Biotin Content of Paediatric Formulae, Early Lactation Milk and Seasonal Bovine Milk Powders by Biosensor Immunoassay

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Abstract

An optical biosensor assay utilising a monoclonal antibody was developed for the quantitation of the biotin content of milk and paediatric formulae. The method provides a reliable estimate of total biotin accomplished by simple aqueous extraction, combined with heat treatment, prior to automated biosensor analysis. The binding assay was configured under inhibition conditions utilising a sensor surface functionalised with biotin and was subjected to single-laboratory validation. Critical assay factors, including calibration parameters, cross-reactivity, non-specific binding and matrix interferences were evaluated systematically. Assay performance parameters including range, detection limits, precision, recovery and bias were estimated. The method was applied to the routine compliance testing of paediatric formulae and the temporal change in the biotin content of early lactation milk and seasonal milk powder. The assay is an expedient alternative to current HPLC, microbiological and proprietary kit-based immunoassay methods for the determination of the biotin content of milk-based foods.

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1. Introduction

Biotin is an essential dietary micronutrient for mammals due to its role as a cofactor for multiple carboxylase enzymes involved in central metabolism. As mammalian cells cannot synthesise biotin, an exogenous supply is mandatory and hence biotin is classified as a vitamin. Biotin is widely distributed in the human diet, either mostly free in milk, vegetables and fruit, or partly protein bound via a covalent lysine linkage as biocytin in animal tissues and plant seeds. Although biotin contains three asymmetric carbons and can therefore exist as eight potential stereoisomers, only naturally occurring D-biotin is biologically active and nutritionally significant. Recent general reviews of the physiology, transport, dietary distribution and nutrition of biotin are available for detailed background information (Mock, 2005; Mock 2006). It has also been reported that, in the lactating dairy cow, biotin is central to metabolic pathways that are specifically involved with milk biosynthesis and hoof integrity (Chen, Wang, Wang, & Liu, 2011; Singh et al., 2011).

Biotin is widely distributed in the food supply, although generally at significantly lower levels compared with other B-group vitamins. Although the biotin content of bovine milk is low, it has generally been reported to be higher than that of human milk. Despite the variable biotin levels reported, human milk is considered to be sufficient to supply the newborn infant with the recommended daily intake of 5–6 μg day^{-1}, as evidenced by the general absence of deficiency syndromes in breast-fed babies. Nonetheless, marginal biotin deficiency is common in normal human pregnancy (Mock, Quirk, & Mock, 2002). It is consistently stated that more than 95% of the biotin in human milk is free and exceeds blood serum concentrations by more than one order of magnitude, suggesting an active transport mechanism that currently remains unidentified, and similar observations have been reported for the cow (Higuchi et al., 2003; Mock, 2005; Mock, Mock, & Langbehn, 1992). As human milk is the primary source of infant nutrition, its composition guides the manufacture of paediatric formulae, which are most commonly based on bovine milk.

Reliable analytical methods for biotin are required to establish population intakes, to support clinical studies and to facilitate food compliance testing programmes. The determination of total biotin in foods containing significant levels of bound biocytin will require an initial proteolytic step to release the analyte to the free biotin pool for final analysis, and such extraction strategies have commonly employed a combination of high temperature acid digestion and enzymatic hydrolysis techniques. In contrast, for milk and milk-based paediatric formulae containing biotin almost exclusively in its free form with no measurable contribution from biocytin, effective extraction strategies can be simplified. Extraction and analysis techniques for the quantitative determination of biotin in foods, including specifically dairy products, have been comprehensively reviewed, with microbiological, chromatographic and ligand-binding assays being

the most prevalent analytical platforms, each with a specific range of attributes (Eitenmiller, Ye, & Landen, 2008; Livaniou et al., 2000; Ploux, 2000; Woollard & Indyk, 2013).

Although traditional microbiological assays remain important for food analysis, high performance liquid chromatography (HPLC) methods have proliferated. In the absence of a selective ultraviolet (UV) chromophore, biotin is generally detected via either coupled post-column derivatisation and fluorescence or, more recently, direct mass spectrometry (MS) (Campos-Gimenez, Trisconi, Kilinc, & Andrieux, 2010; Hayakawa et al., 2009; Höller, Wachter, Wehrli, & Fizet, 2006; Lahely, Ndaw, Arella, & Hasselmann, 1999; Lang, Cheng, & Ma, 2009; Lu et al., 2008; Shang, Zhang, Han, & Sheng, 2009; Thompson, Schmitz, & Pan, 2006).

Labelled biospecific avidin- or streptavidin-binding and immunoassay variants utilising the microtitration plate format have been described for the determination of biotin in biological matrices, food and milk (Bitsch, Salz, & Hötzel, 1989; Finglas, Faulks, & Morgan, 1986; Higuchi et al., 2003; Mock et al., 1992; Reyes, Romero, & de Castro, 2001). Despite being more prevalent in the clinical diagnostic field, these platforms have also provided an analytical strategy for food analysis and offer unique advantages. In general, such techniques are sensitive, specific, rapid and easily implemented for routine food compliance testing, and therefore represent strategic alternatives to traditional microbiological and contemporary HPLC strategies.

