International Dairy Journal 73 (2017) 68–73

Contents lists available at ScienceDirect

International Dairy Journal

journal homepage: www.elsevier.com/locate/idairyj

The β-lactoglobulin content of bovine milk: Development and application of a biosensor immunoassay

Harvey E. Indyk [a, *], Selena Hart [a], Theo Meerkerk [a], Brendon D. Gill [a], David C. Woollard [b]

[a] Fonterra Co-operative Group Ltd, SH 26, Waitoa, New Zealand
[b] Eurofins NZ, Auckland, New Zealand

ARTICLE INFO

Article history:
Received 10 April 2017
Received in revised form
25 May 2017
Accepted 30 May 2017
Available online 12 June 2017

ABSTRACT

An optical biosensor immunoassay exploiting surface plasmon resonance is described for the quantification of β-lactoglobulin in milk. Samples were diluted with buffer, and the protein estimated from binding with a polyclonal antibody immobilised on the sensor surface. Analytical method performance characteristics including range, detection limit, precision and accuracy were determined and reported. The temporal variability in the β-lactoglobulin content of milk from pasture-fed cows during early lactation and across a production season was investigated. The content of β-lactoglobulin decreased from >10 mg mL^{-1} in early colostrum to <5 mg mL^{-1} in mature milk, and the β-lactoglobulin content of skim milk powder trended from 25 to 60 mg g^{-1} across a season. In view of its allergenicity, these data will improve understanding of the expression of innate β-lactoglobulin in the milk of pasture-grazed dairy herds, thereby providing information that is applicable to the formulation of bovine milk-based products.
© 2017 Elsevier Ltd. All rights reserved.

1. Introduction

β-Lactoglobulin (β-Lg) is an 18.4 kDa globular lipocalin protein that consists of 162 amino acid residues with a pI of approximately 5.4 and is the dominant whey protein in bovine milk at a concentration of 2–4 g L^{-1} of skim milk, representing approximately 60% of the total whey protein. It is synthesised within the epithelial cells of the mammary gland from blood precursors and commonly occurs in bovine milk, predominantly in both A and B genetic variants, each differing in two single amino acid residues, with both forms being vulnerable to pH-dependent conformational changes. The physiological functions of β-Lg remain speculative, but may involve hydrophobic ligand transport and uptake, enzyme regulation and neonatal passive immunity, although its over-expression in many, but not all, species suggests that its major role in milk may be nutritional (Farrell et al., 2004; Kontopidis, Holt, & Sawyer, 2004; Madureira, Pereira, Gomes, Pintado, & Malcata, 2007; Qin, Bewley, Creamer, Baker, & Jameson, 1999). The known lability of β-Lg to thermal denaturation has been studied in detail, in view of its importance to commercial milk processing and milk functionality (Anema, 2014; Considine, Patel, Anema, Singh, & Creamer, 2007; Delahaije, Gruppen, van Eijk–van Boxtel, Cornacchia, & Wierenga, 2016; Edwards & Jameson, 2014; Leeb, Götz, Letzel, Cheison, & Kulozik, 2015; Manzo, Nicolai, & Pizzano, 2015).

The recent ability to compare the genome of *Bos taurus* with that of other mammals has thus far been unable to rationalise the notable absence of β-Lg in human milk (Lemay et al., 2009). Native β-Lg, specific β-Lg peptide domains released during gastrointestinal digestion, or β-Lg derived peptide epitopes that are persistent in whey protein hydrolysates are thus considered to be implicated in the incidence of infant allergy to bovine milk, the most prevalent childhood food allergy, which is a significant issue given the predominant use of bovine milk in infant formula manufacture (Bu, Luo, Chen, Liu, & Zhu, 2013; Picariello et al., 2015). The most commonly recommended formula alternative for allergic infants is a product that is promoted as hypoallergenic and is assembled with hydrolysed whey protein, despite the uncertainties and potential risks associated with this claim (Boyle et al., 2016; Caira et al., 2012; Claeys et al., 2014; Docena, Rozenfeld, Fernández, & Fossati, 2002; El-Agamy, 2007).

Food proteins are generally quantified in either allergy surveillance programmes or compositional surveys by various immunoassay, DNA-based and electrophoresis platforms. However, high performance liquid chromatography procedures utilising a range of

* Corresponding author. Tel.: +64 7 889 0899.
E-mail address: harvey.indyk@fonterra.com (H.E. Indyk).

http://dx.doi.org/10.1016/j.idairyj.2017.05.010
0958-6946/© 2017 Elsevier Ltd. All rights reserved.

