $\mu$ L min<sup>-1</sup>, 7 min) on a separately designated chip surface. Following ligand immobilisation, unreacted ester functionalities were deactivated with ethanolamine (1 M, pH 8.5, 10  $\mu$ L min<sup>-1</sup>, 7 min). A reference flow cell surface was prepared similarly by omitting the ligand immobilisation step. Functionalised chips were stored in a sealed container between analyses over silica gel desiccant at 4 °C.

### 2.3. Standards

Bovine BSA was dissolved in water to approximately 5 mg mL<sup>-1</sup>, the concentration accurately determined spectrophotometrically ( $E_{1\text{cm}}^{1\%}$  at 280 nm = 6.6), and stored frozen. An intermediate standard was prepared at 100  $\mu$ g mL<sup>-1</sup> in HBS-EP buffer and sub-aliquots were stored at -18 °C. BSA working standards (10–1000 ng mL<sup>-1</sup>) were prepared daily by sequential dilution from a 1:100 (v/v) fold dilution of the intermediate standard in HBS-EP buffer and used to generate a calibration curve for the estimation of BSA content in unknown milk-based samples.

Stock solutions of other individual milk proteins were separately prepared to ~10 mg mL<sup>-1</sup> in water and were stored frozen, from which working dilutions (1–10  $\mu$ g mL<sup>-1</sup>) were prepared in HBS-EP buffer for the evaluation of antibody cross-reactivity.

### 2.4. Samples

Liquid consumer milks, milk powders, whey protein concentrates, whey protein isolates and a range of infant formulae were obtained from milk processing facilities and commercial sources. Samples were prepared for analysis by initial vortex dissolution in water (1:20, w/v; 10 min) aided by ultrasonication (15 min), followed by serial dilution in HBS-EP buffer to the final dilution level (liquid milk, 1:1000; protein concentrates, 1:50,000; infant and adult nutritional formulae, 1:5000).

Raw bovine milk samples were collected mid-flow from the same quarter of a single 10-year-old Jersey cow (fourth calving) from prior to imminent calving until 28 days post partum and were prepared as described for liquid milk, and extracts were stored at -18 °C until analysis. Skim milk powders were prepared monthly across the 2011–2012 New Zealand production season from pooled pasteurised herd skim milk using a pilot-scale spray drier, sealed in laminated sachets and stored at 4 °C, and sample preparation for analysis was as described for milk powders.

A standard reference material SRM 927e containing certified levels of BSA isolated from bovine blood serum was sourced from the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA) and was prepared for analysis by serial dilution in HBS-EP buffer to 1:100,000 and 1:500,000 (v/v).

### 2.5. Direct biosensor assay

For the optimised direct assay protocol recommended for routine application, BSA working standards or sample extracts were dispensed (100  $\mu$ L) into a 96-well microtitre plate and, under automated instrument control, were injected for 3 min at 10  $\mu$ L min<sup>-1</sup> over an immobilised sheep anti-bovine BSA surface with HBS-EP running buffer at 25 °C. Binding responses relative to the baseline were acquired 30 s after injection termination and were used for the generation of a calibration curve and the interpolation of unknown samples. Surface ligand regeneration was achieved by the sequential injection of 18  $\mu$ L and 10  $\mu$ L of 10 mM glycine-HCl, pH 2.0 at 50  $\mu$ L min<sup>-1</sup>. Dose-response calibration curves were generated by four-parameter fit regression analysis under instrument software control [ $y = R_{hi} - ((R_{hi} - R_{lo})/1 + (Conc/$

$A_1 \cdot A_2$ ], where  $R_{hi}$  = response at infinite concentration,  $R_{lo}$  = response at zero concentration,  $A_1 = B_{50}$ ,  $A_2$  = slope factor and  $Conc$  = concentration ( $\text{ng mL}^{-1}$ ).

