The Analysis of Vitamin B_{12} in Milk and Infant Formula: A Review

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Abstract

Vitamin B_{12} plays a vital role in human metabolism and is an essential vitamin obtained predominantly from food of animal origin. Amongst all animal products, naturally occurring vitamin B_{12} in milk has the highest bioavailability and dairy products are a broad-access source, especially for vegetarian individuals. The dairy industry requires an accurate and highly sensitive detection method for vitamin B_{12}, however, the extremely low concentration and instability of vitamin B_{12} creates challenges in analysis. This review discusses the application of modern instrumental techniques for analysis of vitamin B_{12} in milk as well as a variety of sample preparations, together with their respective advantages and drawbacks.

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1. Introduction

Vitamin B_{12}, an essential micronutrient for humans, has attracted significant scientific interest and is the subject of two separate Nobel Prizes, the first in 1934 for its use in treatment of pernicious anaemia (Minot & Murphy, 1983) and the second for the determination of its crystal structure by Hodgkin et al. (1955). Vitamin B_{12} is the most complicated and the largest vitamin B-group and is the common term for a group of biologically active cobalamins; it comprises four vitamers, adenosylcobalamin (Ado-Cbl), hydroxocobalamin (OH-Cbl), methylcobalamin (Me-Cbl), and cyanocobalamin (CN-Cbl); the latter being the most stable form (Kumar, Chouhan, & Thakur, 2010; see Fig. 1). Vitamin B_{12} has significant metabolic functions in humans and is essential for cell development, energy production, foetus growth, neurological function and haematological development (Combs & McClung, 2017). Ado-Cbl and Me-Cbl are the co-enzyme form of vitamin B_{12} and function in the metabolism of amino acids and single carbon units with the vitamin B_{12}-dependent enzymes methylmalonyl CoA mutase and methionine synthase, respectively (Gueant & Alpers, 2013).

Vitamin B_{12} deficiency can lead to megaloblastic anaemia and/or neuropathy, which is commonly caused by insufficient intake or malabsorption (Truswell, 2007). In the human diet the predominant source of vitamin B_{12} is animal products such as milk, meat, egg, and fish; in these food products naturally occurring vitamin B_{12} is often bound to proteins. Vitamin B_{12}, in the form of CN-Cbl, is added as a fortification to many foods. Several recent publications have suggested that some plant species (*Hippophae rhamnoides*, Elymus spp., and *Inula helenium*) contain vitamin B_{12} as well (Nakos et al., 2017). Vitamin B_{12} is synthesised by certain bacteria that are present in host animals and plants, but in humans these bacteria are located in the colon, so no absorption can occur (Combs & McClung, 2017; Gille & Schmid, 2015; Nakos et al., 2017).

A review focussing on methods for vitamin B_{12} analysis in dairy products has not been published previously, and given the concern over the use of cyanide in analytical laboratories, this review provides a framework for analysts to assess alternative strategies in future method development. The advantages and drawbacks of various sample preparation and instrumental techniques for the determination of vitamin B_{12} in milk and infant formulas are discussed.

2. Vitamin B_{12} properties

Vitamin B_{12} belongs to the so-called cobalt-containing corrinoids in which a cobalt (III) atom at the centre is complexed to four pyrrole rings (Kumar et al., 2010). The lower α-ligand and upper β-ligand of cobalt contribute the difference between biologically active cobalamin and its analogues. The lower ligand is provided by the nitrogen atom of 5,6-dimethylbenzimidazole (DMB), which plays a vital role in vitamin B_{12}-protein binding. The DMB is substituted by a D-ribofuranose moiety with a phosphate

group on its C-3 position. The phosphate is connected to a pyrrole ring through a D-1-amino-2-propanol group and thus a nucleotide loop is formed. The upper ligand (R) differs for each of the four vitamers (Fig. 1); Me-Cbl (R = methyl), OH-Cbl (R = hydroxyl), Ado-Cbl (R = 5' deoxyadenosyl) and CN-Cbl (R = cyano) (Brown, 2005; Kumar et al., 2010).

CN-Cbl is the most stable form of vitamin B_{12}, in contrast to naturally occurring vitamin B_{12} forms, Me-Cbl, OH-Cbl and Ado-Cbl, that are well known for their instability to light and heat exposure. Aqueous solutions of both Me-Cbl and Ado-Cbl are highly sensitive to light and even a few seconds of exposure will cause conversion into the OH-Cbl form. A relatively slow transition to OH-Cbl has been identified for CN-Cbl in aqueous solutions, which occurs over a period of hours. Although OH-Cbl is the most photostable form of vitamin B_{12}, further chemical degradation is highly likely, due to the instability of OH-Cbl in the presence of oxidising agents (Ahmad & Hussain, 1993; Juzeniene & Nizauskaite, 2013; Vaid et al., 2018).

CN-Cbl is relatively stable to non-vigorous thermal treatment at less than 100 °C; while decomposition will occur at temperatures above 210 °C and after prolonged heat treatment time (Nakos et al., 2017).

3. Vitamin B_{12} in milk and infant formula

OH-Cbl is the predominant form of vitamin B_{12} in bovine milk (the milk type under review is bovine milk, unless otherwise indicated) milk, with the minor presence of Me-Cbl and Ado-Cbl (Indyk et al., 2002; Jensen, 1995). These naturally occurring B_{12} vitamers in milk are bound to specific mammalian B_{12}-binding proteins for transport, for example transcobalamin (Fedosov, Petersen, & Nexø, 1996; Watanabe, 2007).