separation mechanisms have become the most commonly used platform for the simultaneous analysis of multiple whey proteins including β-Lg and, when coupled with mass spectrometric detection, provide a confirmatory technique in allergen control (Anema, 2009; Boitz, Fiechter, Seifried, & Mayer, 2015; Bremer, Smits, & Haasnoot, 2009; Bütikofer, Meyer, & Rehberger, 2006; Cucu, Jacxsens, & De Meulenaer, 2013; Elgar et al., 2000; Levieux & Ollier, 1999; Rebe Raz, Liu, Norde, & Bremer, 2010). The limitations of the current analysis techniques that are deployed as part of allergen risk management control of foods have recently been summarised (Walker, Burns, Elliott, Gowland, & Mills, 2016). Optical biosensor platforms that exploit real-time, label-free surface plasmon resonance (SPR) detection have become an established alternative to conventional enzyme-linked immunosorbent assay (ELISA) immunoassay for the quantification of allergenic milk proteins, with minimal sample preparation and high sensitivity (Alves, Barroso, Gonzáles-García, Oliveira, & Delerue-Matos, 2016; Billakanti, Fee, Lane, Kash, & Fredericks, 2010; Indyk, 2009; Indyk, Gill, & Woollard, 2015; Pilolli, Monaci, & Visconti, 2013; Wu et al., 2016).

Depending on the specific formulation, the contribution of β-Lg derived from bovine milk and whey ingredients generally represents the total β-Lg content of commercial milk-based nutritional products. The aim of the present study was to develop and validate an SPR-based biosensor immunoassay and apply it to the estimation of the native, undenatured β-Lg content of bovine milk during early lactation and across a production season. The utility of the analytical method has been demonstrated by generating information regarding the temporal variation in β-Lg content in milk from pasture-fed cows to assist the formulation of bovine milk-based infant and adult nutritional foods.

2. Materials and methods

2.1. Instrumentation

The Biacore® Q optical biosensor, instrument operation and data processing software version 3.0.5 and CM5 sensor chips were from GE Healthcare (Uppsala, Sweden).

2.2. Reagents

1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide-HCl (EDC, 0.4 mol L^{-1}), N-hydroxysuccinimide (NHS, 0.1 mol L^{-1}), ethanolamine-HCl (1 mol L^{-1}, pH 8.5), sodium acetate buffer (10 mmol L^{-1}, pH 4.0, 4.5, 5.0 and 5.5), glycine-HCl buffer pH 2.0 and HBS-EP running buffer (10 mmol L^{-1} HEPES, 150 mmol L^{-1} NaCl, 3.4 mmol L^{-1} EDTA, 0.005% surfactant P20, pH 7.4) were obtained from GE Healthcare (Uppsala, Sweden). All water used was of >18 MΩ resistivity.

Affinity-purified rabbit polyclonal anti-bovine β-Lg antibody (A10-125A, 1.0 mg mL^{-1}, Bethyl Laboratories, Montgomery, TX, USA), affinity-purified sheep polyclonal anti-bovine β-Lg antibody (AbD Serotec 5607-0409, 5.0 mg mL^{-1}, Abacus ALS, Auckland, New Zealand) and rabbit polyclonal anti-bovine β-Lg antibody (GWB-77CA4C, 1.0 mg mL^{-1}, GenWay Biotech, San Diego, CA, USA) were evaluated during method development. Stock antibody solutions were stable for 6 months during storage at 4 °C. Bovine β-Lg A (L7880) and β-Lg B (L8005) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The bovine milk proteins α-casein, β-casein, κ-casein, α-lactalbumin, bovine serum albumin and immunoglobulin G were obtained from Sigma-Aldrich and bovine lactoferrin was isolated from skim milk as described previously (Indyk et al., 2015).

2.3. Biosensor surface preparation

During initial method development, the three anti-bovine β-Lg antibodies were immobilised on separately assigned flow cells of a CM5 sensor chip by standard amine-coupling at 25 °C. Each surface was activated with a mixture of EDC and NHS (1:1, v/v, 10 μL min^{-1}, 7 min) followed by antibody (50 μg mL^{-1} in 10 mmol L^{-1} sodium acetate, pH 5.0, 10 μL min^{-1}, 7 min). Similarly, β-Lg A and β-Lg B were separately immobilised (50 μg mL^{-1} in 10 mmol L^{-1} sodium acetate, pH 5.0, 10 μL min^{-1}, 7 min) on designated flow cells. Following ligand immobilisation, unreacted ester functionalities were deactivated with ethanolamine (1 mol L^{-1}, pH 8.5, 10 μL min^{-1}, 7 min). A reference flow cell surface was prepared similarly by omitting the ligand immobilisation step. Between analyses, functionalised chips were stored in a sealed container over silica gel desiccant at 4 °C.

2.4. Standards

Bovine β-Lg A and β-Lg B stock standards were separately prepared in water, each to approximately 5 mg mL^{-1}, and the concentration was accurately determined spectrophotometrically ($E^{1\%}_{1cm}$ at 280 nm = 9.7). Intermediate standards (1.0 mg mL^{-1}) were separately prepared in HBS-EP buffer and sub-aliquots were stored at −18 °C. Secondary intermediate standards (10 μg mL^{-1}) of each β-Lg were prepared in HBS-EP buffer daily. Calibration standards (10–1000 ng mL^{-1}) of a 1:1 mixture of β-Lg A and β-Lg B were prepared daily in HBS-EP buffer by sequential dilution from the secondary intermediate standards.

