Development of a Novel Cyanide-free Method for Analysis of Vitamin B_{12} in Milk-based Infant Formula

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ABSTRACT

The analysis of vitamin B_{12} in infant formulas requires the use of cyanide during the sample preparation process to convert the three unstable vitamers (hydroxocobalamin, methylcobalamin and adenosylcobalamin) to cyanocobalamin, the most stable form of vitamin B_{12}. The undesirable handling of cyanide for the analyst in the laboratory and the associated safety risk indicates a clear necessity for development of a cyanide-free method for vitamin B_{12} analysis without compromising the analytical quality.

This doctoral research demonstrates the possibility of using cobalamin-derived α-ribazole to represent total vitamin B_{12}, since the α-ribazole exists in all vitamin B_{12} forms removing the necessity for conversion. The absence of a commercial standard of α-ribazole required its in-house isolation from a cyanocobalamin standard. The α-ribazole was released through consecutive acidic hydrolysis and dephosphorylation by alkaline phosphatase. The freed α-ribazole was collected by boronate affinity chromatography and concentrated by lyophilisation. α-Ribazole was identified and characterised by nuclear magnetic resonance spectroscopy and liquid chromatography mass spectrometry to demonstrate its suitability as a standard. This protocol was optimised and adopted in the sample preparation for analysing vitamin B_{12} in infant formulas. Several extra steps were added to eliminate or limit interferences, including protein denaturation and sugar removal using C_{18} solid phase extraction. The final analyte was quantified using hydrophilic interaction liquid chromatography with fluorescence detection.

The single laboratory validation experiment showed that this cyanide-free method is fit for purpose. Analysis of various milk-based infant formulas and comparison with current procedure demonstrated no bias for vitamin B_{12} analysis.

To my parents who gave me life and made me free

十月胎恩重，三生报答轻

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LIST OF ABBREVIATIONS

AAS	Atomic absorption spectrometry
Ado-Cbl	Adenosylcobalamin
ALP	Alkaline phosphatase
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Collaboration
ATCC	American type culture collection
BAC	Boronate affinity chromatography
CA	Cellulose acetate
Cbi	Cobamide
CC BY	Creative commons by
CE	Collision energy
CN-Cbl	Cyanocobalamin
COSY	Correlation spectroscopy
CRM	Certified reference materials
DCN-Cbl	Dicyanocobinamide
DEPT	Distortionless enhancement by polarization transfer
df	Degrees of freedom
DF	Dilution factor
DMB	5, 6- Dimethylbenzimidazole
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DSS	Sodium trimethylsilylpropanesulfonate
EFSA	The European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
ESI^+	Positive electrospray ionisation
FAO	Food and Agriculture Organisation
FLD	Fluorescence detection
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
GR ACS	Guaranteed reagent by American Chemical Society

H-ESI	Heated-electrospray ionisation
HILIC	Hydrophilic interaction liquid chromatography
HLB	Hydrophilic-lipophilic-balanced
HMBC	Heteronuclear multiple bond correlation
HMQC	Heteronuclear multiple quantum coherence
HPLC	High performance liquid chromatography
HRMS	High resolution mass spectrometry
HSS	High-strength silica
ICP-MS	Inductively coupled plasma mass spectrometry
LC	Liquid chromatography
LC-MS	Liquid chromatography mass spectrometry
LC-MS/MS	Liquid chromatography with tandem mass spectrometry
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantitation
MBA	Microbiological assays
Me-Cbl	Methylcobalamin
Mr	Molecular mass
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MW	Molecular weight
NMR	Nuclear magnetic resonance
NTDs	Neural tube defects
OH-Cbl	Hydroxocobalamin
PNP	Purine nucleoside phosphorylase
$pRSD_R$	Predicted RSD of reproducibility
Pseudo-Cbl	Adenylcyanocobamide
PTFE	Polytetrafluoroethylene
QuEChERS	Quick easy cheap effective rugged safe
RDA	Recommended daily amount
RNA	Ribonucleic acid
RP	Reversed phase
RP-HPLC	Reversed-phase high performance liquid chromatography

RSD	Relative standard deviation
RSD_{iR}	Relative standard deviation of intermediate precision
RSD_r	Relative standard deviation of repeatability
RSD_R	Relative standard deviation of reproducibility
RT	Retention time
S/N	Signal noise ratio
SD	Standard deviation
SD_{iR}	Standard deviation of intermediate precision
SD_r	Standard deviation of repeatability
S_N1_CB	Substitution nucleophilic unimolecular conjugate base
S_N2	Substitution nucleophilic bimolecular
S_N2i	Substitution Nucleophilic intramolecular
SRM	Standard reference material
UHPLC	Ultra-high performance liquid chromatography

1 Introduction and General Literature Review*

Vitamin B_{12} plays a vital role in the metabolism of human body cells including DNA synthesis, one-carbon metabolism of fatty acids and amino acids, involvement in functioning of the nervous system and haematological development. The requirement for vitamin B_{12} is at a trace level under normal health conditions and vitamin B_{12} deficiency causes severe adverse physiological effects. Vitamin B_{12} can only be synthesised by bacteria which predominately live in an animal host, therefore animal-origin foods are the main vitamin B_{12} source for humans. The concentrations of vitamin B_{12} in those foods, however, are at extremely low levels. Therefore, both clinical and food industries demand a highly accurate and sensitive detection method for vitamin B_{12}.

There are a number of reported methods for analysis of vitamin B_{12} in milk and other dairy products including infant formula. Microbiological assays, spectrometric methods, biospecific binding based assays and chromatography with various detection methods have been studied and 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 and reducing agents, means that sample preparation is a key factor in the analysis of vitamin B_{12}. There are four different forms of vitamin B_{12}; among them, the relatively stable form is cyanocobalamin which can be converted through reacting other forms with cyanide. Hence, for all the techniques mentioned above, cyanide has been employed to prevent vitamin B_{12} degradation during analyses and provide a convenient measure of vitamin B_{12} as a single form only. However, routine use of cyanide in the industrial laboratory is generally considered undesirable because of health and safety issues.

This thesis describes the development of a cyanide-free method for analysis of vitamin B_{12} in milk-based infant formula. This method uses α-ribazole, the lower moiety of vitamin B_{12}, as a fluorescence marker to quantify vitamin B_{12}. Since

* Part of literature review included in the chapter has been published (Li *et al.*, 2019). A copy of the full article can be found in Appendix A.

α-ribazole is a degradation product of vitamin B_{12}, a pre-conversion to avoid degradation during the analysis is not required. The α-ribazole moiety is cleaved from vitamin B_{12} by acidic and enzymatic hydrolyses subsequently and is analysed by hydrophilic interaction liquid chromatography (HILIC) with fluorescence detection (FLD). The pre-existence of α-ribazole in the sample due to natural degradation means that α-ribazole content in the background must be determined prior to the hydrolysis procedure. Due to the absence of a commercially available α-ribazole standard, in-house production was required. A method by Mattes and Escalante-Semerena (2018) was adopted for isolating α-ribazole from vitamin B_{12} standard. The isolated α-ribazole was characterised and identified by nuclear magnetic resonance (NMR) and mass spectrometry (MS), and the isolation procedure was optimised. A single laboratory validation and a bias study against an AOAC Official Method (Campos-Giménez *et al.*, 2008) were conducted for the new method. In addition, the new method was applied to various infant formula products for validation purposes and as a survey study.

1.1 Vitamin B_{12} Category

Vitamin B_{12} has four vitamers* hydroxocobalamin (OH-Cbl), methylcobalamin (Me-Cbl), adenosylcobalamin (Ado-Cbl) and cyanocobalamin (CN-Cbl). In this literature review, the term vitamin B_{12} indicates all four forms instead of any particular vitamer. A relationship map between vitamin B_{12} and other terms included in following sections is given in **Figure 1.1**.

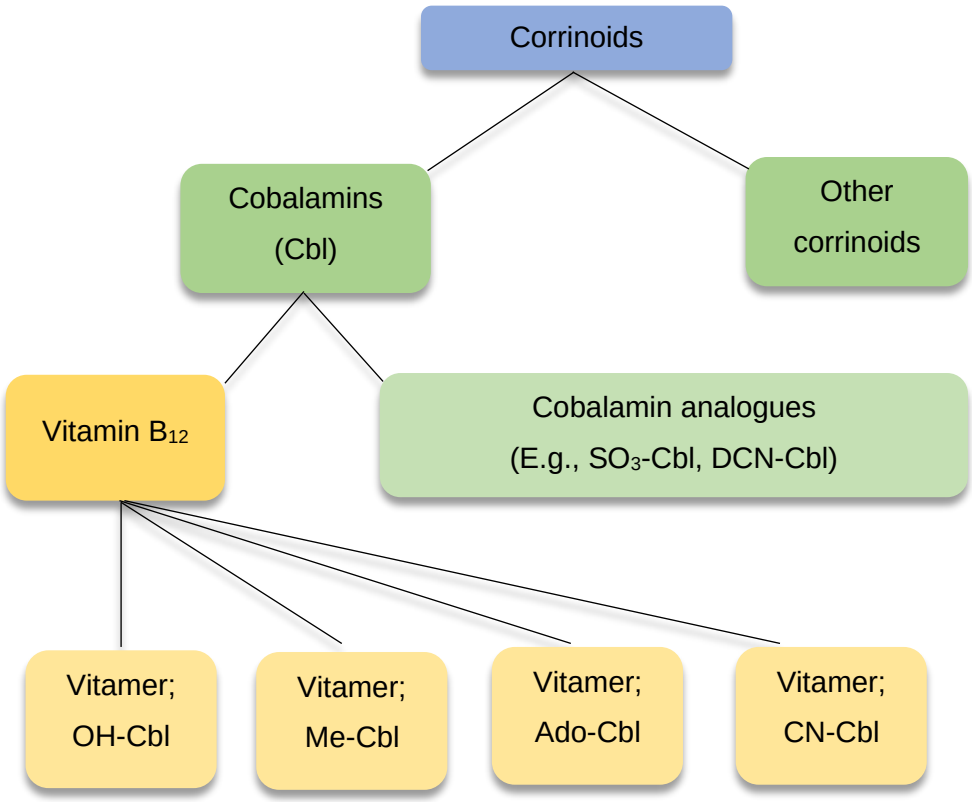


Figure 1.1. Relationship of the terms used, including corrinoids, cobalamins, vitamin B_{12} and vitamer, in this literature review.

1.2 Vitamin B_{12} Biochemistry

Vitamin B_{12}, a water-soluble vitamin, is the most complicated and the largest of the vitamin B group that has been discovered. Even though vitamin B_{12} is a common term used in the nutritional field and usually restricted to CN-Cbl,

* A vitamer of a particular vitamin is one of any of a number of chemical compounds, generally having a similar molecular structure, each of which shows vitamin activity in a vitamin-deficient biological system (Keresztesy, 1944).

vitamin B_{12} is the name for a group of biologically active cobalamins (FAO and WHO 2001; Watanabe, 2007). Since the chemical structure was determined by Dorothy Hodgkin in 1955 (Hodgkin *et al.*, 1955), vitamin B_{12} and its analogues have attracted the interests of scientists, principally due to their structural and functional complexity.

1.2.1 Vitamin B_{12} Structure

Vitamin B_{12} belongs to a group of compounds which all consist of a complex ring system. The general cobalamin structure is shown in **Figure 1.2** The cobalt-containing corrinoids in which a cobalt (III) atom at the centre is complexed to four pyrrole rings (**A**–**D**). Between the pyrroles **B** and **C**, there is one missing methylene bridge (Kasalová *et al.*, 2015).

A lower moiety and upper R groups of cobalt contribute the difference between cobalamin and its analogue forms. The lower ligand of cobalt (III) 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 ribosyl-phosphate group on its C-3 position; this nucleotide analogue loop is linked to the propionic acid of pyrrole **C** by a D-1-amino-2-propanol group. The varying nature of the upper ligand (R group) defines the four main cobalamin forms (vitamers): Me-Cbl (R = CH_3), OH-Cbl (R = OH), Ado-Cbl (R = 5′-deoxyadensoyl) and CN-Cbl (R = CN) (Kasalová *et al.*, 2015; Nakos *et al.*, 2017).

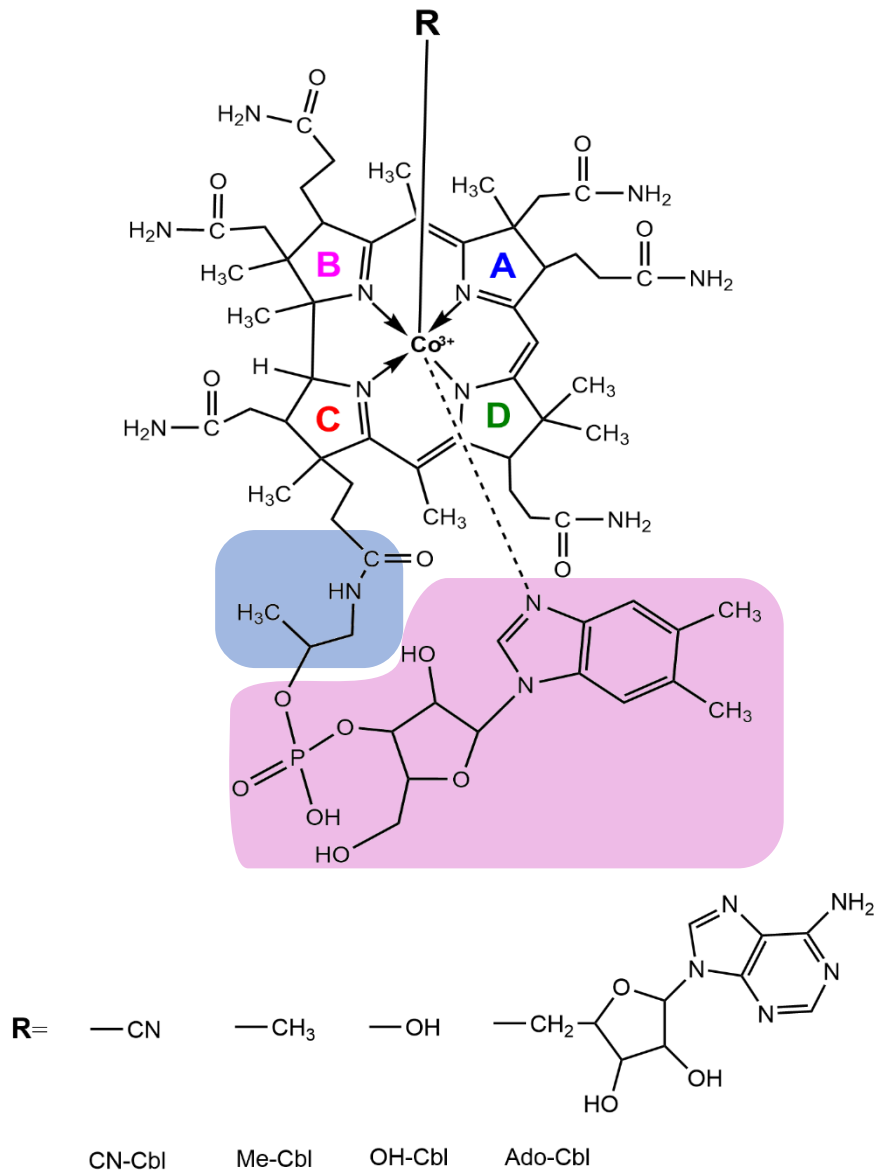


Figure 1.2. Common molecular structure of vitamin B_{12}

1.2.1.1 α-Ribazole-Phosphate and α-Ribazole

The nucleotide analogue, which exists in the structure of vitamin B_{12}, is α-ribazole-3′-phosphate moiety (molecular mass 358.3 g mol^{-1}). It has a benzimidazole ribonucleotide structure in which an imidazole moiety of benzimidazole is *N*-linked to a ribose; additionally, a phosphate group is linked to the C-3 carbon of the ribose. The α-ribazole-3′-phosphate can be converted into α-ribazole (molecular weight 278.3 g mol^{-1}), which is a ribonucleoside analogue, via an enzyme: alkaline phosphatase (ALP). The base moiety of α-ribazole-3′-phosphate and α-ribazole is a DMB (molecular weight 146.2 g mol^{-1}) which contains a dimethyl substituted benzene ring fused to an

imidazole ring**.** As a derivative of benzimidazole, the free form of DMB exhibits fluorescent properties and, in addition, the free forms of α-ribazole and α-ribazole-3′-phosphate also show fluorescence because of the DMB present in their structures.

