A Cyanide-Free Sample Preparation Methodology Prior to Determination of Vitamin B_{12} in Infant Milk Formula Using Hydrophilic Interaction Liquid Chromatography with Fluorescence Detection

Yanan Li[a], Brendon D. Gill[b], Merilyn Manley-Harris[a], and Megan N.C. Grainger*[a]

[a] School of Science, University of Waikato, Private Bag 3105, Hamilton, 3240, New Zealand

[b] Fonterra Co-operative Group Limited, P.O. Box 7, Waitoa 3380, New Zealand

* Corresponding author

Abstract

The analysis of vitamin B_{12} in infant formulas typically requires the use of cyanide during sample preparation to convert the unstable vitamers (hydroxocobalamin, methylcobalamin and adenosylcobalamin) to cyanocobalamin, the most stable form of vitamin B_{12}. To eliminate the risk to laboratory analysts in handling cyanide, alternative strategies are preferred for the analysis of vitamin B_{12}. This research demonstrates the use of cobalamin-derived α-ribazole (a nucleoside moiety of vitamin B_{12}) to determine total vitamin B_{12} content. Infant formula samples underwent protein denaturation and sugar removal with subsequent acidic hydrolysis and dephosphorylation employed to release α-ribazole, which was isolated by boronate affinity chromatography then analysed by hydrophilic interaction liquid chromatography with fluorescence detection. The method was validated using bovine- and ovine milk-based infant formula samples. The newly developed method was linear over the range of 0.65–6.48 ng mL^{-1} with repeatability of 3.78–5.47% relative standard deviation (RSD_r, n = 10) and an intermediate precision of 3.59–10.0% RSD_{iR} (n = 10). The limits of detection and quantitation (LOD and LOQ) were 0.4 and 1.2 µg 100 g^{-1} of dry weight, respectively. Accuracy was 68.9–76.4% and 68.7–80.0% at 50 and 150% of typical B_{12} concentrations in infant formula, respectively. The validated method was applied to eleven infant formulas and no statistical difference ($p = 0.45$, $\alpha = 0.05$) was found when comparing with the results obtained using the AOAC Official Method 2014.02 high performance liquid chromatography with ultraviolet detection that requires the use of cyanide. These results indicate that the newly validated

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method is not only reliable but also offers a safer alternative for routine vitamin B_{12} determination in infant formula while maintaining high accuracy and precision.

1. Introduction

Vitamin B_{12} (cobalamin) plays a vital role in the metabolism of human body cells including DNA synthesis and one-carbon metabolism of fatty acids and amino acids. It is involved in the functioning of the nervous system and haematological development. The requirement for vitamin B_{12} is at trace levels under normal health conditions, with vitamin B_{12} deficiency causing severe adverse physiological effects. Infants that are not breast-fed rely on infant formula to supply all necessary micronutrients, including vitamin B_{12}. Regulatory laboratories and infant formula industries demand highly accurate and sensitive detection methods to detect the relatively low concentrations of vitamin B_{12} in infant formula.

There are a number of methodologies used for determination of vitamin B_{12} in milk and infant formula. Microbiological assay [1], biospecific binding based assay [2] and chromatography with various detection methods [3–6] have typically been applied for the determination of vitamin B_{12} content. The instability of vitamin B_{12} caused by factors such as light, heat, acids, bases, oxidizing agents and reducing agents, means that sample preparation is a key factor in the determination of vitamin B_{12}. There are four different forms of vitamin B_{12} (methylcobalamin, hydroxocobalamin, adenosylcobalamin and cyanocobalamin); among them, the most stable form is cyanocobalamin to which the other vitamers can be converted by reaction with cyanide.

Milk-based infant formula contains all four forms of vitamin B_{12}. Hence, for all of the methodologies mentioned above, as a standard practice, conversion to cyanocobalamin has been employed to prevent vitamin B_{12} degradation during sample preparation and to provide a convenient measure of vitamin B_{12} as a single form.

Despite the fact that routine use of cyanide in the laboratory is considered undesirable because of health and safety issues, a cyanide free method for vitamin B_{12} determination without underrepresentation of unstable forms of vitamin B_{12} has not yet been reported.

All cobalamins belong to the broader corrinoid family that have the common characteristic of a corrin ring containing a central cobalt atom are distinguished from other corrinoids by having lower and upper axial ligands. The various moieties in the nucleotide-like loop on the lower ligand of the corrinoid have rather strong fluorescence in the form of α-ribazole-3'-phosphate (Fig. 1A), α-ribazole (Fig. 1B), or 5,6-dimethylbenzimidazole (DMB; Fig. 1C) each of which potentially can be employed as a fluorescent proxy for vitamin B_{12} determination, thus eliminating the requirement for conversion to cyanocobalamin by cyanide.

The release of the nucleotide analogue, Fig. 1A, can be achieved by acidic and/or alkaline hydrolysis [7,8]. However, it has been suggested that during hydrolysis the phosphate group can migrate to either the C-2′ or C-5′ position resulting in the α-ribazole-3′-phosphate or α-ribazole-5′-phosphate, respectively [6]. This mixture of α-ribazole phosphates is likely to elute at varying times, thus affecting the quantitation and rendering it unsuitable as a potential fluorescent marker.

Cleavage of the bond between ribose and DMB requires stringent conditions [9]. Strong acid and prolonged heating time raises the risk of non-specific degradation. Even though a DMB standard is commercially available, the difficulty of liberating DMB from the vitamin B_{12} led to the rejection of DMB, Fig. 1C, as a marker candidate for this study.

α-Ribazole, Fig. 1B, can be obtained by intense acidic hydrolysis of vitamin B_{12} with prolonged time and temperatures up to 150 °C [8]. However, dephosphorylation of α-ribazole phosphate by enzyme treatment with alkaline phosphatase (ALP; E.C. 3.1.3.1) is another possible method to yield α-ribazole. ALP is a nonspecific phosphomonoesterase that hydrolyses phosphate monoesters at an alkaline pH yielding inorganic phosphate [10,11].

The aim of this work was to develop and validate a method using α-ribazole, as a fluorescent moiety of vitamin B_{12}, to represent total vitamin B_{12}, thereby avoiding pre-conversion to the cyanide form. The fluorescent nature of α-ribazole means that highly sensitive fluorescence detection can be used for vitamin B_{12} determination in infant formulas at a level of parts per billion.