Stock solutions of individual bovine milk proteins (α-casein, β-casein, κ-casein, α-lactalbumin, bovine serum albumin, immunoglobulin G and lactoferrin, approximately 10 mg mL^{-1}) were each prepared in water and were stored frozen, from which working standards (10 μg mL^{-1}) in HBS-EP buffer were used for the evaluation of antibody cross-reactivity.

2.5. Samples

Liquid skim milk, milk powders, whey protein concentrate and isolate powders and infant formula powders were obtained from milk processing facilities and commercial sources. Samples were prepared for analysis by initial vortex dissolution in HBS-EP buffer (1:20, w/v) and ultrasonication (15 min), followed by serial dilution in HBS-EP buffer to the final optimised dilution level depending on the expected β-Lg content (liquid milk, 1:50,000, v/v; protein products, from 1:500,000 to 1:5,000,000, w/v; infant formula, 1:200,000, w/v).

Raw bovine milk samples were collected in early spring (August–September), mid-flow from the same quarter of a single 5-year-old Jersey cow (third calving) during the first 28 days postpartum and were prepared as described for liquid milk, with initial dilution extracts (1:20, v/v) stored at −18 °C until analysis. Skim milk powders were prepared monthly across a production season from pooled pasteurised herd skim milk (predominantly Friesian and Jersey breeds) using a pilot-scale spray drier, were sealed in laminated sachets and were stored at 4 °C until analysis, with sample preparation as described above (1:200,000, w/v).

2.6. Biosensor assay

The direct assay format utilised the tethered rabbit polyclonal anti-bovine β-Lg antibody (A10-125A) as the active surface. Mixed β-Lg A and β-Lg B calibration standards and sample extracts (100 μL) were sequentially injected (5 min at 10 μL min^{-1}) with a running buffer of HBS-EP at 25 °C. Relative binding responses were

measured 30 s after the commencement of the dissociation phase and were used for both assay calibration against protein concentration and quantitation of samples, with β-Lg content expressed as the aggregate of A and B variants. A four-parameter regression [$y = R_{hi}-((R_{hi}-R_{lo})/1 + (Conc/A_1)^{A2})$], where R_{hi} = response at infinite concentration, R_{lo} = response at zero concentration, $A_1 = B_{50}$, A_2 = slope factor and Conc = concentration of β-Lg (ng mL^{-1}), was used to construct the dose–response calibration. Following each cycle, the sensor surface was regenerated by a 12 μL injection of 10 mmol L^{-1} glycine-HCl, pH 2.0 at 50 μL min^{-1}.

3. Results and discussion

3.1. Method development and validation

Several variables, including antibody selection, assay format, ligand affinity, coupling chemistry, buffer conditions, contact time and regeneration technique are important determinants of biosensor immunoassay capability and were each investigated during method development. A high affinity ligand is generally recommended for a concentration immunoassay, and three polyclonal antibodies specific for bovine β-Lg were therefore evaluated by determining their relative binding characteristics to high density β-Lg A and β-Lg B immobilised surfaces. Two antibodies bound significantly and importantly, were progressively inhibited by increasing concentrations of solution β-Lg, whereas one antibody failed to bind. Based on its greater binding response to both immobilised β-Lg variants, complete inhibition by β-Lg in solution (>99% at 10 μg mL^{-1}) and absence of non-specific binding to a control surface, the rabbit polyclonal antibody (A10-125A) was adopted for method development, optimisation and validation.

Given the common presence of both genetic variants of β-Lg in bulk bovine herd milk, the selected anti-bovine β-Lg antibody was confirmed, under both inhibition (immobilised β-Lg) and direct (immobilised antibody) assay formats, to yield equivalent binding responses to the A and B variants. Therefore, although not mandatory for accurate quantification, it was considered expedient to calibrate the routine direct assay format with an equimolar standard mixture of the two β-Lg variants.

Antibody immobilisation via covalent amine coupling chemistry produced an active surface with a high ligand density in the order of 10 kRU (1 RU = refractive index change of 10^{-6}, equivalent to 1 pg mm^{-2} protein), equivalent to 65 fmol of IgG antibody. The binding capacity of the active surface for the target analyte is also a function of ligand orientation, and as the limiting β-Lg binding capacity (R_{max}) was experimentally determined to be greater than 0.25 of theoretical, the described procedure yielded an active ligand surface appropriate for the determination of native β-Lg content using a direct biosensor immunoassay format.