Compared with other vitamins, the content of naturally occurring vitamin B_{12} in foods is at an extremely low level. The concentration of vitamin B_{12} in milk from commercial dairy herds in Canada was 2.3–3.9 mg L^{-1} (Duplessis, Pellerin, Cue, & Girard, 2016). The national food composition database of Denmark and Switzerland found the concentration of vitamin B_{12} in bovine milk was 0.8–4.9 mg L^{-1} (Gille & Schmid, 2015). The vitamin B_{12} content of skim milk in New Zealand was reported as 2–8 mg L^{-1} (Indyk et al., 2002).

Although concentrations of vitamin B_{12} in milk are considerably lower than in meat, its bioavailability is higher (Matte, Guay, & Girard, 2012; Vogiatzoglou et al., 2009). Therefore, for individuals who have vegetarian diets, ingestion of milk and other dairy products can reduce the risk of vitamin B_{12} deficiency since bovine milk is considered an excellent source of vitamin B_{12} (Matte et al., 2012).

Processing of milk, including boiling, microwaving and pasteurisation, can have a negative effect on the vitamin B_{12} content. An estimated 30% and 50% of vitamin B_{12} was lost by boiling for 2–5 and 30 min, respectively, and 50% was lost by 5 min microwave cooking, while 5–10% was lost by pasteurisation (Watanabe, 2007).

However, the concentrations of vitamin B_{12} in pasteurised milk did not significantly decrease during daylight exposure and/or refrigerator storage (Duplessis et al., 2016). Vitamin B_{12} concentrations in fermented milk, in products such as yoghurt, reduced during the fermentation processing and storage due to the presence of *Lactobacillus bulgaricus* and Streptococcus thermophiles, which have been identified as vitamin B_{12} consumers, such that the loss of vitamin B_{12} in prepared and stored yoghurt ranged from 25% to 60% (Gille & Schmid, 2015).

The recommended dietary intakes (RDI) of vitamin B_{12} are low at 2.4 mg per day for adults, a slightly higher intake for pregnancy at 2.6 mg per day and during lactation at 2.8 mg per day. The daily RDIs of vitamin B_{12} for infants, children and teenagers are even lower than the adult RDI of 2.4 mg (D'Ulivo et al., 2017).

Infant formula is normally based on bovine milk and designed as a substitute for human milk with certain modifications including the fortification of vitamin B_{12}, due to loss during manufacture, which can occur at multiple processing steps, especially heat treatments (pasteurisation, sterilisation and evaporation) and drying processing (dry blending and/or spray drying). Vitamin B_{12} is fortified in infant formula as CN-Cbl. The international regulatory minimum amount of vitamin B_{12} in infant formula is 0.025 mg $100kJ^{-1}$ (~0.7 mg L^{-1} prepared feed) with an upper guidance limit of 0.36 mg $100kJ^{-1}$ (~9.8 mg L^{-1} prepared feed) (Codex Alimentarius Commission, 1981). Greibe and Nexo (2016) analysed the vitamin B_{12} content of several commercial infant formulas and found that concentrations were 1.6–3.4 mg L^{-1} prepared feed.

The dairy industry, particularly those sectors involved in the manufacture of infant formula, requires accurate and highly sensitive analytical methods for vitamin B_{12} quantitation. The trace content and complicated physical and chemical characteristics of vitamin B_{12} bring unique challenges to routine product compliance testing of dairy products.

4. Vitamin B_{12} analytical methods

Numerous methods have been developed for vitamin B_{12} determination in milk and infant formula. These include liquid chromatography (LC) methods with varying detection techniques, microbiological assays which employ various B_{12}-dependent microorganisms, spectrometric methods including atomic absorption spectrometry (AAS) and a group of biospecific binding based assays (Karmi, Zayed,

Baraghethi, Qadi, & Ghanem, 2011; Kumar et al., 2010). While various advantages have been identified for each these methods, each also has shortcomings, for instance, being time-consuming, having poor sensitivity, lacking specificity, and/or requiring expensive apparatus. Among these methods, high-performance liquid chromatography (HPLC) methods have most often been used to analyse vitamin B_{12} in dairy products. For all the methods mentioned above, sample preparation plays a key role in the analysis, with the instability of vitamin B_{12} placing limitations on the overall method.

Due to the complex nature of vitamin B_{12} and the form in which it presents in milk and infant formula, sample preparation requires considerable care to ensure the precision of methods. Conventional preparation procedures generally include denaturation of the binding protein, conversion into the most stable form (CN-Cbl) by reaction with cyanide ion, and concentration and separation by a clean-up technique. Conversion to CN-Cbl is a common sample preparation step regardless of what other determination techniques are employed; however, the continued utilisation of cyanide raises health and safety concerns in the workplace.

4.1. Sample preparation vitamin B_{12}

The sample preparation protocol depends on the targeted forms of vitamin B_{12} for analysis; these may be unbound or free B_{12}, including any fortified CN-Cbl in dairy products, or total vitamin B_{12}, which includes protein-bound forms, which are themselves converted into CN-Cbl during sample preparation (Perez-Fernandez, Gentili, Martinelli, Caretti, & Curini, 2016). In consideration of the photosensitivity of vitamin B_{12}, operations under dim light conditions and the use of amber glassware are highly recommended and should be observed throughout sample preparation (Campos-Gimenez, Fontannaz, Trisconi, Kilinc, & Gimenez, 2008; Chamlagain, Edelmann, Kariluoto, Ollilainen, & Piironen, 2015).

4.1.1. Denaturation of binding protein

In milk, naturally occurring vitamin B_{12} presents as bound to protein carriers and the abundant protein content can cause matrix interference. During preparation, the proteins have to be denatured to ensure the further extraction of cobalamin in a free form without protein interference. The following three approaches have been reported: heat treatment, enzymatic proteolysis, and acidic precipitation (Kumar et al., 2010; Perez-Fernandez et al., 2016), and these have been used either individually or in combination.