Biosensor platforms facilitate the development of real-time, non-labelled ligand-binding assays for the quantitation of vitamins, including biotin, in a range of foods. They provide high throughput, rapid and cost-effective strategies that can meet the increasing demands of the food industry and, as with traditional binding assays, biospecific analyte recognition dramatically reduces the need for extensive sample preparation. The most commonly applied biosensor system for the analysis of biotin in supplemented foods utilises surface plasmon resonance (SPR) optics and is routinely applied as a proprietary immunoaffinity-based kit assay, although other transducer detection principles have been exploited (Blake, 2007; Gao et al., 2008; Indyk et al., 2000; Kalman, Caelen, & Svorc, 2006; Kergaravat et al., 2012). The mass change associated with binding at the sensor surface dictates SPR signal intensity, and the low molecular mass of biotin therefore mandates the adoption of an inhibition format that exploits a biospecific detecting protein and a surface functionalised with tethered analyte, yielding an inverse dose-response relationship. The purpose of this study was to i) develop an alternative to the proprietary kit biosensor-SPR immunoassay for routine compliance measurement of the biotin content of paediatric formulae, and ii) apply it to survey the variation in endogenous bovine milk biotin during lactation and across a production season.

2. Materials and methods

2.1. Instrumentation

A Biacore Q optical biosensor and control software version 3.0.4 were used in this study (GE Healthcare, Uppsala, Sweden).

2.2. Chemicals and reagents

1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC, 0.4 M), N-hydroxysuccinimide (NHS, 0.1 M), ethanolamine. HCl (1 M, pH = 8.5), sensor chip CM5 and HBS-EP buffer (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.005% Surfactant Polysorbate 20 [polyoxyethylenesorbitan], pH = 7.4) were obtained from GE Healthcare. D-Biotin was sourced from US Pharmacopeia (Rockville, MD, USA). Ethylenediamine and bovine serum albumin (BSA) were sourced from Sigma-Aldrich (St. Louis, MO, USA). All water used was to 18 MΩ quality.

Antibody diluent buffer (200 mM HEPES, 600 mM NaCl, 13.6 mM EDTA, 1% BSA, 0.02% Surfactant P20) was prepared by dissolving 452 mg of HEPES, 263 mg of NaCl, 38 mg of EDTA.$2H_2O$ (disodium salt), 100 mg of BSA and 15 mL of Surfactant P20 (10%, w/v) in 10 mL of HBS-EP buffer. The pH was adjusted to 7.3 with NaOH and the buffer was filtered (0.22 μm).

A number of commercially available biotin-specific purified antibodies were acquired, including (1) goat polyclonal, 1 mg mL^{-1}, (2) sheep polyclonal, 1 mg mL^{-1}, (3) rabbit polyclonal, 1 mg mL^{-1} (Bethyl Laboratories, Montgomery, TX, USA), (4) monoclonal, 0.2 mg mL^{-1} (Pierce Biotechnology, Rockford, IL, USA) and (5) monoclonal, lyophilised (Novocastra Leica Microsystems, North Ryde, NSW, Australia) prepared at 0.5 mg mL^{-1} by dissolution in 1.0 mL of sterile water (18 MΩ). All antibody solutions were stable for 6 months during storage at 4 °C.

Antibody working solutions were prepared at various dilutions (5.0–10.0 μg mL^{-1}) in diluent buffer during comparative evaluation of the biotin-specific antibodies. The optimised assay utilised the monoclonal antibody from Novocastra Leica at 7.0 μg mL^{-1}.

Biotin stock standard (100 μg mL^{-1}) was prepared by dissolving 10.0 mg in water (40 °C), made to 100 mL and stored at −18 °C (biotin has low solubility of approximately 200 μg mL^{-1} in water, and requires care to ensure complete dissolution). Intermediate I (1.0 μg mL^{-1}) was prepared by diluting 1.0 mL of stock standard in 100 mL of water. Intermediate II (100 ng mL^{-1}) was prepared by diluting 1.0 mL of intermediate I in 10.0 mL of water. Working calibration standards (100, 50, 25, 12.5, 6.25, 3.125 and 1.563 ng mL^{-1}) were prepared by serial dilution of Intermediate II in water.

Regeneration reagent (20 mM NaOH, 0.05% Surfactant P20) was prepared by diluting 100 mM NaOH solution 1:5 v/v with water and adding 50 mL of Surfactant P20 (10%, w/v).

A proprietary AOAC certified kit assay (BR-1003-38)was sourced from GE Healthcare and, in accordance with the manufacturer's instructions, was applied during validation studies.

2.3. Samples

A number of consumer milks and nutritional milk-based paediatric formula powders were obtained from both New Zealand milk processing facilities and commercial sources. A representative lactose-free, soy protein-based paediatric formula powder was also acquired. Raw bovine milk was collected (mid-flow from the same quarter) from a single 10 year-old Jersey cow (fourth calving) between 0.2 day prepartum and 28 days postpartum and was prepared immediately as described below for liquid milk, and the extracts were stored at −18 °C until analysed. 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 and were sealed in laminated sachets and stored at 4 °C until analysis. A certified reference infant/adult milk-based nutritional paediatric formula (SRM 1849a) was obtained from the National Institute of Standards and Technology (NIST), Gaithersburg, MD, USA.