2. Method and materials

2.1. General methods

Chromatographic separation was performed using a Kinetex hydrophilic interaction liquid chromatography (HILIC), 2.6 µm, 4.6 × 150 mm column (Phenomenex, Torrance, CA, USA), maintained at 30 °C, on an UltiMate 3000 High-Performance Liquid Chromatography (HPLC) system configured with an SRD-3200 degasser, HPG-3200SD pump, ACC-300 Autosampler, TCC-3000SD column oven, and FLD-3100 fluorescence detector (Dionex, Waltham, MA, USA). An isocratic elution consisting of 95% acetonitrile and 5% ammonium formate (0.1 mol L^{-1}, pH 3.5) at 1.0 mL min^{-1} was used and 20 µL samples were injected. The total elution time was 10 min. The fluorescence detector (FLD) acquired spectral data at a specific excitation wavelength (251 nm) and emission wavelength (303 nm). The flow cell temperature was 40 °C. Chromeleon 6.8 Chromatography Data System software was used for instrument control and data processing. 1H, 13C, distortionless enhancement polarisation transfer (DEPT), 1H–1H correlation spectroscopy (1H–1H COSY), heteronuclear multiple quantum coherence (HMQC), and heteronuclear

multiple bond coherence (HMBC) NMR spectra were acquired using a JEOL ECZR 600 MHz NMR spectrometer. Samples were dissolved in deuterium oxide (Sigma-Aldrich). Chemical shifts were determined with reference to sodium trimethylsilylpropanesulfonate. Data were processed using Delta NMR Processing and Control software (version 6.1). Mass spectra of Q1 Scan (±) and Product Ion Scan (+) were obtained using a QTRAP 6500 MS/MS system (AB Sciex, Framingham, MA). The *m/z* range was 5–400 Da for both scans. Mobile phase A: ammonia aqueous solution (0.3% v:v, pH = 9.5) and mobile phase B: 100% methanol. Gradient elution comprised [min, (%B)]: 0 (5), 6 (95), 12 (95), 13 (5), 15 (5)] at a flow rate of 0.6 mL min^{-1}. Column temperature was 40 °C. MS parameters were: ion source temperature (200 °C), curtain gas (20 psi); ion spray voltage (5500 V), declustering potential (46 V), entrance potential (10 V) and collision energy (25 V). Samples were dissolved in 10% methanol aqueous solution. Data were processed using Analyst software (version 1.7.2).

2.2. Materials

Cyanocobalamin (96.0–102.0%), magnesium chloride (GR ACS), sodium acetate trihydrate (GR ACS), DMB (99%) and bovine ALP (5000 units mg^{-1}) were purchased from Sigma-Aldrich (St Louis, MO, USA). Sodium hydroxide (≥99%), glacial acetic acid ammonium hydroxide (28–30%), acetonitrile and methanol (HPLC grade) were purchased from Merck (Darmstadt, Germany). Ammonium acetate (GR ACS), ammonium formate (GR ACS), formic acid (≥ 99%), and hydrochloric acid (36%) were purchased from AJAX FineChem (Sydney, Australia). Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Standard Reference Material 1869 Infant/Adult Nutritional Formula II (SRM 1869) was supplied by the National Institute of Standards and Technology (NIST; Gaithersburg, MD, USA). Syringe filters with 0.45 µm polytetrafluorethylene (PTFE) membranes (Biofil, Guangzhou, China) was used for sample filtration. Solid phase extraction (SPE) C18 cartridges (500 mg, 6 mL) were purchased from Phenomenex. Immobilised Boronic Acid Gel (Catalogue No. 20244) was purchased from Thermo Fisher Scientific (Rockford, IL, USA). A centrifuge column (2 mL) was used for packing the boronate affinity chromatography (BAC) column (Thermo Fisher Scientific) with 0.5 mL of Immobilised Boronic Acid Gel. The packed BAC columns were stored at 4 °C.

2.3. Solutions

Sodium hydroxide solutions at concentrations of 3.0 M and 1.5 M were prepared by dissolving sodium hydroxide (60.0 g) in water (type 1) and making to 500 mL and 1 L, respectively, in volumetric flasks. Similarly, 3.0 M or 1.5 M hydrochloric acid solutions were made by diluting concentrated hydrochloric acid (36%, w/w, 123.1 mL) to 500 mL or 1 L, respectively, with water. Sodium acetate solution (0.1 M, pH 4) was prepared by dissolving sodium acetate (13.6 g) into water (950 mL), adjusting pH to 4 with glacial acetic

acid, then making up to 1 L with water. The incubating buffer (0.3 M ammonium acetate, 2 mM magnesium chloride, pH 8.8) was made by dissolving ammonium acetate (23.2 g) and magnesium chloride (0.2 g) into water (900 mL) and adjusting the pH to 8.8 with ammonium hydroxide solution, then adjusting the volume to 1 L with water. Alkaline phosphatase stock solution (>450 units mL 1) was made by dissolving alkaline phosphatase (2 μL) into incubating buffer solution (10 mL). The BAC binding buffer (ammonium acetate, 0.3 M, pH 8.8) was made by dissolving ammonium acetate (11.6 g) into water (450 mL) and adjusting pH to 8.8 by adding ammonium hydroxide solution, then adjusting the volume to 500 mL. The BAC eluent was made by mixing formic acid (1.0 M, 300 mL) with acetonitrile (700 mL). The mobile phase consisted of 95% acetonitrile and 5% ammonium formate with a concentration of 0.1 mol L^{-1} and pH 3.5 adjusted by formic acid. All buffer solutions were stored at 4 °C and discarded after one week.

Cyanocobalamin stock standard solution was prepared by accurately weighing cyanocobalamin (50.0 mg) into a 50 mL volumetric flask and making it to volume with 20% methanol. An intermediate standard solution of cyanocobalamin was prepared by diluting 1.00 mL of stock solution to 100 mL with 10% methanol. The α-ribazole stock standard solution was prepared by re-suspending the in-house isolated α-ribazole (from cyanocobalamin) with dimethyl sulfoxide [12]. Calibration standards of α-ribazole were prepared by diluting this stock standard to the required concentrations using ammonium formate solution (0.1 mol L^{-1}, pH 3.5). The calibration range was 0.65 ng mL^{-1} to 6.48 ng mL^{-1}.

Commercially available milk-based infant formula products were used for the validation, including bovine, ovine and caprine milk-based infant formulas.

2.4. α-Ribazole standard isolation

Due to the commercial unavailability of α-ribazole, a standard was created by isolating it from cyanocobalamin. The isolation procedure for α-ribazole was adapted from the method of Mattes and Escalante-Semerena [12] with modifications of the chemical hydrolysis and elution with BAC for a higher yield. This method employed HCl (1.5 mol L^{-1}) instead of NaOH (2.5 mol L^{-1}) for the release of α-ribazole-phosphate, and acetonitrile: formic acid (1.0 mol L^{-1}, 30:70, v/v) for elution in BAC, instead of formic acid (0.1 mol L^{-1}).

The dried product (approximately 1 mg) was dissolved in deuterium oxide (0.75 mL) with addition of acetic acid (7.5 μL) to ensure a pH of ~3. The solution was mixed by vortex and centrifuged (5 min, 1680 × g). The supernatant was transferred into an NMR tube (5 mm) for NMR analysis.

An aliquot of the isolated product was dissolved in 10% methanol aqueous solution and was analysed by LC-MS/MS.

The yield of α-ribazole was determined by its concentration in DMSO using UV-Vis Spectroscopy and using DMB as a proxy for calculation of the molar absorption coefficient. The molar absorption coefficient of DMB at 280 nm (ε_{280}) was calculated from a series of DMB solutions with known concentrations in DMSO.