Boiling and/or autoclaving the sample in aqueous form at 98–121 °C for at least 30–35 min in the presence of cyanide at pH 4–4.8 (acetate buffer) is the most common form of sample extraction; since this procedure combines the denaturation of binding proteins and conversion of other forms of

vitamin B_{12} into the more stable form CN-Cbl. This procedure has been reported numerous times (Campos-Gimenez, 2014; Chamlagain et al., 2015; Parvin, Azizi, Arjomandi, & Lee, 2018; Repossi, Zironi, Gazzotti, Serraino, & Pagliuca, 2017; Schimpf, Spiegel, Thompson, & Dowell, 2012; Watanabe, Abe, Takenaka, Fujita, & Nakano, 1997; Watanabe & Bito, 2018; Zironi et al., 2013).

There are several reported studies in which an enzymatic proteolysis step was employed prior to heating. Commonly, a proteolytic enzyme, such as pepsin or papain, was added and incubated at 37 °C at pH 4–4.8 for 1.5–3 h. Incubation was ended by continuing a boiling treatment as described above. This modification improved the liberation of bound cobalamin (Campos-Gimenez et al., 2008; Heudi, Kilinc, Fontannaz, & Marley, 2006; Nakos et al., 2017; Watanabe & Bito, 2018). Pakin, Bergaentzle, Aoude-Werner, and Hasselmann (2005) used proteolysis, but without further heating or addition of potassium cyanide, however, the exclusion of cyanide does risk incomplete vitamin B_{12} extraction when immunoaffinity extraction is applied (Campos-Gimenez et al., 2008).

Acidic precipitation has also been applied to release vitamin B_{12} from the vitamin B_{12}-protein complex. Sodium acetate and/or trichloroacetic acid solution have been used at room temperature with a centrifugation step to remove the solid residue (Oprean, Iancu, Radu, Litescu, & Truica, 2011; Perez-Fernandez et al., 2016).

4.1.2. Conversion to a single form of vitamin B_{12}

Based on its relative stability, CN-Cbl has been commonly used, as a standard, for vitamin B_{12} analysis and is the form of vitamin B_{12} reported in nutritional information. The vast majority of published methods for total vitamin B_{12} content determination have utilised dilute cyanide solutions (sodium or potassium cyanide) to achieve conversion of the natural forms of vitamin B_{12} to the more stable CN-Cbl. The amount of cyanide employed depends upon the sample size and generally an excess is present (Kumar et al., 2010). However, the use of cyanide salts in the workplace is undesirable due to their extreme toxicity. Boiling or heating with metabisulphite to convert all naturally occurring forms of vitamin B_{12} into sulphitocobalamin has been suggested to replace the use of cyanide since sulphitocobalamin is as stable as CN-Cbl (Watanabe & Bito, 2018). However, there is no commercially available sulphitocobalamin as a standard, which limits the development of this method; in addition, fortified CN-Cbl cannot be converted due to its stability and hence two forms (SO_3-Cbl and CN-Cbl) need to be analysed (Muhammad, Briggs, & Jones, 1993; Watanabe & Bito, 2018).

The conversion of endogenous vitamin B_{12} forms to CN-Cbl remains a nearly universal procedure used in the determination of vitamin B_{12}. The details for denaturation of binding proteins and conversion into CN-Cbl from the available literature are summarised in Table 1.

4.1.3. Solid phase extraction

For analysis of vitamin B_{12} in dairy products, further purification and concentration steps are essential due to the complexity of the matrix. Solid-phase extraction (SPE) cartridges with various packing materials have been reported for this clean-up purpose. Both C_{18} and C_8 cartridges, with hydrophobic chains coating silica particles, are effective at retaining most organic compounds in the vitamin B_{12} extraction solution (D'Ulivo et al., 2017; Iwase & Ono, 1997; Schimpf et al., 2012; Zhu, Aller, & Kaushik, 2011).

The HLB (hydrophilic-lipophilic-balanced) cartridge is claimed to have a relative non-polar retention capacity three fold higher than silica-based C_{18} sorbent (Waters, 2014). Using HLB, 107% and 93% recovery for CN-Cbl from milk have been reported respectively (Perez-Fernandez et al., 2016; Zironi et al., 2013).

A summary of the SPE procedure details from several literature sources is presented in Table 2.

4.1.4. Immunoaffinity extraction

Although SPE has been widely used in vitamin B_{12} analysis, there are growing concerns regarding its poor selectivity because biologically inactive cobalamin analogues can also be retained and eluted within the SPE step leading to inaccurate results.

An immunoaffinity extraction clean-up, with high selectivity and specificity, can be utilised as a replacement for SPE. This procedure was developed with monoclonal antibody technology whereby antibodies, which are immobilised in a gel suspension packed into a column, bind to the vitamin B_{12} when the sample is loaded onto the column.

There are several vitamin B_{12} methods combining immunoaffinity extraction technique with either HPLC-UV or liquid chromatography combined with tandem mass spectrometry (LC-MS/MS) (Campos-Gimenez, 2014; Campos-Gimenez et al., 2008; Chamlagain et al., 2015; Heudi et al., 2006; Martin, Gimenez, & Konings, 2016; Nakos et al., 2017; Pakin et al., 2005). The common protocol is that the immunoaffinity columns are warmed to ambient temperature prior to use; the storage buffer in the column is drained and then the filtered sample solution loaded and allowed to equilibrate for an appropriate period. The column is then eluted with methanol twice with the columns being dried by air after each elution. To avoid blockage as sample passes through the column, the sample solution must be filtered prior to loading to the column.

The use of immunoaffinity extraction significantly increased the recovered concentration of CN-Cbl, thus providing higher recovery and improved repeatability. The overall recoveries of two methods

using immunoaffinity were 94–100% (Campos-Gimenez et al., 2008) and 93.3–108.3% (Heudi et al., 2006), which are higher than the usual overall recoveries of methods utilising C_{18} or C_8 SPE clean-up.