2.4. Biosensor surface preparation

Surface preparation was performed under static conditions external to the biosensor instrument and followed a protocol analogous to that described previously for the immobilisation of folic acid (Indyk, 2010; Indyk, 2011), with each step terminated by a water wash and nitrogen drying. Activation of the CM5 sensor chip, coupling of ethylenediamine and blocking steps were however extended to 30 min incubation at ambient temperature. NHS (0.1 M, 100 µL) and EDC (0.4 M, 100 µL) were combined, 50 µL of 10 mM biotin in 50 mM borate buffer, pH 8.5 added and the mixture incubated (30 min). Following dilution with 50 mM borate buffer, pH 8.5 (1:1, v/v), the activated biotin was finally coupled to the ethylenediamine-functionalised sensor surface (60 min). A reference surface was prepared similarly, but with omission of the ligand immobilisation step. Between analyses, the functionalized biosensor chip was stored over desiccant at 4 °C in a sealed container.

2.5. Sample preparation

Extraction was performed under conditions of low level, yellow incandescent light. Powdered milk or paediatric formula samples (approximately 1.3 g) were weighed accurately into 50 mL disposable centrifuge tubes and dissolved in approximately 40 mL of warm water (37 °C) with vortex mixing. Liquid

milks (approximately 10.0 g) were similarly diluted to 40 mL with water. Following ultrasonication (30 min), samples were made to volume (50 mL) with water. A 5 mL aliquot was transferred to a glass Kimax tube, capped, treated at 100 °C in a water-bath for 15 min and cooled to ambient temperature. A 2 mL aliquot was transferred to an Eppendorf tube, capped and centrifuged (4500 × g, 15 min), and the supernatant was filtered through a combined 0.45 and 0.22 µm membrane filter. Extracts were further diluted with water depending on the expected concentration.

2.6. Biosensor assay

Reagents and a biotin-immobilised sensor chip were equilibrated to ambient temperature, calibration standards and sample extracts (140 µL) distributed in a 96-well microtitre plate and protected from light. Automated analysis was accomplished under assay parameters optimised for binding signal, sample consumption and temperature stability (running buffer HBS-EP; biotin antibody working solution 7.0 µg mL^{-1}; injection time 110 s; flow rate 40 µL min^{-1}; temperature 25 °C). Under these conditions, biotin antibody working solution was mixed (25%, v/v) with either calibrant or sample extract and 73 µL injected over the sensor surface. Bound antibody was removed with regeneration reagent (23 µL at 50 µL min^{-1}) and the binding response (RU) acquired 30 s after injection relative to the initial baseline (10 s before injection).

Quantitation of the biotin content in the sample extract (ng mL^{-1}) was by interpolation from a seven-level sigmoidal calibration curve fit with a four-parameter logistic regression [y = R_{hi} − ((R_{hi} - R_{lo})/1 + (*Conc*/A_1)^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 (ng mL^{-1}). The sample biotin content was then calculated Biotin (µg hg^{-1}) = Biotin (ng mL^{-1}) x DF x V x 100/W x 1/1000], where DF = dilution factor, V = volume (50), and W = sample weight (g).

2.7. Method validation

Five commercially available biotin-specific antibodies were evaluated as potential binders by comparing their uninhibited binding to a biotin-immobilised surface under identical assay conditions. The specificity of the selected antibody was further characterised by evaluating (i) cross-reactivity against potential interfering compounds, and (ii) extent of binding inhibition by biotin in solution.

The chemistry utilised for covalently tethering biotin to the chip surface was evaluated by estimating the maximum binding capacity (R_{max}) of the surface by repeated injection of the selected antibody without regeneration. The potential for non-specific binding from matrix components was evaluated over both active and reference surfaces.

The immunoassay conditions were optimised with respect to sample preparation, antibody diluent buffer composition, antibody concentration, contact time, flow rate and regeneration protocol. Method performance parameters, including range, sensitivity, detection limits, stability and precision, were determined. Recovery was estimated by standard addition and accuracy was evaluated by (i) investigating the effect of matrix dilution, (ii) compliance with the assigned certified biotin content in NIST SRM 1849A and (iii) comparison over a range of dairy products against a commercially available AOAC International validated kit-based method.

3. Results and discussion

3.1. Method validation

3.1.1. Sample preparation

Innate biotin in bovine milk occurs predominantly unbound and in supplemented milk-based paediatric foods, biotin is almost exclusively present in its free form. Thus, conventional multi-stage extractive hydrolysis treatments, known to partially degrade biotin, are not required for these food types before measurement. The analysis of free biotin in such foods can therefore be considered to be a good estimation of total biotin for compliance monitoring and label claim purposes, and was accomplished by simple aqueous extraction combined with heat treatment to denature milk proteins and release any potentially protein-bound biotin. Similar facile extraction strategies for the quantitation of biotin in milk and paediatric formulae have been reported previously, utilizing various analytical platforms (Hayakawa et al., 2009; Höller et al., 2006; Indyk et al., 2000; Kergaravat et al., 2012; Lang et al., 2009; Lu et al., 2008; Shang et al., 2009; Thompson et al., 2006).