1.2.2 Physical Properties of Vitamin B_{12}

Cobalamins are a group of crystalline substances with red-yellow colour. They display intense absorption spectra above 300 nm due to the presence of the π- π transitions of the corrin nucleus (Iwase and Ono, 1997; Oprean *et al.*, 2011). At ambient temperature, vitamin B_{12} is moderately soluble in water (Combs and McClung, 2017; Juzeniene and Nizauskaite, 2013; Knyazev *et al.*, 2014).

1.2.3 Stability of Vitamin B_{12}

Vitamin B_{12} is well known for its poor stability and sensitivity to light, heat, acids, bases, oxidizing and reducing agents. Overall, CN-Cbl is the most stable form of vitamin B_{12} (Combs and McClung, 2017; Juzeniene and Nizauskaite, 2013; Knyazev *et al.*, 2014).

1.2.3.1 Light Stability of Vitamin B_{12}

Vitamin B_{12} has photosensitivity and, in aqueous solutions, decomposes through light exposure. Aqueous solutions of both Me-Cbl and Ado-Cbl are highly sensitive to light (Ahmad and Hussain, 1993; Juzeniene and Nizauskaite, 2013; Vaid *et al.*, 2018) and even a few seconds of exposure will cause conversion into the OH-Cbl form. A relatively slow transition has been identified with CN-Cbl in aqueous solutions which occurs within hours (Ahmad and Hussain, 1993; Juzeniene and Nizauskaite, 2013). Although OH-Cbl is the most photostable form of vitamin B_{12}, further chemical degradation is highly likely, because of its reducibility in the presence of oxidising agents.

Nakos *et al.* (2017) conducted a stability test with a freshly prepared CN-Cbl standard solution and found that after 24 and 48 hours there were 10.8% and

21.3% losses in concentrations at the limit of quantitation (LOQ) level. Standard solutions with higher concentrations ($>$ 0.5 µg mL^{-1}) can be kept stable for 2–3 days, which showed that the working standard needs to be prepared freshly for analysis.

In a natural environment, such as animal food and the human body, cobalamin is bound to certain proteins, whereas CN-Cbl is present in the free form in fortified foods. These binding proteins serve a protective role against photolysis. De Keukeleire *et al.* (2015) found that the concentration of vitamin B_{12} in human blood samples did not show a clinical difference after up to 24 hours ambient light exposure. Wang *et al.* (2019) concluded that, with the protection of added whey protein (β-lactoglobulin and α-lactalbumin), the photostability of Ado-Cbl and CN-Cbl solution were enhanced by 20%.

1.2.3.2 Thermal Stability of Vitamin B_{12}

Heat treatment can also negatively impact the stability of vitamin B_{12}. Goldstein and Duca (1982) examined the thermal stability of: CN-Cbl and OH-Cbl by using thermogravimetric, derivative thermogravimetric and differential thermal analyses; in addition, CN-Cbl degradation was monitored by IR spectroscopy. The results of CN-Cbl heating test indicated that the removal of the cyan group from the structure occurred at 140–145 °C, at 10 °C min^{-1} heat increase. After heating beyond 230 °C (taking approximately 20 min from ambient temperature) extensive decomposition occurred with significant structural changes, such as, cleavage of the bond between DMB and central cobalt. Further temperature increase led to full degradation resulting in a mixture of phosphorus and cobalt oxides. Similar results were found for OH-Cbl which suggests that the different upper ligands did not affect the thermal stability. Nakos *et al.* (2017) also investigated the thermal stability of CN-Cbl solution at 100 ^{o}C for 5-minute intervals up to one hour. They found that after 15 min heat treatment, there was no content losses, but for longer durations, for example 30–45 min, nearly 10% of CN-Cbl decomposed.

Therefore CN-Cbl is relatively stable to non-vigorous thermal treatment, however, decomposition will occur at temperatures above 150 °C for prolonged

treatment time. During the manufacturing of infant formulas, several heat treatments are employed for safety and dehydration purposes. Temperature can rise up to 200 °C during spray drying (Piñón-Balderrama *et al.*, 2020), which inevitably can lead to loss of natural vitamin B_{12}. Although the exposure to such high temperature is relatively short (less than 60 s) (Fox *et al.*, 2014; Knipschildt and Andersen, 1994), there are multiple different drying steps can contribute to the thermal-inducted degradation effect on vitamin B_{12}. Therefore, supplementation of sensitive vitamins including vitamin B_{12} is often required after the drying process.

1.2.3.3 Effect of pH on the Stability of Vitamin B_{12}

There are reports that pH conditions effect the stability of vitamin B_{12}. The maximum stability of CN-Cbl is at neutral pH (pH = 6–7) (Ahmad and Hussain, 1993). The photolysis rate of vitamin B_{12} varies with pH; Vaid *et al.* (2018) found that Me-Cbl had the highest photolysis rate around pH = 5 and decreased rates under more acidic or more alkaline conditions; CN-Cbl and OH-Cbl showed the same pattern.

1.2.4 Vitamin B_{12} Biosynthesis

There are three main naturally occurring forms of vitamin B_{12}: Me-Cbl, OH-Cbl and Ado-Cbl (Gille and Schmid, 2015). The Ado-Cbl and Me-Cbl are found in the form of coenzymes (enzyme cofactors) in living tissues of mammals and certain bacteria. The two coenzymes covalently bind to proteins and form cobalamin-protein complexes (Martin *et al.*, 2016; Perez-Fernandez *et al.*, 2016). All three natural forms of vitamin B_{12} are present in foods of animal origin, including meat, milk, eggs and fish. Liver is the richest sources of vitamin B_{12} (Combs and McClung, 2017). Several recent publications have suggested that some plant species contain vitamin B_{12} as well (D'Ulivo *et al.*, 2017; Nakos *et al.*, 2017). Recent research indicated that significant contents of vitamin B_{12} have been found in plant sources including *Hippophae rhamnoides* (37 µg 100 g^{-1} dry weight), *Elymus spp* (26 µg 100 g^{-1} dry weight). and *Inula helenium* (11 µg 100g^{-1} dry weight) (Nakos *et al.*, 2017).

Although vitamin B_{12} is found in animals and some plant sources, they are in fact synthesized by bacteria, archaebacteria, and algae which are present in host animals and plants; for example, the ruminal microflora synthesize relatively high levels of vitamin B_{12} compared with other sources (Combs, 1998). In animals, once produced, vitamin B_{12} is absorbed and stored in the liver and muscles of the host or secreted in milk during lactation (Chamlagain *et al.*, 2015; Duplessis *et al.*, 2016; Gille and Schmid, 2015; Matte *et al.*, 2012).

There are naturally occurring CN-Cbl, but in general chemical transformation from other natural vitamers with cyanide is its major synthesis pathway and CN-Cbl is used commercially for addition in fortified foods, nutritional formula and pharmaceutical preparation (Chamlagain *et al.*, 2015; Perez-Fernandez *et al.*, 2016).

1.2.5 Vitamin B_{12} Absorption and Metabolism

The colonic microflora of humans can synthesize vitamin B_{12}, however, it cannot be absorbed by humans due to the long distance between the location of absorption in the small intestine and where the synthesis occurred. Therefore, the essential source of vitamin B_{12} is food consumption. In food, the endogenous cobalamins are bound to proteins and the vitamin is freed from the vitamin B_{12}-protein complex during digestion processes including gastric acidification and proteolysis (Gille and Schmid, 2015; Nohr *et al.*, 2011).

After release from the binding proteins, the free vitamin binds to the R-proteins (haptocorrins) which are glycoproteins secreted in saliva. Subsequently, in the small intestine, the R-protein-vitamin B_{12} bonds are broken by pancreatic protease to liberate the vitamin for further binding with intrinsic factor. The latter is a small glycoprotein secreted by gastric parietal cells. Unlike R-proteins, which can also bind to cobalamin analogues, intrinsic factor only binds to the four biologically active cobalamins and forms a vitamin B_{12}-intrinsic factor complex for the succeeding transport steps (Combs and McClung, 2017; Gille and Schmid, 2015; Herbert, 1988). Subsequently, the vitamin B_{12}-intrinsic factor

complex moves into cells via a carrier-mediated active transport process then the vitamin B_{12} is released from the complex. There are two proteins responsible for the next transportation into the plasma, namely, plasma haptocorrin and transcobalamin. The haptocorrin transports vitamin B_{12} to the liver. On the other hand, transcobalamin forms a complex with vitamin B_{12} called holotranscobalamin which is the active form of vitamin B_{12} in tissue cells (Combs and McClung, 2017; Gille and Schmid, 2015; Quadros *et al.*, 1999). For subsequent metabolism, all forms of vitamin B_{12} are converted into the Me-Cbl and Ado-Cbl forms as coenzymes in the cells (Greibe and Nexo, 2016).

1.3 Importance of Vitamin B_{12}

Each vitamin B_{12} vitamer has significant metabolic functions in humans and are essential for: cell development, energy production, foetus growth and other human physiological functions.

1.3.1 Vitamin B_{12} Coenzyme Functions

In metabolism vitamin B_{12} is actively involved as the previously mentioned coenzyme forms: Me-Cbl and Ado-Cbl. These two compounds function in the metabolism of propionate, amino acid and single-carbon units (Combs and McClung, 2017).

1.3.1.1 Methylcobalamin

Me-Cbl is the coenzyme for methionine synthase which catalyses the regeneration of the essential amino acid methionine by methylation of homocysteine. In order to achieve such a reaction, Me-Cbl transports the methyl group from the donor 5-methytetrahydrofolate to the acceptor homocysteine. Methionine is an essential part of the synthesis of proteins and polyamines, so that a deficiency of Me-Cbl will greatly affect the availability of methionine and the further metabolism of proteins and amino acids. Additionally, methionine is the precursor of *S*-adenosylmethionine which is involved in hundreds of enzymatic reactions and serves as the main donor of methyl groups; these reactions all play key roles in metabolism. A deficiency of

S-adenosylmethionine will influence the synthesis of creatine, phospholipids, and the neurotransmitter acetylcholine, and cause impairments which will be expressed in physiological functions. Moreover, the Me-Cbl and methionine synthase balanced the flux of single-carbon units towards synthesis of DNA and RNA which is essential in single carbon metabolism (Combs and McClung, 2017; Gueant and Alpers, 2013).

1.3.1.2 Adenosylcobalamin

As the coenzyme of methylmalonyl CoA mutase, Ado-Cbl catalyses methylmalonyl CoA to convert into succinyl CoA during the degradation of propionate which is produced from fatty acids. The mechanism of this conversion involves cleaving a C-C bond of the Ado-Cbl and resulting a free radical formed on the Ado-Cbl, which free radical can be transferred to the substrate by an amino acid residue. This reaction is especially significant for ruminants since it contributes to the production of energy (Combs and McClung, 2017).

In human bodies, the lack of vitamin B_{12} will firstly impact methylmalonyl CoA mutase and lead to activity losses which can express as methylmalonic aciduria -like symptoms. The methylmalonic acid accumulated in body can inhibit the tricarboxylic acid cycle then result in damage to glucose and glutamic acid metabolism (Combs and McClung, 2017).

1.3.2 Relationship with Folate

Folate is essential for preventing chromosome breakage and hypomethylation of DNA. In the normal functions of folate, specifically the methylation cycle, vitamin B_{12} is involved to maintain the availability of the methyl group donor *S*-adenosylmethionine. This methylation is vital since it is the process by which DNA determines gene expression. The vitamin B_{12} concentration can positively impact the efficiency of methylation activity. A lower vitamin B_{12} level means that methionine synthase activity is reduced, and the production of *S*-adenosylmethionine is compromised which could reduce DNA methylation. In such case, folate is unable to be used for the conversion of deoxyuridine

monophosphate to deoxythymidine monophosphate in DNA metabolism which results in accumulation of deoxyuridine monophosphate and further risk of DNA damage (Combs and McClung, 2017; Eto and Krumdieck, 1986; Fenech, 2001; Zingg and Jones, 1997).

1.3.3 Vitamin B_{12} Physiological Functions

Vitamin B_{12} status shows a close relationship with some human physiological functions, which are outlined in the sections below.

1.3.3.1 Neurological Function

Vitamin B_{12} is a necessary micronutrient for maintenance of myelin sheaths which surround nerve cells. In addition, vitamin B_{12} has a key role in the synthesis of choline which produces the neurotransmitter acetylcholine. All of these functions contribute to neurological functions primarily peripheral, autonomic nervous and cerebral-spinal function which relate to cognition, sensation and muscular coordination (Combs and McClung, 2017; Stabler, 2013).

1.3.3.2 Haematological Development

Vitamin B_{12} assists supply of the single carbons for red blood cell division in the bone marrow since the single-carbon units come from the synthesis of thymidylate catalysed by methionine synthase (Combs and McClung, 2017).

1.3.3.3 Foetal Development

According to Ray and Blom (2003), there is an association between low vitamin B_{12} levels and the risk of foetal neural tube defects (NTD). The research found that the NTD pregnancies often showed lower vitamin B_{12} in amniotic fluid comparing with healthy groups. It suggests that vitamin B_{12} possibly plays a key role in early foetal development (Combs and McClung, 2017).

1.3.4 Vitamin B_{12} Deficiency

In adult bodies, the estimated total content of vitamin B_{12} ranges between 2 and 3 mg, which is lower than the typical level of other vitamins. Under normal

conditions the required level of vitamin B_{12} can be satisfied by a balanced diet for adults and well-nourished maternal source for an infant. Different from other B vitamins, vitamin B_{12} has a large concentration store in the liver. Therefore, a short term absence of vitamin B_{12} within dietary intake would not cause deficiency (Akcaboy *et al.*, 2015; Aytekin *et al.*, 2018; Gille and Schmid, 2015; Parvin *et al.*, 2018).

The diagnosis of vitamin B_{12} deficiency is controversial. Deficiency is a value less than 200 pg mL^{-1} of total vitamin B_{12} in serum, which is the traditional cut-off for vitamin B_{12} deficiency according to WHO (World Health Organization, 1968). However, there are other biochemical or functional indicators which have been reported and applied as well, such as holoTC, methylmalonic acid, and total homocysteine. Since "*there is no 'gold standard' test to define deficiency*", as quoted from Guidelines of British Society for Haematology, a more comprehensive diagnosis is preferred (Lindenbaum *et al.*, 1994; Smith *et al.*, 2018).

1.3.4.1 Common Causes of Vitamin B_{12} Deficiency

There are two common causes of vitamin B_{12} deficiency: insufficient intake and malabsorption (Truswell, 2007). For individuals who have a strict vegetarian or vegan diet, lack of consumption of animal-origin foods puts them at high risk of vitamin B_{12} deficiency, because, in general, plants do not produce vitamin B_{12}. Likewise, people who live in developing countries and regions are also under high risk due to the inadequate uptake of meat or milk products. Infants who are breast-fed may suffer from deficiency if their mothers are deficient (Akcaboy *et al.*, 2015; FAO and WHO, 2001; Gille and Schmid, 2015; Nohr *et al.*, 2011).

Another common cause of deficiency is malabsorption of vitamin B_{12}. There are various factors which lead to impaired absorption and disturbance of cobalamin metabolism. For instance, an autoimmune disease named pernicious anaemia, hypochlorhydria from atrophic gastritis, drugs and intestinal parasites can cause malabsorption. Pernicious anaemia brings autoimmune destruction of gastric parietal cells which leads to the loss of intrinsic factor. Intrinsic factors are the

transport protein of cobalamin and the lack of them will damage the absorption of vitamin B_{12}. In addition, in hypochlorhydria cases, the absence of hydrochloric acid in stomach can preclude the liberation of vitamin B_{12} from binding proteins, which commonly occurs in elderly population (Food and Agriculture Organization of the United Nations and World Health Organization, 2001; Gille and Schmid, 2015; Truswell, 2007).

