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Analytical Methods

Quantification of water-soluble vitamins in milk-based infant formulae using biosensor-based assays

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ABSTRACT

Vitamin analysis is essential for quality control and development of functional foods. In this study, a biosensor-based technology developed by Biacore AB was evaluated for analysis of water-soluble vitamins B_2, B_{12}, folic acid, biotin, and pantothenic acid used to supplement infant formula samples. Performance parameters such as accuracy, repeatability and recovery for the five vitamins were studied. The repeatability was measured in terms of relative standard deviation (RSD) and $HORRAT_r$ value. The RSD for all vitamins was below 2% and the values of $HORRAT_r$ were 0.16, 0.10, 0.15, 0.11 and 0.22, for B_2, B_{12}, folic acid, biotin, and pantothenic acid, respectively. The recovery of vitamins ranged from 94.7% to 109.1%. Linear analyses indicated that the square of the correlation coefficient (R^2) for B_2, B_{12}, folic acid, biotin, and pantothenic acid were 0.993, 0.997, 0.993, 0.993 and 0.995, respectively. The results showed that the biosensor-based vitamin analysis technology is a sensitive, reliable and realistic alternative to other methods.

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1. Introduction

Vitamins are a broad group of organic components that are minor, but essential, constituents of food required for normal growth, self-maintenance and functioning of animal bodies. In addition, vitamins play an important role in maintaining muscle tone along the lining of the digestive tract and promoting the health of the nervous system, skin, hairs, eyes, mouth, and liver (Arcot & Shrestha, 2005; Ball, 2006; Fenech, 2001; Wardlow & Insel, 1995). Vitamin fortification is one of the key operations for formulation and processing of infant formulas and nutritional supplements. Water-soluble vitamins are used to fortify many kinds of functional foods to meet the nutritional requirements for humans; however, most of them are relatively unstable and can be lost during processing and storage. Quantification of vitamins in these nutritional products is required for the purposes of government regulation, quality control and nutrition labeling. There is a need to develop a specific, rapid and sensitive quantitative assay for the determination of water-soluble vitamin content in both food matrices and premixes.

Traditional methods of water-soluble vitamin (WSV) analysis include microbiological assay (MBA) (Baker, Frank, Khali, DeAngelis, & Hutner, 1986; Chun, Martin, Chen, Ye, & Eitenmiller, 2004; Voigt, Eitenmiller, & Ware, 1979; Watanabe, Takenakd, Aeb, Tamura, & Nakano, 1998; Wilson, Clifford, & Clifford, 1987), high performance liquid chromatography (HPLC) (DeLeenheer, Lambert, & Van BoExlaer, 2000; Eitenmiller & Landen, 1999; Ollilainen, Finglas, van den Berg, & de Froidmont-Gortz, 2001; Osseyi, Wehling, & Albrecht, 2001; Pawlosky & Flanagan, 2001; Rizzolo, Baldo, & Polesello, 1991; Woollard, Indyk, & Christiansen, 2000), in-capillary enzyme reaction methods (Ball, 2006; Okamoto, Nakajima, & Ito, 2003), electrochemical analysis (Kadara, Haggett, & Birch, 2006) and enzyme-linked immunosorbent assay (ELISA) (Finglas, Faulks, Morris, Scott, & Morgan, 1988).

Among these techniques, the most widely used methods are MBA and HPLC. MBAs are based on the growth response of various vitamin-dependent lactobacilli (Baker et al., 1986; Voigt et al., 1979; Watanabe et al., 1998). These assays may use *Lactobacillus plantarum* or *Saccharomyces cerevisiae* for biotin and *Lactobacillus rhamnosus* (formerly *Lactobacillus casei*) or *Streptococcus faecalis* for folic acid, although *S. faecalis* does not respond to methyl folates. *L. plantarum* and *L. rhamnosus* are the most commonly used for analysis of biotin and folic acid, respectively, in foods, because despite variable growth response to different vitamers, they provide optimum specificity and versatility. MBA is in general both sensitive and specific. It can provide a single, lactobacilli-specific estimate of biological activity through the combined response of all vitamers present. However, MBA methods are also time consuming, laborious and can exhibit poor precision (Finglas, Polesello, & Rizzolo, 1993). Compared to an optical biosensor protein-binding assay, analysis of vitamin B_{12} by MBA has previously been

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observed to give higher vitamin determinations, which was due to the reporting of biologically inactive corrinoids (5–20% of reported vitamin content) with the actual vitamin (Indyk et al., 2002). HPLC methods are more reliable, but often not sensitive.

As a part of functional proteomics, biomolecular interaction analysis (BIA) aims to quantify interaction patterns within a protein network in detail. BIA comprises a variety of methods based on physics, biochemistry and molecular biology (Moll et al., 2006). Since the 1990s, commercial biosensors utilizing surface plasmon resonance (SPR) for biomolecular interaction analysis have primarily been applied to characterizing macromolecular interactions (Blake, 2007; Lopez et al., 2003; Malmqvist, 1993; Stenberg, Persson, Roos, & Urbaniczky, 1991). Furthermore, optical biosensor techniques based on SPR now provide a promising method for quantitative analysis of several WSVs. The principle advantages of this instrumental technique compared with other biospecific techniques include real-time measurement, freedom from enzyme or radioisotope labeling requirements, and enhanced precision. The sensor chips used provide reproducible results, stable baselines, high chemical stability and low non-specific binding. Stability is such that sensor chip surfaces can be regenerated for many cycles depending on the nature of the immobilized ligands – 100 is average but as many as 400 are possible. They can also withstand high salt concentrations, extremes of pH and organic solvents. Sensor chips are easily interchangeable between assays providing great flexibility in the research environment.