The selectivity of the active sensor surface was determined by comparing binding responses for individual bovine milk proteins with that for the β-Lg variants. The immobilised polyclonal antibody was demonstrated to be highly specific for the target protein, with minimal cross-reactivity to α-casein, κ-casein and lactoferrin (<2%, <2% and <1% respectively, relative to β-Lg), yielding analytically insignificant responses (<5 RU) for these proteins in diluted milk extracts. Antibody specificity was further demonstrated through a competition format by confirming >98% binding inhibition of β-Lg to ligand when equilibrated with excess antibody in solution.

Immunoassays, irrespective of platform, are susceptible to non-specific binding, which if present, will compromise method reliability. Therefore, the extent of potential non-specific binding of the selected antibody, authentic β-Lg and a range of dairy sample extracts was assessed over a ligand-free carboxymethyldextran surface and, in all cases, was confirmed to be negligible (<3 RU), thereby facilitating a facile dilution of sample in buffer to within the assay working range, without prior lipid removal or additional sample clean-up.

As β-Lg-free bovine milk is unavailable, potential unidentified interfering sample binding constituents were examined following serial dilutions of milk (10,000–50,000) and whey protein concentrates (200,000–10,000,000), (n = 4) over the active antibody-immobilised surface. Independent of dilution level, the measured β-Lg content was consistently within ±10% of the mean value, thereby confirming an absence of significant matrix non-specific binding.

Immunoassay sensitivity may be amplified by utilising the sandwich assay format, whereby in a sequential binding step, the high molecular mass anti-bovine β-Lg antibody is injected over the surface-captured β-Lg prior to surface regeneration, a technique that also facilitated additional evidence of ligand specificity. Although both direct and sandwich assays yielded comparable β-Lg content for milk and whey protein samples, the direct immunoassay format was favoured for routine use, in view of its operational simplicity for the quantification of β-Lg content.

Optimised contact time, flow rate and ligand regeneration conditions were defined for the direct-binding assay used for routine application. A 5 min injection at 10 μL min^{-1} was determined as optimal with respect to detector response and, subsequent to analyte binding, regeneration of the surface ligand was achieved with 10 mmol L^{-1} glycine (pH 2.0) at 50 μL min^{-1}, as verified by a consistent response prior to injection over multiple cycles; repeatability relative standard deviation (RSD_r) = 0.19% (n = 45). Sensorgrams acquired for a single set of β-Lg calibration standards (0–1000 ng mL^{-1}) and a derived dose–response curve are presented in Fig. 1.

The calibration was suitably described by a four-parameter regression over the concentration interval (10–1000 ng mL^{-1}), with an intermediate reproducibility (RSD_{iR}) of 14.8% (n = 11) for a β-Lg concentration equivalent to 50% of the maximum response (B_{50}), as obtained over several independent run sequences and active surfaces. The method detection limit ($MDL = sd \times t_{n-1,\ 0.01}$), as estimated by replicate testing of liquid milk (n = 7), was 0.54 mg mL^{-1}, giving a method limit (ML = MDL × 3.18) of 1.71 mg g^{-1} (Su, 1998).

The RSD_r was estimated for a β-Lg calibrant (250 ng mL^{-1}) to be 1.95% (n = 15). Instrument performance was further evaluated with a calibration control (250 ng mL^{-1}) over several independent sequences and the RSD_{iR} estimated as 5.3% (n = 10). The RSD_r for fluid milk was determined to be 3.28% (n = 7), and the RSD_{iR} was 12.5% (n = 12) for replicate independent fluid milk samples, 10.4% (n = 4) for a milk powder and 7.2% (n = 6) for a whey powder.

Mean recovery was 103.2% as estimated by dosing a skim milk sample with β-Lg at 1×, 2× and 3× the known endogenous content. The contents of β-Lg, measured as the sum of the A and B variants by the biosensor method were assessed against high performance liquid chromatography (Elgar et al., 2000) for a range of dairy products including milk, powdered skim milk, infant formula and several protein products. Despite the fundamentally different analytical principles, both techniques yield similar values of β-Lg content ($y = 0.87x-3.32$, $R^2 = 0.9973$; $p_{0.05} = 0.12$). The measured β-Lg content of a milk–soya-based infant formula certified reference material (CRM) (FAPAS, Fera Science Ltd., York, UK) was 0.175 mg g^{-1}, and was, in the absence of a certified confidence interval, comparable with the assigned value of 0.148 mg g^{-1}. These spike recovery, method comparison and CRM data support the suitability of the proposed biosensor immunoassay technique for the determination of the β-Lg content of dairy products.