1.3.4.2 Syndromes of Vitamin B_{12} Deficiency

In humans, syndromes of vitamin B_{12} deficiency manifest with two main symptoms: megaloblastic anaemia and/or neuropathy. Megaloblastic anaemia occurs in subjects whose absorption of cobalamin is damaged and there are several associated syndromes including: anaemia, enlarged red blood cells, low white blood cell count, sore tongue and infertility. Megaloblastic anaemia is a late symptom for vitamin B_{12} deficiency. In contrast, cases of neuropathy are rather commonly associated with neuromuscular dysfunction symptoms such as: degeneration of the spinal cord, loss of proprioceptive sensation, muscle weakness, gait ataxia, and in rare cases depression and memory loss can be observed (Akcaboy *et al.*, 2015; Aytekin *et al.*, 2018; Gille and Schmid, 2015; Matte *et al.*, 2012; Toh *et al.*, 1997; Truswell, 2007). For foetuses, maternal cobalamin deficiency can impact weight, length and head circumference. In new-borns cases, infants may develop symptoms from age of 2 months up to 12 months (Akcaboy *et al.*, 2015; Dror and Allen, 2008; Muthayya *et al.*, 2006; Yaikhomba *et al.*, 2015).

1.3.4.3 Recommendation of Intake

The recommended dietary intake of vitamin B_{12} varies by age, gender and physiological conditions. In New Zealand, the recommended daily intake (RDI) of vitamin B_{12} is 2.4 µg per day for the population of 14 years and older, 0.9–1.8 µg per day for those between 1 and 13 years old and 0.4–0.5 µg per day for infants (< 12 months) (National Health and Medical Research Council. Australian Government Department of Health and Ageing. New Zealand Ministry of Health, 2006). Worldwide, the recommended daily amount (RDA) is 1.5 µg per day for all gender groups aged 15 plus in the UK. The German

Nutrition Society recommends a higher value of 3 µg per day. The Institute of Medicine (US) has suggested the average daily requirement of vitamin B_{12} is 2 µg for healthy adults and 0.4 µg for infants (Aytekin *et al.*, 2018; D'Ulivo *et al.*, 2017; Dietary reference intakes, 1997; EFSA, 2015; Great Britain: Panel on Dietary Reference Values, 1991; Martin *et al.*, 2016).

Exogenous intake is essential because vitamin B_{12} cannot be synthesised by humans. Animal-food sources including meat, fish, eggs and dairy products are the main and natural vitamin B_{12} source for individuals. However, for vegans and vegetarians, vitamin B_{12} fortified food and vitamin tablets are recommended to lower the deficiency risk.

During pregnancy and lactation, it is recommended that women take vitamin B_{12} supplementation and/or vitamin B_{12} rich foods so infants can also benefit (Allen, 2005; Aytekin *et al.*, 2018; Dror and Allen, 2008).

1.3.5 Bioavailability of Vitamin B_{12}

The bioavailability of vitamin B_{12} varies and depends on several factors including the food source and the forms in which it is present. Several researchers have reported that bioavailability of natural forms of vitamin B_{12} averages around 50% (Perez-Fernandez *et al.*, 2016). In contrast, Gille and Schmid (2015) claimed that synthetic CN-Cbl has low bioavailability of less than 4%. But in other publications, the absorption efficiency of crystalline CN-Cbl administered orally was reported as comparable to natural vitamin B_{12} in food (Ball, 2006).

With regards to the specific sources of intake, vitamin B_{12} presented in milk is more efficiently absorbed than the synthetic form in supplements (Matte *et al.*, 2012). Moreover, Vogiatzoglou *et al.* (2009) investigated the relationship between various intake of food items and vitamin B_{12} concentration in blood plasma. The results revealed that the increased intake of vitamin B_{12} supplemented by milk products or fish led to a significant rise of vitamin B_{12} in plasma which was not observed in cases of egg or meat. In addition,

vitamin B_{12} from dairy products appeared to have higher bioavailability in comparison with fish (Gille and Schmid, 2015).

Herbert (1988) and Matte *et al.* (2012) both found that vitamin B_{12} concentrations in dietary sources correlated negatively with bioavailability in studies for pig and human subjects. The results showed that when the actual amount of vitamin B_{12} consumed increased, the proportion of vitamin B_{12} absorbed decreased. The cause of this phenomenon was that in the small intestine the limited amount of vitamin B_{12}-intrinsic factor receptors became saturated when more than 1.5–2.0 µg vitamin B_{12} was ingested for one intake (Allen, 2010; Scott, 1997).

Although in general the content of vitamin B_{12} in meat, egg and fish are greater than vitamin B_{12} in milk (Ball, 2006), the highest bioavailability was found in milk and related products. There are three factors that are influenced by this finding; firstly the cooking process affects the vitamin B_{12} concentration which causes the actual intake to be less than the original content; secondly, one large ingestion of vitamin B_{12} means that a smaller proportion is absorbed than would be for several repeated low doses because of the limited receptor ability in the small intestine during absorption; and finally for individuals who suffer from reduced gastric secretion the difficulty in digesting foods such as meat can result in a decreased release of the vitamin B_{12} (Bennink and Ono, 1982; Gille and Schmid, 2015; Vogiatzoglou *et al.*, 2009).

1.4 Vitamin B_{12} in Milk

In bovine milk research, findings of the content of vitamin B_{12} vary. A Canadian research group studied the concentration of vitamin B_{12} in milk among and within commercial dairy herds. The results showed that the concentration of vitamin B_{12} in milk ranged from 2.3 to 3.9 µg L^{-1} (Duplessis *et al.*, 2016). The national food composition database of Denmark and Switzerland suggests that the concentration of vitamin B_{12} in bovine milk was 0.08–0.49 µg 100 g^{-1} (Gille and Schmid, 2015). The methods of analysis and the unstable nature of vitamin

B_{12} both contribute to the variability of B_{12} concentration in milk. **Table 1.1** summarises the vitamin B_{12} content in various types of milk.

Table 1.1.Vitamin B_{12} concentration in various types of milk

Reference source	Vitamin B_{12} concentration			
	Cow milk	Sheep milk	Goat milk	Human milk
Gille and Schmid (2015)	0.2–0.7 μg 100 g^{-1}	0.30 μg 100 g^{-1}	0.07 μg 100 g^{-1}	0.05 μg 100 g^{-1}
Duplessis *et al.* (2016)	0.23–0.38 μg 100 mL^{-1}	—	—	—
Graulet and Girard (2017)	0.45 μg 100 g^{-1}	—	—	—
Collins *et al.* (1951)	—	0.14 μg 100 mL^{-1}	0.42 μg 100 mL^{-1}	0.04 μg 100 mL^{-1}
Ramos *et al.* (1994)	—	0.45–1.17 μg 100 mL^{-1}	—	—
Indyk *et al.* (2002)	—	—	0.46–0.71 μg 100 g^{-1}	—
Raynal-Ljutovac *et al.* (2008)	—	—	—	0.04 μg 100 mL^{-1}

Vitamin B_{12} naturally presented in bovine milk is bound to specific transport proteins such as transcobalamin which is one of mammalian vitamin B_{12}-binding proteins (Watanabe, 2007). OH-Cbl is the predominant form of vitamin B_{12} in milk, with the minor presence of Me-Cbl and Ado-Cbl (Indyk *et al.*, 2002).

The processing of milk includes boiling, microwaving and pasteurisation leading to various losses in vitamin B_{12}. An estimated 30% and 50% of vitamin B_{12} was lost by boiling for 2–5 min and 30 min, respectively, and 50% was lost by 5 min microwave cooking and 5–10% lost by pasteurization (Watanabe, 2007). However, the concentrations of vitamin B_{12} in pasteurized milk did not significantly decrease over daylight exposure and/or refrigerator storage (Duplessis *et al.*, 2016). Vitamin B_{12} concentrations in fermented milk like yogurt reduced during the fermentation processing and storage due to the presence of *L. bulgaricus* and *S. thermophilus* which have been identified as vitamin B_{12}

consumers. It is reported that the loss of vitamin B_{12} in prepared and stored yoghurt ranged from 25% to 60% (Gille and Schmid, 2015).

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 as an excellent vitamin B_{12} source according to the Canadian Food Inspection Agency (Matte *et al.*, 2012). However, many vegetarians also choose to exclude dairy from their diets, thus, the risk of vitamin B_{12} deficiency is as significant as in vegans. In this case, vitamin B_{12} supplements and other fortified food products should be introduced into their diets to prevent the deficiency.

1.5 Vitamin B_{12} in Infant Formulas

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}. During the manufacturing of powdered infant formula, several processing technologies are involved such as heat treatments (pasteurisation, sterilisation and evaporation) and drying processing (dry blending and/or spray drying). **Figure 1.3** shows a manufacturing flow chart of infant formula powder. These processing steps all have a negative effect on the content of vitamin B_{12}. Chapman *et al.* (1957) described a study of the effect of processing on B-group vitamins in milk including vitamin B_{12}. They found that approximately 90–100% of vitamin B_{12} was destroyed during the sterilisation (steam autoclave for 30 min either at 107 °C or 111 °C); 70–90% of initial vitamin B_{12} was lost by evaporation; spray drying also caused more than 30% losses of vitamin B_{12} in raw milk. Therefore, after processing, infant formula may contain much fewer natural forms of vitamin B_{12} than the raw milk. (Bezie, 2019) reviewed the effect of different heat treatment on the nutrients of the milk products and found that up to 90% loss of vitamin B_{12} in evaporation process. Spraying drying, even with great care, led to the loss of a third of vitamin B_{12} during the process (Wigzell, 2003). Therefore, vitamin B_{12} supplementation by adding CN-Cbl is used to ensure the final products contain an appropriate amount of vitamin B_{12}. According to the Australian and New Zealand Food Standards Code, the

minimum amount of vitamin B_{12} in infant formula should be 0.025 µg 100 kJ^{-1} (Australia New Zealand Food Standards Code, 2018).

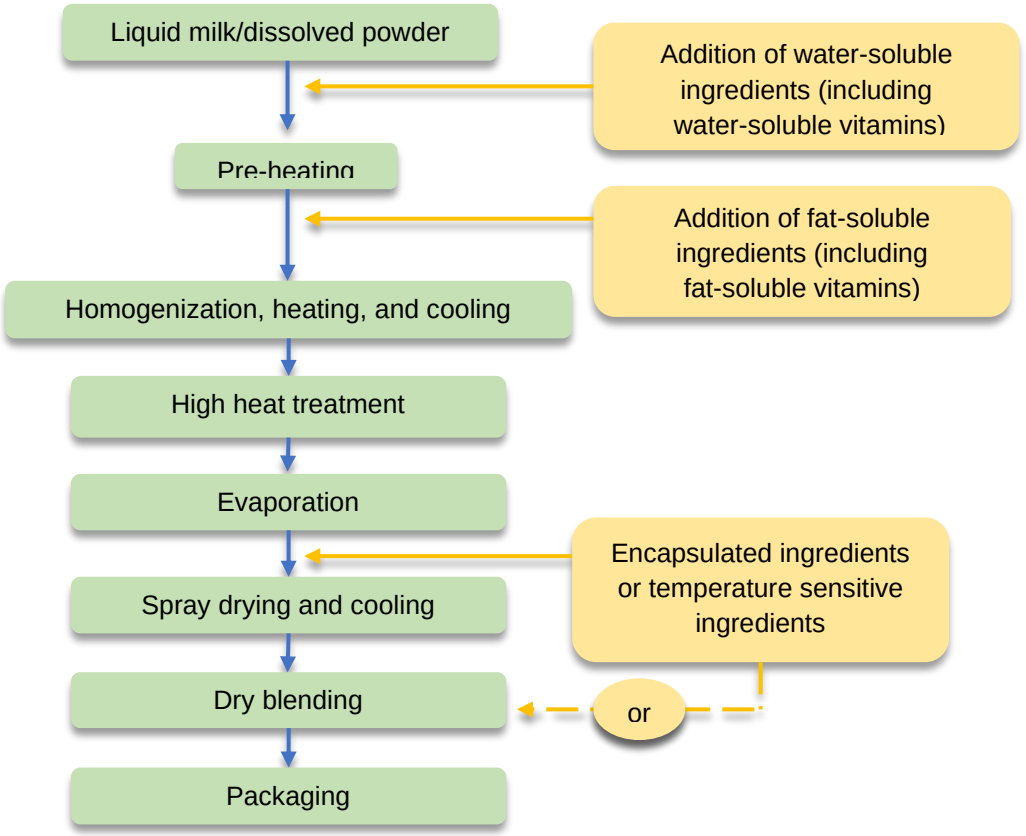


Figure 1.3. Manufacturing flow chart for powdered infant formula

In human milk, the predominant form of vitamin B_{12} is OH-Cbl, which is similar to bovine milk. However, the binding proteins are not the same; vitamin B_{12} is bound to haptocorrin in human milk and to transcobalamin in bovine milk. Although the importance of transcobalamin in milk has not been fully identified, it is believed that transcobalamin can increase the bioavailability of vitamin B_{12} in milk. In infant formula, vitamin B_{12} predominately presents in the free CN-Cbl form. However, there is no known difference in clinical outcome between these two forms of vitamin B_{12} (Greibe and Nexo, 2016). Greibe and Nexo (2016) analysed the vitamin B_{12} content of several commercial infant formulas by an enzyme-linked immunosorbent assay (ELISA); the observed and indicated concentrations of vitamin B_{12} in infant formula (median range) were 0.16–0.34 µg 100 mL^{-1} and 0.14–0.50 µg 100 mL^{-1} prepared feed, respectively (**Table 1.2).**

Table 1.2. Comparison of Vitamin B_{12} content between indicated values and observed results

Product name No. and (status)[b]	Total vitamin B_{12} concentration	
	Indicated[c] µg 100 mL^{-1}	Observed µg 100mL^{-1}
Semper		
1 (Infant formula)	1.60	0.22
2 (Follow-on formula)	0.22	0.24
Nestlé		
3 (Infant formula)	0.15	0.17
4 (Infant formula)	0.14	0.25
5 (Follow-on formula)	0.19	0.21
6 (Follow-on formula)	0.22	0.20
Arla		
7 (Infant formula)	0.25	0.16
8 (Infant formula)	0.19	0.18
9 (Follow-on formula)	0.23	0.23
HIPP		
10 (Infant formula)	0.50	0.34
11 (Follow-on formula)	0.15	0.21

[a] Data extracted from Greibe and Nexo (2016). Licensed under CC BY 4.0
[b] Infant formula for infants aging from birth to 6 months or as follow-on formula for infants aging from 6 months and onwards.
[c] The vitamin B_{12} content indicated on the package.

The difference between the indicated values on nutrition labels and the experimental results may be explained as follows: (i) the indicated values are often typical values, which may not represent the real vitamin B_{12} content of the sample; (ii) considering the poor stability of vitamin B_{12}, degradation can occur during the shelf life; (iii) the analytical methods may not be the same, which can lead to varying results.

According to a Danish study (Mosekilde *et al.*, 2013), the concentration of vitamin B_{12} in human milk depends on the postpartum duration. The human milk collected at two weeks and four months postpartum contained 0.03-0.26 µg 100 mL^{-1} and 0.02–0.09 µg 100 mL^{-1}, respectively. Comparing these results with the vitamin B_{12} content in infant formula mentioned above, the latter contains at least twice the content of vitamin B_{12} found in human milk.

Table 1.3 shows various vitamin contents information of two ordinary infant formula products, which is marketed in New Zealand and Australia; in which vitamin B_{12} has the lowest concentration among all vitamin components, which further indicates analytical challenges at such low level of concentration.

Table 1.3. Vitamins content information of an infant formula

Vitamins	Average quantity per 100ml prepared feed	
	Brand A	Brand B
Vitamin A	69	68
Vitamin B_6	55	56
Vitamin B_{12}	**0.18**	**0.27**
Vitamin C (ascorbic acid)	9000	1900
Vitamin D	1200	940
Vitamin E	800	120
Vitamin K	6.7	6.3
Biotin	2.0	3.4
Niacin (vitamin B_3)	500	536
Folic acid	11	13.4
Pantothenic acid (vitamin B_5)	400	500
Riboflavin (vitamin B_2)	110	160
Thiamine (vitamin B_1)	100	76

[a] This table is extracted from the nutritional information of two different brands of infant formula products (0–6 months) from a local supermarket.

1.6 Analysis of Vitamin B_{12}

Chromatographic methods have been developed for the routine analysis of vitamin B_{12} in dairy foods. In addition, microbiological assay, spectroscopic assay, radioisotopic assay and competitive binding assay have also been reported and applied in the determination of vitamin B_{12} in milk and infant formula. Due to the limited active research in developing new method for vitamin B_{12} analysis, some of the literature reviewed here might appear as historical.

Because of the complexity of vitamin B_{12} and the different forms it presents in milk, sample preparation was essential for achieving high precision of methods.