There are few publications reporting the validity of the SPR-immunoassay for the water-soluble vitamins (Blake, 2007; Caelen, Kalman, & Wahlstrom, 2004; Haughey et al., 2005; Indyk et al., 2000; Kalman, Caelen, & Svorc, 2006). In view of its potential for routine quality control and nutritional labeling applications, in the present study, the BIA technique based on SPR was evaluated for analysis of B_2, B_{12}, biotin, folic acid and pantothenic acid supplemented infant formulas.

In SPR optical biosensor-based immunoassays, light is totally internally reflected at a glass–metal film (typically gold) interface and the reflectance is monitored as a function of angle. At a certain reflected angle (the SPR angle), a minimum in the intensity of the reflected light is observed. This indicates the excitation of surface plasmons at the metal–solution interface. The position of the response is sensitive to changes in refractive index in the vicinity of the metal surface (Englebienne, Hoonacker, & Verhas, 2003). Within SPR biosensors the resonance is expressed in an arbitrary scale of resonance units (RU). A response is defined as the RU difference compared with a baseline, normally fixed at the start of an experimental cycle.

In the present study a Biacore® Q biosensor and Biacore Qflex® vitamin kits were used to quantify WSVs concentrations. One of the key components of Biacore's biosensor technology is the patented gold sensor chip surfaces. The sensor chip surfaces consist of a glass surface coated with a thin layer of gold and this forms the basis for the range of specialized facades designed to optimize the covalent attachment and immobilization of variety of biomolecules. The most commonly used surface is a matrix comprised of carboxymethyl (CM) groups either directly attached to the gold surface or covalently attached via dextran hydrogel linkers of variable length. On sensor chip surfaces where a CM dextran matrix is present there is an alkane thiol linker layer that (i) allows covalent attachment of the CM dextran matrix and (ii) minimizes non-specific binding to the gold layer. This dextran hydrogel layer forms a hydrophilic environment for attached biomolecules, preserving them in a non-denatured state. The CM groups are open to covalent amine, thiol, aldehyde as well as other common coupling chemistries. As the injected sample interacts with the immobilized partners, the refractive index at the interface between the sensor surface and the solution alters to a degree proportional to change in mass at the surface. Thus, when molecules in the test solution bind to a target immobilized on the chip surface the molecular mass increases resulting in an increase in the local refractive index. Conversely when the binding complexes dissociate the mass decreases resulting in a decrease in the refractive index close to the sensor chip surface. Real-time changes in mass on the sensor chip surface are optically detected and reported in the form of a sensor gram (a plot of the resonance angle (in RU) against time).

Biacore's recently AOAC PTM accredited Qflex® vitamin kits are based on an inhibition immunoassay principle. Small analytes such as vitamins (<1000 Da) can be difficult to measure directly with high precision at low concentrations as they generate only small changes in mass. In this case, the inhibition assay has been demonstrated to be accurate, rapid and sensitive (Indyk, 2006).

2. Materials and methods

2.1. Instrument

Biacore Q® instrument was provided by Biacore, AB (Uppsala, Sweden) for this study. Biacore Q control software is a wizard-based tool for immobilization and quantitative as well as qualitative concentration analysis. It was designed to directly report analyte concentration from sensor gram data. Results are presented in the form of a calibration curve together with a table of response values and the corresponding analyte concentrations. As denoted in the respective instrument handbook, the detection limit for vitamins B_2 and B_{12}, folic acid, biotin and pantothenic acid are 85.5 μg/100 g, 0.3 μg/100 g, 5.0 μg/100 g, 5.0 μg/100 g and, 22 μg/100 g, respectively.

2.2. General principle

The general inhibition assay protocol used to analyze the WSVs is as follows. The Biacore Qflex kits are supplied with sensor chips immobilized with the vitamin or a derivative of the vitamin. A known, excess, amount of high molecular weight detecting molecule such as vitamin-specific binding protein or antibody (VBP or A) is added to the sample. The sample solution is injected over the sensor chips, the VBP or A binds to the analyte (vitamin) in the sample in proportion to the amount of analyte (vitamin) present in the sample. Unbound VBP or A will remain in solution available to interact with the analyte (vitamin or vitamin derivative) on the sensor surface. This binding to the surface takes place in a continuous, pulse-free and controlled flow, thereby maintaining constant VBP or A concentration at the chip surface. This means that the degree of response is directly proportional to the contact time between the sample and the surface, i.e., the injection volume. The response is measured as the difference in absolute response obtained immediately before and immediately after the injection of the sample. The higher the vitamin concentration in the sample, the higher the inhibition level and lower the detected response on the biosensor chip. The responses from known standard concentrations are used to generate a calibration curve and unknown samples are determined with reference to the standard curve.