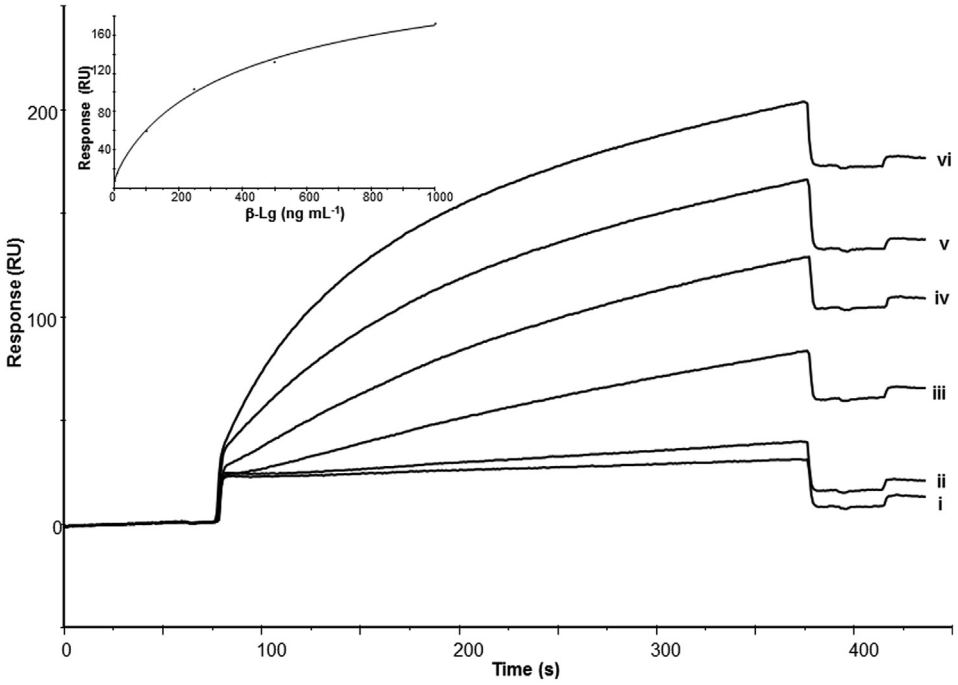

Fig. 1. Overlaid sensorgrams obtained for the concentration series of β-Lg (i) 0 ng mL^{-1}, (ii) 10 ng mL^{-1}, (iii) 100 ng mL^{-1}, (iv) 250 ng mL^{-1}, (v) 500 ng mL^{-1} and (vi) 1000 ng mL^{-1} under optimised direct assay conditions (regeneration phase of each cycle removed for clarity). Inset: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

The binding response stability of the immobilised antibody over multiple within-run injection cycles was further evaluated and the RSD_r of the relative response measured as 1.95% for a 250 ng mL^{-1} β-Lg standard (mean = 89.5 RU, n = 15) and 3.28% (mean = 63.1 RU, n = 15) for fluid milk. An RSD_r for the absolute baseline of 0.19% (mean = 26,196 RU, n = 45) and an average baseline variation of <3 RU $cycle^{-1}$ is evidence for the stability of the immobilised antibody during multiple regenerations. Each of the four addressable sensor chip ligand-activated surfaces performed consistently for >350 injections.

3.2. Application to bovine milk

Raw, unprocessed bovine lacteal composition is variable and significantly influenced by multiple factors including post-partum lactation stage, breed, parity, individual genetics, pathological status, and seasonal feed type, as extensively reported by others (Auldist, Walsh, & Thomson, 1998; McGrath, Fox, McSweeney, & Kelly, 2016). To assess the applicability of the developed biosensor immunoassay, the β-Lg content during the transition from colostrum to mature milk for a single healthy herd-selected Jersey cow over 28 days postpartum was determined, and the mean data obtained from four independent analyses are presented in Table 1. The mean content of β-Lg, measured as the sum of the A and B variants, was found to be significantly higher immediately following parturition (>10 mg mL^{-1}) than in transitional and mature milk (<5 mg mL^{-1}), illustrating a definite physiological response to early lactogenesis in the whey proteome. Although this study was restricted to the milk of a single cow, the observed trend in β-Lg content is consistent with reported data for a wide range of breeds based on alternative radial immunodiffusion, ELISA, electrophoresis and proteomic techniques (Gellrich, Meyer, & Wiedemann, 2014; Levieux & Ollier, 1999; Prosser et al., 2000; Senda, Fukuda, Ishii, & Urashima, 2011). The β-Lg content in mature milk of approximately 4.7 mg mL^{-1} is consistent with the 2–6 mg mL^{-1} range previously reported in multiple studies using immunoassay, chromatographic and electrophoretic techniques (Anema, 2009; Billakanti et al., 2010; Bordin, Cordeiro Raposo, de la Calle, & Rodriguez, 2001; Bütikofer et al., 2006; Ding, Yang, Zhao, Li, & Wang, 2011; Farrell et al., 2004; Kontopidis et al., 2004; Levieux & Ollier, 1999; Mackle, Bryant, Petch, Hill, & Auldist, 1999; McLaren, Auldist, & Prosser, 1998; Prosser et al., 2000).