The conventional preparation procedure for vitamin B_{12} analysis in milk and infant formula is denaturation of binding protein, conversion into stable form (CN-Cbl) by addition of cyanide, concentration and separation by a clean-up technique. However, the utilisation of cyanide has raised safety concerns in relation to the workplace. Cyanides are highly toxic and an exposure to cyanides can lead to histotoxic hypoxia through the disruption of electron transport chain and inhibition of oxygen usage in cells. Also, an acute exposure can cause a coma and cardiac arrest with death following. Therefore, a safer, simplified sample preparation and rapid chromatographic analysis is desirable for both laboratory and industry.

1.6.1 Sample Preparation

The sample preparation step in vitamin B_{12} detection for food, particularly with dairy products due to its emulsion property, is critical to the analysis. Matrix inference and interaction with protein presented in a sample should be removed since they can affect the repeatability and accuracy of analysis. Moreover, vitamin B_{12} is unstable to light, heat, oxidising and reducing reagents. Therefore, proper sample preparation and handling is essential to ensure the reliability of results. The sample preparation protocol depends on the targeted forms of vitamin B_{12} for analysis. In general, there are two expressions of vitamin B_{12} in milk and other dairy products; free vitamin B_{12} normally is CN-Cbl which is supplemented in dairy products such as infant formulas, whereas all forms of cobalamin, including protein-bound cobalamin, converted into CN-Cbl represents the total vitamin B_{12} (Perez-Fernandez *et al.*, 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-Giménez *et al.*, 2008; Chamlagain *et al.*, 2015).

1.6.1.1 Denaturation of Binding Protein

In natural samples, vitamin B_{12} is present bound to protein carriers. During preparation the proteins need to be denatured to ensure the further extraction of

cobalamin in a free form without protein inference. Previous researchers have reported heat or autoclave treatment, enzymatic proteolysis and acidic precipitation (**Table 1.4**) (Kumar *et al.*, 2010; Perez-Fernandez *et al.*, 2016). For milk and other dairy products boiling and/or autoclaving the sample in aqueous form at 98–121 °C for at least 30–35 min in the presence of potassium cyanide (KCN) 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 cobalamin into the more stable form CN-Cbl. This procedure has been applied in many vitamin B_{12} analyses subsequently using high performance liquid chromatography (HPLC) and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) approaches, because CN-Cbl is commercially available as a standard. However, employing potassium cyanide is highly undesirable due to its extreme toxicity (Campos-Gimenez, 2014; Chamlagain *et al.*, 2015; Parvin *et al.*, 2018; Repossi *et al.*, 2017; Schimpf *et al.*, 2012; Watanabe *et al.*, 1997; Watanabe and Bito, 2018; Zironi *et al.*, 2013).

Table 1.4. Summary of extraction procedures for denaturation of binding protein and conversion into cyanocobalamin in the reviewed literature

	Sample Types	Sample Weight	Extraction Procedure	Cyanide Amount	Recovery%	References
Heating/ Boiling/ Autoclave Treatment	Cereals, Infant Formulas, and Poly-vitamin Premixes.	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 and Adults Formulas	30 g, 12% (*w*/*w*)	30 mL sodium acetate solution pH = 4.5 Heated in 105 °C oven for ≥ 60 min	1 mL of 1% potassium cyanide solution	—[c]	Schimpf *et al.* (2012)
	Infant Formula and Adults Formulas	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 of 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.	0.150 mL of 1% potassium cyanide solution	—[c]	Repossi *et al.* (2017)
Enzymatic Treatment	Pig liver, whole egg, beef fillet, salmon, powdered milk	2–8 g	25 mL sodium acetate buffer pH = 4 2 mL pepsin solution (50 mg mL^{-1}), incubated at 37 °C for 3h	—[a]	95–100%	Pakin *et al.* (2005)
	Infant Formula, Cereals	5–25 g	60 mL sodium acetate buffer, pH = 4 1 g pepsin, incubated at 37 °C for 3 hours 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	—[b]	80–93%	Perez-Fernandez *et al.* (2016)

[a] No cyanide applied
[b] This method was designed to analyse four vitamin B_{12} forms separately, thus no need for cyanide conversion
[c] No data available

There are several reported studies in which the heating treatment process was modified to include an enzymatic proteolysis step prior to the heating commonly utilising a proteolytic enzyme such as pepsin or papain. Pepsin or papain was added and incubated at 37 °C for 1.5–3 hours at pH = 4–4.8. Incubation ended by continuing a boiling treatment as mentioned above. This modification improved the liberation of bound cobalamin (Campos-Giménez *et al.*, 2008; Heudi *et al.*, 2006; Nakos *et al.*, 2017; Watanabe and Bito, 2018). Pakin *et al.* (2005) conducted a similar sample extraction but without further heating and addition of potassium cyanide; the exclusion of cyanide has been controversial because of the risk of non-representative results due to the losses during the extraction.

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 commonly used at room temperature with a centrifugation step to remove the solid residue (Oprean *et al.*, 2011; Perez-Fernandez *et al.*, 2016).

1.6.1.2 Conversion to a Single Form of Vitamin B12

CN-Cbl and other natural forms of cobalamins will undergo degradation after light exposure and heating during the quantitative extraction. CN-Cbl is more stable and the decomposition rate is relatively low compared with the other three forms (Me-Cbl, OH-Cbl and Ado-Cbl). Stabilization needs to be employed to ensure the precision of the succeeding quantitative analyses. Based on its relatively good stability, the CN-Cbl form has been used for quantitation of vitamin B_{12} and is reported on nutrition information labels. Currently, the majority of published methods for quantitation of total vitamin B_{12} content achieve CN-Cbl conversion by utilising dilute cyanide solutions (sodium or potassium cyanide). The amount of cyanide depends on the sample size and to which it is added in excess (Kumar *et al.*, 2010). However, due to the toxic nature of cyanide, there are growing ethical concerns about the necessity of including such a potentially fatal substance in the procedure.

Alternatively, boiling or heating with metabisulfite to convert all forms of vitamin B_{12} into sulfitocobalamin (SO_3-Cbl) can be employed to replace the use

of cyanide, since SO_3-Cbl is as stable as CN-Cbl. However, the limitation of this replacement is that there is no commercially available SO_3-Cbl standard. Thus, conversion to CN-Cbl is still the universal procedure used in the procedure of determination of vitamin B_{12}.

1.6.1.3 Solid Phase Extraction and Immunoaffinity Extraction

Excluding the interference from sample matrix, purifying the substance and concentrating the analyte are needed in the sample preparation protocol to ensure the accuracy and precision of analysis methods. Solid phase extraction (SPE) is a common approach to fulfil these demands; this employs solid particle and/or chromatographic packing material placed in a cartridge device to achieve the chemical separation of the various components in the sample. Generally, a sample in solution is loaded onto the SPE cartridge, which may require a pre-conditioning wash, then elution by different strength solvents is employed to separate analytes from the matrices in which non-targeted compounds are not retained.

For vitamin B_{12}* analysis in food products SPE is commonly applied due to the complexity of the food matrix. SPE cartridges with various packing materials have been reported for this clean-up. A summary of the SPE procedure detail from several literature sources is presented in **Table 1.5**. C_{18} and C_8 cartridges are traditional SPE with a hydrophobic, bonded silica sorbent which has the most retentive nature of non-polar compounds. C_{18} and C_8 cartridges are effectively able to retain most organic compounds in the vitamin B_{12} extraction solution. D'Ulivo *et al.* (2017), Iwase and Ono (1997), Schimpf *et al.* (2012) and Zhu *et al.* (2011) have used either a C_{18} or C_8 cartridge as the SPE clean-up protocol, the cartridge was conditioned by methanol or acetonitrile and subsequently ultrapure water prior to the sample solution being loaded, and the cartridge was rinsed by ultrapure water or 5% methanol water solution. Finally, vitamin B_{12} was eluted and collected with 100% methanol or 25–30% acetonitrile.

* The vitamin B_{12} analysis in the section were quantified as cyanocobalamin, unless otherwise indicated.

The HLB (Hydrophilic-Lipophilic-Balanced) cartridge has a relative non-polar retention capacity three-fold higher than silica-based C_{18} sorbent. This SPE procedure has been employed by Repossi *et al.* (2017) and Zironi *et al.* (2013). The HLB cartridge was conditioned by acetonitrile and water in sequence, the sample was loaded and the cartridge washed by water and finally eluted with 50% acetonitrile.

SPE in vitamin B_{12} analysis has been recognised and used as a conventional clean-up procedure. However, there are growing concerns regarding its poor selectivity. Since biologically inactive cobalamin analogues can also be retained and eluted within the SPE step and thus incorporated in further analysis which might lead 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 where antibodies were immobilized in a gel suspension packed in a column. When the sample was loaded on the column the analyte of interest bound to the antibody. There are several vitamin B_{12} determination methods combining immunoaffinity extraction for concentration and either HPLC or LC-MS/MS system for detection. The product EASI-EXTRACT® Vitamin B_{12} (R-Biopharm AG, Glasgow, Scotland) was employed in the following publications: Campos-Giménez *et al.* (2008), Chamlagain *et al.* (2015), Campos-Gimenez (2014), Heudi *et al.* (2006), Martin *et al.* (2016), Nakos *et al.* (2017) and Pakin *et al.* (2005). The common protocol, **Figure 1.4,** 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 mixed well. Air is inserted to dry the column, then the column is eluted with methanol twice (less methanol is used in the second run), the columns being dried by air after each elution. The sample solution must be filtered prior to loading to the column to avoid blockage during sample passing through the column.

Table 1.5. Summary for SPE procedure details in the reviewed literature

SPE Cartridge Type	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 methanol	10 mL water	5 mL water	10 mL 50% acetonitrile in water	88.8–92.2	Iwase and Ono (1997)
C_{18} (2 g/6 mL)	0.5–2 L	10 mL methanol	10 mL water	10 mL water	5 mL methanol	83.2–97.6	Zhu *et al.* (2011)
C_{18}/C_8 (600/900 mg)	20-80 mL	20 mL acetonitrile	10 mL water	5 mL water	4.4 mL 25% or 30% acetonitrile in water	—[a]	Schimpf *et al.* (2012)
C_{18} (500 mg/12 mL)	60 mL	10 mL methanol	10 mL water	10 mL, 5% methanol in water	10 mL methanol	109.7–112	D'Ulivo *et al.* (2017)
HLB (500 mg/6 mL)	10 mL	2 mL acetonitrile	2 mL water	2 mL water	1 mL 50% acetonitrile in water	—[a]	Repossi *et al.* (2017); Zironi *et al.* (2013)

[a] No data available

According to Campos-Giménez *et al.* (2008) and Heudi *et al.* (2006), immunoaffinity extraction concentrated the CN-Cbl, and thus enhanced the strength of the response signal and provided higher recovery and better repeatability. The overall recoveries of these two methods were 94–100% and 93.3–108.3%, respectively. However, the immunoaffinity columns are more sensitive to CN-Cbl than any other form. Campos-Giménez *et al.* (2008) found the recoveries of Me-Cbl, OH-Cbl and Ade-Cbl through immunoaffinity extraction were less than 20%, which indicated the necessity of preceding conversion into CN-Cbl as total vitamin B_{12}. But the reason behind the low recoveries might also be the instability of those three forms of cobalamin, the authors did not present the experimental details and data in their publication, therefore the possibility that instability caused low recoveries cannot be excluded.

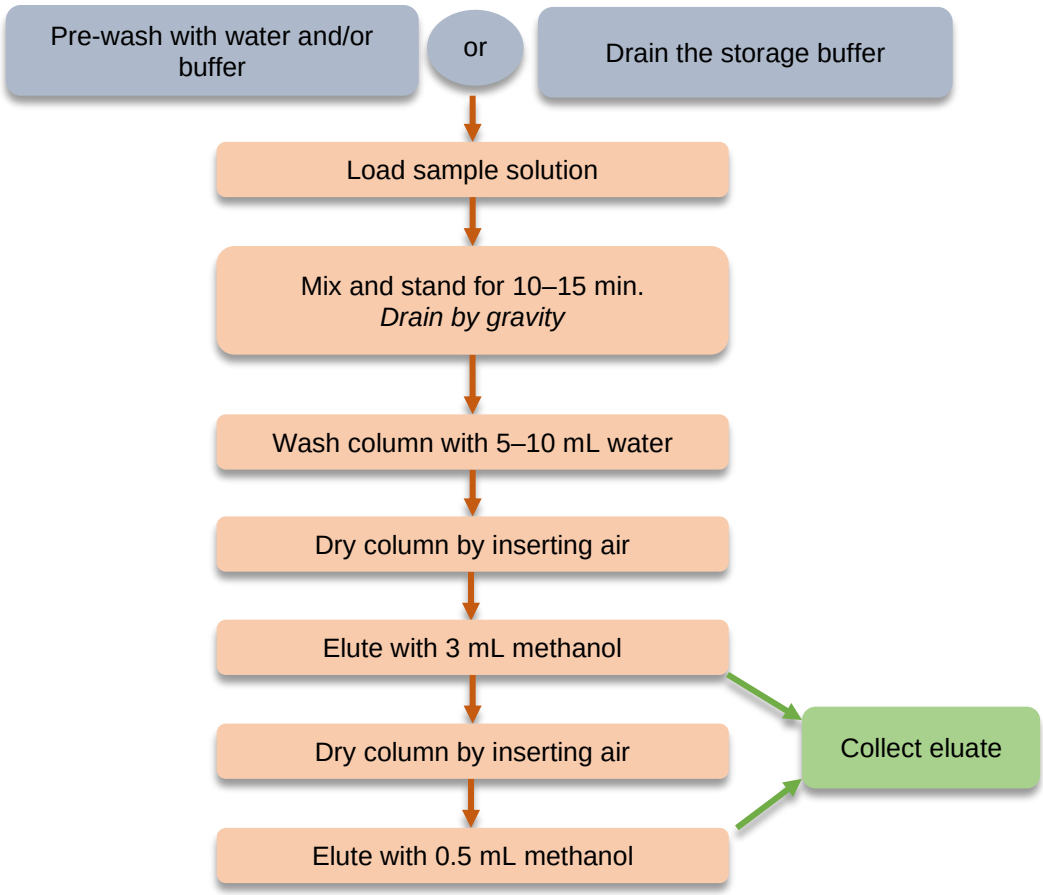


Figure 1.4. General protocol for immunoaffinity extraction

1.6.2 Chromatographic Analysis

In milk and other dairy products, the complex matrices, and the associated analogues and moieties of vitamin B_{12} indicate the requirement of efficient separation, identification and determination. 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. While gas chromatography (GC) has been employed, the vast majority of reported methods were based on HPLC.

1.6.2.1 Liquid Chromatography

Liquid chromatography (LC) has played a significant role in vitamin B_{12} analysis particularly in the food context. A series of parameters in the liquid chromatographic method need to be optimised to achieve the favoured separation; these include the specification of column (stationary phase, length, diameter and particle size of packing material), content of organic solvent in mobile phase, isocratic and gradient elution program, pH of mobile phase, flow rate and temperature of column. Choice of these parameters are affected by vitamin B_{12} solubility in the mobile phase, the sample matrix and the stability of vitamin B_{12} in the system and detector types. Parameters may have an influence on each other which complicates the set-up for an LC method.

Among the various LC methods for vitamin B_{12} determination, C_{18} reversed-phase, which was used in all the literature reviewed, is widely employed for food materials like meats, milk, infant formula and other vitamin B_{12} fortified products. CN-Cbl has been applied as an external standard to measure the total vitamin B_{12} by reversed-phase LC with various detection techniques. The following sections briefly discussed three detectors: Ultra-violet and visible (UV/Vis) detector, fluorescence detector and mass spectrometer.

HPLC-UV/Vis Methods

The instrumental conditions of HPLC-UV/Vis played a vital role in obtaining the desired separation performance and detection sensitivity. A summary of the

methods reviewed is presented in **Table 1.6**. In all methods reviewed below, it can be assumed that CN-Cbl was being analysed unless otherwise indicated.