2.5. Sample preparation

Since α-ribazole is a degradation product of vitamin B_{12} and is present in all vitamin B_{12} forms [6]; this endogenous background contribution must be accounted for when analysing the samples. Therefore, every sample was analysed twice; first for endogenous α-ribazole and then for total α-ribazole. The sample preparation process is illustrated in Fig. 2.

2.5.1. Reconstitution of infant formula samples

Infant formula powder (7.0 g, average from reconstitution instructions on the label) was added to water (50.0 mL) in a 100 mL Schott bottle. The capped bottle was placed in a warm water bath (approximately 35 °C, ensuring temperature remained less than 37 °C) for 1 h and shaken by hand for 30 s every 20 min to ensure full rehydration.

2.5.2. Protein denaturation

Reconstituted infant formula milk (2.0 g) was weighed into a 10 mL glass vial. Sodium acetate solution (0.1 mol L^{-1}, pH 4.0, 5 mL) was added to the vial which was capped and vortexed for 30 s and then heated at 90 °C for 30 min. Subsequently, the vial was cooled in an ice water bath. The extract was centrifuged (30 min, 2947 × g, 4 °C). The aqueous supernatant was removed, and the pellet re-extracted with sodium acetate solution (5 mL). Finally, the combined supernatants were filtered through a 0.45 μm cellulose acetate syringe filter prior to sugar removal by C18 SPE.

2.5.3. Removal of sugars

A C_{18} SPE cartridge was conditioned and equilibrated with methanol (5 mL) followed by sodium acetate solution (0.1 mol L^{-1}, pH 4.0, 5 mL). The filtered extract sample from previous step was applied to the cartridge and allowed to drain by gravity flow. Another 5 mL of sodium acetate solution (0.1 mol L^{-1}, pH 4.0) was used to rinse the empty sample vial and then loaded into the cartridge. Subsequently, air was passed through the cartridge under a vacuum (< 15 bar) for 1 min to dry the cartridge and sorbed material. Finally, 100% methanol (2 × 5 mL) was used to elute the bound vitamin B_{12} by gravity (the cartridge was dried by passing air through between the two methanol elutions). The combined methanol fractions were evaporated to dryness under nitrogen (50 °C).

2.5.4. Acid hydrolysis

To measure total α-ribazole, hydrochloric acid solution (1.5 mol L^{-1}, 1 mL) was added to the dried extract (from section 2.5.3) and the capped vial was vortexed for 1 min and heated (100 °C, 60 min). After cooling

in an ice bath for 30 min, sodium hydroxide solution (1.5 mol L^{-1}, 1 mL) was added to neutralise the hydrochloric acid.

To ensure the sample for endogenous α-ribazole had similar solution conditions to the sample as total α-ribazole after hydrolysis, sodium chloride solution, prepared by mixing hydrochloric acid (1 mL, 1.5 mol L^{-1}) and sodium hydroxide (1 mL, 1.5 mol L^{-1}), were added to the dried extract (from section 2.5.3). The vial was capped and mixed by vortexing (1 min). Since an acid hydrolysis was not required, this solution was ready for dephosphorylation by ALP in the next step.

2.5.5. Alkaline phosphatase dephosphorylation

The incubating buffer (ammonium acetate, 0.3 mol L^{-1}; magnesium chloride, 2 mmol L^{-1}; pH 8.8; 2 mL) and an ALP stock solution (50 µL) were added to both the acid-hydrolysed sample (total α-ribazole) and the non-hydrolysed sample (endogenous α-ribazole) and mixed well by vortexing for 30 s. The resultant solutions were incubated at 37 °C overnight (16 h).

2.5.6. Boronate affinity chromatography clean-up

The α-ribazole was separated from the hydrolysate mixture by passing the solution through a boronate affinity column. Prior to sample loading, the column was equilibrated with binding buffer ammonium acetate (0.3 mol L^{-1}, pH 8.8, 3 mL). The column was capped and shaken by hand for 30 s to mix the gel with the binding buffer and allowed to stand for 15 min with mixing by shaking for 30 s every 5 min to convert the gel into the basic form. The binding buffer was discharged from the column by gravity flow and the process repeated. The hydrolysate sample was loaded onto the column, mixed by shaking by hand (30 s) and was allowed to stand for 15 min with mixing by shaking for 30 s every 5 min. The solution was drained by gravity flow and the column was washed with ammonium acetate (0.3 mol L^{-1}, pH 8.8, 3 mL). Finally, acetonitrile: formic acid (1.0 mol L^{-1}, 30:70, v/v, 5 mL) was used to elute the retained α-ribazole. The eluate was evaporated under dry nitrogen (60 °C), re-suspended by adding 1 mL of acetonitrile, filtered with a 0.45 µm PTFE syringe filter, and analysed by HILIC-FLD. The used column was regenerated by washing with the same eluent (5 mL) and binding buffer (5 mL).

2.6. Identification and quantitation

The α-ribazole peak in the chromatograms of the sample solution was identified by comparing with the retention time and the fluorescence spectrum of the α-ribazole standard solution.

The concentration of vitamin B_{12} (C_{B12} in µg 100 g^{-1} of dry weight of infant formula) was calculated by Equation (1):

$$C_{B_{12}} = \left[\left(\frac{c_t \times v_t}{m_t}\right) - \left(\frac{c_e \times v_e}{m_e}\right)\right] \times \frac{M_{B_{12}}}{M_{\alpha-R} \times 10} \quad (1)$$

where c_t and c_e are, respectively, the concentrations of total and endogenous α-ribazole, in the sample solution obtained from HILIC-FLD analysis using external calibration (in ng mL^{-1}), v_t and v_e are volumes (1.0 mL) of the sample solutions analysed (for total and endogenous α-ribazole, respectively) and m_t and m_e are masses of infant formula sample that underwent analysis for total and endogenous α-ribazole, respectively, which can be calculated by Equation (2):

$$m_t \text{ or } m_e = \frac{m_{powder}}{m_{powder} + m_{water}} \times m_{prep} \quad (2)$$

where m_{powder} is the mass of infant formula powder used for reconstitution (g), m_{water} is the mass of water used for reconstitution (g) and m_{prep} is the mass of the sample taken of prepared reconstituted milk (g).

2.7. Method validation

α-Ribazole working standard solutions without sample matrix (range 0.65–6.48 ng mL^{-1}) with six different concentrations were prepared and analysed (n = 3).

The LOD and LOQ of the method were determined by the standard deviation (σ) of a low vitamin B_{12} content infant formula sample (n = 10) since it is difficult to acquire a vitamin B_{12}-free matrix. LOD and LOQ were determined by measuring low analyte content samples with a vitamin B_{12} content greater than LOQ, but less than ten times the final calculated LOQ. LOD was calculated as (10/3) × σ and LOQ was 10 × σ [13].

The accuracy of the method was assessed using Standard Reference Material (SRM) 1869. Recovery experiments were conducted on triplicate preparations for each sample. Vitamin B_{12} (cyanocobalamin) standard at either low or high concentration (0.25 or 0.75 μg mL^{-1}, 20 μL) was spiked into the sample after accurately weighing the reconstituted infant formula sample then mixed well before carrying out sample preparation.