## 2.6. Method validation

Four commercially available BSA-specific antibodies were initially evaluated for an SPR-based biosensor immunoassay by comparing their binding isotherms over a BSA-immobilised surface. The specificity of immobilised anti-BSA antibodies were further evaluated by determination of cross-reactivity against individual milk proteins, and the maximum binding capacity ( $R_{max}$ ) was estimated by sequential injection of BSA without regeneration. Antibody specificity was further confirmed under optimised direct assay conditions by competition with the same protein in solution.

To further evaluate the specificity of the optimised direct assay format, the measured BSA content of a range of samples was compared against results obtained from both sandwich-enhancement and inhibition formats. For the sandwich assay, a second anti-bovine BSA antibody ( $50 \mu\text{g mL}^{-1}$ ) was injected over bound BSA for 3 min at  $10 \mu\text{L min}^{-1}$  immediately following the described direct assay protocol. The binding responses acquired 30 s after the end of the secondary injection were measured relative to the primary binding response and were used for the generation of a calibration curve and the interpolation of unknown samples. For the inhibition assay, BSA calibrants or sample extracts were mixed (1:1) under instrument control with sheep anti-BSA ( $1.0 \mu\text{g mL}^{-1}$ ) prior to injection for 3.5 min at  $10 \mu\text{L min}^{-1}$  over an immobilised BSA surface with HBS-EP running buffer. The binding response relative to the baseline was acquired 30 s after injection termination and was used for the generation of a calibration curve and the interpolation of unknown samples. For both assay formats, surface regeneration was accomplished as described for the direct assay format.

Non-specific binding of BSA and sample matrix components was quantified over an unfunctionalised reference surface, and the recommended direct immunoassay protocol was optimised for sample preparation, contact time, flow rate, stability and surface regeneration procedure. Analytical performance attributes with respect to range, detection limits, sensitivity, precision and recovery were defined, and method accuracy assessed against both a certified reference material and a liquid chromatographic technique (Elgar et al., 2000).

## 3. Results and discussion

### 3.1. Binding protein: selection and properties

Proteins of high affinity for target analyte are favoured for quantitative immunoassays, and four candidate commercially available BSA-specific polyclonal antibodies (pAb) of variable affinity were initially evaluated through binding to a high density BSA-immobilised surface under identical inhibition assay conditions (pAb:BSA ratio, contact time, flow rate and regeneration), as illustrated in Fig. 1. Whereas three antibody preparations demonstrated significant uninhibited binding responses, and were effectively inhibited by BSA in solution in a predicted dose–response manner, one antibody failed to bind to the BSA surface.

Following their individual immobilisation to equivalent ligand levels (approximately 10 kRU), the four antibodies were further characterised by injection of BSA in a direct-binding assay format, as presented in Fig. 2. The binding characteristics of the four immobilised antibodies under direct assay conditions were consistent with observations under inhibition assay conditions, with the same antibody demonstrating an absence of binding to

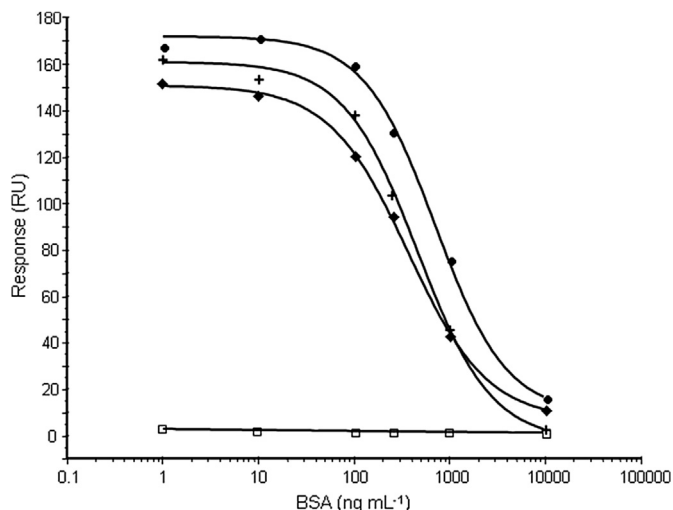


Fig. 1. Binding response (RU) of polyclonal anti-BSA antibodies injected over a single BSA-immobilised sensor surface under inhibition assay conditions: (●) rabbit (Sigma,  $2.5 \mu\text{g mL}^{-1}$ ); (+) sheep (Bethyl,  $1.0 \mu\text{g mL}^{-1}$ ); (◆) rabbit (Bethyl,  $1.0 \mu\text{g mL}^{-1}$ ); (□) goat (Santa Cruz,  $5.0 \mu\text{g mL}^{-1}$ ).