Albala-Hurtado *et al.* (1997) developed a HPLC-UV method for water-soluble vitamins, including B_{12}, in infant milk. 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 acid-trimethylamine buffer (pH = 3.6) at room temperature. The retention time of vitamin B_{12} (CN-Cbl) ranged from 18–20 min, the LOQ was 0.3 µg mL^{-1} and recoveries of CN-Cbl in powdered and liquid milk were 77.23 ± 3.31% and 76.81 ± 2.98%, respectively. These were the lowest recoveries compared with other vitamins detected at the same time and the LOQ was insufficiently sensitive for the low content of vitamin B_{12} in non-supplemented milk, as average content is less than 4 ng g^{-1} (Gille and 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 heptansulfonic acid in methanol and 1% acetic acid (30:70). The total run time was 40 min. As with Albala-Hurtado and co-workers' research, the analysis was performed at room temperature, 25 °C and detected by UV at 361 nm. However, the latter did not conduct any recovery experiments to validate their method, in this case, the accuracy and precision need to be further confirmed.

Table 1.6. Summary for detailed LC conditions of HPLC-UV/Vis methods in the reviewed literature

Wavelength (nm)	LC Column	Flow rate (mL min^{-1})	Temperature (°C)	Injection volume (µL)	Mobile phase	References
UV 361	C_{18} 250 × 4.6 mm, 5 µm	1	Room temperature	20	15% methanol + 5 mM Octane-sulfonic acid + 0.5% TEA + 2.4% acetic acid (pH = 3.6)	Albala-Hurtado *et al.* (1997)
UV 361	C_{18} 250 × 4.6 mm, 5 µm	1	25	20	5 mM heptanesulfonic acid in methanol: 1% acetic acid = 30: 70	Oprean *et al.* (2011)
UV 361	C_{18} 150 × 3.0 mm	0.25	—[a]	100	A: 0.025% TFA in water (pH = 2.6) B: Acetonitrile	Heudi *et al.* (2006)
UV 361	C_{18} 150 or 125 ×3.0 mm	0.25	—[a]	100	A: 0.025% TFA in water B: 0.025% TFA in Acetonitrile	Campos-Giménez *et al.* (2008)
UV 361	C_{18} 100 × 2.1 mm, 1.7 µm	0.4	—[a]	50	A: 0.025% TFA in water B: 0.025% TFA in Acetonitrile	Campos-Gimenez (2014)
UV 361	C_{18} 100 × 4.6 mm, 2.6 µm	1	30	100	A: 0.025% TFA in water B: Acetonitrile	Nakos *et al.* (2017)
Vis 550	C_{18} 150 × 4.6 mm, 5 µm	1	40	200	50 mM KH_2PO_4 (pH = 2): Acetonitrile = 90:10	Iwase and Ono (1997)
Vis 550	C_{18} 100 × 4.6 mm, 3 µm	1	—[a]	2	A: 0.4% TFA in water B: 0.4% TFA in 25% Acetonitrile C: 0.4% TFA in 75% Acetonitrile	Schimpf *et al.* (2012)

[a] No data available

While the two methods mentioned above used isocratic elution, other researchers applied a gradient elution 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 with gradient elution steps. A 150 × 3.0 mm C_{18} column was employed to perform the separation at a relatively slow flow rate, 250 µL min^{-1}. Vitamin B_{12} was determined by UV detection at 361 nm. The results obtained from this method gave a 94–100% recovery. The run time was reduced to 30 min comparing to Oprean *et al.* (2011). In addition, the instrumental LOQ was estimated to be 10 ng mL^{-1}, which enabled the quantification of 2.5 ng g^{-1} of vitamin B_{12} content in samples and the procedure was relatively sensitive for fortified samples, such as infant formula. Subsequently, Campos-Giménez *et al.* (2008) adopted and modified the method by Heudi *et al* with narrow-bore columns (150 × 3.0 mm or 125 × 3.0 mm) and a large injection volume (100 µL) with gradient elution to shorten analysis time while maintaining the sensitivity. The instrumental limit of detection (LOD) was 3 ng mL^{-1} approximately, the overall method detection and quantitation limits were 1.0 ng g^{-1} and 3.0 ng g^{-1}, which also established the suitability of the method for fortified food products. This method was subsequently granted First Action status and designated AOAC Official Method 2011.08 (Campos-Gimenez *et al.*, 2012).

This method was subsequently improved by Campos-Gimenez (2014) with an introduction of ultra-HPLC (UHPLC) system and a narrow UHPLC column (100 × 2.1 mm, 1.7 µm). Although the same mobile phase, 0.025% TFA in water and 0.025% TFA in acetonitrile, were employed, a shorter gradient elution program, decreased the analysis time to only half of that previously required. The flow rate was increased from 0.25 mL min^{-1} to 0.4 mL min^{-1}. In addition, the injection volume was halved to 50 µL. According to the report, LOQ was estimated at 0.13 ng g^{-1}, respectively and recoveries were between 87.8–98.3%, which has proven to be applicable for the measurement of vitamin B_{12} in various forms of: infant, adult and paediatric formulas. This method was granted AOAC Official Method status AOAC 2014.02. The enhanced resolution of the

UHPLC system contributed to the high sensitivity and time-saving performance of this method.

Since CN-Cbl has an absorption maxima at 550 nm, Iwase and Ono (1997) analysed vitamin B_{12} at this wavelength, instead of the previously used 361 nm and separating the compounds on a slightly shorter C_{18} column (150 × 4.6 mm, 5 µm) with temperature set at 40 °C. Additionally, 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, was used. 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% with 12 min run time. 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, were used to separate the CN-Cbl. Gradient elution was used with a run time of 30 min. The LOQ was 0.8 ng g^{-1} of the reconstituted product (25 g of powder + 200 g of water); 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 nutritional supplements.

Nakos *et al.* (2017) developed a HPLC-UV method for vitamin B_{12} in plant samples by performing the separation on a C_{18} column (100 × 4.6 mm, 2.6 µm) at 30 °C. A mobile phase consisting of 0.025% TFA water solution and acetonitrile were used for a gradient elution at a flow rate of 1.0 mL min^{-1}. The analyte was detected at 361 nm. The LOQ of the method was 1.4 µg mL^{-1}. According to the authors, the vitamin B_{12} content in a sea buckthorn (*Hippophae rhamnoides*) sample was 37 µg 100 g^{-1}, in a couch grass (*Elymus*) sample was 26 µg 100 g^{-1} and in an elecampane (*Inula helenium*) was 11 µg 100 g^{-1}. This indicated satisfactory content of vitamin B_{12} in plant sources from different families despite conventional agreement that vitamin B_{12} cannot be found in plants. The possible explanation was in those tested plants which

actinobacteria *Frankia alni* is symbiotic to and the vitamin B_{12} produced by the actinobacteria and eventually accumulated in the plants.

Several similar conditions and tendencies can be noticed between the reviewed methods. Acidic buffer was used in the mobile phase for all methods, which suggests that the low pH increased retention of CN-Cbl on the stationary phase in order to achieve better separation from matrix interference. A combination of water and another less polar organic solvent such as methanol or acetonitrile were common choices for mobile phase components. Moreover, a gradient elution was preferred due to the possibility of a shorter analysis time. Although CN-Cbl has specific absorption at 550 nm in the visible wavelength range the majority of reported methods still detected in the ultraviolet at 361 nm due to its higher absorptivity. The UV-vis spectrum of CN-Cbl with maxima at 361 nm and 550 nm is shown in **Figure 1.5**; the greater absorptivity at 361 nm would increase the sensitivity of the method. The C_{18} column was a standard LC column choice however, C_{18} columns with various specifications were applied in vitamin B_{12} analyses. As expected, when considering the accuracy, precision and analysis time of each method, a narrow-bore column with smaller inner diameter and particle size delivered better results.

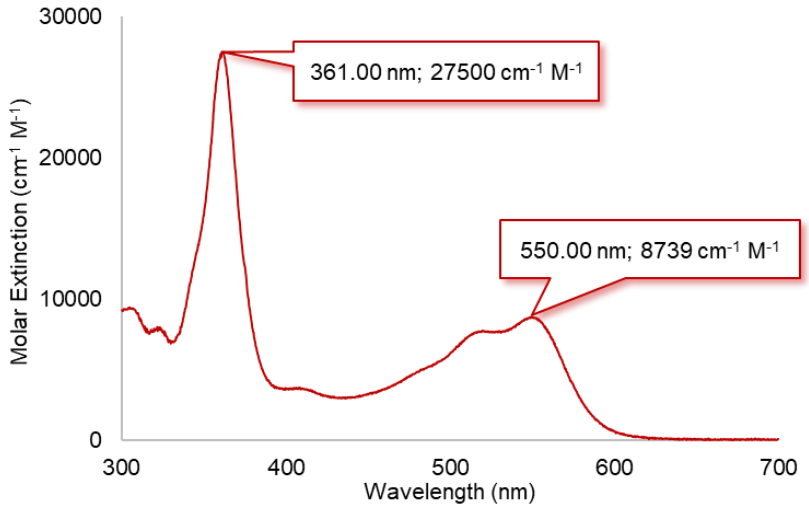


Figure 1.5. Absorption of cyanocobalamin between 300 nm and 700 nm.

Based on the review of the research mentioned above, HPLC-UV/Vis has been a dominant approach in analysis of vitamin B_{12} in milk and infant formula. With continuing refinements coupled with improvements in technology, results (**Table 1.7.**) from reported vitamin B_{12} analyses have shown significant improvement in detection and quantitation limits, recoveries of standard addition and precision.

CN-Cbl was always the stable standard of vitamin B_{12} used in quantitative analysis with HPLC-UV/Vis; thus, conversion into the cyano-form of cobalamin was necessary, which required the undesirable involvement of cyanide. Moreover, the low sensitivity for trace vitamin B_{12} analysis in non-supplemented food products was another drawback of HPLC-UV/Vis methods.

HPLC-FLD Methods

Li *et al.* (2000) reported a method for quantifying vitamin B_{12} in multivitamin tablets using HPLC-FLD. The separation was achieved on a C_{18} column (300 × 3.9 mm, 10 µm) and with an isocratic elution using 30% aqueous methanol solution at flow rate of 0.8 mL min^{-1}. The FLD was performed at maximum excitation/emission wavelength: 275 nm/305 nm. While the instrumental LOD of this method (0.1 ng mL^{-1}) was lower than most of HPLC-UV/Vis methods reviewed in the previous section, the targeted sample of this method was multivitamin tablets rather than complex food matrices. It means that the very less interference would be present. To apply such method to milk and infant formulas, appropriate sample preparation is necessary and the form conversion to CN-Cbl is unavoidable.

Pakin *et al.* (2005) described a vitamin B_{12} assay method by detecting the α-ribazole (fluorescent marker) with HPLC-FLD. Food samples were incubated with pepsin (at 37 °C, pH = 4 for 3 hours) to denature the protein and to obtain total vitamin B_{12} content. Vitamin B_{12} was concentrated by passing through an immunoaffinity column and purified from the pre-existing metabolic fragments of vitamin B_{12}; which contained the fluorescent marker. The concentrate was hydrolysed with 2.5 M sodium hydroxide (100 °C, 15 min) and ALP (37 °C, pH = 8,16 hours) to release the fluorescent fragment, α-ribazole. Reversed phase chromatography was used to isolate the marker.

Table 1.7. Summary of limits of detection and quantitation recovery and relative standard deviation of HPLC-UV/Vis methods in the reviewed literature

Wavelength used	LOD[ab]	LOQ[ab]	Recovery (%)	RSD (%) of method precision[c]	References
361 nm	–[d]	3×10^2 ng mL^{-1}[a]	73.83–81.54	5.75[e], 5.12[f]	Albala-Hurtado *et al.* (1997)
	3 ng mL^{-1}[a]	2.5 ng g^{-1}[b]	94–100	3.2	Heudi *et al.* (2006)
	1.0 ng g^{-1}[b]	3.0 ng g^{-1}[b]	93.3–108.3	2.1	Campos-Giménez *et al.* (2008)
	0.08 ng g^{-1}[b]	0.13 ng g^{-1}[b]	87.8–98.3	2.7–8.2[g]	Campos-Gimenez (2014)
	4 ng mL^{-1}[a]	14 ng mL^{-1}[a]	80–100	≥ 0.7	Nakos *et al.* (2017)
550 nm	0.5 ng mL^{-1}[a]	–[d]	88.8–92.2	2.7	Iwase and Ono (1997)
	0.2 ng g^{-1}[b]	0.8 ng g^{-1}[b]	–[d]	–[d]	Schimpf *et al.* (2012)

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

The separation was performed on a reversed phase (RP) C_{18} column (250 mm × 4 mm, 5 µm) with mobile phases consisting of methanol and water in gradient elution. α-ribazole was fluorometrically detected at an excitation wavelength of 250 nm and an emission wavelength of 312 nm. However, an α-ribazole standard is not commercially available, therefore a series of CN-Cbl standard solutions were converted to α-ribazole via the procedure described above to produce the external calibration.

Pakin *et al.* (2005), who studied vitamin B_{12} in food samples including pig liver, chicken egg, beef fillet, salmon and powdered milk, obtained a satisfactory recovery (95–100%) and repeatability (RSD% 1.0–5.4%). The LOQ of the method was 3 ng g^{-1}. Although this published method did not employ cyanide during the extraction for "*the need to handle potassium cyanide appeared quite undesirable*" (Pakin *et al.*, 2005). However, the immunoaffinity column extraction was more specific to CN-Cbl and less than 20% of the other forms of cobalamin were recovered after passing through the immunoaffinity column without pre-conversion to the cyanide form (Campos-Giménez *et al.*, 2008). Since immunoaffinity column without the conversion by cyanide will underestimate the total vitamin B_{12} content. Moreover, CN-Cbl was the recovery standard added which will not reflect the poor recovery of other three forms of vitamin B_{12}. Therefore, although this method has potential, a reliable clean-up procedure targeting all vitamin B_{12} forms needs to be developed and the commercialisation of an α-ribazole standard should be encouraged.

LC-MS/MS Methods

In recent years, LC-MS/MS has been used in analysis of vitamin B_{12} content in food and other natural products. This method has the advantage of high sensitivity and selectivity for detection of products where vitamin B_{12} naturally occurs at trace levels.

In LC-MS/MS systems, HPLC or UHPLC is employed to separate analytes from other matrix interferences, a particular ion (precursor ion) is chosen to be subjected to tandem mass spectrometry to produce secondary ion fragments (product ions). The selected pairs of ion fragments are specific to the target

molecules. In multiple reaction monitoring (MRM) mode, a series of parameters can be optimised to obtain high performance; these parameters commonly include collision energy, capillary voltage, temperature of source and desolvation, flow rate of drying gas and collision gas. The combination of parameters may be dependent upon manufacturers.

Zironi *et al.* (2013) developed a method on a UHPLC-MS/MS system for determination of vitamin B_{12} in milk and dairy products. The separation was established on a RP C_{18} column, high-strength silica (HSS) T3 (50 × 2.1 mm, 1.8 µm) with guard column, at 45 °C and the analyte eluted with 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}. It was noticeable that the total analysis time was only 5 minutes which is the benefit from the UHPLC as well as the sharper narrower peaks which gave higher sensitivity. Detection was in positive electrospray ionisation (ESI^+) mode with monitoring two pairs of transitions; *m/z* 678.3–147.1 and *m/z* 678.3–359.3; The detection parameters of ion fragmentation for this and other reports are presented in **Table 1.8**. Drying gas and collision gas were nitrogen at 12.5 L min^{-1} and argon at 0.35 mL min^{-1}, respectively. Capillary voltage was 3200 V and desolvation temperature was 350 °C. This method was successfully applied to a raw milk sample and its quantitation limit was 2 ng g^{-1}, which suggested that this method was more sensitive than using HPLC-UV for liquid milk samples by Van Wyk and Britz (2010) (LOQ = 5 ng mL^{-1}). Subsequently, Repossi *et al.* (2017) adopted the method by Zironi *et al.* (2013) to study the degradation of vitamin B_{12} during ricotta cheese shelf-life with the MS instrumental conditions remaining the same.

Kakitani *et al.* (2014) applied a UHPLC-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 solution (A) and 0.3% formic acid in acetonitrile (B) and conducted under a gradient elution mode at 0.2 mL min^{-1} flow rate; ESI^+ was used in MS detection. Under MRM

mode, a precursor ion *m/z* 678.5 and product ion *m/z* 147.1 and *m/z* 359.1 were selected for monitoring. Desolvation gas temperature was 250 °C and flow rate was 15 L min^{-1}. Although the LOD and LOQ were 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 beverages and supplements.