Bias was determined by replicate analyses over two days (n = 12) by the same analyst. Overall mean was calculated and compared (95% confidence interval) to the certified reference value.

The precision was determined by means of repeatability (within-day) and intermediate precision (between-day). Three infant formula samples (1.5–3.7 μg 100 g^{-1} of dry weight of infant formula) were processed in duplicate each day and repeated for five days by the same analyst. The relative standard deviation of repeatability (RSD_r) and intermediate precision (RSD_{iR}) were calculated to evaluate precision performance.

Eleven infant formula products were selected for the comparison study. All samples were analysed by this HILIC-FLD method and the AOAC method that requires cyanide [3] with a paired t-test conducted ($\alpha = 0.05$). The statistical analysis was conducted using Microsoft Excel software (version 2303).

1. Results and discussion

1.1. Isolation of α-ribazole as a fluorescent marker

The isolated α-ribazole was characterised by NMR and LC-MS/MS. ^{1}H and ^{13}C chemical shifts were virtually identical with those reported in the literature [14]. The isolated α-ribazole standard was subjected to mass spectrometry for further confirmation. The existence of the m/z = 279.1 peak in the Q1 scan was consistent with the $[M+H]^+$ ion for α-ribazole (MW = 278.3); a fragment ion peak m/z = 147.1 was shown in the product ion scan, which matched the DMB fragment (MW = 146.1) with proton transfer under positive mode. The absence of ions around m/z = 358 in the Q1 scan under positive or negative modes indicated that the isolated product did not contain α-ribazole-phosphate.

The average calculated ε_{280} of DMB in DMSO was 6136 mol^{-1} L cm^{-1}, which is similar to previously reported values (6027 and 6090 mol^{-1} L cm^{-1}) [12, 13]. Total production of α-ribazole was 7.92 mg from 50.0 mg of cyanocobalamin (77.2%). The yield rate was increased from 67% reported by the method of Mattes and Escalante-Semerena [12] due to the modification made on using a HCl as the hydrolysis reagent and introduction of organic modifier (acetonitrile) into eluent composition in BAC.

1.1. Vitamin B_{12} extraction and clean-up

1.1.1. Denaturation of protein

Protein-bound vitamin B_{12} must be released as a free form before further conversion and analysis. It is also necessary to remove protein to limit the matrix effect. A combined acid and heat protein denaturation method was employed since this is the most common way to precipitate protein in milk products. A sodium acetate buffer (0.1 mol L^{-1}, pH = 4.0) was chosen for this method based on literature [3–6, 15–17]. To investigate the extraction efficiency for the vitamin B_{12} freed from its bound protein, the reconstituted infant formula sample was spiked with a cyanocobalamin standard (20 ng) before the sample was prepared as described in Section 2.5 with single or double extraction by sodium acetate buffer. A spiked procedural blank (cyanocobalamin standard) was included for comparison. The results are summarised in Table 1A single cycle of protein denaturation showed a vitamin B_{12} recovery of 59.0 ± 2.6% (n = 2). After a double cycle, the recovery was increased to 73.9 ± 7.1% (n = 2), which matched with the recovery results of the spiked procedural blank: 69.9 ± 2.4% (n = 2), suggesting that this represents the maximum extraction

efficiency for vitamin B_{12} from sample matrices. Therefore, a double step protein denaturation was used for this method.

1.1.2. Sugar removal to prevent browning

Lactose is the primary reducing sugar in infant formula, if this and other reducing sugars are not removed from the extract, discolouration of the sample solution will occur with production of yellow/brown pigment during the acid hydrolysis stage caused by the Maillard reaction. This browning can cause several problems for the later preparation steps, including competition for binding sites on the boronic acid gel from the compounds responsible for the colouration, large interferences in the chromatogram due to the intensive colour and complicated fluorescence of compounds produced during the Maillard reaction [18]. Therefore, it is necessary to remove the sugar before the chemical hydrolysis.

Reversed phase SPE can separate sugar (lactose) from vitamin B_{12} due to their different strength interactions with the stationary phase. Sugars are not strongly retained on the SPE cartridges due to their high hydrophilicity, whereas vitamin B_{12} is a large molecule and has multiple hydrophobic groups leading to longer retention. C18 SPE has been used and was often reported in vitamin B_{12} determination until it was replaced by immunoaffinity extraction, which has higher selectivity for cyanocobalamin but less selectivity towards other forms of vitamin B_{12}. There are reports of using C18 SPE to retain vitamin B_{12} with satisfactory recovery for all four vitamin B_{12} forms [19, 20]. Therefore, C18 SPE was chosen for the current method. An eluent of 100% methanol was used to recover the retained vitamin B_{12} from the sorbent.

2.2. The release of α-ribazole and concentration

2.2.1. Release of α-ribazole-phosphate by acid hydrolysis

Several chemical hydrolysis procedures for cyanocobalamin have been reported in the literature. The method has evolved based upon that developed by Pakin et al. [6], with modifications made to the hydrolysis procedure, including the method of Mattes and Escalante-Semerena [12]. Both methods employed alkaline hydrolysis using sodium hydroxide (2.5 mol L^{-1}) at 100 °C for 15 min [6] or at 80–90 °C for 75 min [12]. Acid hydrolysis approaches have also been reported [7]. To maximise α-ribazole yield, acidic and basic hydrolyses were compared. Only the chemical hydrolysis step was modified while the conditions in the enzymatic dephosphorylation step remained constant. Cyanocobalamin standard (40 ng) was hydrolysed by HCl or NaOH at various concentrations. The results showed that, under the same conditions of time and temperature, acidic hydrolysis produced more α-ribazole- phosphate than the alkaline method. Such observation was not aligned with the conclusions of Pakin et al. [6], who claimed that alkaline hydrolysis (NaOH) produced higher levels of α-ribazole-phosphate than acidic hydrolysis (HCl). Base hydrolysis of cobalt complexes often takes place faster than the corresponding acid hydrolysis [21].

However, the comparison experiment of Pakin et al. [6] was conducted only for 5 min which was short than the current study (up to 120 min). Therefore, a higher initial reaction rate might not necessarily suggest a higher total yield. Based on the comparative results from the current study, acid hydrolysis was selected and further optimised for concentration and treatment duration. Within the concentration range investigated (0.4–4.0 mol L^{-1} HCl), 1.5 mol L^{-1} HCl showed the best yield. Heating duration was altered from 15 to 120 min; the α-ribazole yield reached a plateau after 30 min of hydrolysis and a 60 min heating duration was chosen to ensure maximum hydrolysis was achieved. Acidic hydrolysis has the added advantage of causing less colouration by degradation of reducing sugars [22] since sugar cannot be totally eliminated even with clean-up by C18 SPE.