injected BSA. On the basis of its higher affinity for BSA and its superior performance relative to the other candidate proteins under both assay formats, the affinity-purified sheep polyclonal anti-bovine BSA antibody was selected for further evaluation, consistent with a previously reported SPR multiplex assay for whey proteins (Billakanti et al., 2010).

High surface binding capacity was achieved by immobilisation of the selected antibody via covalent amine coupling, a technique characterised by random antibody paratope orientation, resulting in a ligand density of approximately 10,000 response units (RU, where 1 RU is a change in refractive index of  $10^{-6}$ , equivalent to  $1 \text{ pg mm}^2$  protein), representing approximately 65 fmol protein. As analyte binding capacity also depends on ligand orientation, the maximum BSA binding capacity  $R_{max}$  was estimated to be  $> 0.3$  of the theoretical value under direct assay conditions, and confirms that the recommended immobilisation protocol generated a ligand surface suitable for the quantitation of BSA.

Specificity was evaluated under direct assay conditions through estimation of the extent of inhibition by antibody in solution. Thus, when authentic BSA or diluted milk containing BSA at

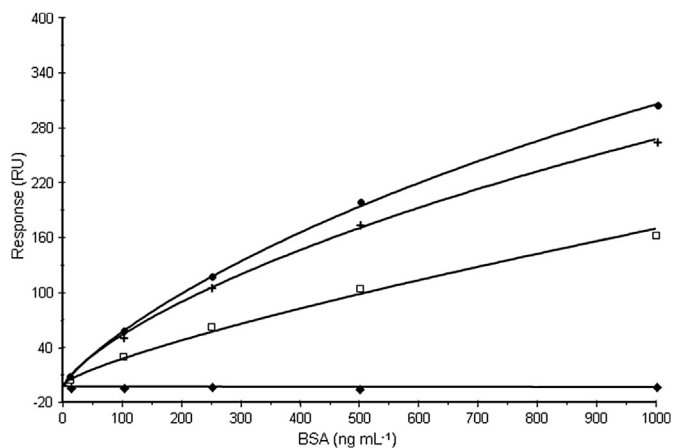


Fig. 2. Binding response (RU) of BSA injected over four independent anti-BSA immobilised flow cells under direct assay conditions: (+) rabbit (Sigma); (●) sheep (Bethyl); (□) rabbit (Bethyl); (◆) goat (Santa Cruz).

approximately  $5 \text{ pmol mL}^{-1}$  was equilibrated with excess antibody (approximately  $250 \text{ pmol mL}^{-1}$ ), binding to the functionalised surface was inhibited by  $> 99\%$ , confirming both specificity and absence of non-specific binding.

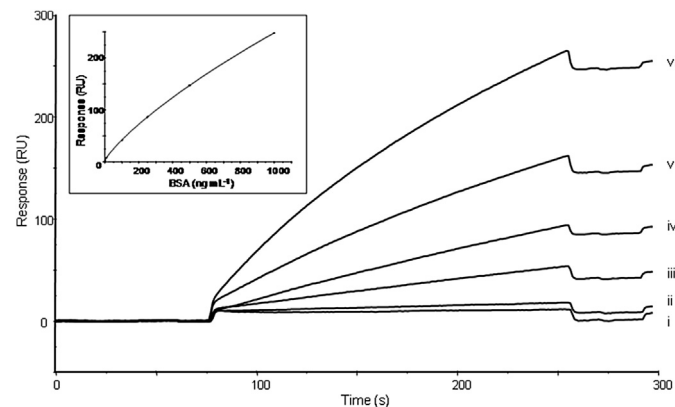
Cross-reactivity of the selected immobilised anti-BSA polyclonal antibody towards authentic bovine milk proteins was assessed, and binding was found to be essentially specific for BSA. A minor cross-reactivity to  $\kappa$ -casein and lactoferrin ( $<2.0\%$  and  $<1.5\%$  respectively, relative to BSA) was observed, and responses for these proteins at concentrations equivalent to diluted milk samples of  $<6 \text{ RU}$  represent a potential analytical bias of 2–4% for a typical estimation of BSA content.

