FOOD COMPOSITION AND ADDITIVES

Determination of Vitamin B$_{12}$ in Milk Products and Selected Foods by Optical Biosensor Protein-Binding Assay: Method Comparison

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Biomolecular interaction analysis was evaluated for the automated determination of vitamin B$_{12}$ in a range of foods. The analytical technique was configured as a biosensor-based, nonlabeled inhibition protein-binding assay using nonintrinsic R-protein. Sample extraction conditions were optimized, and both ligand specificity and nonspecific binding considerations were evaluated. Performance parameters included a quantitation range of 0.08–2.40 ng/mL, recoveries of 89–106%, agreement against assigned reference values for 3 independent certified food reference materials, and a mean between-laboratory reproducibility relative standard deviation of 4.9%. The proposed method was compared with reference microbiological and radioisotope protein-binding methods for a range of food samples. A wide selection of milks, infant formulas, meats, and liver were evaluated for their vitamin B$_{12}$ content. The influence of season was studied in herd milk, early lactation was followed for a single animal, and the cobalamin content of bovine, caprine, and ovine milks was compared.

V itamin B$_{12}$ (Cbl) is an important cofactor for 3 cobalamin-dependent enzymes involved in red blood cell synthesis, fatty acid metabolism, and mediation of folate availability. Specifically, the most significant coenzyme functions are involved with methionine synthase, methylmalonyl-CoA mutase, and a ribonucleotide reductase (1). Clinical indicators of deficiency include pernicious anaemia and neurological pathologies. Vitamin B$_{12}$ refers generically to the cobalt-containing cobalamins with vitamin activity and

excludes nonactive cobinamide analogs, which may also be present in certain foods and tissues.

The predominant forms of cobalamin present in mammalian milk, animal tissues, and most foods include hydroxocobalamin (OH-Cbl), the 2 coenzyme forms, methylcobalamin (Me-Cbl) and adenosylcobalamin (Ado-Cbl), and the nonnaturally occurring cyanocobalamin (CN-Cbl) used for food supplementation. Although Cbl analogs predominate over Cbl in the alimentary tract of ruminants and in maternal plasma, they occur in milk and animal tissues only in traces, and the naturally occurring vitamin is protein bound in all mammalian milks (2). A recent Cbl speciation study confirmed the absence of Cbl analogs in milk and noted that secretory mechanisms by which gastric intrinsic factor-, serum transcobalamin-, or salivary haptocorrin-bound Cbl is absorbed via specific mammary gland receptors and ultimately expressed in milk, remain largely speculative (3). Meat and animal organ tissue, especially liver, are excellent dietary sources of vitamin B$_{12}$, with Cbl originating from intestinal flora and coprophagy in the case of herbivorous animals (4). Although several shellfish species and fermented foods contain substantial levels of inactive Cbl analogs, they do not occur to a significant extent in foods normally consumed by humans (5, 6).

Extraction is a critical factor and may influence results even more than analytical measurement technique. This is principally a consequence of the variable extent of protein binding, the existence of multiple vitamers, and their relative stabilities. Extraction protocols are generally designed to liberate protein-bound endogenous cobalamins present in foods and convert them to the stable CN-Cbl by heating at 100 C in mildly acidic buffer containing excess cyanide. Because OH-Cbl, CN-Cbl, and Ado-Cbl are reportedly converted into dicyanocobalamin (diCN-Cbl) with excess cyanide, while Me-Cbl remains unchanged, the appropriateness of utilizing CN-Cbl as a calibrant during determination has been considered (7).

Analytical techniques for evaluating either clinical status or food content are generally based on traditional microbiological assay (MBA), competitive protein-binding assay (PBA), or less commonly, liquid chromatography (LC). In
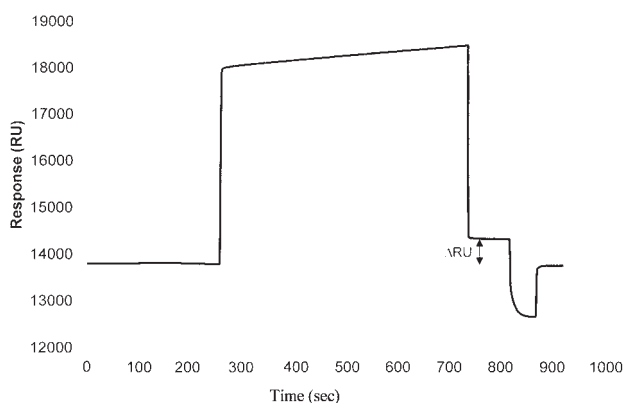
Figure 1. Sensorgram of vitamin B_{12} standard (0.08 ng/mL). ΔRU = relative response.

comparison to rational chromatographic methods, biospecific techniques are arguably empirical in nature, where the value determined is influenced by the specificity of the detecting species. There are issues with the assay of vitamin B_{12} content in certain foods, and it has been estimated that 5–20% of reported B_{12} content may be biologically inactive corrinoids (1, 8). MBA of vitamin B_{12}-dependent microorganisms is the most sensitive technique available and has been widely used for determining Cbl content of milk, formula, and foods, despite its recognized lengthy and manipulative attributes and relatively poor precision (8–12). In addition, issues of specificity exist, because the commonly used *Lactobacillus delbrueckii* can use both vitamin B_{12} and inactive purine-substituted cobalamin analogues (except cobinamide), as well as thymidine and deoxyribonucleosides (4, 6, 13).