2.2.2. Release of α-ribazole by alkaline phosphatase enzymatic hydrolysis

In the literature describing the use of ALP enzyme on α-ribazolephosphate, the incubation buffer had pH values of 8 [6] or 8.8 [12], while the manufacturer of the ALP enzyme states that the activity was determined at pH = 9.8 [23]. Therefore, these three incubation pH levels, 8.0, 8.8 and 9.8 (adjusted by ammonium hydroxide), were optimized and it was determined that using ammonium acetate (0.3 mol L^{-1}) and magnesium chloride (2 mmol L^{-1}) with a pH of 8.8 produced the best yields of α-ribazole. The vitamin B_{12} content in prepared infant formulas are up to 3.4 ng mL^{-1}, which is equivalent to 2.5×10^{-6} µmol per 1 mL of prepared feed [24–26]. The overnight (16 h) incubation period was employed for convenience as the sample preparation requires two days to complete and this is a suitable place to halt the process.

2.2.3. Boronate affinity chromatography clean-up

Boronic acid gel (0.5 mL) was packed in an empty centrifuge column and this has a theoretical capacity to retain 55 µmol of adenosine monophosphate (equivalent to 55 µmol of α-ribazole) at pH 8.8 according to the manufacturer. In addition to retaining α-ribazole (approximately 2.5×10^{-6} µmol of vitamin B_{12} in 1 mL of prepared infant formula), the gel will also retain other cis-diol containing compounds present in milk and the infant formulas, for example, ribose containing nucleosides and nucleotides, which may be up to 0.079 µmol mL^{-1} [27]. The selected quantity of gel has sufficient capacity to retain the entire α-ribazole yield from 2 g of reconstituted infant formula sample in this work in the presence of the other cis-diol containing compounds.

To optimise the elution of α-ribazole from boronic acid gel, the concentration of formic acid, choice of organic modifiers, and volume of eluent were tested. Recovery was improved with a higher formic acid concentration (1.0 mol L^{-1}). When pH adjustment alone proved insufficient, acetonitrile and methanol were evaluated as organic modifiers to suppress the secondary binding mechanism, that is hydrophobic interaction, in the binding step. Acetonitrile was found to be more effective than methanol. An eluent of

30% acetonitrile and 70% formic acid (1.0 mol L^{-1}) was found to be optimal. Maximum recovery was achieved with 4 mL of this eluent, which was then selected for eluting α-ribazole from the BAC column.

2.3. Chromatography separation

As reported, α-ribazole can be retained by reversed phase (RP)-HPLC [6,28]. During the determination of α-ribazole in the infant formula sample, a co-eluting peak was observed in the chromatogram when the determination was carried out by C18 RP-HPLC with fluorescence detection. A series of chromatographic variables were investigated in an attempt to solve the poor resolution problem; however, satisfactory resolution could not be achieved with RP-HPLC. An alternative separation mode was required to solve this resolution issue.

As an ionisable species, protonated α-ribazole shows the potential to be retained by hydrophilic interaction using a HILIC column, which offers an alternative separation mechanism for polar compounds including charged substances, by partitioning of polar compounds in the water-rich layer near the stationary phase, which leads to the retention of these analytes. The pKa value of α-ribazole (conjugate acid) is around 6 [14]. Under acidic conditions, it becomes protonated and positively-charged, which promotes hydrophilicity and retention on a HILIC column. The chromatograms of infant formula samples and the α-ribazole standard by HILIC-FLD with a mobile phase consisting of acetonitrile (95%) and 100 mM ammonium formate (pH 3.5) (5%) are shown in Fig. 3; in the infant formula sample, a single peak was observed at retention time 3.66 min that matched the retention time of the α-ribazole standard (3.65 min). There was no visible co-eluting peak or peak shoulder on the α-ribazole signal in the chromatogram of the infant formula sample. Additionally, since the analyte of interest had a lower retention time than on the C18 column, the total instrumental analysis time was significantly shortened (10 min) from using C18 column. A further advantage of this method is that it does not require a gradient elution to achieve sufficient separation.

2.4. Method validation results

Linearity was assessed as part of the single laboratory validation study and conducted in standard solution without a matrix consistent with other methods [3–5], Table 2. A calibration curve consisting of six α-ribazole standard solutions across the expected concentration range in samples were analysed (n = 3). The linear regression produced a squared correlation coefficient r^2 within acceptable level (> 0.99). Residuals versus standards showed a random scatter of points around zero, indicating a linear fit was appropriate.

Precision was assessed at two levels: repeatability and intermediate precision. The former was measured by analysing duplicate pairs (n = 10) of samples covering three infant formulas. Intermediate precision was determined by testing the same sample on five different days (n = 10) (Table 3). HorRat values for

repeatability were 0.3–0.4, which fall in the typically acceptable range of 0.3–1.3 [13]. The intermediate precision RSD_{iR} values were 3.59–10.0%, which were lower than the predicated RSD of reproducibility ($pRSD_R$) values calculated using the Horwitz equation ($2 \times c^{-0.15}$, where c represents the concentration as a mass fraction). This aligns with the expected trend that intermediate precision should fall between repeatability and reproducibility.

The LOD and LOQ were calculated as 0.4 and 1.2 μg 100 g^{-1} of dry weight, respectively. The typical vitamin B_{12} content in milk-based infant formula ranges from 1.33 to 2.00 μg 100 g^{-1} [29]. The LOQ of this new method is sufficiently low to measure the typical vitamin B_{12} concentration accurately, demonstrating the method's sensitivity and suitability for routine analysis of infant formula.

A recovery study was conducted on three different infant formula products and the results are summarised in Table 4. The samples (n = 6) were spiked with vitamin B_{12} at 2 and 6 μg 100 g^{-1} of dry weight and had recoveries ranging between 68.7 and 80.0%. Both spiked and unspiked sample were analysed by the same method. The recovery results were within the limits of 60–115% at the 10 μg kg^{-1} concentration level stated in Guidelines for Standard Method Performance Requirements [30].

A bias study was conducted on SRM 1869 by replicate (n = 12) tests. The mean value was at 4.00 μg 100 g^{-1} with a standard deviation of 0.59. The calculated 95% confidence interval for the method (3.63–4.48 μg 100 g^{-1}) overlaps with SRM certified values (4.47 ± 0.49 μg 100 g−1), and a t-test (α = 0.05) showed that there was no significant difference (p = 0.12), demonstrating that this method produces results that are consistent with certified values.

The recoveries and bias indicate that this method can accurately measure vitamin B_{12} in infant formula, which is known to be challenging due to its high fat and protein content, and the low vitamin B_{12} concentration.

2.5. Comparison with AOAC method results

The newly validated method was applied to various infant formula products for different stages of infant development manufactured from three popular milk origins: ovine, bovine and caprine. The vitamin B_{12} content results are shown in Table 5 together with label values and results obtained by an external laboratory using an AOAC official method (using cyanide to convert all vitamin B_{12} forms to cyanocobalamin) based upon the method described by Campos-Giménez et al. [3].

The label value from the nutritional information table is the typical concentration of vitamin B_{12}, hence, the observed concentration may be greater than labelled due to manufacturers deliberately producing nutrient overages to ensure that the required minimum levels are met throughout the shelf-life of the formula. (e.g., Table 5, bovine milk-based infant formula 1 and caprine milk-based follow-on formula 2).