3.1.2. Biosensor surface

Biotin was immobilised by covalent coupling of its NHS-activated valeric acid carboxylate terminal group to an amine-modified carboxymethyldextran sensor surface. The described immobilisation protocol yields a sensor chip with four independently available active flowcells. The efficacy of immobilization was evidenced by a maximum antibody binding capacity (R_{max}) of approximately 20,000 RU (1 RU = 1 pg mm^{-2}), a value equivalent to approximately 37 pg of surface-coupled biotin per channel. This high capacity surface facilitates concentration analysis under mass-transport limiting conditions, as illustrated by antibody binding that proceeds at a constant rate. Under routine uninhibited concentration assay conditions, less than 10% of the active surface is bound by antibody.

3.1.3. Antibody specificity

Although all of the commercially available affinity-purified antibodies bound to the biotin-immobilised surface at comparable levels, the rabbit polyclonal antibody could not be removed despite rigorous regeneration, thereby precluding a dose-response relationship. Both the sheep and goat polyclonal antibodies were poorly inhibited by biotin in solution. Although both monoclonal antibodies were effectively inhibited by biotin in solution, one displayed unacceptable levels of residual non-specific binding. These observations are illustrated in Figure 1.

On the basis of its favourable dose-response relationship (Figure 1, curve b), the commercially available monoclonal antibody (Novocastra Leica) was selected for further assay development. Antibody specificity was characterised by cross-reactivity (CR) against potentially interfering milk components relative to biotin. These data are presented in Table 1, where CR (%) was estimated by comparing IC_{50} values derived from normalised analyte calibration curves with that for biotin under optimised biosensor assay conditions ($CR\% = IC_{50,analyte}/IC_{50,biotin} \times 100$).

The moderate cross-reactivity to biocytin is analytically unimportant, given the absence of this form of biotin in milk. Although lumichrome, a photochemically induced derivative of riboflavin, shares a common epitope with biotin and is therefore a potentially significant interferent, the low level incandescent light conditions completely prevented its formation during analysis. The formation of lumichrome in milk products prior to analysis is prevented given the physiological pH of milk and the exclusion of UV light during both milk collection and spray-drying processes (Palanuk & Warthesen, 1988). Nonetheless, the incorporation of riboflavin binding protein during sample extraction demonstrated an analytically insignificant influence (< 2.5%) on biotin levels estimated by the described method. This confirms the absence of both innate and extractive lumichrome, as has been reported previously (Indyk et al., 2000).

Specificity of the selected antibody was further demonstrated by determining the extent of binding inhibition when equilibrated with biotin in solution. Inhibition was confirmed at > 99.5% at a solution biotin concentration of 100 µg mL^{-1}, and verified both antibody specificity and the absence of non-specific binding.

3.1.4. Immunoassay conditions

The optimised immunoassay exploits the inhibition of high molecular mass antibody binding to the immobilised surface by biotin, and binding equilibrium in solution has been confirmed prior to injection over the active surface.

Since calibrants and sample extracts were prepared in water, it was necessary to dilute the antibody in the prescribed high ionic strength diluent, both to buffer against potential sample pH variation and to generate a positive refractive index bulk shift upon injection. In addition, both BSA and surfactant were confirmed to stabilise the antibody, prevent adsorption and attenuate assay sensitivity.

Antibodies are commonly exploited as the detecting species in inhibition assays despite their bivalency, which requires both binding sites to be occupied for effective inhibition. Antibody affinity and concentration typically determine the working range of an inhibition assay, and Figure 2 illustrates this relationship for the described biotin assay, from which an antibody concentration of 7 μg mL^{-1} was selected.

Regeneration of the biotin-immobilised surface following antibody binding was accomplished effectively under dilute basic conditions, as demonstrated by the stable baseline response after 50 cycles of uninhibited binding of antibody (relative standard deviation, RSD_r = 0.06%). The optimised regeneration protocol facilitated a high stability surface, with negligible loss of antibody binding capacity and was comparable with those typically employed for low molecular mass ligands.

3.1.5. Immunoassay performance

The four-parameter logistic function reliably fitted the observed sigmoidal dose-response relationship and was consistent with general immunoassays. Multiple dose-response calibration curves established a quantitation range of 2.5–75 ng mL^{-1} and the limit of detection from replicate (n = 7) analysis of uninhibited antibody was estimated to be 0.5 ng mL^{-1} (3 sd), equivalent to 2 mg hg^{-1} for a typical paediatric formula. This value was comparable with the method detection limit (sd × $t_{[n-1,\ 0.01]}$) of 5.8 mg hg^{-1}, as estimated from replicate analysis of a product containing low biotin levels. The reproducibility of the mean IC_{50} value (17.67 ng mL^{-1}) derived from replicate (n = 20) biotin calibration curves was 5.61% RSD_{iR}, and for the control calibrant was 2.60% RSD_{iR} (n = 10). Further, the reliability of the regression model was assessed by evaluating the bias and recovery of calculated versus actual calibration concentration values, which were demonstrated to be < 3% and 98–103% (n = 16) respectively.