2.3. Chemicals

Proprietary Qflex kits for vitamins B_2 and B_{12}, folic acid, biotin, and pantothenic acid were used in the present study. The kits are AOAC performance tested methods (AOAC license numbers 010604, 010605, 080201, 010606, and 090601, respectively) and were supplied by Biacore AB (Uppsala, Sweden). All other reagents including citric acid monohydrate, disodium hydrogen orthophosphate dihydrate ($Na_2HPO_4 \cdot 2H_2O$), hydrochloric acid, sodium hydroxide, acetonitrile, glacial acetic acid, sodium dodecyl sulfate

(SDS), sodium cyanide, sodium chloride, ethanol (absolute; BDH, Poole, UK), cyanocobalamin, >99% (Sigma–Aldrich, NSW, Australia) and bovine serum albumin (BSA) (Sigma–Aldrich) were of analytical grade. All water used was Millipore filtered with a resistance of 18.2 mΩ. All of the chemicals, reagents and sensor chips used were equilibrated to room temperature before use.

(a) *Qflex® vitamin B_2 kit*: The kit includes a vitamin B_2-immobilized sensor chip, the binding protein RBP, vitamin B_2 calibration solution (100 μg/mL vitamin B_2), running buffer (HBS-EP buffer), conditioning solution, regeneration solution, 96-well microtiter plates, adhesive foil strips and plastic disposable vials with penetrable seals.
(b) *Qflex® vitamin B_{12} kit*: The kit includes a vitamin B_{12}-immobilized sensor chip, vitamin B_{12} binding protein (VBP), vitamin B_{12} calibration solution. Other reagents and materials are as described for vitamin B_2.
(c) *Qflex® folic acid kit*: The kit includes a folic acid-immobilized sensor chip, anti-folic acid antibody, folic acid calibration solution (100 μg/mL). Other reagents and materials are as described for vitamin B_2.
(d) *Qflex® biotin kit*: The kit includes a biotin-immobilized sensor chip, biotin binding protein (BBP), biotin calibration solution (100 ng/mL). Other reagents and materials are as described for vitamin B_2.
(e) *Qflex® pantothenic acid kit*: The kit includes a pantothenic acid-immobilized sensor chip, pantothenic acid binding protein (PABP), pantothenic acid calibration solution (1 mg/mL). Other reagents and materials are as described for vitamin B_2.

2.4. Reagent preparation

2.4.1. Vitamin B_2

1. Calibrant/extraction buffer (phosphate-citrate buffer, pH 7.0 ± 0.05): This solution was used to prepare standards and for sample extraction. This buffer is composed of 900 mL of 0.2 M disodium hydrogen orthophosphate dihydrate solution and 200 mL of 0.1 M citric acid solution. The pH was adjusted to 7.0 ± 0.05 by adding either acid or base as necessary.
2. Vitamin B_2 calibration solutions: Prepared by diluting B_2 calibration stock solution to different concentrations (500, 300, 150, 50 ng/mL) with the above phosphate-citrate buffer.
3. Vitamin B_2 binding protein (RBP): Prepared by diluting B_2 binding protein stock solution 5-fold using HBS-EP buffer.
4. Vitamin B_2 regeneration solution: 100 mM hydrochloric acid solution.

2.4.2. Vitamin B_{12}

1. Calibrant buffer: 0.5 g of bovine serum albumin (BSA) was added to 50 mL phosphate-citrate buffer (pH 4.5). The mixture was stirred for at least 30 min until the BSA was fully dissolved. The solution was filtered through a 0.22 μm membrane.
2. Vitamin B_{12} extraction buffer: Prepared by diluting 0.2% sodium cyanide solution 40-fold with phosphate-citrate buffer (pH 4.5).
3. Vitamin B_{12} calibration solution: Vitamin B_{12} calibration solution was diluted with calibration buffer to concentrations of 2.40, 1.60, 0.80 and 0.08 ng/mL.
4. Vitamin B_{12} binding protein (VBP): Prepared by diluting the binding protein stock solution 10-fold using the vitamin binding protein diluent supplied.
5. Vitamin B_{12} regeneration solution: Prepared by mixing 0.25% sodium dodecyl sulfate solution and 150 mM solution of sodium hydroxide in 1:1 (v/v) ratio thoroughly.

2.4.3. Folic acid

1. Calibrant/extraction buffer: deionized water (pH 6.0–8.0).
2. Folic acid calibration solution: 2.50 ± 0.02 g of folic acid calibration stock solution diluted to 100 mL in a volumetric flask with deionized water at room temperature. This solution is further diluted with deionized water to prepare 100 mL of 100 ng/mL solution by adding 3.99 ± 0.02 g of the above solution. This solution was diluted with deionized water to get concentrations 33.3, 11.1, 3.70, 1.23 ng/mL.
3. Anti-folic acid antibody: Anti-folic acid antibody was used as supplied in the Qflex® folic acid kit.

Other reagents such as regeneration solution, conditioning solution and running buffer (10 mM HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% Surfactant P20) were used as supplied.

2.4.4. Biotin

1. Calibrant/extraction buffer: deionised water (pH 6.0–8.0).
2. Biotin calibration solution: The calibration solutions were prepared by serially diluting room temperature equilibrated biotin calibration stock solution with deionized water. The concentrations of biotin in calibration solutions were 100.00, 33.30, 11.10, 3.70 and 1.23 ng/mL.
3. Biotin binding protein: Biotin binding protein was used as supplied in Qflex® biotin kit.

Other reagents such as regeneration solution, conditioning solution and running buffer (10 mM HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% Surfactant P20) were used as supplied. All the samples and calibration solutions were protected from light to avoid cross-reactivity with lumichrome (a degradation product produced when riboflavin is exposed to light).