Radioisotope-PBA techniques (RPBA; also described as radioisotope dilution assay) have largely met the need for rapid analysis of clinical samples and have been applied to various milks, foods, and dietary supplements (7, 10, 14–17). The assay is based on competition for a B_{12} specific ligand (intrinsic factor, nonintrinsic factor, or transcobalamin-II) with high affinity for the vitamin (K_D: 10^{-10}–10^{-16}M). In view of earlier reported evidence of the unreliability of PBA attributed to either variable response, nonspecific binding or metabolically inactive cobalamins, the specificity of the binding protein is critical to the accuracy of the technique, and a purified intrinsic factor is now usually recommended (4, 6). Direct comparisons between radioisotope-PBA and MBA have shown variable, yet comparable correlations in several studies provided rigorous extraction schemes are used, although the relative substrate specificity between intrinsic factor and *L. delbrueckii* is doubtless a significant factor (6, 8, 10, 11, 14). Homogeneous enzyme-linked PBA (18) and heterogeneous enzyme-linked 96-well microtiter plate PBA techniques have also been reported for vitamin B_{12} with nonintrinsic R-protein as Cbl ligand (19, 20). Although the haptocorrin R-protein binds all corrinoids (cobalamins and inactive analogs) compared to intrinsic factor (only cobalamins), it is appropriate for CN-Cbl fortified foods and exhibits greater solu-

tion stability than intrinsic factor (18, 20, 21). More recently, a fluorescence-PBA assay (22) and chemiluminescence system (5) have been described. Immunoassay techniques, although feasible, have been limited because of difficulties in generating hapten-specific B_{12}-active antibodies.

LC remains somewhat limited for the determination of endogenous vitamin B_{12} in foods, primarily due to a lack of specificity and sensitivity and the difficulty in accounting for all potentially bioactive forms present. Nevertheless, direct LC determination has been reported for infant milks (23), milk (24), and fatty foods (25), while combined LC-radioassay has been more commonly advocated for the speciation of cobalamins in milk (3, 26).

Recent developments in affinity-based immunosensor techniques exploit the potential for analyte detection in foods through coupling of the antibody–antigen interaction via optical, piezoelectric, or electrochemical signal transducer (27). The development of any immunosensor requires immobilization of either antibody or antigen to the transducer surface, often fabricated with a gold support layer. Biomolecular interaction analysis (BIA) is a biosensor-based technique and its general principles have been reviewed (28, 29). It involves the continuous, nonlabeled monitoring of a sensor-bound, ligand–analyte interaction via surface plasmon resonance (SPR) optics. To date, instrumentation configured with angular SPR optics has been used predominantly for estimating the binding kinetics, affinities, and structure–activity correlations of biomolecular interactions, but has also been increasingly applied to quantitative immunoassay of food components. Generally, immobilization is achieved covalently via a flexible hydrophilic carboxymethyldextran polymer linked to an alkylthiol-modified gold surface (CM5 sensor chip), thereby facilitating multiple regeneration of the biospecific ligand. Attributes of this technique have been reported for the analysis of toxins (30, 31), antibiotics (32, 33), and antimicrobials (34), while its multidimensional potential has been described for BIA-mass spectrometry (MS; 35) and LC-SPR (36).

Whereas the detection of high molecular mass analytes (>2 kDa) is facile by direct SPR, which is sensitive to both mass and refractive index changes at a sensor surface, low molecular mass compounds cannot be determined in the direct mode with the sensitivity and precision required for concentration analysis (37). Alternative optical techniques, such as reflectometric interference spectroscopy and flow injection absorptiometry, have been recently applied to the direct and nonlabeled detection of such low mass analytes (38, 39).

The routine, automated application of BIA-SPR using monoclonal antibodies in an indirect, inhibition assay format, has been described for determination of low molecular mass folic acid in supplemented foods (40) and both biotin and folate in milk and infant formula (41). The high affinity and specificity of Cbl binding proteins may also be exploited with this instrumental technique and a nonlabeled, inhibition PBA developed for the assay of vitamin B_{12} in milk, formula, and foods. In view of its potential for routine compliance and nutritional labeling, the technique was evaluated with reference to conventional methods and results are reported. The influ-

ence of season and lactation on the vitamin B_{12} content of bovine milk from extensively grazed herds was also evaluated.

Experimental

Apparatus

(**a**) *BiacoreQ^{TM}*.—Biacore AB, Uppsala, Sweden.

(**b**) *Autoclave*.—121 C, 15 psi.

(**c**) *Autoclavable vials*.—30 mL, disposable, with screw-capped lids.

(**d**) *Graduated glass Kimax tubes*.—25 mL.

(**e**) *Millex-GS syringe filters*.—0.22 m (Millipore, Bedford, MA).

(**f**) *Leuer-tipped plastic syringes*.—5 mL, disposable.

(**g**) *Volumetric flasks*.—10–1000 mL.

(**h**) *Variable volume micropipets*.—10–100 and 100–1000 L.

(**i**) *Brown vials, with teflon screw caps*.—1.8 mL.

Reagents

(**a**) *Vitamin B_{12} Qflex Kit*.—Biacore AB; includes CM5 sensor chip, porcine nonintrinsic factor (R-protein), amine-modified B_{12}-derivative, 96-well microtiter plates, adhesive foil strips, plastic disposable vials with penetrable seals, glass vials (4 mL), HBS-EP buffer (10mM Hepes, 150mM NaCl, 3.4mM EDTA, 0.005% surfactant P20, pH 7.4).