Repeatability and intermediate reproducibility (different days, analysts, sensor surface and calibrations, single biosensor instrument) estimates of an in-house quality control paediatric formula sample were 3.62% RSD_r (n = 7) and 4.96% RSD_{iR} (n = 11) respectively, and replicate analysis of SRM NIST 1849a yielded an intermediate reproducibility of 5.42% RSD_{iR} (n = 9). The Horwitz function represents a performance parameter for the reproducibility of an analytical method, and the measured $HorRat_r$ (observed RSD_r/predicted RSD_r, where predicted $RSD_r = 0.67 \times 2^{(1-0.5 \log C)}$) values for these two products were 0.28

and 0.38 respectively, and comply with the accepted guidance limits of 0.3–1.3 for repeatability (Chen & Eitenmiller, 2007; Horwitz & Albert, 2006).

As for ligand-binding concentration assays in general, SPR-based methods are vulnerable to non-specific interactions that can be associated with complex food matrices, thereby compromising accuracy (Situ, Wylie, Douglas, & Elliott, 2008). Such potential non-specific binding was investigated by injecting a range of sample extracts over a biotin-immobilised surface in the absence of antibody, with the observed binding levels insignificant compared with the analytical signal (< 2.0%). Non-specific binding of antibody and sample extracts to the non biotin-immobilised reference surface was also investigated and found to be analytically negligible (< 0.6%).

The potential for matrix-derived non-specific binding interferences was further evaluated by serial dilution of the extracts from four independent paediatric formulae over the active surface. Biotin concentrations were determined at five different dilutions yielding binding responses within the working range. The calculated contents were all within ± 10% from the mean (Chen & Eitenmiller, 2007) providing further confidence that the binding response was specifically a function of analyte concentration.

Performance over sequential replicate analyses demonstrates the stability of the immobilised ligand with respect to binding capacity. The within-run repeatability of the relative binding response was 1.05% RSD_r (n = 15) for uninhibited antibody, 1.25% RSD_r for a control biotin calibrant (n = 15), 1.34% RSD_r (n = 15) for a typical infant formula extract and 0.06% RSD_r (n = 45) for the absolute baseline, with a baseline drift of approximately 0.6 RU $cycle^{-1}$. Overall, a biotin-immobilised surface was found to be durable over at least 400 injections for each of the four flow cells available on a single sensor chip.

The recovery of added biotin at 50, 100 and 200% was assessed with three independent products of different composition, and was estimated from the slope of the linear regression of measured biotin against added biotin. Over the three addition levels, recovery was 91–104, 85–99 and 91–102% for each of the three products, with r^2 values > 0.995. Recovery is a function of analyte concentration, and these values were within the established guidelines of 75–120% (AOAC International, 2002).

Accuracy of the overall method was further evidenced through analysis of SRM 1849a, for which the mean was found to be 202 ± 11 μg hg^{-1} (n = 9), and was within the assigned certified value of 199 ± 13 μg hg^{-1}, the latter being the combined mean of results from NIST and collaborating laboratories derived from HPLC-MS, HPLC-MS^2, HPLC-fluorescence, microbiological and ligand-binding methods.

Comparability of quantitative data from independent analytical methods provides evidence for the accuracy of a proposed method, and the described method was therefore further evaluated over a range

of paediatric formulae by comparison with an AOAC validated proprietary kit-based biosensor assay that has independently been demonstrated to yield statistically equivalent data to that obtained from the reference microbiological assay (Indyk et al., 2000). Figure 3 presents these data graphically, yielding a linear regression slope of 0.9716 (r^2 = 0.995), an intercept that incorporated zero and a paired two-tail t-test that confirmed an absence of significant difference (p = 0.17).

3.2. Application

3.2.1. Paediatric compliance

Human milk is the primary evolutionary source of infant nutrition and therefore directs the composition of manufactured breast milk substitutes. With reference to both the reported biotin content of human milk and the absence of consensus reference intakes, biotin levels in paediatric formulae of 1.5–10.0 µg 100 $kcal^{-1}$ are recommended under regulation to meet the nutritional requirements of infants during the first months of life (MacLean et al., 2010). The rapid and reliable determination of the biotin content of manufactured paediatric formulae is therefore a strategic requirement for regulatory compliance. In the present study, bovine milk-based and soy-based formulae were subjected to a facile extraction scheme that was appropriate for the analysis of biotin present predominantly in its free innate and supplemented form. Compliance with the assigned certified content of an SRM and consistency with specification intervals for a range of products, as presented in Table 2, provides confidence in the reliability of the method.

Comparable simplified extraction strategies for the analysis of biotin in paediatric formulae have been reported (Hayakawa et al., 2009; Höller et al., 2006; Indyk et al., 2000; Kergaravat et al., 2012; Lang et al., 2009; Lu et al., 2008; Shang et al., 2009; Thompson et al., 2006).

3.2.2. Bovine milk lactation

In mammals, physiological expression of milk components is influenced by the stage of lactation, with a generally declining concentration as lactogenesis proceeds from colostrum to mature milk (Michaelidou, 2008). The described biosensor immunoassay was applied to investigate the predominant free biotin content during the progression from early bovine colostrum and Figure 4 illustrates the temporal trend for an individual lactating animal over the first 28 days post-parturition.