Table 1.8. Summary of transitions and collision energy of multiple reaction monitoring

Analytes	Precursor ion (m/z)	Product ion (m/z)	Collision energy (V)	References
CN-Cbl	678.36	147.10	43	Repossi *et al.* (2017); Zironi *et al.* (2013)
	678.36	359.30	23	
CN-Cbl	678.50	147.10	49	Kakitani *et al.* (2014)
	678.50	359.15	25	
CN-Cbl	678	147	40	Lee *et al.* (2015)
	678	359	24	
OH-Cbl	665	359	35	Perez-Fernandez *et al.* (2016)
	665	147	75	
Ado-Cbl	527	164	25	
	527	147	48	
CN-Cbl	678	359	36	
	678	147	65	
Me-Cbl	665	147	60	
	673	147	60	

[a] The precursor ion m/z 678 is the doubly-protonated molecular ion $[M+2H]^{2+}$for cyanocobalamin (CN-Cbl); m/z 665 is the ion derived from loss of the upper ligand of the corrin macrocycle $[Cbl+H]^{2+}$ for hydroxocobalamin (OH-Cbl) and methylcobalamin (Me-Cbl); m/z 527 is the triply protonated molecular ion $[M+3H]^{3+}$ for adenosylcobalamin (Ado-Cbl); m/z 673 is the doubly-protonated molecular ion $[M+2H]^{2+}$for Me-Cbl. The product ion m/z 147 corresponds to protonated 5,6-dimethylbenzimidazole; m/z 359 is the protonated α-ribazole-phosphate moiety (Perez-Fernandez *et al.*, 2016).

Chamlagain *et al.* (2015) employed UHPLC technology in analysis of active vitamin B_{12} in cells of *Propionibacterium spp.* and fermented cereal matrices. They conducted a comparison of two LC columns, HSS T3 (100 × 2.1 mm,

1.8 µm) and ethylene bridged hybrid (100 × 2.1 mm, 1.7 µm) and concluded the former had better performance in respect of peak height, peak resolution, number of theoretical plates and separation from an interfering substance. Mass spectrometry (ESI^+) was conducted to confirm the *in situ* production of vitamin B_{12}. The capillary voltage was 4500 V, temperature and flow rate of drying gas were 300 °C and 8 L min^{-1}, respectively. Helium was used as the collision gas. The pseudomolecular ion 1356 m/z ($[M+H]^+$) was chosen to characterise CN-Cbl.

Lee *et al.* (2015) reported a LC-MS/MS method to determine vitamin B_{12} in infant and toddler milk formulas by using HLB cartridges (200 mg, 6 mL) to concentrate and collect CN-Cbl in the sample. No cyanide was used for the vitamin B_{12} form conversion and only CN-Cbl were analysed. The chromatographic separation was performed on an UG 120V C_{18} Column (250 × 1.5 mm, 5 µm) with a gradient elution of mobile phase A (20 mM ammonium formate in water) and B (100% acetonitrile). The flow rate was 0.2 mL min^{-1} and column temperature were maintained at 35 °C. Mass spectrometry (ESI^+) detection was conducted with at 5000 V spray voltage and 350 °C source temperature. Gas flow was 10 L min^{-1} and nebuliser gas pressure was 50 psi. Two quantitative ion pairs: *m/z* 147/678 and *m/z* 359/678 (precursor/product ion) were chosen to determine the CN-Cbl. No internal standard was used and the LOD and LOQ were 0.03 and 0.1 ng mL^{-1}. However, the determination method for the LOD and LOQ were not clarified by the authors, hence, it is difficult to compare it with other methods. A method detection limit of 0.2 ng g^{-1} was also reported by these authors, which was lower than the LOD values reported by Campos-Giménez *et al.* (2008) (1 ng g^{-1}) who used HPLC-UV to analyse vitamin B_{12} in the powdered milk products including infant formulas. But there were a few inconsistencies regarding the value and unit of this method detection limit in their manuscript.

Perez-Fernandez *et al.* (2016) described a LC-MS/MS method for the determination of four forms of cobalamin in bovine milk. The different cobalamins separated on a RP C_{18} column (250 × 4.6 mm, 5 µm); the mobile

phases consisted of 5 mM formic acid in water and 5 mM formic acid in acetonitrile at a flow rate of 0.8 mL min^{-1}. A gradient elution mode was applied. Detection of the four selected cobalamins was performed using ESI^+. Air was used as drying gas and nebulizer gas and nitrogen was the collision gas. The detection parameters of ion fragmentation are summarised in **Table 1.8**. LOQs of the OH-Cbl (0.61 ng mL^{-1}) and CN-Cbl (0.64 ng mL^{-1}) were lower than Ado-Cbl (2.69 ng mL^{-1}) and Me-Cbl (1.30 ng mL^{-1}), which is understandable as Ado-Cbl and Me-Cbl are more instable than the other two.

Dicyanocobinamide (DCN-Cbl) and methotrexate have often been applied as an internal standard in the LC-MS/MS methods. DCN-Cbl is an analogue of CN-Cbl where the lower ligand (α-ribazole-phosphate) of the cobalt ion was freed from the main structure and replaced by a cyan group. The collision energies used for DCN-Cbl was slightly higher than for vitamin B_{12}. In the contrast, methotrexate is a dicarboxylic acid and smaller molecule (454.5 Da) than vitamin B_{12} and DCN-Cbl. The collision energy was relatively lower for both production ions (*m/z* 175.1 and 308.2). The transitions and collision energies are summarised in **Table 1.9**. The precursor ion *m/z* 455.2 is the pronated pseudomolecular ion $[M+H]^+$ (exact mass of methotrexate is 454); *m/z* 1016 is the ion derived for loss of a cyano group of the corrin macrocycle $[Cbl+H]^+$ (molecular mass of DCN-Cbl is 1041 Da).

Table 1.9. Summary of multiple reaction monitoring transitions and collision energies for two internal standards

Internal standard	Precursor ion (*m/z*)	Product ion (*m/z*)	CE[a] (V)	References
Methotrexate	455.2	175.1	39	Zironi *et al.* (2013)
	455.2	308.2	19	
DCN-Cbl[b]	1016	931	82	Perez-Fernandez *et al.* (2016)

[a] CE = collision energy

[b] DCN-Cbl = dicyanocobinamide

Overall, currently published LC-MS/MS methods have higher sensitivity and specificity than UV or visible detectors and fluorescence detectors. However, as with the HPLC-UV/Vis methods, a conversion to CN-Cbl form by addition of

cyanide during sample preparation was still required to avoid degradation which might occur in the analysis process.

1.6.2.2 Gas Chromatography

Direct gas chromatographic assay for cobalamin in food is rarely reported due to its low volatility. Sundin and Allen (1992) described a method for the identification of the nucleoside moiety of cobalamin and its analogues present in rabbit kidney samples by using gas chromatography-mass spectrometry (GC-MS). Cobalamin and cobalamin analogues were incubated with potassium cyanide and isolated from samples by affinity chromatography. The nucleoside analogue moieties of cobalamin and its analogues were hydrolysed and isolated by using a mixed bed column. After oxidation, reduction and derivatisation of the nucleoside analogues, it was analysed by GC-MS with selected ion monitoring. As expected, this method also involved cyanide for conversion of all cobalamins into the single form, CN-Cbl. Moreover, the sample treatment of this method was a time-consuming process as multiple steps were applied, which increases cost and the chance for error.

1.6.3 Microbiological Assay

Shorb (1947) observed that *Lactobacillus lactis* will not fully grow in the absence of vitamin B_{12}. Subsequently, several other vitamin B_{12}-dependent microorganisms have been identified. Therefore, a microbiological assay has been developed for the determination of vitamin B_{12} in milk and infant formulas and is the most sensitive method (LOQ: 1.0–20.0 pg mL^{-1}) (AOAC International, 2023b). 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). **Table 1.10** shows available organisms for the vitamin B_{12} assay. Currently, the microbiological assay using *Lactobacillus delbrueckii* subsp. *lactis* American Type Culture Collection (ATCC) 7830 to analyse vitamin B_{12} in food has been widely used (Watanabe and 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, showed similar growth for *Lactobacillus delbrueckii* ATCC 7830 as vitamin B_{12} (Watanabe and Bito, 2018). According to Ball (2006), the poor selectivity of microbiological assay can result in overestimation by 5–30%.

Table 1.10. Organisms for vitamin B_{12} microbiological assay

Organism name	Organism
Escherichia coli mutant 113	Bacteria
Lactobacillus leichmannii 326	Bacteria
Lactobacillus delbrueckii ATCC 7830	Bacteria
Euglena gracilis Z alga	Protozoa
Ochromonas malhamensis	Protozoa
Arthrobacter pascens (Lochhead 38)	Bacteria

Additionally, prior to the microbiological assay, a KCN-boiling extraction was conducted to convert all vitamin B_{12} into CN-Cbl form due to the instability of other cobalamin forms (Bito *et al.*, 2016). To avoid the involvement of highly toxic potassium cyanide, sodium metabisulfite ($Na_2S_2O_5$) was used to replace potassium cyanide. Sulfitocobalamin (SO_3-Cbl), produced with $Na_2S_2O_5$-boiling extraction, has identical activity to CN-Cbl in the growth of vitamin B_{12}-dependent bacteria. However, a sulfitocobalamin standard is not commercially available and in-house synthesis is required (Watanabe and Bito, 2018).

1.6.4 Spectroscopic Methods

1.6.4.1 Ultraviolet-Visible Spectrophotometry

Ultraviolet-visible spectrophotometry methods have been used for the detection of vitamin B_{12} content. In this method, CN-Cbl is often chosen as a standard to represent the total vitamin B_{12}. Therefore, a pre-conversion is required to transfer other B_{12} vitamers into CN-Cbl. However, sensitivity is an issue when analysing samples with low vitamin B_{12} concentrations and complex matrices (Kumar *et al.*, 2010). Thus, UV-visible methods are not adequate for vitamin B_{12}

analysis in food samples, including milk and infant formula; but suitable for larger quantities of purified samples such as vitamin B_{12} in multivitamin tablets.

1.6.4.2 Atomic Absorption Spectrometry

Atomic absorption spectrometry (AAS) has also been applied in the determination of vitamin B_{12} in biological samples and vitamin B_{12} tablets by analysing the cobalt at 241 nm. As with other studies, CN-Cbl was determined. However, since the AAS method analyses the total cobalt content, cobalt from other sources than vitamin B_{12} can cause an erroneously overvalued result (Diaz, 1972; Suzuki *et al.*, 1979). Moreover, the complexity of the food matrix is another challenge for vitamin B_{12} analysis in food samples when utilising an atomic absorption method.

1.6.4.3 Inductively Couple Plasma-Mass Spectrometry

Inductively Couple Plasma-Mass Spectrometry (ICP-MS) is another technique which has been used to indirectly determine the content of vitamin B_{12} by measuring the complexed cobalt in cobalamins. ICP-MS, has excellent sensitivity for trace analysis, often is coupled with LC for separation. Raju *et al.* (2013) described a LC-ICP-MS method for the quantitation of vitamin B_{12} in supplemented breakfast cereals and multivitamin tablets. Liquid chromatography was used to separate different vitamin B_{12} forms and separate the ionic cobalt, since free cobalt presented in samples would result in an overestimation if the total cobalt was measured. The separation was performed on an Atlantis T3 C_{18} column (150 mm × 2.1 mm, 3 µm) and the mobile phase consisted of 20 mM EDTA and 25% methanol in water. After the LC separation, the individual vitamers were measured as ^{59}Co by ICP-MS and the results of the individual vitamers were summed to give vitamin B_{12}. Although ^{59}Co is 100% abundant, the exitance of several polyatomic interferences, for example, $^{43}Ca^{16}O^{+}$, $^{42}Ca^{16}O^{1}H^{+}$, $^{24}Mg^{35}Cl^{+}$, $^{36}Ar^{23}Na^{+}$ and $^{40}Ar^{18}O^{1}H^{+}$, can cause issues, especially for high Ca content in milk matrices. The LOD of this method was less than 1 ng g^{-1}, which was sufficiently sensitive for dietary supplements and fortified products. However, food matrices involved in this method were less complicated than milk and other dairy products. No cyanide was used in this

method as CN-Cbl was the targeted vitamin B_{12} form during the analysis, but it was not the same case for milk and infant formulas where other instable vitamers were presented as well. Therefore, if applying such a method to milk products to quantify vitamin B_{12}, the risk of underestimating the content would be increased.

1.6.5 Competitive Binding Assay

Competitive binding assay is an alternative to the conventional vitamin B_{12} analysis methods. It utilises the biospecific binding between either vitamin B_{12} and antibodies or vitamin B_{12} natural occurring proteins to achieve the determination.

1.6.5.1 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. ELISA is performed in plastic wells in a microtiter plates (Kumar *et al.*, 2010). Zhu *et al.* (2011) described an analysis method of vitamin B_{12} in seawater by using ELISA; an antibody directed against vitamin B_{12} was immobilized on the surface of a 96-microwell plate; free antigen (vitamin B_{12} standard or samples) and enzyme-labelled antigen, horseradish peroxidase labelled vitamin B_{12}, were added into each microtiter well to competitively bind to the limited binding sites of the antibodies. Subsequently, the unbound antigens were removed, and a horseradish peroxidase substrate solution was added to produce the absorbance of horseradish peroxidase-vitamin B_{12} conjugates. Since the measured absorbance was produced by labelled vitamin B_{12}, the resulting signal was inversely proportional to the concentration of vitamin B_{12} in samples or standards. In addition, a SPE clean-up procedure was required prior to the ELISA analysis. However, more sample preparation steps are required for complex food samples including the conversion into CN-Cbl. In addition, difficulties in generating specific vitamin B_{12}-active antibodies have limited deployment of ELISA techniques.

1.6.5.2 Radioisotopic Dilution Assay

The radioisotopic dilution assay is also based on the competitive binding occurring between the vitamin B_{12} in the sample and a radioisotopic reference (^{57}Co-labeled vitamin B_{12}) for the vitamin B_{12} binding sites upon a protein; R-protein or intrinsic factor (Kumar *et al.*, 2010).

Commercial kits are available for the analysis of vitamin B_{12}. However, there is concern about the reliability of this method, since the R-proteins used in the kits can bind other inactive cobalamins. Although intrinsic factor has higher specificity to cobalamin than R-proteins, in some kits, the contamination of intrinsic factor by R-proteins often occurs. Thus, purified intrinsic factor kits are required for the use of radioisotopic dilution assay, but the cost is relatively high (Kumar *et al.*, 2010). The radioisotopic dilution assay is often applied for clinical samples, such as serum, rather than food samples. In addition, conversion to the cyano-form using cyanide is essential for the determination (Kolhouse *et al.*, 1978).

1.6.5.3 Biosensor-based Assay

Biosensor technology has been utilised for the analysis of vitamin B_{12}. This technology is also based on the interaction between vitamin B_{12} and its binding protein. A fixed amount of specific binding protein was added to the sample; vitamin B_{12} standard was immobilized to a transduce surface (a sensor chip, usually a modified gold surface); when analyte solution containing vitamin B_{12}-protein complex and the unbound proteins flowed past the sensor chip surface, the binding interaction was 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%. However, in this report, total vitamin B_{12} content was expressed as CN-Cbl as well; an extraction buffer containing sodium cyanide was used during sample preparation to convert all vitamin B_{12} into CN-Cbl form.

1.7 Rationale of Research and Thesis Outline

Regardless of what determination techniques were employed, boiling with sodium or potassium cyanide was a common sample preparation procedure for vitamin B_{12} analysis in the majority of the literature reviewed here. There appears to be no active research concerned in the development of a cyanide-free method for vitamin B_{12} in milk and infant formula.

This review of vitamin B_{12} analysis methods indicates that developing a novel method without using cyanide is essential to fill the gaps in the safety of current vitamin B_{12} analyses. The proposed method must compare favourably for precision, recovery, LOD and LOQ plus cost with methods that employ cyanide or laboratories will not adopt it.