However, immunoaffinity columns are more specific for CN-Cbl than any other form. Campos-Gimenez et al. (2008) found that the recoveries of Me-Cbl, OH-Cbl and Ado-Cbl through immunoaffinity extraction were less than 20%, which reinforces the requirement of preceding conversion into CN-Cbl as total vitamin B_{12}. Another possible reason behind the poor recoveries for these three vitamers might be their instability, and careful experimental design would be needed to exclude this possibility. Moreover, during an analysis of a fermented malt extract, which was purified by immunoaffinity extraction, Chamlagain et al. (2015) found that a biologically inactive cobalamin analogue, pseudovitamin B_{12}, could be retained and eluted from the immunoaffinity column as well. This example indicates that immunoaffinity extraction might have the same unwanted retention issue as C_{18} SPE.

4.2. Chromatographic methods

In recent years, numerous chromatographic methods have been developed for the qualitative and quantitative analysis of vitamin B_{12} in milk and infant formula products. The vast majority of reported methods were based on HPLC, which has been adopted into numerous laboratories as a routine assay method for vitamin B_{12}. Reversed-phase (RPLC) is the most common stationary phase employed; generally, CN-Cbl is used as an external standard to measure the total vitamin B_{12}. A series of parameters must be optimised to achieve the favoured separation in RPLC, these include: the content of organic solvent in the mobile phase, the choice of isocratic or gradient elution program, the pH of the mobile phase, flowrate, temperature of the column and specification of the column (stationary phase, length, internal diameter and particle size of packing material).

4.2.1. HPLC with ultra violet or visible detection

An HPLC-ultra violet (UV) method was developed for water soluble vitamins, including vitamin B_{12}, in infant formula using a C_{18} column (250 × 4.6 mm, 5 µm) and isocratic elution with a mobile phase of 15% methanol in water with octanesulfonic acidtrimethylamine buffer (pH = 3.6) at room temperature (Albala-Hurtado, Veciana-Nogues, Izquierdo-Pulido, & Marine-Font, 1997). The retention time of CN-Cbl ranged from 18 to 20 min, the limit of quantitation (LOQ) was 0.3 µg mL^{-1} and recoveries of CN-Cbl in powdered and liquid milk were 77.2 ± 3.3% and 76.8 ± 2.9%, respectively. These were the lowest recoveries compared with other vitamins detected in parallel and the LOQ was insufficiently sensitive to quantify the low content of vitamin B_{12} in non-fortified milk, in which the average content is less than 4 ng g^{-1} (Gille & Schmid, 2015). Oprean et al. (2011) determined vitamin B_{12} content in goat milk by HPLC-UV with a similar C_{18} column; the experiment was carried out isocratically with a

two component mobile phase of 5 mM heptanesulfonic acid in methanol and 1% acetic acid (30:70) at room temperature (25 °C) with UV detection at 361 nm. However, since no recovery experiment was conducted for the method validation, the accuracy and precision need to be further confirmed.

Gradient elution has been reported throughout the literature to achieve better separation and shorter analysis times. Heudi et al. (2006) used a mobile phase consisting of 0.025% trifluoroacetic acid (TFA) aqueous solution at pH = 2.6 and acetonitrile. A 150 × 3.0 mm C_{18} column was employed to perform the separation at a relatively slow flow rate of 250 mL min^{-1}; UV detection at 361 nm was used. The results, thus obtained, gave a 94–100% recovery and the instrumental limit of detection (LOD) and LOQ were estimated to be 3 ng mL^{-1} and 10 ng mL^{-1} respectively, which is appropriate for the quantitation of vitamin B_{12} content in samples. Subsequently, Campos-Gimenez et al. (2008) modified this method utilising the same columns but with a large injection volume (100 mL) and a faster gradient elution to shorten chromatography analysis time while maintaining the sensitivity. The instrumental LOD was the same at 3 ng mL^{-1} and the overall method detection and quantitation limits were 1.0 ng g^{-1} and 3.0 ng g^{-1}. Thus, this modified method would be suitable for fortified food products, but the increased injection volume led to a proportional increase in both signal and noise (Campos-Gimenez et al., 2012).

This method was improved by Campos-Gimenez (2014) with introduction of ultra-HPLC (UHPLC) allowing use of a shorter column with smaller particle size (100 × 2.1 mm, 1.7 µm). Although the same mobile phase, 0.025% TFA in water and 0.025% TFA in acetonitrile, was employed, the advantages of UHPLC allowed the flow rate to be increased from 0.25 to 0.4 mL min^{-1} and permitted a shorter gradient elution program decreasing the analysis time to half that previously required; the injection volume was also halved to 50 mL. The reported LOD and LOQ were estimated at 0.08 ng g^{-1} and 0.13 ng g^{-1}, respectively and recoveries were between 87.8 and 98.3%, which are appropriate for the measurement of vitamin B_{12} in various types of infant, adult and paediatric formulas. The enhanced resolution and more rapid throughput of the ultra-HPLC system contributed to the high sensitivity and time-saving performance of this method.

Since CN-Cbl has an absorption maximum at 550 nm, Iwase and Ono (1997) analysed vitamin B_{12} at this wavelength, separating the compounds on a C_{18} column (150 × 4.6 mm, 5 µm) with temperature set at 40 °C and using an isocratic mobile phase, consisting of a 50 mM potassium dihydrogen phosphate (KH_2PO_4) buffer solution (pH = 2.1 with phosphoric acid) and acetonitrile, at a ratio of 90:10, respectively. The detection limit was 0.5 ng mL^{-1} at a signal/noise (S/N) ratio of 3:1 and the recovery was over 90%. The same detection wavelength was chosen by Schimpf et al. (2012) with a gradient elution. The separation was performed on a 100 × 4.6 mm, 3 µm C_{18} column and mobile phases consisting of A (0.4% triethylamine (TEA) in water), B (0.4% TEA and 25% acetonitrile in water)

and C (0.4% TEA and 75% acetonitrile in water) at pH = 5–7. The LOQ was 0.8 ng g^{-1}; due to the considerably enhanced sensitivity, this method was designated as AOAC Official Method 2011.10 for vitamin B_{12} in infant formula and adult nutritionals.