A minor, but distinct physiological response to early lactogenesis was apparent, with almost threefold higher biotin content in early colostrum relative to the stable levels found in mature milk (> day 6). The estimated biotin content of mature milk (3–4 µg hg^{-1}) was consistent with levels previously reported (2.0–4.5 µg hg^{-1}) (Bitsch et al., 1989; Hayakawa et al., 2009; Higuchi et al., 2003; Höller et al., 2006; Indyk et al.,

2000; Woollard & Indyk, 2013). However, there have been very few reports of the biotin content in early lactation milk; a study on human milk indicated an increase between days 8 and 36 postpartum (Mock, Mock, & Stratton, 1997), whereas a study on bovine milk over a much longer time period revealed a decline from 0–80 days to 81–200 days postpartum (Higuchi et al., 2003). The expression of biotin in human milk is largely dependent on maternal diet, while, in contrast, ruminal physiology is a greater factor for bovine milk. The higher levels of biotin in early bovine colostrum (< day 5) may provide an essential supply to the calf until it is able to support its own rumen functionality.

3.2.3. Bovine milk seasonality

As a perishable food, bovine milk is commonly converted to powder form to facilitate storage and trade and thereby extend its utility in the human diet. Dairy husbandry techniques in New Zealand are based on extensive pasture grazing, which allows for the monitoring of natural seasonal changes in herd milk. In this study, biotin levels in low heat skim milk powder were estimated over an entire production season, as illustrated in Figure 5.

Biotin levels were reported on an as-is basis, given that the moisture content was consistent across the production period. The minor seasonal variation in biotin provides evidence for the view that rumen microbial biochemistry and milk maturity, rather than exogenous diet, are probably controlling criteria during ruminant lactation across the season. Nonetheless, the minor trend may be a consequence of systematic herd calving, which is intended to maximise lactating cows at peak early summer grass growth.

The biotin content of low heat skim milk powder was consistent with previously reported data for fluid milk, as expressed on a solids-not-fat basis (Bitsch et al., 1989; Hayakawa et al., 2009; Higuchi et al., 2003; Höller et al., 2006; Indyk et al., 2000; Woollard & Indyk, 2013). This supports the view that modern spray drying initiates negligible losses of biotin and therefore milk powder composition reflects, with reasonable accuracy, the contributions of raw milk.

4. Conclusions

The development, optimisation and single-laboratory validation of an optical biosensor for the quantitation of biotin in milk and paediatric formulae were described. A simple sample extraction combined with an automated monoclonal antibody-based inhibition assay format was demonstrated to be advantageous for the routine compliance monitoring of paediatric formulae in comparison with alternative HPLC, microbiological and proprietary kit-based biosensor analysis techniques commonly employed by industry, regulatory and contract laboratories. The method was further applied to the temporal change in the biotin content of early lactation milk, indicating a minor, but distinct physiological response to early lactogenesis,

and a survey of seasonal milk powder supported the view that rumen biochemistry and milk maturity are dominant factors.immunoassay

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Table 1. Cross-reactivity (CR) of anti-biotin antibody

Compound	IC_{50}[a]	CR (%)[b]
Biotin	19	100
Biocytin	166	11
Riboflavin	549	3
Lumichrome	43	44
Folic acid	e	0

[a] IC_{50} = half-maximal inhibitory concentration.

[b] CR (%) = IC_{50},analyte/IC_{50},biotin x 100.

Table 2. Biotin concentration of milk, paediatric formulae and adult nutritionals

Product (powders)	Biotin concentration (μg hg^{-1})	
	Biosensor[a]	Declared interval[b]
NIST SRM 1849a	201.7 (0.8)	186–212
Follow-on formula	54.0 (2.4)	24–67
Growing-up formula	96.3 (7.5)	58–136
Infant formula	31.9 (5.1)	11–49
Growing-up formula	50.6 (3.4)	26–72
Infant formula	29.0 (6.4)	13–48
Adult nutritional	93.5 (2.8)	50–100
Follow-on formula	33.6 (5.3)	24–67
Adult nutritional	86.0 (4.3)	50–100
Follow-on formula	31.3 (5.4)	10–50
Soy-based infant formula	12.1 (3.4)	10–20
Non-supplemented whole milk	22.5 (5.8)	na[c]

[a] Biotin levels are the means of independent triplicates (RSD%) by the described biosensor assay.

[b] Declared minimum and maximum range based on product specification.

[c] na = not applicable.

SRM NIST 1849a certified value = 199 ± 13 expanded uncertainty at 95% confidence.