The thesis is made up of 8 chapters, which are divided as follows: **Chapter 1** reviewed the physical-chemical properties of vitamin B_{12} as well as its importance. This chapter focused on analysis methods for vitamin B_{12} in the literature. **Chapter 2** summaries the general materials, equipment and methods used in the work. **Chapter 3** described the isolation procedure of α-ribazole from CN-Cbl through chemical and enzymatic hydrolysis and boronate affinity chromatography clean-up. The characterisation by NMR and liquid chromatography mass spectrometry (LC-MS) was included. **Chapter 4** discussed optimisation of isolation procedure for α-ribazole from CN-Cbl. **Chapter 5** described the extraction procedure of cobalamin-derived α-ribazole in milk and infant formulas, which were based on the optimised condition from the previous chapter with additional steps prior to the hydrolysis: protein denaturation and sugar removal. **Chapter 6** detailed the instrumental analysis of α-ribazole in infant formula samples and compared HILIC with reserved phase chromatography. **Chapter 7** contained the validation of the developed method for performance assessment by system suitability and single laboratory validation study, including a comparison study between candidate method and an AOAC standard method for vitamin B_{12} analysis in infant formulas. **Chapter 8** concluded this work and presented a cost-benefit analysis and suggestions on potential future research.

2 General Materials and Equipment

Materials and general methods used throughout this research are summarised in this chapter. Specific methods, materials and equipment are stated in the corresponding chapters.

2.1 General Materials

Cyanocobalamin (Product No. C3607, 96.0–102.0%), magnesium chloride (GR ACS) and sodium acetate trihydrate (GR ACS) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Sodium hydroxide (≥ 99%) and glacial acetic acid 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 was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Ammonium hydroxide solution (28–30%), acetonitrile and methanol (HPLC grade) were purchased from Merck. Immobilised Boronic Acid Gel™ (Catalogue No. 20244) was purchased from Thermo Scientific (Rockford, IL, USA).

All water used in this work was distilled and deionised water with resistivity ≥ 18 MΩ, supplied by a Milli-Q integral water purification system (Merck, Germany).

2.2 Solution Preparation

Sodium hydroxide solution (3.0 M or 1.5 M) was prepared by dissolving sodium hydroxide (59.98 g) into water in a 500 mL or 1 L volumetric flask, respectively. Hydrochloric acid solution (3.0 M or 1.5 M) was made by diluting concentrated hydrochloric acid (36% *w/w*, 123.14 mL) made up to volume (500 mL or 1 L) with water.

Sodium acetate solution (0.1 M pH = 4) was prepared by dissolving sodium acetate (13.61 g) into water (950 mL) adjusting pH to 4 with glacial acetic acid then made up to 1 L with water.
Alkaline phosphatase (ALP) bovine, recombinant, expressed in *Pichia pastoris,* 5000 units mg^{-1} (1 unit will hydrolyse one µmol of 4-nitrophenyl phosphate per minute at pH = 9.8 at 37 °C) was supplied by Sigma-Aldrich (Product No. P8361, 0.45 mg μL^{-1}). ALP stock solution (> 450 units mL^{-1}) was made by dissolving ALP (2 µL) into incubating buffer solution (10 mL). The stock solution was stored at 4 °C.

The incubating buffer (ammonium acetate 0.3 M: magnesium chloride, 2 mM; pH = 8.8) was made by dissolving ammonium acetate (23.23 g) and magnesium chloride (0.19 g) into water (900 mL) and adjusting pH to 8.8 with ammonium hydroxide solution, then adjusting the volume to 1 L with water.

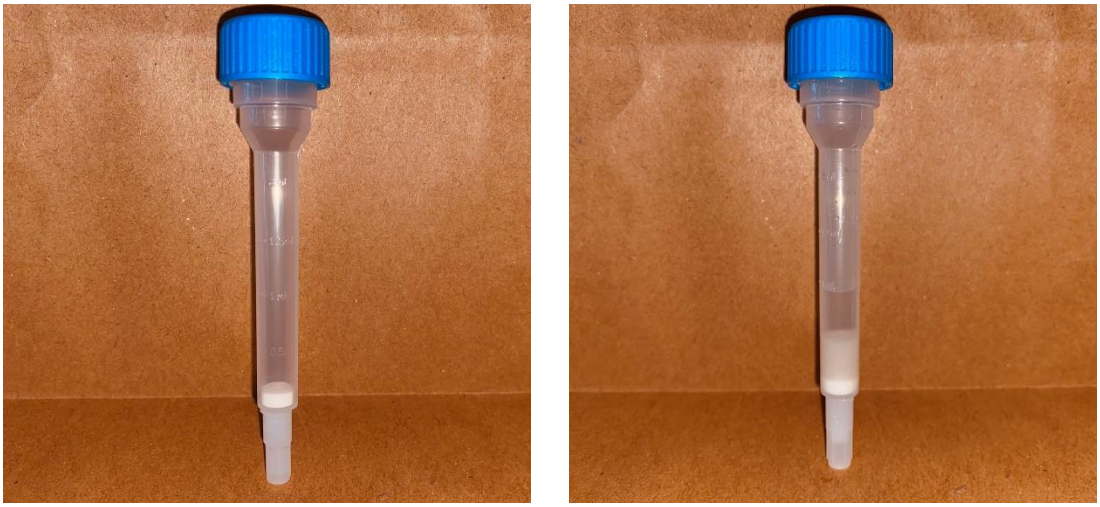
The boronate affinity chromatography (BAC) binding buffer (ammonium acetate, 0.3 M, pH = 8.8) was made by dissolving ammonium acetate (11.56 g) into water (450 mL) and adjusting pH to 8.8 by adding ammonium hydroxide solution, then adjusting the volume to 500 mL. The BAC eluent was made by mixing formic acid (1.0 M, 300 mL) with acetonitrile (700 mL).

All buffer solutions were stored at 4 °C and discarded after one week.

2.3 General Equipment

A model pH 700 pH meter (Eutech, Singapore) was used for pH measurement. Heating treatment was conducted in a Dri™-Block DB-3D heater (Techne, United Kingdom). Incubation was carried out in a Thermotec 2000 oven (Contherm, New Zealand). A model 5810R centrifuge (Eppendorf, Germany) was used with polypropylene centrifuge tubes, 50 mL (Greiner Bio-One, Austria). 10 mL and 1 mL single-use syringes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and syringe filters with 0.45 µm polytetrafluorethylene (PTFE) membranes (Biofil, China) and 0.45 µm cellulose acetate (CA) membranes (Sartorius, Germany) were used for sample filtration.

A centrifuge column (2 mL) was used for packing the BAC column (Catalogue No. 89896, Thermo Fisher Scientific, Rockford, IL, USA). **Figure 2.1** shows the empty centrifuge column (left) and packed BAC column with the boronate affinity gel (right). The packed BAC columns are stored at 4 °C.



(a) (b)

Figure 2.1. Boronate affinity chromatography column

2.4 HPLC System

The HPLC system used an UltiMate 3000 system configured with an SRD-3200 degasser, an HPG-3200SD pump, a TCC-3000SD column oven, and an FLD-3100 fluorescence detector (Dionex, Waltham, MA, USA), together with either a Rheodyne-8125 sample injector equipped with a 10 µL injection loop (IDEX Health & Science, Rohnert Park, CA, USA) or an ACC-300 Autosampler.

2.4.1 RP-HPLC

Chromatographic separation was performed with a Gemini C_{18}, 5 µm, 4.6 m × 250 mm column (Part No. 00G-4435-E0, Phenomenex, Torrance, CA, USA) using the HPLC system described in **Section 2.4**. Chromeleon 6.8 Chromatography Data System software was used for instrument control and data processing.

Mobile phase A (ammonia aqueous solution, 0.3% *v*:*v*, pH = 9.5) was prepared by diluting concentrated ammonium hydroxide solution (1500 µL) into water (490 mL), adjusting pH to 9.5 with acetic acid, then making up to 500 mL with water. Mobile phase B was 100% methanol. Considering the potential for pH

change and microbial growth occurring in the buffered mobile phase, mobile phase A was prepared daily.

2.4.2 HILIC

HILIC separation was performed using a Kinetex HILIC, 2.6 µm, 4.6 × 150 mm column (Part No. 00F-4461-E0, Phenomenex, Torrance, CA) with using the HPLC system described in **Section 2.4**. Chromeleon 6.8 Chromatography Data System software was used for instrument control and data processing.

Mobile phase A (ammonium formate, 100 mM, pH = 3.5) was prepared by dissolving ammonium formate (3.1528 g) in water (490 mL), adjusting pH to 3.5 with formic acid, then making up to 500 mL with water. Mobile phase B was 100% acetonitrile. Considering the potential for pH change and microbial growth occurring in the buffered mobile phase, mobile phase A was prepared daily. The elution was performed under isocratic conditions with 95% of mobile phase B at 1.0 mL min^{-1}. The column temperature was 30 °C.

2.4.3 FLD

The fluorescence detector acquired spectral data at a specific excitation wavelength (251 nm) and emission wavelength (303 nm). The flow cell temperature was 40 °C.

2.5 Standard Solutions

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 1.000 mL of stock solution to 100 mL with 10% methanol.

The α-ribazole stock standard solution was prepared by re-suspending the previously isolated α-ribazole (**Chapter 3**) with dimethyl sulfoxide. Calibration standards of α-ribazole were prepared by diluting this stock standard to the

required concentrations using ammonia aqueous solution (0.3% *v:v*, pH = 9.5) for RP-HPLC mode or acetonitrile (100%) for HILIC mode. The calibration range was 0.65 ng mL^{-1} to 6.48 ng mL^{-1}.

2.6 Infant Formula Samples

The infant formula samples tested in the body of work were purchased in the local market. A variety of brands and types were included: first-stage infant formula, second-stage follow-on formula and toddler-milk formula. **Table 2.1** shows the labelled vitamin B_{12} content.

Table 2.1. Vitamin B_{12} content from the nutrition information label of the infant formula samples tested

Infant formula sample	Labelled vitamin B_{12} content (µg 100 g^{-1})[a]
Ovine milk-based follow-on formula 1	1.68
Bovine milk-based infant formula 1	1.50
Bovine milk-based infant formula 2	1.60
Bovine milk-based follow-on formula 1	2.69
Bovine milk-based follow-on formula 2	2.58
Bovine milk-based toddler-milk formula 1	1.52
Bovine milk-based toddler-milk formula 2	1.28
Caprine milk-based infant formula 1	1.38
Caprine milk-based infant formula 2	1.49
Caprine milk-based follow-on formula 1	1.28
Caprine milk-based follow-on formula 2	2.59

[a] In the case of the content labelled as in µg 100 mL^{-1} prepared feeding, vitamin B_{12} content was converted to µg 100 g^{-1} based on the reconstitution instruction

3 Isolation and Identification of α-Ribazole from Cyanocobalamin by Boronate Affinity Chromatography

Vitamin B_{12} (cobalamin) itself appears to have weak fluorescence which is not reliable enough for quantitative measurement. However, the various moieties in the nucleotide loop on the lower ligand of the corrinoid have rather strong fluorescence in the form of either α-ribazole-3′-phosphate, α-ribazole or 5, 6-dimethylbenzimidazole (DMB) each of which potentially can be employed as a fluorescence marker for vitamin B_{12} analysis, thus eliminating the requirement for conversion to cyanocobalamin (CN-Cbl) by cyanide.

3.1 α-Ribazole-3′-Phosphate

The nucleotide analogue, which exists in the structure of vitamin B_{12}, is α-ribazole-3′-phosphate moiety. It has a benzimidazole ribonucleotide structure in which an imidazole moiety of benzimidazole is *N*-linked to a ribose; additionally, a phosphate group is linked to the C-3 carbon of the ribose, **Figure 3.1.**

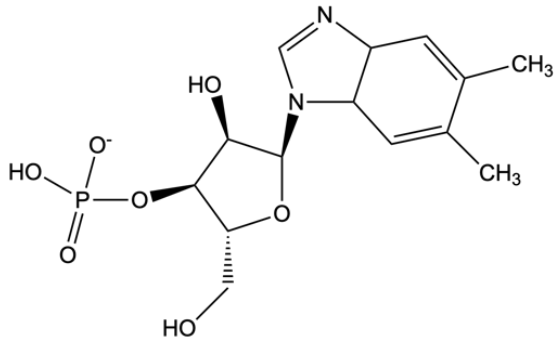


Figure 3.1. Structure of α-ribazole-3'-phosphate

The release of the nucleotide analogue can be achieved by acidic and/or alkaline hydrolysis. During early research on vitamin B_{12}, Buchanan *et al.* (1950) stated that the benzimidazole nucleotide analogue (α-ribazole-3′-phosphate) was isolated from the hydrolysates of acid hydrolysis of vitamin B_{12}. Buchanan used 100 °C for 1–20 hours with 1 M hydrochloric acid

which was a relatively mild condition. Subsequently, Armitage *et al.* (1953) claimed similar hydrolysis products had been found after vitamin B_{12} was hydrolysed with sodium or barium hydroxide. 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 analogues α-ribazole-3′-phosphate or α-ribazole-5′-phosphate, respectively (Pakin *et al.*, 2005). This mixture of α-ribazole-phosphates is likely to elute at varying times thus affecting the quantitation and rendering it unsuitable as a potential fluorescence marker.

3.2 α-Ribazole

The α-ribazole (**Figure 3.2**) in the nucleotide analogue of vitamin B_{12} is a unique fragment which has been obtained by further acidic hydrolysis of the α-ribazole-phosphate produced as mentioned above or vigorous acidic hydrolysis of the initial vitamin B_{12} with prolonged time and higher temperature up to 150 °C (Buchanan *et al.*, 1950). Dephosphorylation of α-ribazole-phosphate through 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, which hydrolyses phosphate monoesters at an alkaline pH and produces inorganic phosphate (Coleman and Gettins, 1983; Sharma *et al.*, 2014). The liberated α-ribazole can be employed as a fluorescence marker for vitamin B_{12} analysis and the problem of analogues is removed.

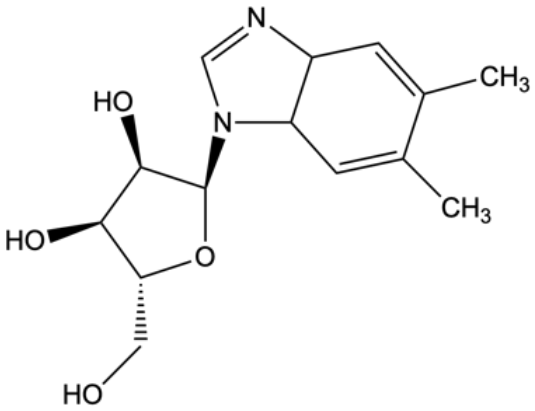


Figure 3.2. Structure of α-ribazole

3.3 5, 6-Dimethylbenzimidazole

The base moiety of α-ribazole-3′-phosphate and α-ribazole is a DMB which contains a dimethyl substituted benzene ring fused to an imidazole ring **Figure 3.3.**

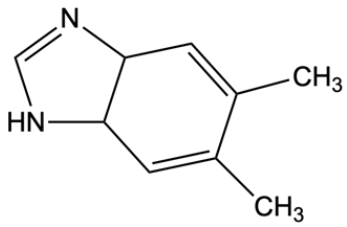


Figure 3.3. Structure of 5,6-dimethylbenzimidazole

Cleavage of the bond between ribose and DMB requires the most vigorous conditions (Bonnett *et al.*, 1964). Brink and Folkers (1950) reported using 6 *N* hydrochloric acid at 150°C for 20 hours to produce DMB from vitamin B_{12}. Beaven *et al.* (1949) reported a similar acidic hydrolysis condition using 20% hydrochloric acid (approx. 6.8 *N*) at 100°C for 15 hours. However, harsh acid and prolonged heating time are not desirable because of the risk of non-specific degradation.

Another possible way to cleave off the DMB is with an enzymatic hydrolysis using purine-nucleoside phosphorylase (PNP) (E.C. 2.4.2.1), which can reversibly catalyse the cleavage of the glycosidic bond in a ribonucleoside or deoxyribonucleoside in the presence of inorganic phosphate to yield purine base and ribose(deoxyribose)-1-phosphate (Bzowska *et al.*, 2000). The general scheme for the action of PNP is shown in **Scheme 3.1.**

β-Purine nucleoside + Phosphate ↔ purine base + α-D-pentose-1-phosphate

Scheme 3.1. The phosphorolysis reaction of β-purine nucleoside catalysed by PNP at the presene of inorganic phosphate to produce purine base and α-D-pentose-1-phosphate. This reaction can be reversed towards a nucleoside synthesis direction.