(**b**) *Amine coupling kit*.—Biacore AB.

(**c**) *Sodium dodecyl sulfate (SDS)*.—0.5% (w/v).

(**d**) *Surfactant P20*.—Biacore AB.

(**e**) *Cyanocobalamin, >99%*.—V2876 (Sigma-Aldrich, NSW, Australia).

(**f**) *Bovine serum albumin (BSA)*.—A6793 (Sigma-Aldrich).

(**g**) *Chemicals*.—Citric acid monohydrate, disodium hydrogen phosphate, sodium cyanide, sodium chloride, sodium hydroxide, and ethanol (absolute; BDH, Poole, UK).

(**h**) *Water*.—Purified to 18 M .

Reagent Preparation

(**a**) *B_{12} binding protein diluent*.—0.2M Na_2HPO_4 + citric acid, pH 7.6; 0.6M NaCl; 1% BSA; 0.1% surfactant P20. Dissolve 1.78 g Na_2HPO_4 + $2H_2O$ in 30 mL water. Add 1.755 g NaCl and dissolve. Add 0.5 g BSA and dissolve with gentle stirring. Adjust to pH 7.6 with citric acid (0.1M), filter (0.22 m) into volumetric flask (50 mL), add 0.5 mL surfactant P20 and dilute to volume. Store at room temperature (RT). Stable for 2 weeks.

(**b**) *B_{12} binding protein solution*.—Reconstitute lyophilized binding protein in 1.5 mL diluent, divide into 4 aliquots (350 L), and store at –18 C. Prior to use, thaw an aliquot and dilute with a further 3150 L diluent.

(**c**) *Regeneration solution*.—(0.25% SDS + 75mM NaOH.) Mix 10 mL SDS solution with 10 mL 150mM NaOH solution. Store at RT. Stable for 1 month.

(**d**) *Extraction buffer*.—(0.2M Na_2HPO_4 + citric acid, pH 4.5; 0.005% [w/v] NaCN.) Mix 450 mL 0.2M Na_2HPO_4 solution (35.6 g Na_2HPO_4 + $2H_2O$ in 1 L water) and 550 mL 0.1M citric acid (21.0 g citric acid monohydrate in 1 L water)

Table 1. Comparison of extraction conditions for vitamin B_{12} (μg/100 g)[a]

Sample[b]	BIA extraction[c]	MBA extraction[d]
Infant formula	4.0	3.8
Infant formula	3.8	3.7
Infant formula	6.1	6.2
Infant formula	3.0	3.2
Skim milk	4.3	4.2
Skim milk	3.0	3.2

[a] Measured by reference microbiological assay (mean of duplicates). BIA = biomolecular interaction analysis; MBA = microbiological assay.

[b] Samples were anhydrous powders.

[c] 1 g samples, 20 mL phosphate (0.1M)—citric (0.05M) buffer, 0.001 g NaCN, pH 4.5, 121 C, 25 min.

[d] 1 g sample, 15 mL acetate (0.3M) buffer, 0.004 g NaCN, pH 4.6, 121 C, 15 min.

and adjust to pH 4.5. Weigh 0.05 g NaCN into volumetric flask (1 L) and dissolve in buffer to volume. Store at 4°C. Stable for 1 month.

(**e**) *Calibrant buffer*.—0.2M Na_2HPO_4 + citric acid, pH 4.5; 1% BSA. Dissolve 2.5 g BSA in 50 mL buffer (0.2M Na_2HPO_4 + citric acid, pH 4.5). Filter (0.22 m) into volumetric flask (250 mL) and dilute to volume with buffer (0.2M Na_2HPO_4 + citric acid, pH 4.5). Store at 4 C. Stable for 1 month.

(**f**) *B_{12} stock*.—240 g/mL. Dissolve 60.0 mg cyanocobalamin (Mw = 1355.4) and dilute to volume in ethanol (25%, v/v) in volumetric flask (250 mL). Calculate accurate concentration spectrophotometrically ($_{361}$ = 28.1 10^3) and purity (A_{361}:A_{278} = 1.80 0.10; A_{361}:A_{550} = 3.25 0.10; 4, 6, 8). Store at 4 C. Stable for 6 months.

(**g**) *B_{12} intermediate I*.—2.4 g/mL. Dilute 0.500 mL stock with calibrant buffer to 50.0 mL. Prepare fresh for each analytical run.

(**h**) *B_{12} intermediate II*.—24 ng/mL. Dilute 0.500 mL intermediate I with calibrant buffer to 50.0 mL.

(**i**) *B_{12} calibrants*.—2.40, 1.60, 0.80, and 0.08 ng/mL. Serially dilute from intermediate II with calibrant buffer.

Sensor Immobilization

Vitamin B_{12} was immobilized by a standard amine-coupling procedure under instrument control. Briefly, the CM5 sensor surface was activated with a combined *N*-ethyl-*N* -(dimethylaminopropyl) carbodiimide and *N*-hydroxysuccinamide (EDC-NHS) reagent (10 L/min, 7 min); B_{12} derivative was coupled to the activated surface (5 L/min, 7 min), and ethanolamine was used to deactivate unreacted ester functionalities (10 L/min, 3 min). Following immobilization, the chip was stored between analyses over dessicant at 4 C in a sealed container.