Dairy husbandry in New Zealand exploits an extensive pasture grazing and seasonal calving practice that is designed to maximise milk production around efficient pasture utilisation, a regime that inherently produces seasonal variation in bovine milk composition (Auldist et al., 1998). As milk is commonly converted to powder form to facilitate storage and extend its value in the human diet, the content of β-Lg in low heat skim milk powder produced monthly from pooled pasteurised herd skim milk was followed over an entire production season, with the mean data obtained from three independent analyses presented in Table 2. There was a significant seasonal trend in β-Lg content, with a minimum (approximately 25 mg g^{-1}) during the late spring–early summer (November–December) and a maximum (approximately 60 mg g^{-1}) during late

Table 1
Content of β-lactoglobulin in raw bovine milk from a single Jersey cow during early lactation.[a]

Day	β-Lactoglobulin (mg mL^{-1})	
	Mean	RSD_{iR} (%)
0	11.5	12.2
1	6.9	12.4
2	6.1	8.0
3	5.9	13.6
5	5.1	12.7
7	5.3	12.8
14	4.2	4.7
21	5.0	16.6
28	4.8	8.9

[a] Day refers to days postpartum; mean and intermediate reproducibility relative standard deviation from 4 replicate analyses.

Table 2
Content of β-lactoglobulin in skim milk powder manufactured from pooled herd milk over a season.[a]

Month	β-Lactoglobulin (mg g^{-1})	
	Mean	RSD_{iR} (%)
Aug	51.2	7.6
Sep	53.9	8.2
Oct	39.0	8.6
Nov	25.9	7.4
Dec	27.7	5.4
Feb	45.5	7.7
Mar	55.4	7.7
Apr	56.6	10.0
May	58.3	9.0
Jun	53.4	11.1
Jul	42.6	7.8
Aug	47.3	10.2

[a] Month refers to the month of production of skim milk powder from raw milk; mean and intermediate reproducibility relative standard deviation from 3 replicate analyses.

autumn (April–May), a pattern that is consistent with the general increase in total protein as a function of lactation stage reported previously for milk from New Zealand pasture-fed cows (Auldist, Napper, & Kolver, 2000). In common with alternative immunoassay techniques, the described biosensor technique does not distinguish between the A and B polymorphs of β-Lg in the milk from heterozygous cows and, although the influence of β-Lg phenotype on bovine milk protein composition has been reported, the segregation of herds by β-Lg phenotype is not common practice (Prosser et al., 2000).

The described biosensor immunoassay has potential to be deployed for the routine quantification of the dominant allergen β-Lg in bovine milk products, ingredients or foods containing cow's milk, as demonstrated during the evaluation of method accuracy for a limited range of milk-based products. This analytical strategy is consistent with the increasing application of immuno-biosensor platforms as an alternative to ELISA for the analysis of food allergens, as has been recently reviewed (Alves et al., 2016).

Compared with conventional immunoassay techniques, label-free optical SPR-based biosensor immunoassays offer advantages of regenerative reusability of the active surface, automation and elimination of the potential influence of a label on binding specificity. Affinity-based immunoassay techniques uniquely provide the inherent attribute of specificity for the conformational state of the native target protein, a characteristic that is dependent on the immunogen exploited during antibody production. The described SPR-based biosensor method will therefore yield quantitative information on the content of soluble native, undenatured β-Lg, a feature that differentiates it from alternative chromatographic techniques, especially when applied to allergen testing of heat-treated foods (Abbott et al., 2010; Bordin et al., 2001).

4. Conclusions

The described direct format biosensor immunoassay for the quantification of β-Lg in bovine milk that exploits an immobilised commercially available polyclonal antibody is rapid, sensitive, precise and accurate, and has also been proven to be reliable for a range of milk-derived products. The method facilitated the determination of both common β-Lg variants in bovine milk, and allowed an evaluation of the influence of lactation and season on the lacteal expression of this protein. The data presented provide information regarding the expression of this major whey protein in bovine milk under extensively pasture-grazed conditions, and will facilitate enhanced control of infant formula manufacture with respect to the content of this protein.

References

Abbott, M., Hayward, S., Ross, W., Godefroy, S. B., Ulberth, F., Van Hengel, A., et al. (2010). Validation procedures for quantitative food allergen ELISA methods: Community guidance and best practices. *Journal of AOAC International, 93*, 442–450.

Alves, R. C., Barroso, M. F., Gonzáles-García, M. B., Oliveira, M. B. P. P., & Delerue-Matos, M. C. (2016). New trends in food allergens detection: Towards biosensing strategies. *Critical Reviews in Food Science and Nutrition, 56*, 2304–2319.

Anema, S. G. (2009). The use of "lab-on-a-chip" microfluidic SDS electrophoresis technology for the separation and quantification of milk proteins. *International Dairy Journal, 19*, 198–204.

Anema, S. G. (2014). The whey proteins in milk: Thermal denaturation, physical interactions, and effects on the functional properties of milk. In H. Singh, M. Boland, & A. K. Thompson (Eds.), *Milk proteins: From expression to food* (2nd ed., pp. 269–318). London, UK: Academic Press.