To compare the performance of the newly developed method (HILIC-FLD) with the AOAC method, a paired t-test was performed on the vitamin B_{12} content of infant formula products (n = 11). The p-value from the paired t-test (α = 0.05) of 0.47 shows that there is no statistical evidence to allow the null hypothesis to be rejected hence, no significant difference was found between the results from the AOAC method and this work. Therefore, this cyanide-free method can be used as an alternative method for vitamin B_{12} without compromising the quantitation performance.

3. Conclusion

The present study has developed and validated an indirect quantitation approach to determine vitamin B_{12} in the milk-based infant formulas that precludes the need for cyanide. The quantitative analysis of vitamin B_{12} in infant formula was achieved by detecting cobalamin derived α-ribazole using HILIC-FLD. This method achieved the aim to be cyanide-free in sample preparation without causing underrepresentation of other natural forms of vitamin B_{12} (adenosylcobalamin, hydroxocobalamin and methylcobalamin) by removing the need for conversion. This work demonstrates that quantitation of α-ribazole is fit for purpose as an indirect method for determining vitamin B_{12}. It provides an effective and practical alternative, offering advantages in terms of safety during sample preparation. Detection of α-ribazole was chosen because of its fluorescence, which allows detection at low levels, and exclusivity to vitamin B_{12}, which prevents interferences. Further validation across a broader range of matrices is recommended to explore this method's wider applicability.

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CRediT authorship contribution statement

Yanan Li: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. Brendon D. Gill: Writing – review & editing, Validation, Conceptualization. Merilyn Manley-Harris: Writing – review & editing, Supervision, Resources. Megan N.C. Grainger: Writing – review & editing, Supervision, Resources.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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References

[1] AOAC International, Cobalamin (Vitamin B_{12} Activity) in Milk-Based Infant Formula. Turbidimetric Method. AOAC Official Method, 986.23, Oxford University Press, New York, NY, USA, 2023.

[2] H.E. Indyk, B.S. Persson, M.C.B. Caselunghe, A. Moberg, E. Filonzi, D.C. Woollard, Determination of vitamin B_{12} in milk products and selected foods by optical biosensor protein-binding assay: method comparison, *J. AOAC Int.* 85 (2002) 72–81, https://doi.org/10.1093/jaoac/85.1.72.

[3] E. Campos-Giménez, P. Fontannaz, M. Trisconi, T. Kilinc, C. Gimenez, Determination of vitamin B_{12} in food products by liquid chromatography/UV detection with immunoaffinity extraction: single-laboratory validation, *J. AOAC Int.* 91 (2008) 786–793, https://doi.org/10.1093/jaoac/91.4.786.

[4] E. Campos-Gimenez, Improved AOAC first action 2011.08 for the analysis of vitamin B_{12} in infant formula and adult/pediatric formulas: first action 2014.02, *J. AOAC Int.* 97 (2014) 1397–1402, https://doi.org/10.5740/jaoacint.14-119.

[5] O. Heudi, T. Kilinc, P. Fontannaz, E. Marley, Determination of vitamin B_{12} in food products and in premixes by reversed-phase high performance liquid chromatography and immunoaffinity extraction, *J. Chromatogr. A* 1101 (2006) 63–68, https://doi.org/10.1016/j.chroma.2005.09.059.

[6] C. Pakin, M. Bergaentzlé, D. Aoudé-Werner, C. Hasselmann, α-Ribazole, a fluorescent marker for the liquid chromatographic determination of vitamin B_{12} in foodstuffs, *J. Chromatogr. A* 1081 (2005) 182–189, https://doi.org/10.1016/j.chroma.2005.05.066.

[7] J.B. Armitage, J.R. Cannon, A.W. Johnson, L.F.J. Parker, E.L. Smith, W.H. Stafford, A.R. Todd, Chemistry of the vitamin B_{12} group. III. The course of hydrolytic degradations, *J. Chem. Soc*. (1953) 3849–3864, https://doi.org/10.1039/jr9530003849.

[8] J.G. Buchanan, A.W. Johnson, J.A. Mills, A.R. Todd, Chemistry of the vitamin B_{12} group. I. Acid hydrolysis studies. Isolation of a phosphorus-containing degradation product, *J. Chem. Soc.* (1950) 2845–2855, https://doi.org/10.1039/jr9500002845.

[9] R. Bonnett, J.A. Raleigh, D.G. Redman, The hydrolysis of vitamin B_{12}. Studies with model amides, *J. Am. Chem. Soc.* 87 (1964) 1600–1607, https://doi.org/10.1021/ja01085a032.

[10] U. Sharma, D. Pal, R. Prasad, Alkaline phosphatase: an overview, *Indian J. Clin. Biochem.* 29 (2014) 269–278, https://doi.org/10.1007/s12291-013-0408-y.

[11] J.E. Coleman, P. Gettins, Alkaline phosphatase, solution structure, and mechanism, *Adv. Enzymol. Relat. Areas Mol. Biol.* 55 (1983) 381–452.

[12] T.A. Mattes, J.C. Escalante-Semerena, Facile isolation of alpha-ribazole from vitamin B_{12} hydrolysates using boronate affinity chromatography, *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.* 1090 (2018) 52–55, https://doi.org/10.1016/j.jchromb.2018.05.022.

[13] AOAC International, Guidelines for Dietary Supplements and Botanicals, Oxford University Press, New York, NY, USA, 2023.

[14] K.L. Brown, J.M. Hakimi, Heteronuclear NMR studies of cobalamins. 4. α-Ribazole-3'-phosphate and the nucleotide loop of base-on cobalamins, *J. Am. Chem. Soc.* 108 (1986) 496–503, 00263a023.

[15] V. Perez-Fernandez, A. Gentili, A. Martinelli, F. Caretti, R. Curini, Evaluation of oxidized buckypaper as material for the solid phase extraction of cobalamins from milk: its efficacy as individual and support sorbent of a hydrophilic-lipophilic balance copolymer, *J. Chromatogr. A* 1428 (2016) 255–266, https://doi.org/10.1016/j.chroma.2015.07.109.

[16] A. Repossi, E. Zironi, T. Gazzotti, A. Serraino, G. Pagliuca, Vitamin B_{12} determination in milk, whey and different by-products of ricotta cheese production by ultra performance liquid chromatography coupled with tandem mass spectrometry, *Ital. J. Food Saf.* 6 (2017), https://doi.org/10.4081/ijfs.2017.6795.Article 6795.

[17] K. Schimpf, R. Spiegel, L. Thompson, D. Dowell, Determination of vitamin B_{12} in infant formula and adult nutritionals by HPLC: first action 2011.10, *J. AOAC Int.* 95 (2012) 313–318, https://doi.org/10.5740/jaoacint.CS2011_10.

[18] H. Nursten, Maillard reaction, in: H. Roginski (Ed.), Encyclopedia of Dairy Sciences, Elsevier, Oxford, 2002, pp. 1657–1672.

[19] K.R. Heal, L.T. Carlson, A.H. Devol, E.V. Armbrust, J.W. Moffett, D.A. Stahl, A. E. Ingalls, Determination of four forms of vitamin B_{12} and other B vitamins in seawater by liquid chromatography/tandem mass spectrometry, *Rapid Commun.Mass Spectrom.* 28 (2014) 2398–2404, https://doi.org/10.1002/rcm.7040.