2.4.5. Pantothenic acid

1. Extraction/calibrant buffer: Same as in vitamin B_2 (pH 7.0).
2. Pantothenic acid calibration solution: Prepared by diluting pantothenic acid calibration stock solution (1 mg/mL) to intermediate solution I (50 μg/ml) by dispensing 500 μL into a 10 mL volumetric flask and dilute to 10 mL with the calibrant buffer. Working calibration solutions are prepared by further diluting intermediate solution I with calibrant buffer to 5000, 1500, 500, 100 and 10 ng/mL.
3. Pantothenic acid binding protein: Prepared by mixing one part binding protein stock solution and four parts HBS-EP buffer.
4. Pantothenic acid regeneration solution: The 20% acetonitrile/80 mM sodium hydroxide solution was prepared by mixing 4 mL of 0.1 M NaOH and 1 mL of acetonitrile.

2.5. Samples

Samples (n = 10) included a range of powdered infant formula products selected for their varying proximate composition and content of supplemental B_2, B_{12}, folic acid, biotin and pantothenic acid. They were obtained from local markets or provided by Shijiazhuang Sanlu Group (PR China). Infant formula samples 2, 4, 7, 8, 9 were milk based. Samples 1, 3, and 10 were enzymatically hydrolyzed reduced minerals whey protein concentrate based, sample 5 was lactose free milk protein isolate based and sample 6 was partially hydrolyzed nonfat milk based. In addition, a certified infant formula – standard reference material (SRM) 1846 (National Institute of Standards and Technology, 2006) was included in order to evaluate accuracy of the method. SRM 1846 is milk-

based infant formula powder prepared by Analytical Systems Research Corporation, Indianapolis, IN.

2.6. Extraction

The entire extraction and analysis were performed under conditions of low level yellow incandescent light. After extraction, all samples were filtered through a 0.45 μm filter followed in sequence with a 0.22 μm syringe driven filter cartridge. Enough filtrate was collected to fill the microtitre well used for the analysis.

2.6.1. Vitamin B_2

About 1 g of homogeneous infant formula was weighed into a 100 mL amber colored Erlenmeyer flask. About 45 mL of extraction buffer was added to the flask. The flask was covered with a stopper and sample was dispersed thoroughly. The mixture was stirred vigorously using a magnetic stir bar for 30 min. The extracts were quantitatively transferred to a 50 mL volumetric flask and made to volume with the extraction buffer.

2.6.2. Vitamin B_{12}

About 2 g of homogeneous infant formula sample was weighed into 100 mL amber colored Erlenmeyer flask. About 45 mL of extraction buffer was added to the flask. Flask was covered with a stopper and sample was dispersed thoroughly. The stopper was removed and the flask was covered with aluminum foil. The mixture was autoclaved at 121 °C for 25 min to denature any vitamin B_{12} binding proteins in the sample, releasing the vitamin for analysis. Sample extracts were cooled to ambient temperature, mixed thoroughly, and quantitatively transferred to 50 mL volumetric flask. The volume was made up with extraction buffer.

2.6.3. Folic acid

About 1 g of homogeneous infant formula sample was weighed into 100 mL amber colored Erlenmeyer flask. About 20–40 mL of extraction buffer (deionized water) was added to the flask. Flask was covered with a stopper and sample was dispersed thoroughly. The stopper was removed and the flask was covered with aluminum foil. The mixture was autoclaved at 121 °C for 10 min to improve vitamin recovery. The extract is cooled to ambient temperature and its pH was adjusted to 6.0–8.0 with dilute sodium hydroxide. The extract is centrifuged (15,000*g* for 10 min).

2.6.4. Biotin

About 1 g of homogeneous infant formula sample was weighed into 100 mL amber colored Erlenmeyer flask. About 20–40 mL of extraction buffer (deionized water) was added to the flask. The flask was covered with a stopper and the sample was dispersed thoroughly. The stopper was removed and the flask was covered with aluminum foil. The mixture was sonicated for 15 min to improve vitamin recovery. The pH of the extract was adjusted to 6.0–8.0 with dilute sodium hydroxide. The extract is centrifuged (15,000*g* for 10 min).

2.6.5. Pantothenic acid

About 1 g of sample is weighed into a 50 mL graduated centrifuge tube. Seven milliliters of water was slowly added to the tube while stirring so that the sample did not stick to the sides of the centrifuge tube. One milliliter of 3% acetic acid was added and made up to 10 mL with water. The mixture was agitated on a roller mixer for 20 min and centrifuged at 5000 rpm for 15 min. Five milliliters of supernatant was carefully pipetted into a 50 mL volumetric flask and made up to volume using the extraction buffer.

2.7. Analysis

Calibration solutions and sample extracts (about 200 μL) were dispensed (in duplicate) into the appropriate wells of a 96-well microtiter plate and covered with light-protective adhesive foil. The sensor chip and microfluidics system were equilibrated with HBS-EP buffer and respective vitamin binding protein, conditioning solution and regeneration solutions were positioned in the reagent rack. Following registration of samples and optimized assay parameters, including flow rate, contact time, regeneration time, system conditioning, and data processing functions, the automated protocol was initiated. The dynamic ranges of these assay parameters are dependent on the experimental conditions and the molecular weights of the interaction partners. The flow rates of the five vitamins are 40, 20, 40, 40 and 25 μL/min for vitamin B_2, B_{12}, folic acid, biotin and pantothenic acid, respectively. The contact time and the regeneration time are 30–480 s and 30–60 s, respectively. The injection volumes are ranged from 22 μL to 177 μL and analysis time per sample is about 2–10 min.