A summary of the experimental parameters, which were used in the methods reviewed above, is presented in Table 3. Certain conclusions can be drawn; acidic buffer is always present in the mobile phase to achieve better separation of CN-Cbl from matrix interference on the non-polar stationary phase; a combination of water and another less polar organic solvent such as methanol or acetonitrile are common choices for mobile phase components as would be expected for reversed phase chromatography and gradient elution is preferred due to the possibility of a shorter analysis time.

Although CN-Cbl has specific absorption at 550 nm visible wavelength, the majority of reported methods still detected in the ultraviolet at 361 nm presumably because the greater absorptivity at this wavelength increases the sensitivity of the method.

A C_{18} reversed phase column was a standard LC column choice; however, C_{18} columns with various specifications were applied in vitamin B_{12} analyses, as expected, a narrow-bore column with smaller inner diameter and particle size delivered better results with respect to accuracy, precision and run time.

HPLC with UV detection has dominated the literature on analysis of vitamin B_{12} in milk and infant formula and with continuing refinements and improvements in technology; results have shown significant improvement in detection and quantitation limits, recoveries and precision (Table 4).

4.2.2. HPLC with fluorescence detection

Pakin et al. (2005) described a vitamin B_{12} assay method by detecting the α-ribazole moiety after HPLC with a fluorescence detector. Food samples were incubated with pepsin (at 37 °C, pH = 4 for 3 h) to denature the protein and to obtain total vitamin B_{12} content. Vitamin B_{12} was concentrated by passing through an immunoaffinity column and separated from the pre-existing metabolic fragments of vitamin B_{12}, which also contained the fluorescent marker. The concentrate was hydrolysed with 2.5 M sodium hydroxide (at 100 °C for 15 min) and incubated with alkaline phosphatase (37 °C, pH = 8 for 16 h) to release the fluorescent fragment: α-ribazole. Reversed phase chromatography was used to isolate the marker. The separation was performed on a C_{18} column (250 × 4 mm, 5 µm) with mobile phases consisting of methanol and water in gradient elution. α-Ribazole was fluorimetrically detected at an excitation wavelength of 250 nm and an emission wavelength of 312 nm. However, α-ribazole is not available commercially; therefore, a CN-Cbl standard solution was used in the pre-column conversion to produce α-ribazole for the external calibration. Food samples including powdered milk

were analysed and satisfactory recovery (95–100%), repeatability (1.0–5.4 RSD%), and LOQ (3 ng g^{-1}) were obtained.

Exclusion of cyanide from the sample preparation was a novelty in this study; using degradation products (α-ribazole) of vitamin B_{12} as a marker overcame the issue caused by the instability of vitamin B_{12}, which addressed the safety concern of using cyanide. Although the poor selectivity of immunoaffinity columns did not affect the overall recovery test results since the CN-Cbl was spiked, a reliable clean-up procedure ought to be developed and the commercialisation of an α-ribazole standard should be encouraged.

4.2.3. Liquid chromatography with tandem mass spectrometry

LC-MS/MS has been used in analysis of vitamin B_{12} content in milk and related products. This method has the advantage of high sensitivity and selectivity in cases in which naturally occurring vitamin B_{12}, at trace levels, is to be analysed.

The lack of an inexpensive isotope labelled CN-Cbl for use as internal standard has resulted in substitution with alternative non-labelled internal standards of which dicyanocobinamide (DCN-Cbl) and methotrexate have commonly been selected for LC-MS/MS methods. Zironi et al. (2013) developed a method on a UPLC-MS/MS system for determination of vitamin B_{12} in milk and dairy products using methotrexate as an internal standard. The separation was established on a C_{18} column, high-strength silica T3 (50 × 2.1 mm, 1.8 µm) with guard column, at 45 °C and the analyte was eluted with a mobile phase consisting of water and 0.1% formic acid in acetonitrile by a gradient elution mode at a flow rate of 0.3 mL min^{-1}. Detection was in positive electrospray ionisation (ESI^+) mode, monitoring two pairs of transitions; 678.3/147.1 *m/z* and 678.3/359.3 *m/z*. The quantitation limit of this method was 2 ng g^{-1}, and the method was successfully applied to a raw milk sample. Subsequently, Repossi et al. (2017) adopted this method to study the degradation of vitamin B_{12} during ricotta cheese shelf-life with the instrumental conditions remaining the same.

Kakitani et al. (2014) applied a UPLC-MS/MS approach for determination of water-soluble vitamins in beverages and dietary supplements. A C_{18} column (150 × 2.0 mm, 3 µm) was used to perform separation at 40 °C. The mobile phase consisted of 0.05% formic acid in 5 mM ammonium formate (NH_4HCO_2) aqueous solution (A) and 0.3% formic acid in acetonitrile (B) with gradient elution at 0.2 mL min_{-1}; ESI^+ was used in MS detection with a precursor ion 678.50 *m/z* and product ions 147.1 *m/z* and 359.1 *m/z* selected for monitoring. Although the LOQ was not noted in this publication, according to the linear calibration range, the lowest concentration of vitamin B_{12} standard was 5 ng g^{-1}, which was satisfactory considering that this method was designed for simultaneous analysis of 15 water-soluble vitamins in fortified beverage and supplements.