Samples

Infant formula powders were selected for their varying proximate composition and content of supplemental CN-Cbl. In ad-

Table 2. Comparison of methods for vitamin B_{12} content (μg/100 g)[a]

Sample[b]	BIA	MBA	RPBA	Declared[c]
NIST SRM 1846 infant formula	3.98 (7.60, 14)	3.71 (5.75, 15)	3.0 (7.2, 5)	3.9 0.3
NIST SRM 2383 food composite	0.51 (13.7, 8)	0.37 (19.2, 2)	0.35 (14.6, 3)	0.44 0.19
NIST SRM 8435 wholemilk	2.38 (9.24, 5)	1.77 (9.98, 2)	1.6 (7.79, 8)	1.7 0.3
Control infant formula	4.82 (4.13, 23)	4.50 (6.39, 77)	3.82 (4.69, 5)	2.0–6.4 (2.0)
Infant formula	7.35 (5.58, 13)	7.2 (3.2, 4)	6.1 (4.2, 5)	1.7–7.0 (2.0)
Infant formula	9.43 (4.28, 12)	9.2 (6.0, 3)	8.9 (8.5, 5)	5.0–13.0 (4.7)
Skim milk	3.67 (7.46, 6)	3.0 (1.9, 3)	3.0 (4.37, 5)	NA[d]
Skim milk	5.87 (3.78, 4)	6.1 (2.3, 3)	5.3 (4.30, 6))	NA[d]
Goat milk	0.71 (8.80, 5)	0.66 (16.6, 4)	0.46 (14.8, 5)	NA[d]
Beef (minced)	1.32 (8.77, 4)	1.85 (8.27, 2)	1.47 (3.93, 3)	NA[d]
Beef (topside)	2.39 (2.40, 4)	2.59 (8.94, 2)	2.73 (4.23, 3)	NA[d]
Beef (rump)	3.52 (4.73, 4)	3.55 (14.46, 2)	3.86 (5.38, 3)	NA[d]
Liver (sheep)	102 (7.43, 3)	121 (4.5, 2)	91 (6.0, 2)	NA[d]

[a] Data expressed as mean (RSD%, n). BIA = biomolecular interaction analysis; MBA = microbiological assay; RPBA = radioisotope protein-binding assay.

[b] Milk and infant formulas are anhydrous powders.

[c] Reference values reported for NIST SRMs; range (label claim) reported for formulas.

[d] NA = Not applicable.

dition, a certified reference infant formula (NIST SRM 1846, National Institute of Standards & Technology, Gaithersburg, MD) was included to evaluate method accuracy.

Whole milk powders, fluid milks, and cereal-based foods were obtained from retail sources. Market fluid and powdered skim and whole milks were representative of predominantly Friesian-Jersey cross supply herds. One production site was selected and skim milk powders were sampled at monthly intervals across an entire season.

Bovine, caprine, and ovine milks were obtained from commercial sources, and human milk was pooled from 5 healthy donors (1–10 weeks postpartum). Raw bovine milk was collected from a single 4 year-old Jersey (2nd calving) between days –1 prepartum and +19 postpartum. Aliquots (5.0 mL) were pipetted into autoclavable vials, and extraction buffer (20 mL) was dispensed with mixing on a vortex mixer and frozen (–18 C) until analyzed.

Various beef cuts and sheep liver were sourced and the NIST SRM 2383 baby food composite and NIST SRM 8435 milk powder were used as additional controls during method evaluation.

Extraction

The entire extraction and analysis were performed under conditions of low level, yellow incandescent light. Dried milk, infant formula, or cereal-based foods (0.5–1.5 g), fluid milks (5.0 mL), baby food composite or meat (0.5–2.0 g), or liver (0.25 g) was weighed accurately into an autoclavable vial and extraction buffer (20 mL) added with vortex mixing. After standing for 30 min, the samples were autoclaved at 121 C for 25 min at 15 psi and then cooled to ambient temperature in a water bath. Extracts were transferred quantitatively to a 25 mL graduated test tube and diluted to volume with extraction buffer. The supernatant was clarified through a 0.22 m syringe filter, collecting ca 1.0 mL. Extracts of foods containing an elevated content of vitamin B_{12} (e.g., liver) require secondary dilution before filtration.

Analysis

Reagents and immobilized sensor chip were allowed to equilibrate to ambient temperature before use. Calibration standards and sample extracts (200 L) 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 Cbl-binding-protein, and regeneration solutions were positioned in the reagent rack. Following registration of samples and optimized assay parameters, including flow rate (20 L/min), injection time (480 s), regeneration time (51 s), system conditioning, and data processing functions, the automated schedule was initiated. Each injection cycle required 17 min, with a 40-sample schedule completed in 14 h, including calibration standards. Total vitamin B_{12} content in foods was expressed as cyanocobalamin.

Comparative Analysis

Several samples were analyzed by alternative MBA and radioassay.

Microbiological Assay

MBA protocols were based on established reference procedures developed for foods (8, 12) and modified for use with infant formulas. Samples (ca 1.0 g) were weighed accurately, in duplicate, into autoclavable vials and dissolved in 5 mL warm water (37°C) followed by NaCN (0.4 mL, 1%, w/v) and stored at –20 C before analysis. After thawing, sodium acetate buffer (10 mL, 0.4M, pH 4.6) was added; samples were then autoclaved at 121 C and 15 psi for 15 min and cooled, and contents were transferred into beakers (100 mL). The pH was adjusted to 6.2 with NaOH (10%, w/v), and the contents were transferred into a volumetric flask (100 mL) and diluted to volume. The solution was filtered, the first fraction was discarded and the filtrate was collected. Following appropriate serial dilution of sample extract, 4-level subaliquots were prepared in appropriately supplemented basal media (pH 6.2), and the assay was formatted using *L. delbrueckii* (ATCC 7830) with absorbance at 600 nm.