Vitamin calibration solutions were used without any pretreatment. The analysis procedure of sample SRM 1846 is exactly the same as the experimental infant formula samples.

2.8. Method performance parameters

Accuracy: Accuracy was evaluated by analyzing the reference sample SRM 1846, through comparison between the analytical values and the accepted values obtained from reference samples.

Recovery: Based on the AOAC guidelines for recovery evaluation (AOAC, 1998), a recovery study was designed to measure the added amount of vitamin in a sample. Five infant formulas were each used to prepare 2 samples spiked with a known amount of vitamin standard for a total of 10 samples. Samples 2, 3, 7, 8 and 9 were spiked with vitamin B_2, Samples 3, 4, 6, 7 and 8 were spiked with pantothenic acid. SRM 1846 was spiked with biotin, folic acid and vitamin B_{12}. Spiked controls were run to verify the assay would enable optimal recovery without the matrix. All samples for vitamin B_2 recovery analysis were spiked at the levels of 100–500 μg/100 g. Spiking levels for pantothenic acid, biotin, folic acid and B_{12} were 1000–5000 μg/100 g, 18.5–166.5 μg/100 g, 18.5–166.5 μg/100 g and 2–8 μg/100 g respectively. Recovery was calculated by the following equation (AOAC, 2002):

$$R\ (\%) = \left(\frac{C_s - C_p}{C_a}\right) \cdot 100$$

where R (%) is the percent recovery of added standard; C_s is vitamin concentration in the spiked sample; C_p is vitamin concentration in the unspiked sample; and C_a is the amount of vitamin standard added.

Repeatability precision: Precision measured within a laboratory is designated as repeatability precision (% RSD_r), including simultaneous and consecutive replicates (Horwitz, 2003).

$$\mathrm{RSD}\ (\%) = \left(\frac{\mathrm{SD}}{\mathrm{mean}}\right) \cdot 100$$

where RSD is relative standard deviation and SD is standard deviation.

Repeatability analysis was performed by analyzing one sample multiple times.

$HORRAT_r$ calculation: The Horwitz criterion of acceptance, determined as the $HORRAT_r$ value, was used to evaluate the precision of the repeatability measurement. The acceptable $HORRAT_r$ value is 0.3–1.3 for single laboratory precision (Horwitz, 2003). Calculations were conducted using the following equations:

$$\mathrm{RSD}_{r,\mathrm{observed}}\ (\%) = \left(\frac{\mathrm{SD}}{\mathrm{mean}}\right) \cdot 100; \quad \mathrm{RSD}_{r,\mathrm{predicted}}\ (\%) = \frac{2}{3}(2^{1-0.5\log C})$$

where C is the analyte concentration expressed as a mass fraction: g/g.

$$\mathrm{HORRAT_r} = \frac{\mathrm{RSD}\ (\%)_{\mathrm{r,observed}}}{\mathrm{RSD}\ (\%)_{\mathrm{r,predicted}}}$$

Intermediate precision: Intermediate precision of the method was determined by analyzing the same sample on three separate days.

2.9. Statistical analysis

The data gathered were analyzed for correlation and regression in accordance with standard statistical methods. The variability of the assay was expressed as the RSD in the same run or between runs performed on different days (inter-assay variability) (SAS, 1999).

3. Results and discussion

Vitamin fortification is necessary within defined ranges to meet the nutritional requirements of infants and is a practice subject to rigorous regulatory control over product composition and labeling. This study was designed to evaluate an optical biosensor-based immunoassay for quantification of water-soluble vitamins (B_2, B_{12}, folic acid, biotin, and pantothenic acid) in infant formula products. The assays were evaluated for intermediate precision, repeatability and recovery analyses.

Table 1
The average recovery at different spiking levels for B_2, B_{12}, biotin, folic acid and pantothenic acid analysis

Spiking level (μg/100 g)	Average recovery (%) (n = 10)	Lowest recovery (%)	Highest recovery (%)	RSD (%)
Vitamin B_2				
100	98.2 ± 6.51	90.0	110.0	6.63
300	109.1 ± 10.91	93.3	118.3	10.00
500	103.6 ± 2.59	100.0	108.0	2.50
100–500	103.6 ± 7.82	93.3	118.3	7.55
Vitamin B_{12}				
2	100.0 ± 2.97	97.5	107.9	2.97
4	97.6 ± 1.34	95.3	99.0	1.37
8	98.1 ± 1.88	95.8	101.1	1.92
2–8	98.6 ± 2.46	95.3	107.9	2.50
Folic acid				
18.5	100.2 ± 3.12	96.6	104.9	2.33
55.5	101.7 ± 3.73	96.1	104.4	2.40
166.5	102.6 ± 4.06	95.5	109.0	4.33
18.5–166.5	101.5 ± 3.56	95.5	109.0	3.07
Biotin				
18.5	99.5 ± 2.33	95.3	104.4	3.14
55.5	100.9 ± 2.44	93.9	106.2	3.70
166.5	100.0 ± 4.44	90.7	105.8	4.06
18.5–166.5	100.1 ± 3.11	93.9	106.2	3.56
Pantothenic acid				
1000	108.5 ± 7.18	92.0	117.0	6.62
3000	94.7 ± 5.32	87.0	104.7	5.62
5000	95.2 ± 1.69	93.20	99.2	1.78
1000–5000	99.5 ± 8.23	87.0	117.0	8.27

3.1. Recovery studies

The results of the recovery studies are presented in Table 1. The range of average recovery was 94.7–109.1% with RSD ranging from 1.78% to 10.00%. The average recovery for vitamin B_2, B_{12}, folic acid, biotin and pantothenic acid are 103.6%, 98.6%, 101.5%, 100.1% and 99.5%, respectively. The optical biosensor-based methodology produced acceptable recovery on all samples tested.