### 3.2. Method development and validation

Immunoassays, including that based on the described SPR biosensor platform, are potentially vulnerable to non-specific binding interferences that therefore require investigation (Breault-Turcot, Chaurand, & Masson, 2014; Situ, Wylie, Douglas, & Elliott, 2008). Binding to the carboxymethyl dextran support due to BSA and sample matrix components was therefore evaluated over the reference surface and non-specific binding responses for BSA standards ( $<1 \text{ RU}$ ) and for a range of milks, whey protein concentrates and infant formulae ( $<5 \text{ RU}$ ) were confirmed to be analytically insignificant. Given the absence of milk devoid of BSA, any possible binding interferences due to unknown milk constituents was further evaluated by analysis of multiple ( $n = 4$ ) dilutions of milk, whey concentrate, and paediatric formula over the anti-BSA tethered surface. BSA levels were found to be independent of dilution with a relative standard deviation (RSD) of  $<5.0\%$  and calculated contents within  $\pm 10\%$  of the mean, providing further evidence that analytical response is specifically a function of BSA concentration (Chen & Eitenmiller, 2007). These trials also served to define appropriate dilution levels for routine method application.

Sensorgrams and a typical dose–response curve under the described direct assay conditions are illustrated in Fig. 3. That the initial binding rate was found to be slightly dependent on flow rate between  $10$  and  $75 \mu\text{L min}^{-1}$  suggested a partial mass diffusion influence, and the recommended end-point binding level measurement strategy was therefore found to be optimal for the reliable quantitation of BSA.

The sandwich immunoassay mode was evaluated for its potential to enhance assay sensitivity relative to the direct assay mode



**Fig. 3.** Overlaid sensorgrams obtained for the concentration series of BSA at (i)  $0 \text{ ng mL}^{-1}$ , (ii)  $10 \text{ ng mL}^{-1}$ , (iii)  $100 \text{ ng mL}^{-1}$ , (iv)  $250 \text{ ng mL}^{-1}$ , (v)  $500 \text{ ng mL}^{-1}$ , and (vi)  $1000 \text{ ng mL}^{-1}$  under optimised direct assay conditions (regeneration phase of each cycle removed for clarity). Inset: dose–response calibration derived from relative binding response (RU).

and as a further verification of antibody specificity. As expected, the same polyclonal anti-bovine BSA antibody employed as the secondary reagent generated an enhanced analytical signal due to the higher molecular mass of IgG relative to that of surface-captured BSA, with the analysis of milk and whey protein concentrate samples yielding equivalent BSA levels by both techniques. Due to increased analysis time and complexity and despite its enhanced sensitivity, the sandwich assay was not implemented for routine application.

In view of the high molecular mass of proteins and the dependence of the SPR response on the refractive index change at the sensor interface associated with the binding event, biosensor-based protein immunoassays may be performed under either direct or inhibition configuration. In general, although the inhibition assay (immobilised target protein) is considered to be more versatile with respect to modifying assay sensitivity, the direct assay (immobilised antibody) provides a single reagent format, a requirement for very small quantities of antibody and a wider working range (Bremer et al., 2009). Analysis under inhibition assay conditions yielded the expected inverse dose–response calibration and was applied to the analysis of several milk, whey protein isolate, and paediatric formula samples covering a range of BSA content. The BSA levels were comparable with those achieved by direct assay with an estimated method bias of  $<10\%$  ( $p_{0.05} = 0.1$ ), and a linear regression equation of  $y = 1.01x + 0.22$  ( $r^2 = 0.9986$ ). The absence of non-specific binding from sample matrix components under inhibition assay conditions was confirmed by injecting extracts in the absence of antibody ( $<4 \text{ RU}$ ). Despite the suitability of the inhibition assay protocol, the direct immunoassay format, in view of its practical advantages, was recommended for routine application to the quantitation of BSA.