Auldist, M. J., Napper, A. R., & Kolver, E. S. (2000). Contribution of nutrition to seasonal variation of milk composition in New Zealand Friesian and US Holstein dairy cows. *Asian–Australasian Journal of Animal Science, 13*(Suppl. A), 513–516.

Auldist, M. J., Walsh, B. J., & Thomson, N. A. (1998). Seasonal and lactational influences on bovine milk composition in New Zealand. *Journal of Dairy Research, 65*, 401–411.

Billakanti, J. M., Fee, C. J., Lane, F. R., Kash, A. S., & Fredericks, R. (2010). Simultaneous, quantitative detection of five whey proteins in multiple samples by surface plasmon resonance. *International Dairy Journal, 20*, 96–105.

Boitz, L. I., Fiechter, G., Seifried, R. K., & Mayer, H. K. (2015). A novel ultra-high performance liquid chromatography method for the rapid determination of β-lactoglobulin as heat load indicator in commercial milk samples. *Journal of Chromatography A, 1386*, 98–102.

Bordin, G., Cordeiro Raposo, F., de la Calle, B., & Rodriguez, A. R. (2001). Identification and quantification of major bovine milk proteins by liquid chromatography. *Journal of Chromatography A, 928*, 63–76.

Boyle, R. J., Ierodiakonou, D., Khan, T., Chivinge, J., Robinson, Z., Geoghegan, N., et al. (2016). Hydrolysed formula and risk of allergic or autoimmune disease: Systematic review and meta-analysis. *British Medical Journal, 352*, i974.

Bremer, M. G. E. G., Smits, N. G. E., & Haasnoot, W. (2009). Biosensor immunoassay for traces of hazelnut protein in olive oil. *Analytical and Bioanalytical Chemistry, 395*, 119–126.

Bu, G., Luo, Y., Chen, F., Liu, K., & Zhu, T. (2013). Milk processing as a tool to reduce cow's milk allergenicity: A mini-review. *Dairy Science and Technology, 93*, 211–223.

Bütikofer, U., Meyer, J., & Rehberger, B. (2006). Determination of the percentage of α-lactalbumin and β-lactoglobulin of total milk protein in raw and heat treated skim milk. *Milchwissenschaft, 61*, 263–266.

Caira, S., Pizzano, R., Picariello, G., Pinto, G., Cuollo, M., Chianese, L., et al. (2012). Allergenicity of milk proteins. In W. L. Hurley (Ed.), *Milk protein* (pp. 173–214). Intech. http://www.intechopen.com/books/milk-protein.

Claeys, W. L., Verraes, C., Cardoen, S., De Block, J., Huyghebaert, A., Raes, K., et al. (2014). Consumption of raw or heated milk from different species: An evaluation of the nutritional and potential health benefits. *Food Control, 42*, 188–201.

Considine, T., Patel, H. A., Anema, S. G., Singh, H., & Creamer, L. K. (2007). Interactions of milk proteins during heat and high hydrostatic pressure treatments—a review. *Innovative Food Science and Emerging Technologies, 8*, 1–23.

Cucu, T., Jacxsens, L., & De Meulenaer, B. (2013). Analysis to support allergen risk management: Which way to go? *Journal of Agriculture and Food Chemistry, 61*, 5624–5633.

Delahaije, R. J. B. M., Gruppen, H., van Eijk–van Boxtel, E. L., Cornacchia, L., & Wierenga, P. A. (2016). Controlling the ratio between native-like, non-native-like, and aggregated β-lactoglobulin after heat treatment. *Journal of Agriculture and Food Chemistry, 64*, 4362–4370.

Ding, X., Yang, Y., Zhao, S., Li, Y., & Wang, Z. (2011). Analysis of α-lactalbumin, β-lactoglobulin A and B in whey protein powder, colostrum, raw milk, and infant formula by CE and LC. *Dairy Science and Technology, 91*, 213–225.

Docena, G., Rozenfeld, P., Fernández, R., & Fossati, C. A. (2002). Evaluation of the residual antigenicity and allergenicity of cow's milk substitutes by in vitro tests. *Allergy, 57*, 83–91.

Edwards, P. J. B., & Jameson, G. B. (2014). Structure and stability of whey proteins. In H. Singh, M. Boland, & A. K. Thompson (Eds.), *Milk proteins: From expression to food* (2nd ed., pp. 201–242). London, UK: Academic Press.

El-Agamy, E. I. (2007). The challenge of cow milk protein allergy. *Small Ruminant Research, 68*, 64–72.

Elgar, D. F., Norris, C. S., Ayers, J. S., Pritchard, M., Otter, D. E., & Palmano, K. P. (2000). Simultaneous separation and quantitation of the major bovine whey proteins including proteose peptone and caseinomacropeptide by reversed-phase high-performance liquid chromatography on polystyrene-divinylbenzene. *Journal of Chromatography A, 878*, 183–196.