3.2. Repeatability of optical biosensor-based immunoassay

Intra-assay variances were evaluated by the repeatability study. One infant formula sample was analyzed 13 times for the five vitamins. The values of RSD were 1.73%, 1.76%, 1.15%, 2.13% and 1.94%, for B_2, B_{12}, folic acid, biotin and pantothenic acid, respectively. The results indicated that optical biosensor-based technology is very reliable with regards to repeatability. When outliers are excluded, the $\mathrm{HORRAT_r}$ value for vitamins analyzed in the present study ranged from 0.10 to 0.22 being significantly below 0.3, indicating acceptable precision for the analysis of the vitamins.

3.3. Intermediate precision of optical biosensor-based immunoassay

Inter-assay variances were evaluated to know the intermediate precision of the assay. We analyzed five vitamins in three infant formula samples on 3 different days. The RSD for each vitamin in three different samples is presented in Table 2 along with $\mathrm{HORRAT_r}$ values for intermediate precision of the method. The results show that the optical biosensor-based immunoassays have good intermediate precision. $\mathrm{HORRAT_r}$ values for vitamin B_2, folic acid, biotin and pantothenic acid are within the acceptable range of 0.3–1.3 (Horwitz, 2003) and that for vitamin B_{12} is below the lower limit of acceptable range which may be due to its lower standard deviation. These results demonstrate the satisfactory precision of the method at the analyte levels present.

3.4. Linear analyses

Linear analyses for the water-soluble vitamins were performed based on the results of analysis of five infant formula samples (in triplicate) at all spiking levels. Confidence of linearity is closely related to the rate of recovery. Fig. 1 shows that the correlation coefficients for the five vitamins analyzed were satisfactory ($R^2 \geqslant 0.9927$). The results of linear analyses indicate that the optical biosensor method is very reliable for quantifying these five water-soluble vitamins.

3.5. Analysis of standard reference sample

The standard reference sample SRM 1846 was also analyzed using the same method for comparing our results. The results of

Table 2
The intermediate precision of the five water-soluble vitamins using optical biosensor-based immunoassay

Vitamin	Sample 1			Sample 2			Sample 3		
	SD (n = 3)	RSD (%)	Horwitz ratio	SD (n = 3)	RSD (%)	Horwitz ratio	SD (n = 3)	RSD (%)	Horwitz ratio
B_2	84.85	6.33	0.59	41.63	2.69	0.30	33.63	5.32	0.44
B_{12}	0.035	0.63	0.025	0.23	3.99	0.16	0.63	3.99	0.15
Folic acid	5.30	3.50	0.24	6.01	4.86	0.33	2.12	1.77	0.30
Biotin	1.09	4.89	0.30	2.58	6.6	0.30	1.46	4.75	0.11
Pantothenic acid	105.83	1.22	0.30	153.73	2.79	0.32	221.88	4.36	0.49