Two important assay conditions are injection contact time and surface ligand regeneration. Contact time was optimised for the selected immobilised antibody under direct-binding assay conditions. As predicted, increasing the contact time from 2 to 4 min shifted the calibration curve to lower  $B_{50}$  values (BSA concentration yielding 50% maximum response). Although measured BSA concentrations in milk and whey protein concentrate samples were independent of contact time ( $<2.5\%$  RSD), a 3-min injection was determined to be optimal for the recommended sample dilution levels. Regeneration of the antibody-immobilised surface following binding of BSA was routinely accomplished with glycine ( $10 \text{ mM}$ ) at pH 2.0, as demonstrated by the stable baseline response after 45 cycles ( $RSD_r = 0.28\%$ ).

In common with biospecific immunoassay techniques in general, the specificity and sensitivity of the SPR detection platform facilitates a minimal sample preparation protocol compared with alternative non-immunoassay analytical techniques. Thus, the described biosensor immunoassay for BSA is expedited following direct sample dilution in running buffer without the requirement for any further clean-up strategies.

### 3.3. Method performance

Over the calibration range ( $10$ – $1000 \text{ ng mL}^{-1}$ ) illustrated in Fig. 3, a four-parameter logistic regression effectively summarised the dose–response association, with acceptable intermediate precision of the estimated  $B_{50}$  value over multiple assays and three independently immobilised chip surfaces ( $RSD = 9.8\%$ ,  $n = 12$ ). The instrumental limit of detection (response  $+ 3 \text{ sd}$  of blank) was estimated to be  $2.5 \text{ ng mL}^{-1}$  ( $n = 7$ ), and the method detection limit ( $MDL = \text{sd} \times t_{n-1, 0.01}$ ) determined by analysis of a fluid milk sample in replicate ( $n = 7$ ) was  $0.02 \text{ mg g}^{-1}$ , with a calculated method limit ( $ML = MDL \times 3.18$ ) of  $0.06 \text{ mg g}^{-1}$  (Su, 1998).

Instrument repeatability, evaluated by replicate injection of a BSA calibrant ( $100 \text{ ng mL}^{-1}$ ), yielded an  $RSD_r$  of 1.9%. Instrumental

precision was further estimated with a single-level control BSA calibration standard ( $250 \text{ ng mL}^{-1}$ ) over separate sequential assays yielding an  $\text{RSD}_{\text{IR}}$  of 3.7% ( $n = 12$ ). Although significant sample dilution was required to achieve optimal analytical responses proximate to the  $B_{50}$  value, the repeatability  $\text{RSD}_r$  for milk, infant formula and whey protein were 2.3, 6.5 and 3.1% respectively ( $n = 7$ ), while intermediate precision  $\text{RSD}_{\text{IR}}$  estimated over multiple assays was 12.1% ( $n = 13$ ) for fluid milk, 10.7% ( $n = 6$ ) for a control infant formula powder and 8.9% ( $n = 7$ ) for a whey protein concentrate powder.