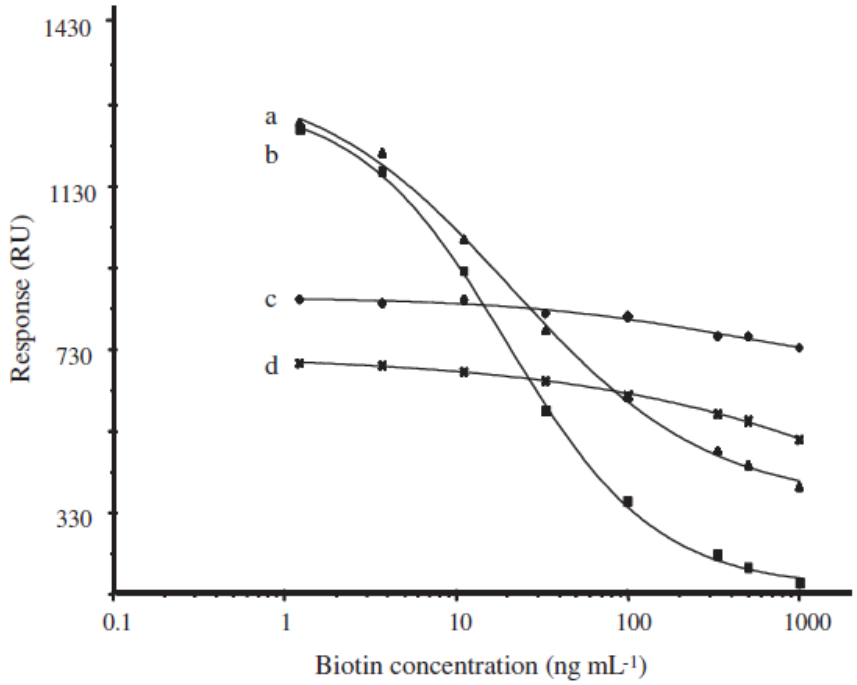

Figure 1. Binding and inhibition of commercially available antibodies by biotin (1.23–1000 ng mL^{-1}) over biotin-immobilised sensor surface. Conditions as described in *Materials and Methods*. (a) Pierce monoclonal antibody; (b) Novocastra Leica monoclonal antibody; (c) Bethyl sheep polyclonal antibody; (d) Bethyl goat polyclonal antibody

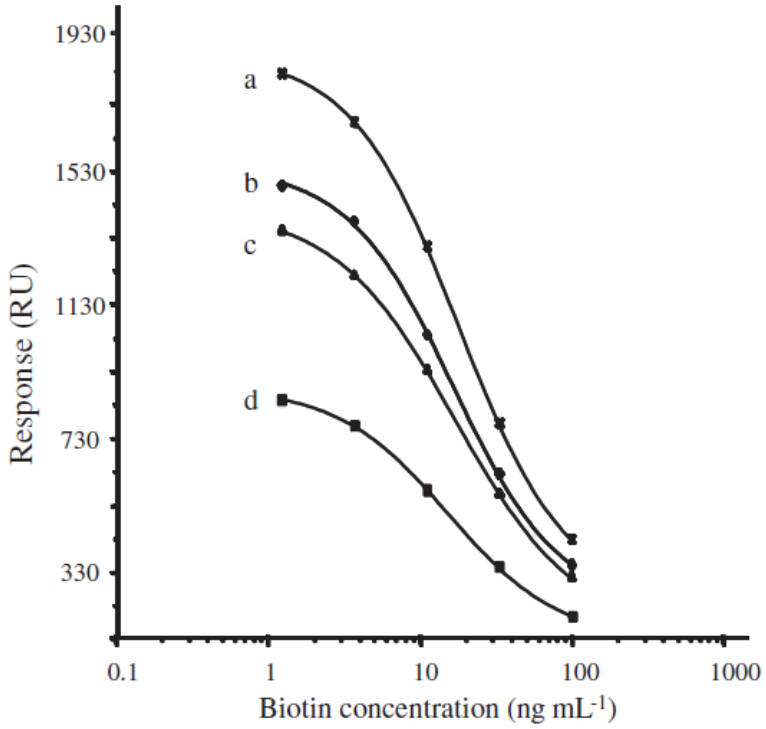

Figure 2. Influence of selected antibody (Novocastra Leica) concentration on sensitivity and working range. Dose-response of biotin with monoclonal antibody concentration (IC50 ng mL^{-1}) of (a) 10.0 µg mL^{-1} (18.2), (b) 8.5 µg mL^{-1} (16.7), (c) 7.5 µg mL^{-1} (17.2) and (d) 5.0 µg mL^{-1} (15.6) over immobilised biotin under the described inhibition assay conditions