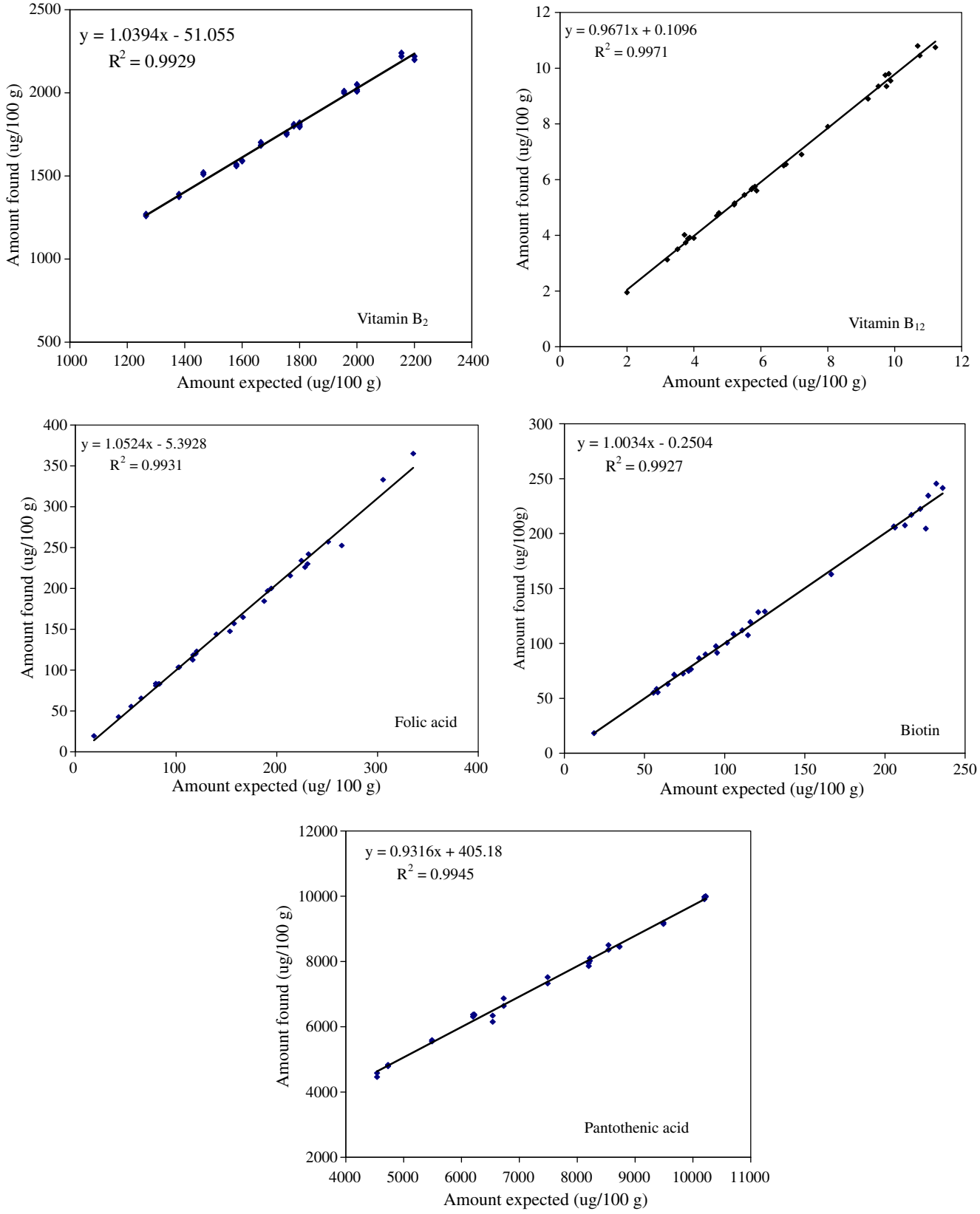

Fig. 1. Linearity of vitamin B_2, vitamin B_{12}, folic acid, biotin and pantothenic acid analyses.

this study were compared with the results obtained using the microbiological method for inter-laboratory certification studies (Table 3, National Institute of Standards and Technology, 2006). For all the vitamins tested the concentration of vitamins in SRM 1846 was similar to and within the assigned reference values found by the inter-laboratory certification study.

3.6. Analysis of water-soluble vitamins in infant formula

After the evaluation of optical biosensor-based technology for vitamin analysis, 10 different commercial samples of infant formula were analyzed. The results were presented in Table 4. They indicate that the values obtained from the assay were much higher than those reported on the nutrition label. Commonly, the amount of vitamins added for fortification is more than infants require, minimizing loss during processing. Innate forms of the WSVs will also be detected during the analysis which will contribute to measured levels exceeding the nutrition label claims. Unknown matrix effects and differing responses for racemic forms or other natural vitamers may also affect the results and may be the reason for the inflated values.

The Biacore Qflex® kits used in this study for the inhibition assays performed are selective, but not totally specific. The cross-reactivity of these five vitamins with other vitamins and analogous compounds are presented in Table 5 as reported by Blake (2007) and Indyk et al. (2000) and indicated in the instrument handbook (Biacore, 2004).

In vitamin analysis the extraction process is a very important step. Investigators have used a variety of solvents for vitamin analysis, but many possess certain disadvantages which render them inefficient or unselective. Pantothenic acid exists in foods largely in its free form. For B_2 and pantothenic acid, the extraction buffer is a phosphate-citrate buffer with pH 7.0. This is consistent with other studies showing that good extraction solvents are water aqueous solution containing a small amount of acid. (Gonthier, Fayol, Viollet, & Hartmann, 1998; Stuart, Elsa, & Mccollum, 1935).

Vitamin B_{12} may exist in different forms in food among which cyanocobalamin is the most stable form and is the form normally used for fortification of food. Endogenous vitamin B_{12} in milk is associated with the haptocorrin R-binder glycoprotein. The optimized extraction procedure used in the present study is consistent with standard protocols, which liberate protein-bound cobalamins and convert endogenous multiforms to stable cyanocobalamin. If other forms of the vitamin B_{12} are present, cyanide in the extraction buffer will transform them to cyanocobalamin.

Table 3
Comparison of the actual analyzed values to the reference values of infant formula RSM 1846 (μg/100 g)

Vitamin	Reference value[a] ($n = 6$)	Actual analyzed value ($n = 6$)
B_2	1740 ± 100	1670 ± 32.25
B_{12}	3.9 ± 0.3	4.19 ± 0.1
Folic acid	129 ± 28	133 ± 1.94
Biotin	41.1 ± 6.6	43.10 ± 2.35
Pantothenic acid	4870 ± 730	4240 ± 71.83

[a] Microbiological assay (National Institute of Standards & Technology, 2006).