Perez-Fernandez et al. (2016) described a LC-MS/MS method for the determination of four forms of cobalamin in bovine milk using DCN-Cbl as an internal standard. The different cobalamins separated on a C_{18} column (250 × 4.6 mm, 5 µm); the mobile phases consisted of 5 mM aqueous formic acid (A) and 5 mM formic acid in acetonitrile (B) with gradient elution at 0.8 mL min^{-1}. Detection of the four selected cobalamins was performed using ESI^+.

Currently published LC-MS/MS methods have higher sensitivity and specificity than HPLC coupled with UV or visible detectors; however, as with the HPLC-UV/Vis method, a conversion to CN-Cbl form by addition of cyanide during sample preparation was still required to avoid degradation which otherwise might have occurred during the analysis process. Another consideration is that the cost of LC-MS/MS instrumentation is considerably higher than HPLC-UV/Vis methods.

4.3. Microbiological assay

Shorb (1947) observed that Lactobacillus lactis would not fully grow in the absence of vitamin B_{12} and subsequently, several other vitamin B_{12}-dependent microorganisms have been identified, including *Escherichia coli*, *Euglena gracilis* and *Ochromonas malhamensis* (Cook & Ellis, 1968). A microbiological assay (MBA) has been developed for the determination of vitamin B_{12} and is the most sensitive method with response range from 1.0 to 10.0 pg mL^{-1} (Skeggs, 1967, as cited in Ball, 1994). In the milk and infant formulas context, the recommended microbiological method in Codex STAN234-1999 is AOAC 986.23 which uses *Lactobacillus leichmannii* as test microorganism (Codex Alimentarius Commission,1999; Walsh, 2014). The assay is based on measurement of growth of a vitamin B_{12}-dependent microorganism, which is presented with vitamin B_{12} samples (Kumar et al., 2010). A microbiological assay using *Lactobacillus delbrueckii* subsp. lactis American Type Culture Collection (ATCC) 7830 to analyse vitamin B_{12} in food has also been used (Watanabe & Bito, 2018).

However, these vitamin B_{12}-dependent microorganisms are not only sensitive to the biologically active cobalamin but also to other inactive vitamin B_{12} analogues, which can lead to an overestimated result. For example, adenylcyanocobamide (Pseudo-Cbl), an analogue of cobalamin, which is commonly found in food, supported similar growth for *L. delbrueckii* ATCC 7830 as did vitamin B_{12} (Berman, Yacowitz, & Weiser, 1956) and the poor selectivity of MBA can result in overestimation by 5–30% (Ball, 2006).

A preparation and extraction including conversion to CN-Cbl is required prior to the microbiological assay. To avoid the involvement of highly toxic potassium cyanide, sodium metabisulphite has been used to replace potassium cyanide in the microbiological assay. Sulphitocobalamin (SO_3-Cbl), produced with sodium metabisulphite boiling extraction, has identical activity to CN-Cbl in the growth

of B_{12}-dependent bacteria (Muhammad et al., 1993). However, a sulphitocobalamin standard is not commercially available and in-house synthesis is required (Watanabe & Bito, 2018).

4.4. Inductively coupled plasma-mass spectrometry

Inductively coupled plasma-mass spectrometry (ICP-MS) is an alternative technique used to determine indirectly the content of vitamin B_{12} by measuring the complexed cobalt in cobalamins (Baker & Miller-Ihli, 2000). An ICP-MS method has not yet been applied to milk or infant formula samples; however, this technique offers a cyanide-free instrumental analysis of vitamin B_{12} and thus it is likely, in the near future, that it will be applied to these matrices for the purpose of quantitative analysis. Raju, Yu, Schiel, and Long (2013) described a LC-ICP-MS method for the quantitation of vitamin B_{12} in fortified breakfast cereals and multivitamin tablets. Liquid chromatography was used to separate ionic cobalt from complexed cobalt in vitamin B_{12}, since free cobalt presented in samples will result in an overestimation if the total cobalt was measured. The separation was performed on an Atlantis T3 C_{18} column (150 × 2.1 mm, 3 µm) and the mobile phase consisted of 20 mM EDTA and 25% methanol in water. After chromatographic separation, vitamin B_{12} was measured as ^{59}Co by ICP-MS. The recovery was 97.3 ± 0.9% and the LOD of this method was less than 1 ng g^{-1}, which was sufficiently sensitive for dietary supplements and fortified products. Cobalt is monoisotopic with ^{59}Co at 100% natural abundance; however, this mass has potential interferences from $^{43}Ca^{16}O$, $^{42}Ca^{16}O^{1}H$, $^{36}Ar^{23}Na$, $^{40}Ar^{18}O^{1}H$, $^{24}Mg^{35}Cl$ that need to be taken into consideration due to the presence of the argon plasma and the high concentration of calcium in milk; alternative reaction gases which would shift these interferences have yet to be investigated. ICP instruments coupled with triple quadrupole mass spectrometers are becoming more readily available and these may improve sensitivity to the point where the techniques could be employed with milk.

4.5. Ligand binding assay

Ligand binding assays have been applied to vitamin B_{12} analysis. These utilise a biospecific binding between vitamin B_{12} and either antibodies or natural occurring proteins to achieve the determination.

4.5.1. Enzyme-linked immunosorbent assay

The enzyme-linked immunosorbent assay (ELISA) is a biospecific method based on the specific interaction between an antibody and its antigen, and is typically performed in a microtiter plate (Alcock, Finglas, & Morgan, 1992; Kumar et al., 2010). Generally, an antibody directly against vitamin B_{12} is immobilised on the surface of a microtitration plate; free antigen (vitamin B_{12} standard or samples) and enzyme-labelled antigen (horseradish peroxidase (HRP) labelled vitamin B_{12}) are added into each microtiter well to competitively bind to the limited binding sites of those antibodies

previously mentioned. Subsequently, the unbound antigens are removed and a HRP substrate solution added so that HRP-B_{12} conjugates generate an absorbing product. Since the measured absorbance is produced by labelled vitamin B_{12}, the resulting signal is inversely proportional to the concentration of vitamin B_{12} in samples or standards (Zhu et al., 2011). ELISA has been applied to milk for vitamin B_{12} estimation (Sharma, Rajput, Dogra, & Tomar, 2007).