Radioisotope Protein-Binding Assay

Samples (ca 0.25 g) were dissolved in saline (10 mL, 0.9%, w/v) and stored at –18 C until analyzed. Analysis was performed using a combined folate + vitamin B_{12} kit (SimulTRAC-S, ICN Pharmaceuticals, Orangeburg, NY) following extraction with cyanide-containing buffer (100 C, 15 min) containing ^{57}Co-B_{12} tracer. Determination was based on competitive binding after incubation with affinity-purified porcine intrinsic factor.

Results

During the vitamin B_{12} inhibition assay, a fixed concentration of binding protein was equilibrated with sample and injected over the CN-Cbl immobilized sensor surface. The data output is in the form of a sensorgram, which records analyte binding in response units (RU) versus time, where 1 RU = 1 pg/mm^2 surface-bound protein. A typical sensorgram is represented in Figure 1, illustrating the significant events corresponding to baseline equilibration, association phase, nonsteady state response plateau, regeneration of sensor surface, and stabilization before subsequent injection cycle. The relative binding response is acquired (RU) and concentration of vitamin B_{12} interpolated from a 4-parameter fit calibration regression, with response inversely related to analyte level.

Because the SPR-evanescent wave extends beyond the dextran layer (ca 100 nm), the extent of abrupt signal response upon injection is dependent on the refractive index difference between sample and running buffer. Preliminary trials have demonstrated that because SPR response is independent of preinjection time, the association kinetics between R-protein and Cbl in solution is rapid enough to attain equilibrium binding before injection over the immobilized surface.

Evaluation of the SPR response over an activated and blocked, but nonimmobilized surface demonstrated minimal nonspecific binding to carboxymethyldextran. Several representative food extracts elicited very low response (ca 15 RU)
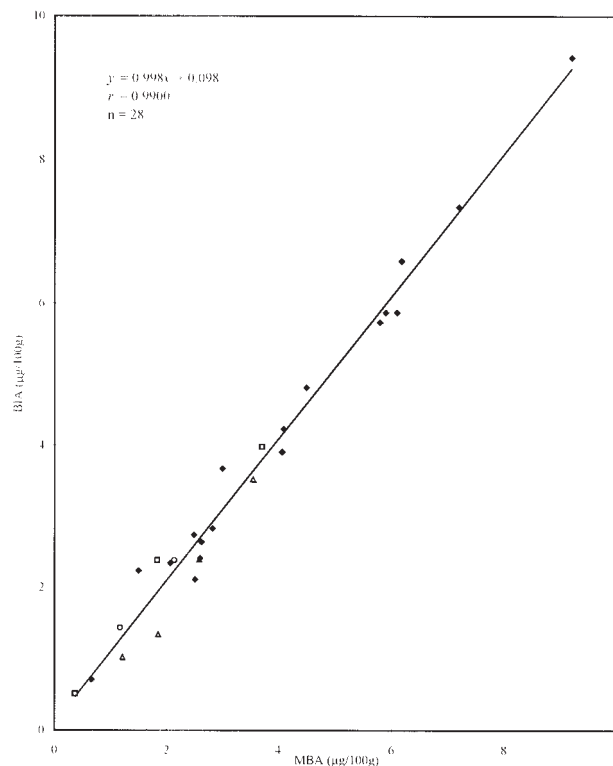

Figure 2. Comparison of vitamin B_{12} levels in foods determined by BIA and MBA. ♦ = milk or infant formula, Δ = meat or liver, □ = NIST SRM, O = cereal.

in the absence of R-protein, relative to the response under total inhibition conditions of about 4 ng/mL (ca 10 RU). Control experiments have shown that this minimal nonspecific binding is largely attributable to BSA. Also, under the described assay conditions, cobinamide (as CN_2-Cbn) will inhibit nonintrinsic factor to an extent comparable to CN-Cbl.

Maximum binding capacity (R_{max}) values of ca 8–10 kRU and noninhibited binding response of ca 500 RU indicate a maximum surface coverage of ca 5% under assay conditions. In addition, capture efficiency of R-protein may be estimated as ca 1.5%, based on the ratio between maximum R-protein bound to the flowcell (ca 5 fmole) and total exposure (ca 340 fmole). Such apparently low capture efficiency ensures that sample is not rapidly depleted of protein during the association phase.

Dose–response calibration curves established quantitation ranges for CN-Cbl of 0.08–2.40 ng/mL. Limits of detection (LOD;   and quantitation (LOQ; 10) were determined from the response of uninhibited binding protein and measured 0.06 and 0.20 ng/mL, respectively.