Biotin is the most stable among the water-soluble vitamins. It is commonly found in two forms: the free vitamin and the protein-bound coenzyme form. For folic acid, however, the most common method to fortify infant foods is to use the pharmaceutical form of the vitamin. In the present study, distilled water is used for extracting biotin and folic acid with a satisfactory recovery rate. The accepted protocol for folic acid extraction uses a very dilute aqueous

Table 5
Cross-reactivity of the five vitamins with other vitamins and analogous compounds

Compound	Cross-reactivity (%)
Vitamin B_2	100
Riboflavin 5′-monohosphate	18.6
Flavin adenine dinucleotide (FAD)	None
Lumichrome	26.5
Lumiflavin	51.0
Biotin	None
Folic acid	None
Pantothenic acid	None
Coenzyme A	None
Vitamin B_{12}	
Cyanocobalamin	100
Cobinamide	35
Folic acid	
Folic acid	100
5-Methyl-tetrahydrofolic acid	100[a]
Dihydrofolic acid	17
Tetrahydrofolic acid	8
5-Formyl tetrahydrofolic acid	None
Biotin	
Biotin	100
Biocytin	10
Biotinyl-4-amidobenzoic acid	37
Luminchrom	38
Riboflavin	None
Pantothenic acid	
Pantothenic acid	100
Pantoyl taurine	3.6
Biotin	2.8
Ribofalvin	<0.7
Vitamin B_{12}	<0.7
Folic acid	<0.7
Thiamine	<0.7
Pantoyl lactone	<0.7
Co-enzyme A	<0.7
Pantethine	<0.7
Panthenol	<0.7
β-Alanine	<0.7
Glycine	<0.7
Lactic acid	<0.7
Glutamic acid	<0.7
Malic acid	<0.7

[a] In presence of ascorbate (1%, w/v) (Biacore, 2004; Blake, 2007; Indyk et al., 2000).

Table 4
Contents of water-soluble vitamins in infant formula samples (μg/100 g)

Infant formula	B_2		B_{12}		Folic acid		Biotin		Pantothenic acid	
	Measured value ($n = 2$)	Nutrition label	Measured value ($n = 2$)	Nutrition label	Measured value ($n = 2$)	Nutrition label	Measured value ($n = 2$)	Nutrition label	Measured value ($n = 2$)	Nutrition label
1	1180 ± 0	651.2	5.3 ± 0.02	1.53	137.5 ± 2.12	69.77	42.7 ± 0.35	20.46	5060 ± 14.14	2093
2	1280 ± 28.28	697.7	6.0 ± 0.04	1.16	155.0 ± 0	69.77	36.2 ± 1.13	20.46	4470 ± 28.28	2093
3	1500 ± 7.07	651.2	5.2 ± 0.02	1.53	140.5 ± 0.71	69.77	41.2 ± 0.28	20.46	3540 ± 28.28	2093
4	1305 ± 21.21	658.8	5.6 ± 0.32	1.41	128.0 ± 2.83	75.29	24.9 ± 1.27	14.12	4490 ± 56.57	2353
5	637 ± 16.97	658.8	4.2 ± 0.04	1.41	125.5 ± 0.71	75.29	27.5 ± 0.64	14.12	6060 ± 42.43	2353
6	971 ± 55.86	651.2	4.7 ± 0.37	1.39	133.0 ± 4.24	74.42	24.4 ± 0.78	13.95	3730 ± 56.57	2353
7	1655 ± 7.07	⩾500	6.0 ± 0.42	⩾1.2	51.6 ± 0.21	⩾40	23.5 ± 0.71	⩾9.0	5200 ± 56.57	⩾2000
8	1165 ± 7.07	⩾500	5.7 ± 0.40	⩾1.1	41.9 ± 0.85	⩾23	30.7 ± 0.49	⩾8.0	5220 ± 28.28	⩾1700
9	1700 ± 56.57	⩾500	5.1 ± 0.06	⩾1.0	34.6 ± 1.91	⩾25	28.6 ± 0.71	⩾9.0	8480 ± 134.35	⩾1600
10	1190 ± 0	⩾500	5.4 ± 0.03	⩾1.1	34.2 ± 0.85	⩾23	31.5 ± 0.57	⩾8.0	7540 ± 113.14	⩾1700

antioxidant solution. Added antioxidants such as ascorbate and 2-mercaptoethanol prevent the endogenous form of folic acid from being oxidized and therefore undetected (Arcot & Shrestha, 2005; Indyk et al., 2000; Kall, Norgaard, Pederson, & Leth, 2000). In the present study, no ascorbate was added to the extraction buffer because it was already present in all fortified infant formulas used.

Biomolecular interaction analysis technology enables savings in time and labor. One advantage of this instrumental technique is the ability to achieve reliable, accurate results without the use of enzymes or radioisotope labeling. The sample preparation scheme is simple and wizard driven software facilitated the process from sample injection to data analysis and reporting. Typically the total time required from sample preparation to generation of results is less than 12 h.

4. Conclusions

The present study demonstrates the suitability of the optical biosensor-based technique for routine compliance monitoring of vitamin supplemented infant formulas. Spiking, recovery, intermediate precision, repeatability analyses and SRM 1846 analyses indicate the method is reliable and accurate. The WSV concentrations detected in the infant formula samples were significantly above the detection limit of the instrument, allowing quantitative analysis of the analyte.

Ease of use and fully automated data analysis come together with selectivity, precision and repeatability to render this technique a practical alternative to established techniques. The BIA technique works with high selectivity and sensitivity. The sample preparation procedure is simple, the injection and analysis technique is fully automated which will overcome time-consuming procedures and reduce the risks of experimental errors. This instrumental technique provides a highly accurate method for rapid and precise analysis of WSVs. New developments such as binding proteins with greater specificity and smaller multichannel devices should help to further its acceptance as a powerful analytical tool.

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