The accuracy of a method may be evaluated based on data obtained from recovery, method comparison and analysis of an appropriate SRM. Recovery was estimated at 103.5% (95.4–108.2%), over two independent immobilised surfaces, through the addition of BSA to skim milk at  $0.5 \times$ ,  $1.0 \times$  and  $1.5 \times$  natural levels. BSA levels derived from the biosensor immunoassay were compared with data from a reversed-phase liquid chromatography (RPLC) method (Elgar et al., 2000) for dairy products including liquid skim milk, spray-dried milk, paediatric formulae and whey protein fractions, with the data correlation illustrated in Fig. 4. Although these methods exploit inherently dissimilar strategies, the biosensor immunoassay provides a comparable estimate of BSA content relative to the RPLC method ( $p_{0.05} = 0.073$ ). Thus, the requirement for casein removal prior to RPLC, and the potential for the biosensor immunoassay to distinguish native from denatured BSA, differentiate the attributes of these divergent analytical techniques (Dupont, Rolet-Repecaud, & Muller-Renaud, 2004; Elgar et al., 2000). NIST SRM 927e is a standard reference material containing BSA isolated from bovine blood and prepared in buffer with assigned values based on isotope dilution LC-MS/MS (certified:  $67.38 \pm 1.38 \text{ mg mL}^{-1}$ ) and biuret (reference:  $69.58 \pm 1.30 \text{ mg mL}^{-1}$ ) methods. The biosensor immunoassay yielded a BSA content of  $68.3 \pm 2.7 \text{ mg mL}^{-1}$  ( $n = 4$ ), confirming an absence of bias based on equivalence with SRM assigned values.

Replicate within-run analysis demonstrates the stability of the tethered immunoglobulin with respect to binding capacity. Thus, the repeatability  $\text{RSD}_r$  of the relative binding response was 2.03% for a control BSA calibrant ( $n = 15$ ) and 2.07% ( $n = 15$ ) for a liquid milk extract. The repeatability  $\text{RSD}_r$  of the absolute baseline response was measured as 0.28% ( $n = 45$ ), with a mean baseline drift of  $<3 \text{ RU cycle}^{-1}$  confirming both ligand stability and the efficacy of the regeneration procedure. It was further demonstrated

that a single anti-bovine BSA immobilised flow cell surface was stable over  $> 400$  injections across multiple storage cycles at  $4^\circ\text{C}$ .

### 3.4. Method applications

The inherent specificity and sensitivity of the described SPR-based biosensor immunoassay supported its application to bovine milk products, subsequent to a facile sample preparation that involved direct dilution in buffer.

The BSA content of consumer milk acquired over the southern hemisphere collection period (August 2013–March 2014) was estimated to be  $0.23 \text{ mg mL}^{-1}$  (range  $0.19$ – $0.27 \text{ mg mL}^{-1}$ ;  $n = 14$ ), with a minor but consistent increasing trend over time. Such levels are consistent with a range of  $0.1$ – $0.9 \text{ mg mL}^{-1}$  previously reported by several alternative analytical methods (Auld et al., 1998; Barłowska, Litwińczuk, Brodziak, & Chabuz, 2012; Billakanti et al., 2010; Farrell et al., 2004; Levieux & Ollier, 1999; Lieske et al., 2005; Marincic et al., 1999; Poutrel, Caffin, & Rainard, 1983). Although it is known that breed, lactation stage, diet and health status influence bovine milk protein levels, it is likely that analytical variability is probably a greater factor in the range of reported bovine milk BSA content (Elgar et al., 2000). As expected, a similar seasonally dependent minor increasing trend was confirmed in skim milk powder manufactured over the 2011–2012 production season, with a mean of  $2.3 \text{ mg g}^{-1}$  (range  $1.7$ – $3.2 \text{ mg g}^{-1}$ ;  $n = 12$ ).

The composition of mammalian milk is influenced by the physiological stage of lactation, and the biosensor immunoassay was therefore applied to study bovine BSA content during the transition from colostrum to milk. Fig. 5 illustrates the trend for a single pasture-fed lactating cow during the 28 days subsequent to parturition. The mean content of BSA was found to be significantly higher in early colostrum ( $< 1$  day) ( $0.64 \text{ mg mL}^{-1}$ ) than in transitional and mature milk ( $0.12 \text{ mg mL}^{-1}$ ) illustrating a considerable physiological response to early lactogenesis, an observation consistent with reported data based on the radial immunodiffusion technique (Levieux & Ollier, 1999; Ostensson, 1993). The elevated levels of BSA during lactogenesis in very early colostrum have been attributed to increased mammary gland epithelial permeability to components that originate in blood serum (Auld et al., 1998; Ostensson, 1993).