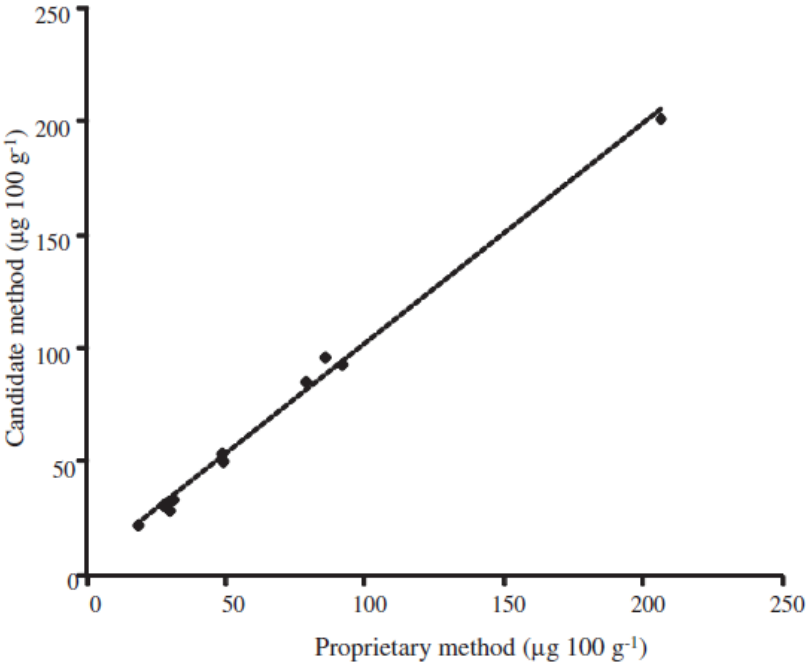

Figure 3. Comparison of the biotin content (µg hg^{-1}) of milk and paediatric formulae estimated by the described candidate and proprietary kit-based biosensor immunoassays

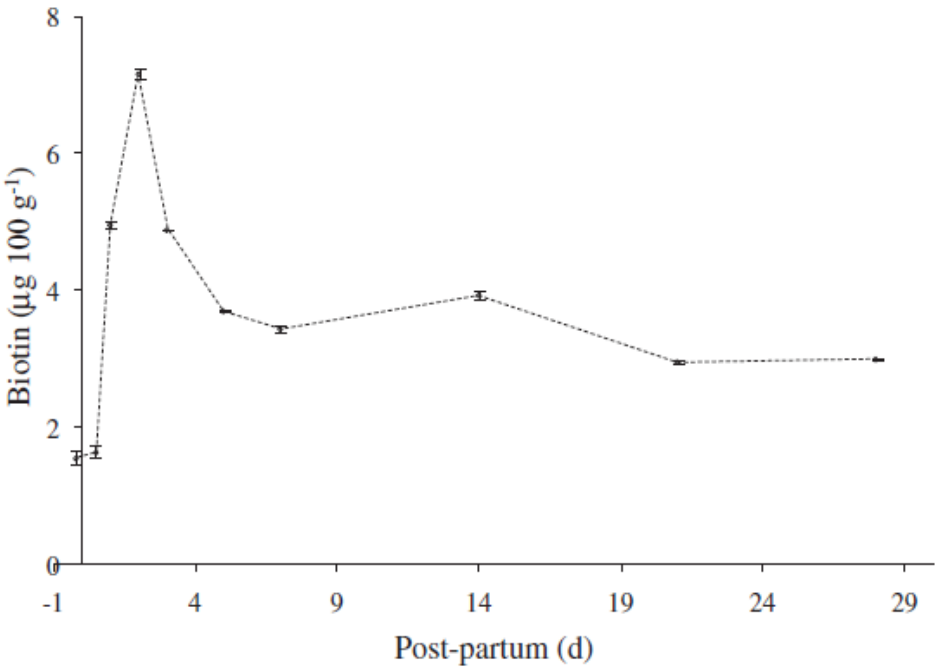

Figure 4. Biotin content of early lactation milk (0.2 day prepartum to 28 days postpartum) acquired from a Jersey breed cow. Each value represents the mean of duplicate analyses with 1 standard deviation error bar

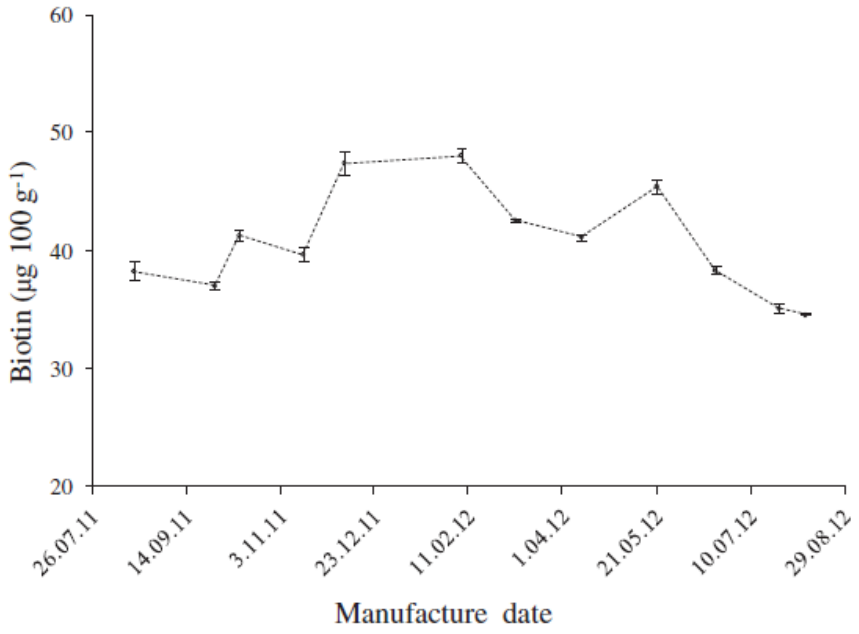

Figure 5. Biotin content of skim milk powder across a production season (August 2011–August 2012) processed by a single New Zealand spray-drying production facility. Each value represents the mean of duplicate analyses with 1 standard deviation error bar