Multiple sample preparation steps are required for complex food samples including conversion into CN-Cbl and SPE. In addition, difficulties in generating specific B_{12}-active antibodies have limited ELISA techniques.

4.5.2. Biosensor-based assay

Biosensor technology has been utilised for the analysis of vitamin B_{12}, which is also based on the interaction between vitamin B_{12} and its binding protein. A fixed amount of specific binding protein is added to the sample; vitamin B_{12} standard is immobilised to a sensor chip, usually a modified gold surface; when analyte solution containing vitamin B_{12}-protein complex and the unbound proteins flows past the sensor chip surface, the binding interaction is monitored via surface plasmon resonance (SPR) optics in an indirect, inhibition format. Indyk et al. (2002) described an analysis method of vitamin B_{12} by utilising a biosensor-SPR technique for a range of foods including milk and infant formula; with recoveries of 89–106%.

5. Conclusions

Regardless of what determination techniques were ultimately employed, boiling with sodium or potassium cyanide is a common sample preparation procedure for vitamin B_{12} analysis in the vast majority of the literature and HPLC combined with UV/vis detection of the resultant CN-Cbl is commonly used for analysis of prepared samples in laboratories of dairy industries across the world.

However, there are still opportunities for future development, for example, the exclusion of cyanide, the sensitivity of analysis for non-fortified milk and the ease of sample preparation. Instability and the trace content level of vitamin B_{12} in non-fortified foods are the greatest challenges faced during the analysis, and addressing these issues is important for any proposed analytical technique. For example, developing methods with fluorescence or mass spectrometric detection would be worthwhile due to the high sensitivity of these two detectors; as would the application of an ICP-MS method.

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Table 1. Summary of procedure details for denaturation of binding protein and conversion into cyanocobalamin in the reviewed literature

	Sample Types	Sample Amount	Extraction Procedure	Cyanide Amount	Recovery%	References
Heating/ Boiling/ Autoclave Treatment	Infant Formula	30 g	50 mL sodium acetate solution. Boiling water bath for 30 min or autoclave 30 min at 100 °C	1 mL 1% sodium cyanide solution	93.3–108.3%	Campos-Giménez et al. (2008)
	Infant Formula	30 g, 12% (w/w)	30 mL sodium acetate solution pH 4.5 Heated in 105 °C oven for ≥ 60 min	1 mL 1% potassium cyanide solution	—[a]	Schimpf et al. (2012)
	Infant Formula	60 g, 12.5% (w/w)	25 mL sodium acetate solution. Boiling water bath for 30 min or autoclave 30 min at 100 °C	1 mL 1% sodium cyanide solution	87.8–98.3%	Campos-Gimenez (2014)
	Milk	5 mL	5 mL of sodium acetate buffer, pH 4 Water bath for 60 min at 90 °C.	0.15 mL 1% potassium cyanide solution	—[a]	Repossi et al. (2017)
Enzymatic Treatment	Powdered milk	2–8 g	25 mL sodium acetate buffer, pH 4 2 mL pepsin solution (5 mg mL^{-1}), incubated at 37 °C for 3 h	na[b]	95–100%	Pakin et al. (2005)
	Infant Formula	5–25 g	60 mL sodium acetate buffer, pH 4 1 g pepsin, incubated at 37 °C for 3h Heated for 35 min at 100 °C	1 mL 1% sodium cyanide solution	94–100%	Heudi et al. (2006)
Acidic Precipitation	Milk	4 mL	6 mL of 50 mM sodium acetate buffer pH 4.6	na[b]	80–93%	Perez-Fernandez et al. (2016)

[a] No data available
[b] No cyanide applied

Table 2. Summary for SPE procedure details in the reviewed literature

SPE Cartridge Type (Bed weight / volume)	Sample Filtrates Amount	Conditioning Solvent 1	Conditioning Solvent 2	Washing Solvent	Eluting Solvent	Recovery%	References
C_{18} (500 mg / 3 mL)	10 mL	5 mL MeOH	10 mL H_2O	5 mL H_2O	10 mL 50% MeCN in H2O	88.8–92.2%	Iwase and Ono (1997)
C_{18} (2 g / 6 mL)	0.5–2 L	10 mL MeOH	10 mL H_2O	10 mL H_2O	5 mL MeOH	83.2–97.6%	Zhu et al. (2011)
C_{18} / C_8 (600 or 900 mg)	20–80 mL	20 mL MeCN	10 mL H_2O	5 mL H_2O	4.4 mL 25% or 30% MeCN in H_2O	—[a]	Schimpf et al. (2012)
C_{18} (500 mg /12 mL)	60 mL	10 mL MeOH	10 mL H_2O	10 mL, 5% MeOH in H_2O	10 mL MeOH	109.7–112%	D'Ulivo et al. (2017)
HLB (500 mg /6 mL)	10 mL	2 mL MeCN	2 mL H_2O	2 mL H_2O	1 mL 50% MeCN in H_2O	107%	Repossi et al. (2017); Zironi et al. (2013)

[a] No data available

Table 3. Summary for detailed LC conditions of HPLC-UV/Vis methods in the reviewed literature