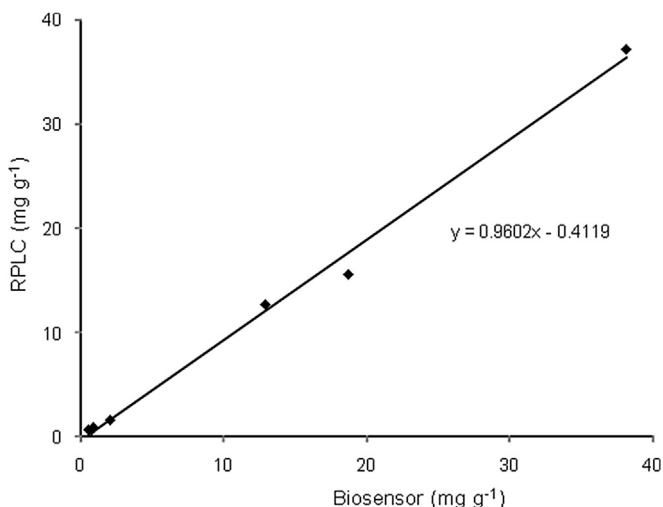


Fig. 4. BSA levels ( $\text{mg g}^{-1}$ ) estimated in a range of milk products by the SPR-based immunoassay compared with those determined by RPLC ( $n = 2$ ).

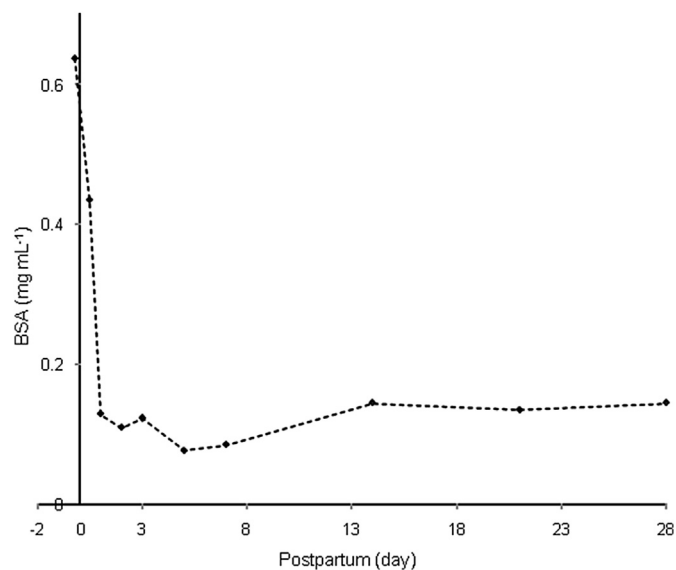


Fig. 5. BSA content ( $\text{mg mL}^{-1}$ ) of early lactation milk acquired from a single Jersey cow. Each value represents the mean of duplicate analyses under the recommended direct assay conditions.

In view of the reported association with IDDM and allergenicity, the potential for the described quantitative biosensor immunoassay for BSA was evaluated in a range of bovine milk products (Table 1). The method was demonstrated to be appropriate for the routine quality and compliance release of paediatric formulae, and the evaluation of raw ingredients utilised in their production, and may be implemented as part of food safety surveillance. It should be noted that, in common with all analytical techniques for allergens, the complete absence of potentially allergenic BSA cannot be guaranteed, unless the product is based on hypoallergenic ingredients.

Protein biological function is critically dependent on three-dimensional folded structure and the native state generally corresponds to the most thermodynamically stable conformation under physiological conditions (Price, 2000). Thermal denaturation of milk whey proteins has been comprehensively studied, with focus on  $\alpha$ -lactalbumin,  $\beta$ -lactoglobulin, IgG, lactoferrin and lactoperoxidase. In general, reported data for reaction order vary between 1 and 3, probably due to the wide range of experimental conditions and analytical methods used (Anema, 2014; Anema & McKenna, 1996; Anema, Stockmann, & Lowe, 2005; Aprodu, Stănciuc, Dumitrascu, Răpeanu, & Stanciu, 2014; Hinrichs & Rademacher, 2005; Indyk et al., 2007; Mayayo et al., 2014; Riera et al., 2014). Whey protein denaturation is considered to proceed through a multistage process, initiated through a reversible, non-covalent unfolding that generally complies with first-order kinetics, followed by subsequent irreversible non-unimolecular aggregation pathways with reaction order highly dependent on environmental reaction conditions.