A range of samples was subjected to both BIA and MBA extraction techniques, and vitamin B_{12} was estimated by MBA (Table 1). Despite differences in extraction conditions (buffer type, pH, ionic strength, cyanide concentration, and heating time), both extracts yielded equivalent vitamin B_{12} content, as determined by the reference MBA technique
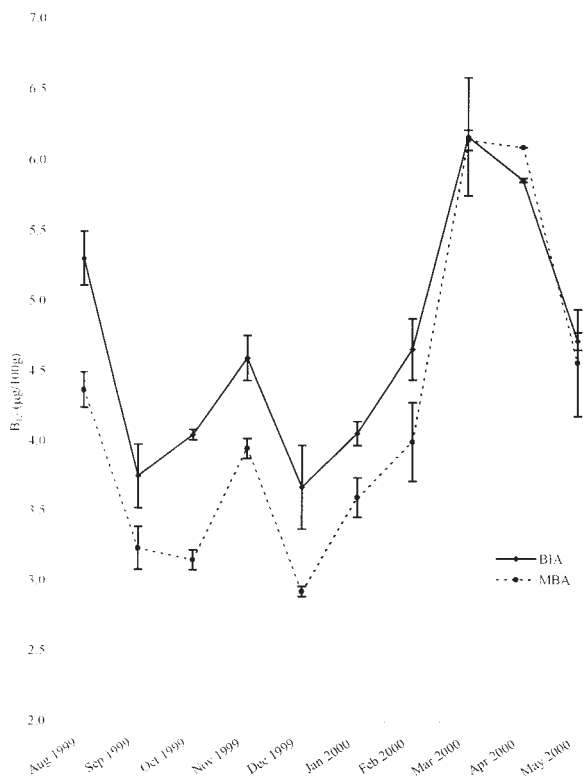
Figure 3.   Seasonal variation in vitamin B$_{12}$ content in skim milk powder.

(paired, 2-tail t-test: $p = 0.822$), suggesting that either extraction scheme facilitates quantitative recovery.

Mean recovery of the BIA method was estimated by duplicate standard additions of CN-Cbl at 3 levels (0.5, 1.0, and 1.5 ng/mL) to 3 infant formulas and one goat milk, and measured 89–106%. Further evidence of quantitative recovery was based on replicate analysis of the certified infant formula NIST SRM 1846, for which the mean and expanded uncertainty of BIA values measured 3.98   0.20   g/100 g ($n = 13$). This value is within the assigned reference value of 3.9   0.3   g/100 g based on an interlaboratory certification study using MBA ($n = 6$), where expanded uncertainty is defined as a 95% confidence interval incorporating an inhomogeneity contribution of the SRM (42). For the baby food composite NIST SRM 2383, these values were 0.51   0.05   g/100 g ($n = 8$) compared with a certified value of 0.44   0.19   g/100 g.

Intralaboratory precision was estimated for BIA through between-run replicate analyses of an infant formula powder (RSD_R, % = 4.15, mean = 4.83   g/100 g, $n = 21$), the NIST SRM 2383 (RSD_R, % = 13.7, mean = 0.51   g/100 g, $n = 8$) and at endogenous levels in a whole milk powder (RSD_R, % = 5.98, mean = 3.94   g/100 g, $n = 8$). The intralaboratory HORRAT values for these and other samples derived from BIA (Table 2) ranged from 0.13 to 0.70 (mean = 0.38), indicating acceptable method performance. Interlaboratory

parameters were evaluated from BIA data obtained by 2 independent laboratories for both the in-house control ($x = 4.83$ g/100 g; RSD_R, % = 4.15 versus $x = 4.69$ g/100 g; RSD_R, % = 3.09) and NIST SRM 1846 ($x = 3.98$ g/100 g; RSD_R, % = 7.60 versus $x = 3.78$ g/100 g; RSD_R, % = 5.03) infant formulas.

Comparability of data based on independent analytical techniques is indicative of an unbiased estimate of analyte level. A range of infant formulas, milks, meats, liver, and SRMs was therefore tested by BIA, MBA, and RPBA techniques (Table 2).

BIA yielded data statistically equivalent to the reference MBA (paired, 2-tail t-test, $p = 0.32$) and $r = 0.9922$; RPBA estimations were generally low ($p < 0.05$) with an overall bias of ca 8% relative to MBA. Results for the SRMs were generally consistent with values assigned from interlaboratory certification based on the exclusive use of MBA techniques. Estimated values obtained for formulas complied with expected specification ranges and were typically higher than declared levels, consistent with formulation overages generally recommended during infant formula production.

Figure 2 illustrates a direct comparison of BIA with independent MBA for an extended range of samples, including additional infant formulas and cereal-based products. Both methods demonstrated statistical equivalence for vitamin B$_{12}$, with the paired 2-tail t-test confirming an absence of significant difference ($p = 0.12$). Linear regression (unweighted) resulted in confidence intervals of slope and intercept including the ideal values of 1 and 0, respectively, confirming the absence of significant bias.

A survey of the potential influence of season on the vitamin B$_{12}$ content of milk was investigated in skim milk powder produced from exclusively pasture-grazed herds. Both BIA and MBA techniques were used independently (Figure 3). Both measurement techniques yielded similar data, confirming a seasonal variation of vitamin B$_{12}$ levels by a factor of approximately 2, with maxima coinciding with spring calving (August) and late summer (March–April).

Changes in vitamin B$_{12}$ content during early bovine lactation were investigated in the milk of an individual animal from precolostrum (–1 day) to mature milk (+19 days) postparturition. Both BIA and MBA techniques were used independently (Figure 4). Both analytical techniques yielded equivalent data, and revealed a significant physiological response to parturition, with a 5-fold variation in early colostrum levels relative to mature milk.