LC Column (Length × Diameter, Particle size)	Flow rate ($mL \cdot min^{-1}$)	Temperature	Mobile phase	Wavelength	References
C_{18} 250 × 4.6 mm, 5 µm	1	RT[a]	15% methanol + 5 mM Octane-sulfonic acid + 0.5% TEA + 2.4% AcOH (pH 3.6)	UV 361 nm	Albala-Hurtado et al. (1997)
C_{18} 250 × 4.6 mm, 5 µm	1	25 °C	5 mM heptanesulfonic acid in MeOH: 1% AcOH = 30: 70	UV 361 nm	Oprean et al. (2011)
C_{18} 150 × 3.0 mm	0.25	—[b]	A: 0.025% TFA in H_2O (pH 2.6) B: MeCN	UV 361 nm	Heudi et al. (2006)
C_{18} 150 or 125 × 3.0 mm	0.25	—[b]	A: 0.025% TFA in H_2O B: 0.025% TFA in MeCN	UV 361 nm	Campos-Giménez et al. (2008)
C_{18} 100 × 2.1 mm, 1.7 µm	0.4	—[b]	A: 0.025% TFA in H_2O B: 0.025% TFA in MeCN	UV 361 nm	Campos-Gimenez (2014)
C_{18} 100 × 4.6 mm, 2.6 µm	1	30 °C	A: 0.025% TFA in H_2O B: MeCN	UV 361 nm	Nakos et al. (2017)
C_{18} 150 × 4.6 mm, 5 µm	1	40 °C	50 mM KH_2PO_4 (pH 2): MeCN = 90:10	Vis 550 nm	Iwase and Ono (1997)
C_{18} 100 × 4.6 mm, 3 µm	1	—[b]	A: 0.4% TFA in H_2O B: 0.4% TFA in 25% MeCN C: 0.4% TFA in 75% MeCN	Vis 550 nm	Schimpf et al. (2012)

[a] Room temperature

[b] No data available

Table 4. Summary of LOD, LOQ, recovery and precision of HPLC-UV/Vis methods in the reviewed literature

Wavelength used	LOD	LOQ	Recovery	Repeatability (RSD%)	References
361 nm	—[a]	3×10^2 ng mL^{-1} [b]	73.83–81.54%	5.75%[c], 5.12%[d]	Albala-Hurtado et al. (1997)
	3 ng mL^{-1} [b]	10 ng mL^{-1} [b]	94–100%	3.2%	Heudi et al. (2006)
	1.0 ng g^{-1} [e]	3.0 ng g^{-1} [e]	93.3–108.3%	2.1%	Campos-Giménez et al. (2008)
	0.08 ng g^{-1}[e]	0.13 ng g^{-1} [e]	87.8–98.3%	2.7–8.2%[f]	Campos-Gimenez (2014)
	4 ng mL^{-1} [b]	14 ng mL^{-1} [b]	80–100%	≥ 0.7%	Nakos et al. (2017)
550 nm	0.5 ng mL^{-1} [b]	—[a]	88.8–92.2%	2.7%	Iwase and Ono (1997)
	0.2 ng g^{-1} [e]	0.8 ng g^{-1}[e]	—[a]	—[a]	Schimpf et al. (2012)

[a] No data available
[b] LOD and LOQ are expressed as instrumental limit (ng mL^{-1})
[c] RSD of method precision in powdered milk
[d] RSD of method precision in liquid milk
[e] LOD and LOQ are expressed as overall method limit (ng g^{-1})
[f] Range for different samples
LOD = limit of detection, LOQ = limit of quantitation, RSD = relative standard deviation

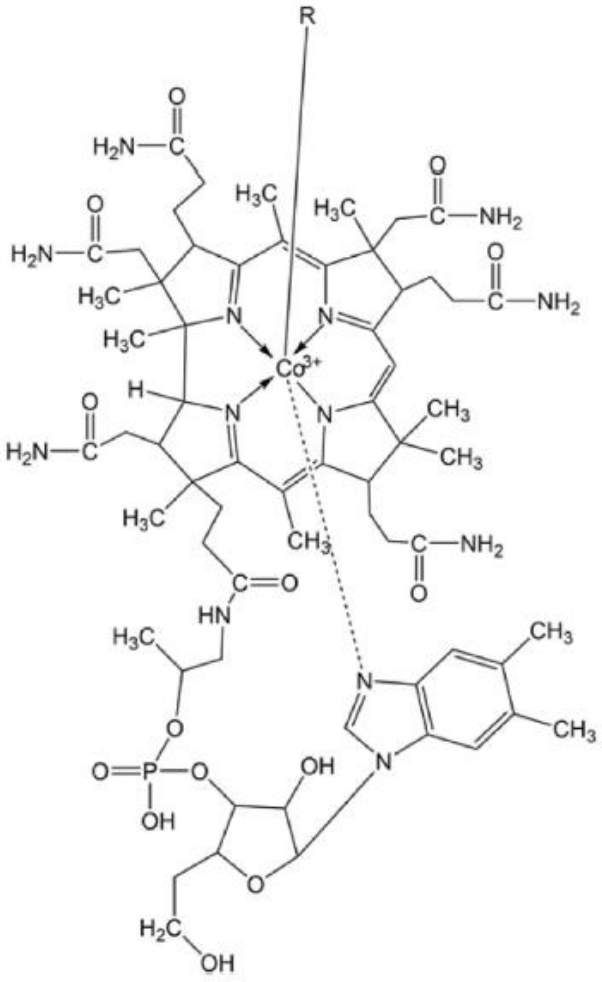

Fig 1. Chemical structure of vitamin B_{12}. The nature of the R group defines the four vitamin B12 forms: methylcobalamin (R = CH_3; Me-Cbl), hydroxocobalamin (R = OH; OH-Cbl), adenosylcobalamin (R = 5'-deoxyadenosyl; Ado-Cbl) and cyanocobalamin (R = CN; CN-Cbl). CN-Cbl is relatively stable to non-vigorous thermal treatment at less than 100 °C; while decomposition will occur at temperatures above 210 °C and after prolonged heat treatment time (Nakos et al., 2017)