Farrell, H. M., Jr., Jiminez-Flores, R., Bleck, G. T., Brown, E. M., Butler, J. E., Creamer, L. K., et al. (2004). Nomenclature of the proteins of cows' milk—Sixth revision. *Journal of Dairy Science, 87*, 1641–1674.

Gellrich, K., Meyer, H. H. D., & Wiedemann, S. (2014). Composition of major proteins in cow milk differing in mean protein concentration during the first 155 days of lactation and the influence of season as well as short-term restricted feeding in early and mid-lactation. *Czech Journal of Animal Science, 59*, 97–106.

Indyk, H. E. (2009). Development and application of an optical biosensor immunoassay for α-lactalbumin in bovine milk. *International Dairy Journal, 19*, 36–42.

Indyk, H. E., Gill, B. D., & Woollard, D. C. (2015). An optical biosensor-based immunoassay for the determination of bovine serum albumin in milk and milk products. *International Dairy Journal, 47*, 72–78.

Kontopidis, G., Holt, C., & Sawyer, L. (2004). β-Lactoglobulin: Binding properties, structure, and function. *Journal of Dairy Science, 87*, 785–796.

Leeb, E., Götz, A., Letzel, T., Cheison, S. C., & Kulozik, U. (2015). Influence of denaturation and aggregation of β-lactoglobulin on its tryptic hydrolysis and the release of functional peptides. *Food Chemistry, 187*, 545–554.

Lemay, D. G., Lynn, D. J., Martin, W. F., Neville, M. C., Casey, T. M., Rincon, G., et al. (2009). The bovine lactation genome: Insights into the evolution of mammalian milk. *Genome Biology, 10*. Article 43.

Levieux, D., & Ollier, A. (1999). Bovine immunoglobulin G, β-lactoglobulin, α-lactalbumin and serum albumin in colostrum and milk during the early post partum period. *Journal of Dairy Research, 66*, 421–430.

Mackle, T. R., Bryant, A. M., Petch, S. F., Hill, J. P., & Auldist, M. J. (1999). Nutritional influences on the composition of milk from cows of different protein phenotypes in New Zealand. *Journal of Dairy Science, 82*, 172–180.

Madureira, A. R., Pereira, C. I., Gomes, A. M. P., Pintado, M. E., & Malcata, F. X. (2007). Bovine whey proteins—Overview on their main biological properties. *Food Research International, 40*, 1197–1211.

Manzo, C., Nicolai, M. A., & Pizzano, R. (2015). Thermal markers arising from changes in the protein component of milk. *Food Control, 51*, 251–255.

McGrath, B. A., Fox, P. F., McSweeney, P. L. H., & Kelly, A. L. (2016). Composition and properties of bovine colostrum: A review. *Dairy Science and Technology, 96*, 133–158.

McLaren, R. D., Auldist, M. J., & Prosser, C. G. (1998). Diurnal variation in the protein composition of bovine milk. *Proceedings of the New Zealand Society of Animal Production, 58*, 49–51.

Picariello, G., Miralles, B., Mamone, G., Sánchez-Rivera, L., Recio, I., Addeo, F., et al. (2015). Role of intestinal brush border peptidases in the simulated digestion of milk proteins. *Molecular Nutrition and Food Research, 59*, 948–956.

Pilolli, R., Monaci, L., & Visconti, A. (2013). Advances in biosensor development based on integrating nanotechnology and applied to food-allergen management. *Trends in Analytical Chemistry, 47*, 12–26.

Prosser, C. G., Turner, S.-A., McLaren, R. D., Langley, B., L'Huillier, P. J., Molan, P., et al. (2000). Milk whey protein concentration and mRNA associated with β-lactoglobulin phenotype. *Journal of Dairy Research, 67*, 287–293.

Qin, B. Y., Bewley, M. C., Creamer, L. K., Baker, E. N., & Jameson, G. B. (1999). Functional implications of structural differences between variants A and B of bovine β-lactoglobulin. *Protein Science, 8*, 75–83.

Rebe Raz, S., Liu, H., Norde, W., & Bremer, M. G. E. G. (2010). Food allergens profiling with an imaging surface plasmon resonance-based biosensor. *Analytical Chemistry, 82*, 8485–8491.

Senda, A., Fukuda, K., Ishii, T., & Urashima, T. (2011). Changes in the bovine whey proteome during the early lactation period. *Animal Science Journal, 82*, 698–706.

Su, G. C. C. (1998). A comparison of statistical and empirical detection limits. *Journal of AOAC International, 81*, 105–110.

Walker, M. J., Burns, D. T., Elliott, C. T., Gowland, M. H., & Mills, E. N. C. (2016). Is food allergen analysis flawed? Health and supply chain risks and a proposed framework to address urgent analytical needs. *Analyst, 141*, 24–35.

Wu, X., Li, Y., Liu, B., Feng, Y., He, W., Liu, Z., et al. (2016). Two-site antibody immunoanalytical detection of food allergens by surface plasmon resonance. *Food Analytical Methods, 9*, 582–588.