As the conformational integrity of BSA in paediatric milk-based formulae has potential implications for both IDDM and allergenicity, its resistance to denaturation during industrial processing is of importance (Marincic et al., 1999). The multiple disulphide bond content renders BSA relatively vulnerable to thermal denaturation (Madureira et al., 2007) and the subsequent loss of conformational epitopes reduces interaction with the anti-BSA polyclonal antibody. Consequently, the SPR-based biosensor immunoassay described technique will yield quantitative information on the content of native undenatured BSA, and indeed this analytical platform has been demonstrated to be a valuable technique to complement existing analytical methods for estimating protein denaturation (Anema, 2014). The biosensor immunoassay was therefore applied in a preliminary assessment of the thermal denaturation of BSA in buffer ( $10 \mu\text{g mL}^{-1}$ ) and in diluted skim milk (1:10, v/v). At  $70^\circ\text{C}$  and  $80^\circ\text{C}$ , the loss of native protein was approximately 30 and 80% respectively over 4 h. At  $80^\circ\text{C}$ , the loss of native BSA conformed to a reaction order of 1 over the initial time period (1 h), consistent with

reversible unfolding. An overall reaction order closer to 2 was observed over the entire 4 h, which probably reflects irreversible aggregation of the unfolded protein.

These results are consistent with previous studies using a wide range of analytical techniques that have reported on the thermal denaturation of BSA and proposed a mechanism involving initial unfolding exposing the interior single cysteine and further mediation by thiol–disulphide exchange (Anema, 2014; Aoki, Sato, Nagaoka, Kamada, & Hiramatsu, 1973; Cao, Yang, Shi, Liu, & Wang, 2008; Edwards & Jameson, 2014; Haque, Aldred, Chen, Barrow, & Adhikari, 2014; Oldfield, Singh, & Taylor, 2005; Paulsson, Hegg, & Castberg, 1985; Rüegg, Moor, & Blanc, 1977; Savadkoochi, Bannikova, Kasapis, & Adhikari, 2014; Takeda, Wada, Yamamoto, Moriyama, & Aoki, 1989).

#### 4. Conclusions

The analytical performance characteristics of biosensor-based immunoassay methods are determined by affinity, ligand selection, immobilisation chemistry, buffer conditions, contact time and regeneration protocol. Sheep polyclonal anti-bovine BSA provided an analyte-binding surface that was stable to multiple regeneration and yielded low detection limits. The sensitivity of SPR detection facilitated high sample dilution and minimal non-specific binding. The described real-time, label-free automated biosensor immunoassay for the quantitation of BSA in bovine milk products and paediatric formulae is rapid, sensitive, precise and accurate, and provides data comparable to that from an independent HPLC method.

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**Table 1**

Bovine serum albumin levels ( $\text{mg g}^{-1}$ ) in dairy products as determined by a surface plasmon resonance-based biosensor assay.<sup>a</sup>

Sample	Mean	Relative standard deviation (%)	n
SM	0.23	10.71	14
SMP	1.20	4.14	4
IF	1.05	10.69	8
RWP	2.11	2.39	4
WP	0.65	12.06	4
WPC	21.61	8.21	12
CWPC	14.13	11.71	4
WPI	40.30	7.37	4

<sup>a</sup> Abbreviations are: SM, liquid skim milk; SMP, skim milk powder; IF, infant formula powder; RWP, rennet whey powder; WP, demineralised (90%) whey powder; WPC, whey protein concentrate (>80% protein); CWPC, cheese whey protein concentrate; WPI, whey protein isolate; n, number of independent analyses of each sample.

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