A limited comparison of the vitamin B$_{12}$ content of domestic mammalian species milks was conducted with pooled, fluid samples. Bovine milk ranged 0.2–0.8   g/100 mL, ovine milk 0.4–0.5   g/100 mL, caprine milk 0.07–0.09   g/100 mL, and human milk was below the limit of detection.

Discussion

Vitamin fortification of infant formulas is allowed within defined ranges to meet the nutritional requirements of the nonbreastfed infant and, for vitamin B$_{12}$, is achieved exclusively through addition of CN-Cbl, which is presumed to re-

main unbound. By contrast, 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 the stable cyanocobalamin. Comparative factorial studies have confirmed that critical extraction parameters influence recovery of vitamin B_{12} in milk, and that under optimized conditions, RPBA with intrinsic factor may yield data equivalent to MBA using *L. delbrueckii* (10).

The highly sensitive MBA is regarded as the reference method for analysis of vitamin B_{12} in foods, despite its inherent performance limitations and manipulative character. Although microtiter plate formats and cryogenic preservation techniques have overcome some of these generic limitations, such protocols have been applied primarily to the analysis of food folates (43–45). Alternative methods based on labeled protein-binding assays (enzyme or radioisotope) and chromatographic techniques have, despite their relative attributes, not fulfilled the practical requirements for routine application in the area of food labeling and compliance analysis.

The described biosensor-based protein-binding assay uses SPR to detect surface binding events and thereby facilitate a nonlabeled, real-time, and automated instrumental technique. As with labeled immunoassay and PBA methods, an inhibition assay protocol is generally used for low molecular weight analytes (<2 kDa) that do not directly elicit a significant SPR optical response. A high concentration of immobilized vitamin B_{12} ligand (R_{max} ca 10 000 RU) and low flow rate (20 L/min) favor the mass transfer limiting conditions generally advocated for concentration measurements. With high affinity systems, these conditions lead to low dissociation rates at the surface and an almost linear binding response during association. Further, analytical detection limits for the described SPR detection technique are dominated by both the high affinity of the Cbl:R-protein interaction ($K_D \leq 10^{-10}$M) and the molecular size of the glycoprotein ligand (ca 100 000 Da).

Despite the high affinities of the several vitamin B_{12} binding proteins involved in uptake and transport of Cbl (46), the affinity characteristics of the surface-bound analyte:ligand interaction facilitated the analytically essential multiregeneration potential of the Cbl-immobilized sensor. Thus, replicate analysis ($n = 40$) of a control sample over 200 injection cycles yielded an RSD_R of <5%, with a baseline response drift of <1 RU/cycle consistent with negligible variation of absolute signal over time.

As for immunoassay, it is imperative to demonstrate during validation of protein-binding assay techniques, that binding interferences are analytically insignificant, especially for label-free detection systems (27, 47). A minimal SPR response under nonspecific binding conditions has confirmed the specificity of the described BIA assay. Further, the addition of CM-dextran to sample extracts did not elicit a moderated response, further indicating insignificant nonspecific binding at the sensor surface. Low susceptibility to nonspecific adsorption is a characteristic property of the hydrophilic carboxymethyldextran layer, through which covalent immobilization establishes the interfacial-recognition layer of SPR-based immunosensors (48, 49).

There was significant correlation between the 3 analytical techniques, despite the radically different specificities of the diverse Cbl detector species. The purified intrinsic factor used in the RPBA exhibits the highest specificity for cobalamins, which probably accounts for the generally systematic and slightly lower results obtained with this technique. Similar observations during comparison of intrinsic factor-based assays with MBA have been reported, although not always consistently (5, 10, 11, 14, 16, 21, 50). As a haptocorrin, R-protein is less selective than intrinsic factor in recognizing physiological forms of B_{12} in view of its cross-reactivity with endogenous nonactive cobinamides. It has, therefore, been less commonly used in PBA (21), although its availability and enhanced conformational stability in solution confer analytical advantages (18, 20). R-protein is, however, generally considered the predominant natural binder in milk for Cbl, although a recent study reported the significance of transcobalamin in bovine milk (51). It is speculated to be either synthesized in the mammary gland, or actively transferred from serum to mammary epithelial cells during lactation. The equimolar binding properties, binding constants, and relative affinities of this and related Cbl-binding glycoproteins have been characterized and reported previously (46, 52, 53).

Equivalence between the haptocorrin-based BIA and *L. delbrueckii*-based MBA for the foods investigated suggests both insignificant levels of B_{12} analogs, as previously confirmed for milk (3), and that potential interferences to MBA from deoxyribosides are insignificant in those foods. Because this biosensor-based study has further confirmed that R-protein is inhibited to a comparable extent by either CN-Cbl or CN_2-Cbn, the equivalence between analytical methods represents further evidence for negligible content of the biologically inactive cobinamides in the foods selected.

Although the BIA technique was assessed primarily for application to routine analysis of CN-Cbl supplemented infant formulas, it was also evaluated for measurement of endogenous Cbl in milk and other foods. In bovine milk, the predominant form is OH-Cbl, with contributions from Me- and Ado-Cbl, all of which are associated with the R-binder glycoprotein (26, 54). In this study, vitamin B_{12} content in mature bovine milk ranged from 0.2 to 0.8 g/100 mL, which compares with published values of 0.15–0.9 g/100 mL obtained by a variety of analytical techniques (2, 5, 9, 10, 11, 22, 26, 50, 51, 55).