[20] M. Okbamichael, S.A. Sañudo-Wilhelmy, A new method for the determination of Vitamin B_{12} in seawater, *Anal. Chim. Acta* 517 (2004) 33–38, https://doi.org/10.1016/j.aca.2004.05.020.

[21] T. Croguennec, Non-enzymatic browning, in: R. Jeantet, T. Croguennec, P. Schuck, G. Brulé (Eds.), *Handbook of Food Science and Technology* 1, John Wiley & Sons, Inc., Hoboken, NJ, USA, 2016, pp. 133–157, https://doi.org/10.1002/9781119268659.ch5.

[22] Y. Baran, P. Comba, G.A. Lawrance, Trends in base hydrolysis rates of chlorocobalt (III) complexes of a series of related pentaamine ligands, *Inorg. React. Mech*. 4 (2002) 31–47, https://doi.org/10.1080/1028662021000062509.

[23] Sigma-Aldrich, Alkaline phosphatase. https://www.sigmaaldrich.com/NZ/en/technical-documents/technical-article/protein-biology/western-blotting/alkaline-phosphatase#bap. (Accessed 18 May 2022).

[24] D. Gille, A. Schmid, Vitamin B_{12} in meat and dairy products, *Nutr. Rev.* 73 (2015) 106–115, https://doi.org/10.1093/nutrit/nuu011.

[25] E. Greibe, E. Nexo, Forms and amounts of vitamin B_{12} in infant formula: a pilot study, *PLoS One* 11 (2016), https://doi.org/10.1371/journal.pone.0165458.Article 0165458.

[26] G.F.M. Ball, *Vitamins in Foods Analysis, Bioavailability, and Stability*, first ed., Taylor & Francis Group, Boca Raton, FL, USA, 2005.

[27] B.D. Gill, H.E. Indyk, M. Manley-Harris, Determination of total potentially available nucleosides in bovine, caprine, and ovine milk, *Int. Dairy J.* 24 (2012) 40–43, 2011.11.011.

[28] T.A. Mattes, J.C. Escalante-Semerena, Salmonella enterica synthesizes 5,6-dimethylbenzimidazolyl-(DMB)-α-riboside. Why some Firmicutes do not require the canonical DMB activation system to synthesize adenosylcobalamin, *Mol. Microbiol.* 103 (2017) 269–281, https://doi.org/10.1111/mmi.13555.

[29] US Department of Agriculture (USDA), Agricultural Research Service; Nutrient Data Laboratory, USDA National Nutrient Database For Standard Reference, Legacy, 2018.

[30] AOAC International, Guidelines for Standard Method Performance Requirements, Oxford University Press, New York, NY, USA, 2023.

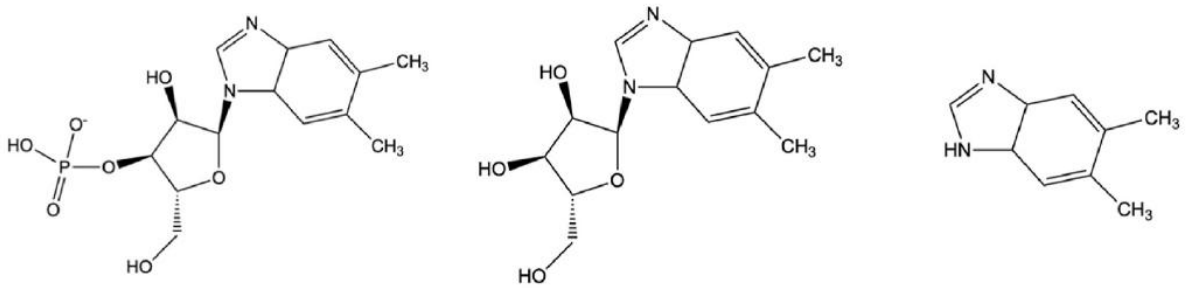

Figure 1. Fig. 1. Structure of (A) α-ribazole-3'-phosphate, (B) α-ribazole and (C) 5,6-dimethylbenzimidazole

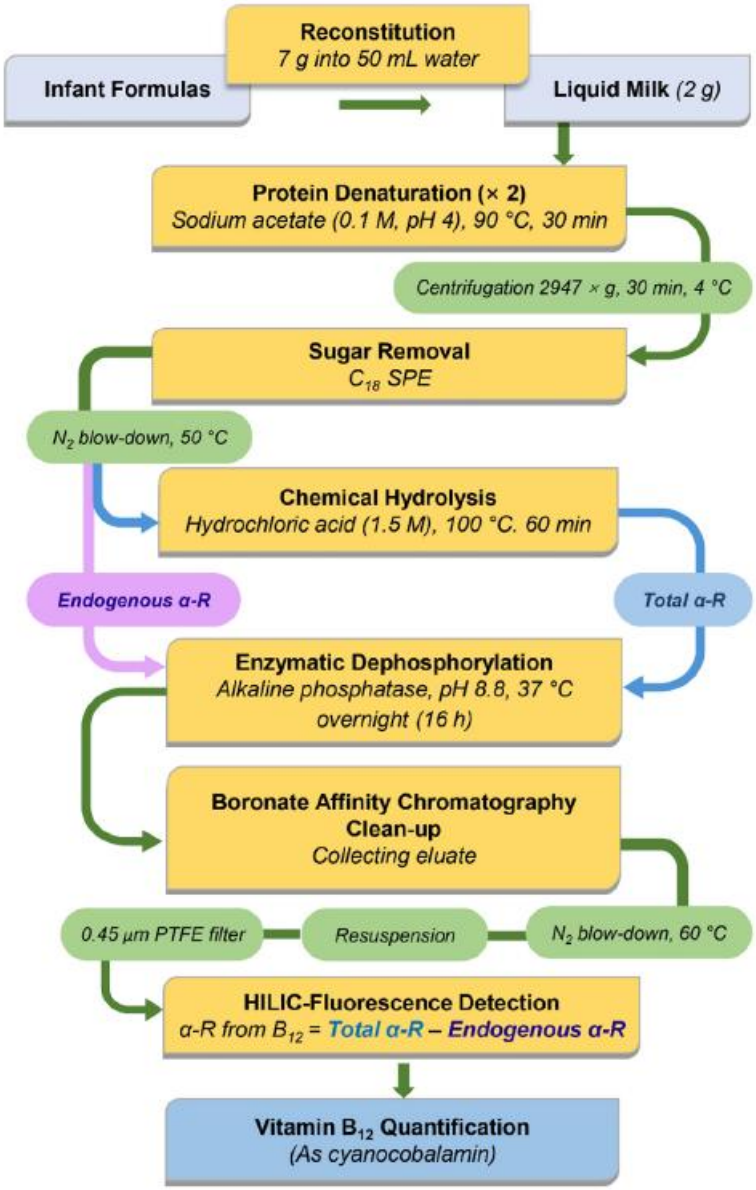

Figure 2. Flow chart of cyanide-free sample preparation for determination of vitamin B_{12} in milk-based infant formulas using HILIC-FLD. The final α-ribazole (α-R) content is calculated by subtracting the endogenous a-R (pink pathway, bypassing chemical hydrolysis step) from the total α-R (blue pathway). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

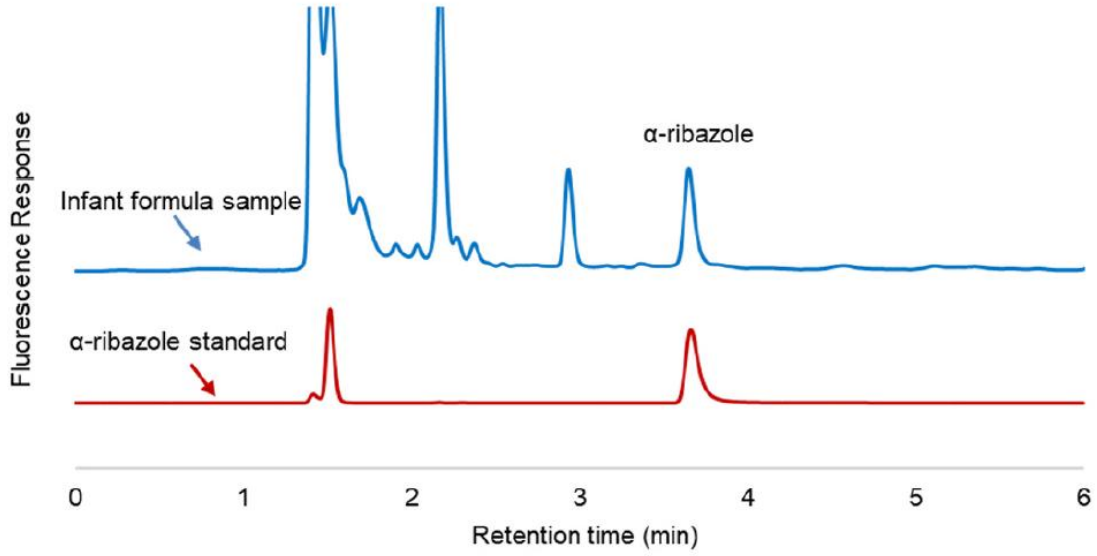

Figure 3. Chromatogram of infant formula sample (top) and α-ribazole standard (bottom) by HILIC-FLD. Separation was using a Kinetex HILIC, 2.6 µm, 4.6 × 150 mm. Mobile phase: 95% acetonitrile and 5% 100 mmol L^{-1} ammonium formate (pH 3.5); flow rate, 1.0 mL min^{-1}; column temperature, 30 °C; excitation/emission wavelength, 251 nm/303 nm.

Table 1. Recovery results from protein denaturation[a]

Protein denaturation and Vitamin B12 extraction cycles	Recovery ± SD (%)	
	Sample	Spiked procedural blank
Single	59.0 ± 2.6	70.2 ± 3.1
Double	73. 9 ± 7.1	69.9 ± 2.4

[a] Values are the mean of two replicates ± standard deviation. The spiked procedural blank was cyanocobalamin standard in water (2.0 g)

Table 2. HILIC-FLD α-ribazole method validation summary: linear regression

α-Ribazole[a]	Linear regression equation	r^2
Rep 1	y = 109.67x – 62.035	0.9946
Rep 2	y = 106.13x – 53.017	0.9963
Rep 3	y = 105.80x – 55.865	0.9969

[a] Range 0.65–6.48 ng mL^{-1}

Table 3. HILIC-FLD α-ribazole method validation summary: precision measurements[a]

Product description	Vitamin B12 content (µg 100 g⁻¹ of dry weight of infant formula powder)		
	Bovine milk based infant formula 1	Bovine milk based infant formula 2	Ovine milk-based infant formula
Day 1 dup 1	2.87	3.80	1.25
Day 1 dup 2	3.15	3.83	1.40
Day 2 dup 1	3.07	3.84	1.38
Day 2 dup 2	3.25	3.46	1.46
Day 3 dup 1	2.98	3.61	1.74
Day 3 dup 2	3.08	3.59	1.74
Day 4 dup 1	3.10	3.66	1.48
Day 4 dup 2	2.98	3.47	1.54
Day 5 dup 1	2.85	3.76	1.55
Day 5 dup 2	3.00	3.65	1.36
Mean	3.03	3.67	1.49
SD_r	0.12	0.14	0.08
RSD_r (%)	4.13	3.78	5.47
HorRat	0.3	0.3	0.4
RSD_{iR} (%)	3.84	3.59	10.0

a Abbreviations are: SDr, 95% confidence interval of repeatability SD; RSDr, repeatability RSD% (n = 10); HorRat = RSDr/pRSDr (pRSDr = predicated repeatability RSD), where pRSDr = 1/2 (pRSDR), pRSDR = 2c⁻¹ 0.15, c is concentration as a mass fraction; RSDiR, intermediate precision RSD% (n = 10).

Table 4. Comparison of vitamin B_{12} content of infant formulas from product label, AOAC method and HILIC-FLD method

Sample types	Recovery (%)	
	50% level	150% level
Bovine milk based infant formula 1	68.9 (4.6)	68.7 (3.4)
Bovine milk based infant formula 2	76.4 (6.3)	80.0 (7.7)
Ovine milk-based infant formula	74.3 (13.4)	76.4 (7.1)

[a] Samples were spiked at 50% and 150% of typical concentrations of vitamin B_{12} (cyanocobalamin) in infant formula. Values are the mean (standard deviation) of six replicates.

Table 5. Summary of recovery result for α-ribazole using the HILIC-FLD method[a]

Sample types	Vitamin B_{12} content (μg 100 g^{-1})				
	HILIC-FLD concentration	Product label concentration[a]	% Difference (label –HILIC-FLD)	AOAC method[b] concentration	% Difference (AOAC –HILIC-FLD)
Ovine milk-based follow-on formula 1	1.47	1.68	13%	1.77	17%
Bovine milk-based infant formula 1	3.37	1.50	−125%	3.94	14%
Bovine milk-based infant formula 2	1.86	1.60	−16%	1.81	−3%
Bovine milk-based follow-on formula 1	3.06	2.69	−14%	2.93	−4%
Bovine milk-based follow-on formula 2	2.95	2.58	−14%	2.93	−6%
Bovine milk-based toddler-milk formula 1	1.46	1.52	4%	1.24	−18%
Bovine milk-based toddler-milk formula 2	0.98	1.28	23%	1.18	17%
Caprine milk-based infant formula 1	1.67	1.38	−21%	1.74	4%
Caprine milk-based infant formula 2	2.04	1.49	−37%	2.09	2%
Caprine milk-based follow-on formula 1	1.18	1.28	8%	0.99	−19%
Caprine milk-based follow-on formula 2	4.47	2.59	−73%	4.73	5%

[a] Extracted from the nutrition information table of each product. This is the typical concentration but is likely to be over to ensure minimum levels are met throughout the shelf-life of the formula.

[b] Adapted from Campos-Giménez et al. [3].