The vitamin B_{12} content in caprine milk was significantly lower than bovine milk and ranged from 0.07 to 0.10 g/100 mL, which compares with published values of 0.04–0.07 g/100 mL (56). Mature ovine milk contained levels comparable to those of bovine milk and ranged from 0.4 to 0.5 g/100 mL, consistent with the limited published values of 0.3–1.0 g/100 mL (16, 56, 57). Human milk is known to contain vitamin B_{12} at the much lower levels of 0.02–0.04 g/100 mL (3, 17, 58), which is below the detection limit of the currently described SPR technique, although MBA yielded data comparable to those reported in the literature

(0.02 g/100 mL). Further, evidence of a characteristically detectable nonspecific interference compounded the difficulty in estimating such low levels of Cbl in human milk by BIA.

Any potential influence of breed, pasture, or indoor feeding regimens on bovine milk Cbl levels have generally been inconsistent and minor, unless a cobalt deficiency existed (2). The present biosensor-based study investigated seasonal influences on the content of vitamin B_{12} in bovine milk derived from exclusively pasture-fed animals and where herd lactation is synchronized to commence in early spring. The results indicate an early decline in Cbl content following commencement of herd lactation, a relatively stable mid-season, and an increase during progression of southern hemisphere summer. It is acknowledged that ruminants have no dietary source of vitamin B_{12} and are dependent on its production by bacteria that inhabit the rumen and utilize cobalt derived from the diet (59). It would therefore seem likely that any seasonal impact on the expression of this vitamin in ruminant milk acts indirectly through moderation of biochemical events in the rumen.

The vitamin B_{12} content of both bovine and ovine colostrum has been reported as ca 5–10-fold greater than that of mature milk, with levels falling rapidly over the first days postpartum and then remaining stable throughout the milking period (2, 16). More variable results have been reported for human colostrum, where, in contrast to ruminant physiology, regulated Cbl expression in human milk is also dependent on maternal diet (17, 58). The present biosensor-based study of a single lactating cow confirms a significant 4–8-fold temporal relationship in the period immediately prior to parturition and during the postpartum transition from colostrum to mature milk. The relatively high levels of vitamin B_{12} in early bovine colostrum plausibly provides an essential source of this vitamin to the calf immediately following parturition and until it is able to both establish cobalt sufficiency and support rumen functionality.

Muscle and organ meats, especially liver, are excellent sources of vitamin B_{12}. In the present study, different muscle meats yielded a range (1.3–3.9 g/100 g) consistent with published values (4, 5, 11, 22). In a representative sample of sheep liver, the result obtained by biosensor assay (1.02 g/g) is also consistent with typical published reports (4, 5, 14, 50).

NIST has addressed the absence of food-matrix SRMs for vitamins and has recently produced 3 materials (1846, 8435, and 2383) representative of infant formula, milk, and a food composite, respectively. The Community Bureau of Reference is also actively involved in providing reference foods of certified vitamin content (50), because such materials are acknowledged to be necessary during the validation of analytical methods intended to ensure compliance and establish traceable compositional information.

Conclusions

With radio-ligand assays not generally considered reliable for foods, the absence of a commercial source of the R-protein enzyme conjugate, and the sensitivity limitations of LC techniques, it has been reported that MBA will continue to be the
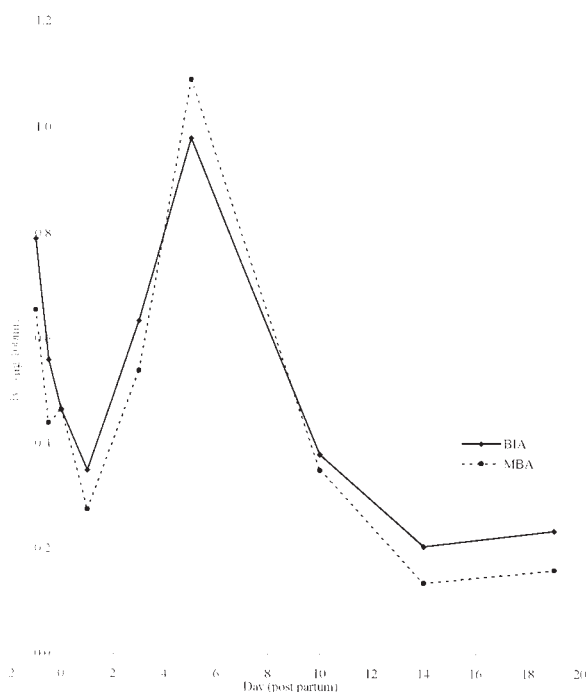
Figure 4. Variation in vitamin B_{12} content during early lactation of a single cow.

method of choice for food analysis (60). However, the present study demonstrates the suitability of the biosensor:SPR-based technique for routine compliance monitoring of vitamin B_{12}-supplemented infant formulas, as well as analysis of vitamin B_{12} at endogenous levels in selected foods. Established performance parameters and its inherently nonlabeled, rapid, and automated attributes qualify this technique as a practical alternative to established techniques. Although in common with IA, PBA, and MBA, BIA is an inherently Cbl group-specific technique, and therefore, unlike LC, cannot speciate the various cobalamins present in a food, it does offer significant advantages for the estimation of total vitamin B_{12} in such foods. The biosensor procedure is robust, thus facilitating its adoption in food laboratories involved in routine surveillance programs.

Acknowledgments

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