PNP can be classified into two main groups based on molecular mass (Mr), low-Mr trimeric PNPs (~80–100 kDa) are derived mainly from mammals and

high-Mr hexameric PNPs (~110–160 kDa) are predominantly found in bacteria. The former is more specific to 6-oxo purines (such as guanine, hypoxanthine), their nucleosides and some analogues, however, PNP from bacteria has borderline specificity and catalyses substrates involving 6-oxo purines as well as 6-amino purines. For instance, adenosine is a natural substrate for most bacterial PNP but not preferred by mammalian PNP (Zimmerman *et al.*, 1971). Additionally, high-Mr PNPs accept more analogues than low-Mr PNPs (Bzowska *et al.*, 2000). The phosphorolysis of benzimidazole riboside mentioned earlier was catalysed by PNP from *E*. *Coli* (Bzowska *et al.*, 1990).

PNPs have a broad optimal pH and temperature range. Both vary with the origin of the enzyme and the nature of substrates. Optimal pH from 5 to 11 has been reported while the majority of them sit between 6.5 and 8.5. (Barsacchi *et al.*, 1992; Glantz and Lewis, 1978; Kamel *et al.*, 2020; Lee *et al.*, 2001; Zhu *et al.*, 2013). The optimal temperature range is even broader ranging from 10–100 °C, with ambient temperature and body temperature being common choices for PNPs of mammalian origin while some thermally stable PNPs can tolerate temperatures up to 120 °C (Cacciapuoti *et al.*, 2007; Li *et al.*, 2008; Prokopowicz *et al.*, 2018; Wen *et al.*, 2018).

There are reports of applying PNP to 1-(β-D-ribofuranosyl)-benzimidazole (**Figure 3.4**) to catalyse the phosphorolysis, and subsequent production of benzimidazole (Bzowska *et al.*, 1990). This is the only reported substrate structurally close to 5,6-dimethyl-1-α-D-ribofuranosyl-benzimidazole (α-ribazole).

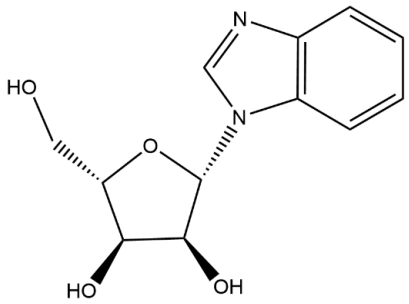


Figure 3.4. Structure of 1-(β-D-ribofuranosyl)-benzimidazole

However, the α configuration could potentially prevent PNP attaching to the substrate and result in no catalysis. In this study, a pilot test of PNP on the α-ribazole showed no production of DMB after the incubation. Even though DMB standard is commercially available, the difficulty of liberating DMB from the vitamin B_{12} led to the rejection of DMB as a marker candidate for this study.

In conclusion, α-ribazole was the chosen fluorescence marker. However, α-ribazole is not commercially available as a standard. Synthesis of α-ribazole has been reported (Cheong *et al.*, 2001; Stevens *et al.*, 1968); however, the number of precursors required, the complicated synthesis schemes involved and the low yield (~62%) (Stevens *et al.*, 1968) made the synthesis pathway undesirable for this project. Mattes and Escalante-Semerena (2018) reported a simple isolation protocol for α-ribazole from CN-Cbl utilising hydrolysis followed by boronate affinity chromatography BAC for its separation. This chapter details a procedure adapted from the method by Mattes and Escalante-Semerena (2018) and α-ribazole was successfully isolated. The product was identified and characterised by NMR and LC-MS.

3.4 Experimental

3.4.1 Materials and General Methods

All materials, solution preparation and general methods are stated in **Chapter 2**.

3.4.1.1 NMR

^{1}H, ^{13}C, distortionless enhancement polarisation transfer (DEPT), ^{1}H-^{1}H correlation spectroscopy (^{1}H-^{1}H COSY), heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple bond coherence (HMBC) spectra were acquired using a JEOL ECZR 600 MHz NMR spectrometer. Samples were dissolved in deuterium oxide. Data was processed using Delta NMR Processing and Control software (version 6.1).

3.4.1.2 LC-MS

Mass spectra of Q1 Scan and Product Ion Scan were obtained using a QTRAP 6500 MS/MS system (AB Sciex, Framingham, MA) coupled to a LC system configured with a LC-30AD Binary Gradient pump, a SIL-30AC autosampler, a CTO-20AC column oven and a CBM-20A system controller (Shimadzu, Japan). The chromatographic separation was achieved on a Gemini C_{18}, 5 µm, 4.6 m × 250 mm column (Part No. 00G-4435-E0, Phenomenex, Torrance, CA, USA). Mobile phase A: ammonia aqueous solution (0.3% *v:v*, pH = 9.5) and mobile phase B: 100% methanol. Gradient elution (min, (%B)): 0 (5), 6 (95), 12 (95), 13 (5), 15 (5). Flow rate: 0.6 mL min^{-1}. Column temperature was 40 °C. MS parameters: ion source temperature (200 °C), curtain gas (20.00 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 was processed using Analyst software (version 1.7.2).

3.4.1.3 UV Spectroscopy

UV absorbance was measured on a Cary 300 UV/Vis spectrometer (Agilent Technologies, Santa Clara, CA) The samples were dissolved in dimethyl sulfoxide (DMSO). Absorbance was measured in quartz cuvettes. Data was processed using Cary WinUV software (version 4.20).

3.4.2 α-Ribazole Isolation Protocol

3.4.2.1 Chemical Hydrolysis of Cyanocobalamin

Cyanocobalamin (50 mg) was dissolved into water (5 mL) and hydrochloric acid (3.0 M, 5 mL) was added. The solution was heated (95–100 °C) for 90 min. Subsequently, the solution was cooled to room temperature before neutralisation by sodium hydroxide (3.0 M, 5 mL). The resulting solution was ready for dephosphorylation by ALP.

3.4.2.2 Dephosphorylation of α-ribazole-phosphate

The hydrolysate from **Section 3.4.2.1**, containing α-ribazole-phosphate, underwent enzymatic hydrolysis with the addition of incubating buffer (ammonium acetate, 0.3 M: magnesium chloride, 2 mM; pH = 8.8, 15 mL) and

ALP stock solution (200 µL). The resulting mixture was incubated at 34 °C overnight (at least 16 hours).

3.4.2.3 Boronate Affinity Chromatography Clean-up

The resultant α-ribazole was concentrated by passing through a boronate affinity column. Immobilised Boronic Acid Gel™ (1.5 mL) was packed into an empty centrifuge column and equilibrated with binding buffer, ammonium acetate (0.3 M, pH = 8.8, 6 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 buffer was discharged from the column by gravity flow and the process repeated with binding buffer (6 mL). The hydrolysate was applied to 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. Then the solution was removed by gravity flow, and the column was washed with ammonium acetate (0.3 M, pH = 8.8) until the flow-through solution was colourless. The column was washed with water (1 mL) to remove the ammonium acetate buffer residue. Acetonitrile: 1.0 M formic acid (30:70 *v*:*v*, 15 mL) was used to elute the retained α-ribazole, which was collected for lyophilisation. The used column was regenerated by washing with the same eluent (6 mL) and binding buffer (6 mL). Although there is no data from the manufacturer to demonstrate the limit of regeneration number, Mattes and Escalante-Semerena (2018) reported the packed column could be reused for 10 times. In this work, the columns were regenerated up to 10 times and stored at 4 °C.

3.4.2.4 Product Lyophilisation

The eluent was diluted with 30 mL of water and pre-frozen at −18 °C before being placed into a freeze-dryer at −60°C under 0.01 kPa overnight.

3.4.2.5 Identification and Characterisation

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, 5000 rpm). 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 to confirm the identity.

3.4.2.6 Yield and Quantitation

The dried product from **Section 3.4.2.4**. was dissolved and made up to 10 mL with DMSO. A further 25-fold dilution was made with DMSO and loaded into a quartz cuvette to measure the absorbance at 280 nm. The total yield of α-ribazole was determined by absorption using a modified Beer-Lambert law (**Equation 3.1**).

$$c = \frac{A_{280}}{\varepsilon_{280} \times l} \times DF \qquad \textit{Equation 3.1}$$

Where c is the molar concentration (mol L^{-1}) of initial α-ribazole solution, A_{280} is the absorbance of α-ribazole at 280 nm in DMSO, l is the light pathway, equal to the inner distance width of a standard cuvette (1 cm), DF is the dilution factor and $\varepsilon 280$ (mol^{-1} L cm^{-1}) is the molar absorption coefficient of the aglycone DMB at 280 nm which will not be affected by the presence of the ribose. The final mass of the total isolated α-ribazole was calculated using **Equation 3.2,** where m is the total mass (g) of α-ribazole, c is the molar concentration (mol L^{-1}) from **Equation 3.1** and V is the volume (L) of the α-ribazole stock solution.

$$m = M \times c \times V \qquad \textit{Equation 3.2}$$

3.5 Results and Discussion

3.5.1 Characterisation by NMR*

The ^{1}H and ^{13}C spectra were well-matched with the work by Brown and Hakimi (1986) who reported NMR studies of α-ribazole and both sets of chemical shift data are shown in **Table 3.1**. The difference between the two sets ^{13}C of data is

* The α-ribazole sample used for characterisation was obtained as stated in Section 3.4.2.

probably due to differences in the fields of the spectrometers and pH of samples. Brown and Hakimi (1986) used internal standard sodium trimethylsilylpropanesulfonate (DSS) for their chemical shift reference, while the current work did not employ a reference compound. However, after a numerical adjustment by the addition of the difference between the highest field signal peaks of the two ^{13}C spectra to all chemical shift data, the adjusted data shows almost no difference compared to Brown and Hakimi (1986), apart from the reversal of the assignments of the two methyl groups on the aglycone.

The deduced structure consists of α-D-ribofuranose *N*-linked to a 5,6-dimethylbenzimidazole aglycone and matched the structure of α-ribazole (5,6-dimethyl-1-α-D-ribofuranosyl-1H-benzimidazole) (**Figure 3.5**). The overall structure was fully assigned by ^{13}C (**Figure 3.6**), ^{1}H (**Figure 3.7**), DEPT (**Figure 3.8**), HMQC (**Figure 3.9**), ^{1}H-^{1}H-COSY (**Figure 3.10**), and ^{13}C-^{1}H HMBC (**Figure 3.11**) and ^{15}N-^{1}H HMBC (**Figure 3.17**)

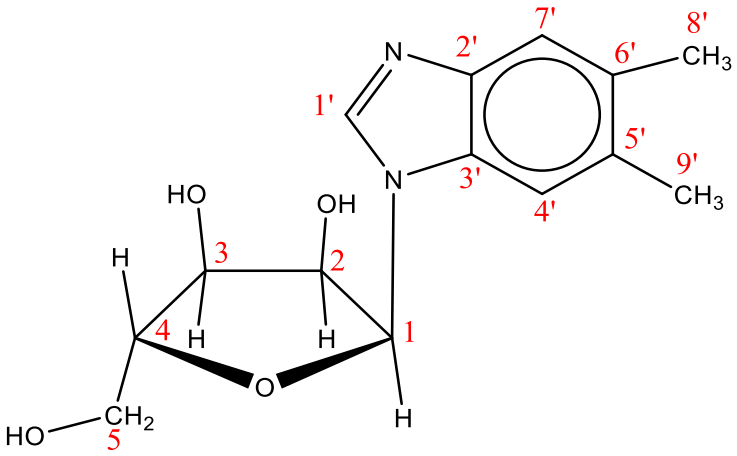


Figure 3.5. The numbering of carbons on α-ribazole

Table 3.1. The chemical shift data of isolated α-ribazole compared with Brown and Hakimi (1986)

	^{13}C			^{1}H	
	This work[a]	Adjusted data[b]	Literature[c]	This work[a]	Literature[c]
1	87.45	*90.08*	90.081	6.39	6.475
2	71.62	*74.25*	74.130	4.63	4.788
3	70.51	*73.14*	73.372	4.30	4.465
4	85.24	*87.87*	88.035	4.37	4.556
5	61.08	*63.71*	63.851	3.66(d, 12.4Hz) 3.80 (d,12.4 Hz)	3.842(d,12.49Hz) H-5a 3.983(d,12.49 Hz) H-5b
1′	138.25	*140.88*	140.791	9.06	9.206
2′	129.04	*131.67*	131.317	-	-
3′	128.69	*131.32*	131.099	-	-
4′	112.41	*115.04*	114.938	7.42	7.445
5′	137.18	*139.81*	139.835	-	-
6′	137.39	*140.02*	140.038	-	-
7′	114.10	*116.73*	116.517	7.47	7.503
8′	19.52	*22.15*	22.354	2.26	2.383
9′	19.71	*22.34*	22.173	2.28	2.362

[a] In 1% acetic acid in D_2O.
[b] The adjustment was made by adding the difference between the highest field signal of the two sets of spectra to this work's data.
[c] In D_2O (pD 3.0). All chemical shifts relative to internal DSS, adapted from (Brown and Hakimi, 1986)

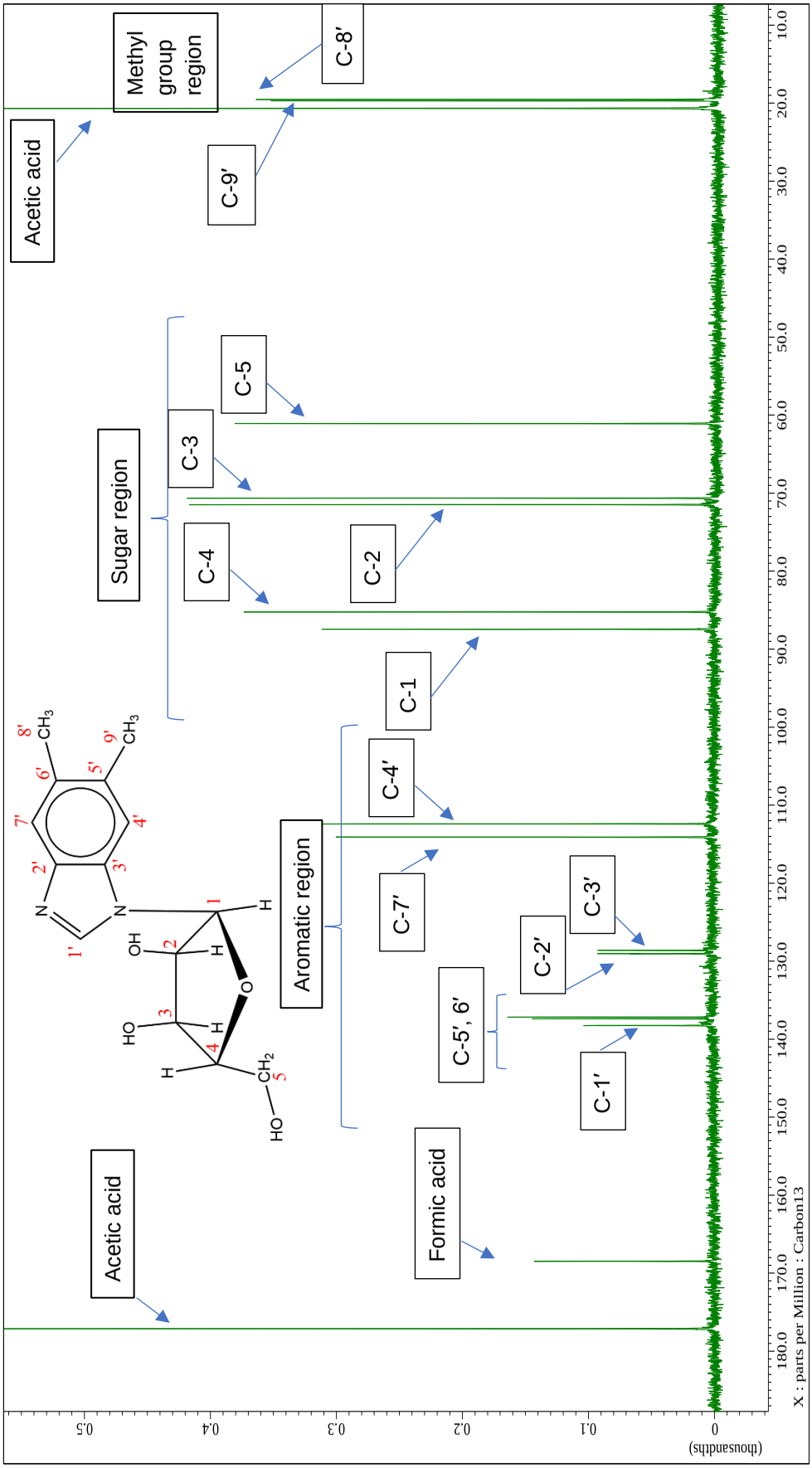
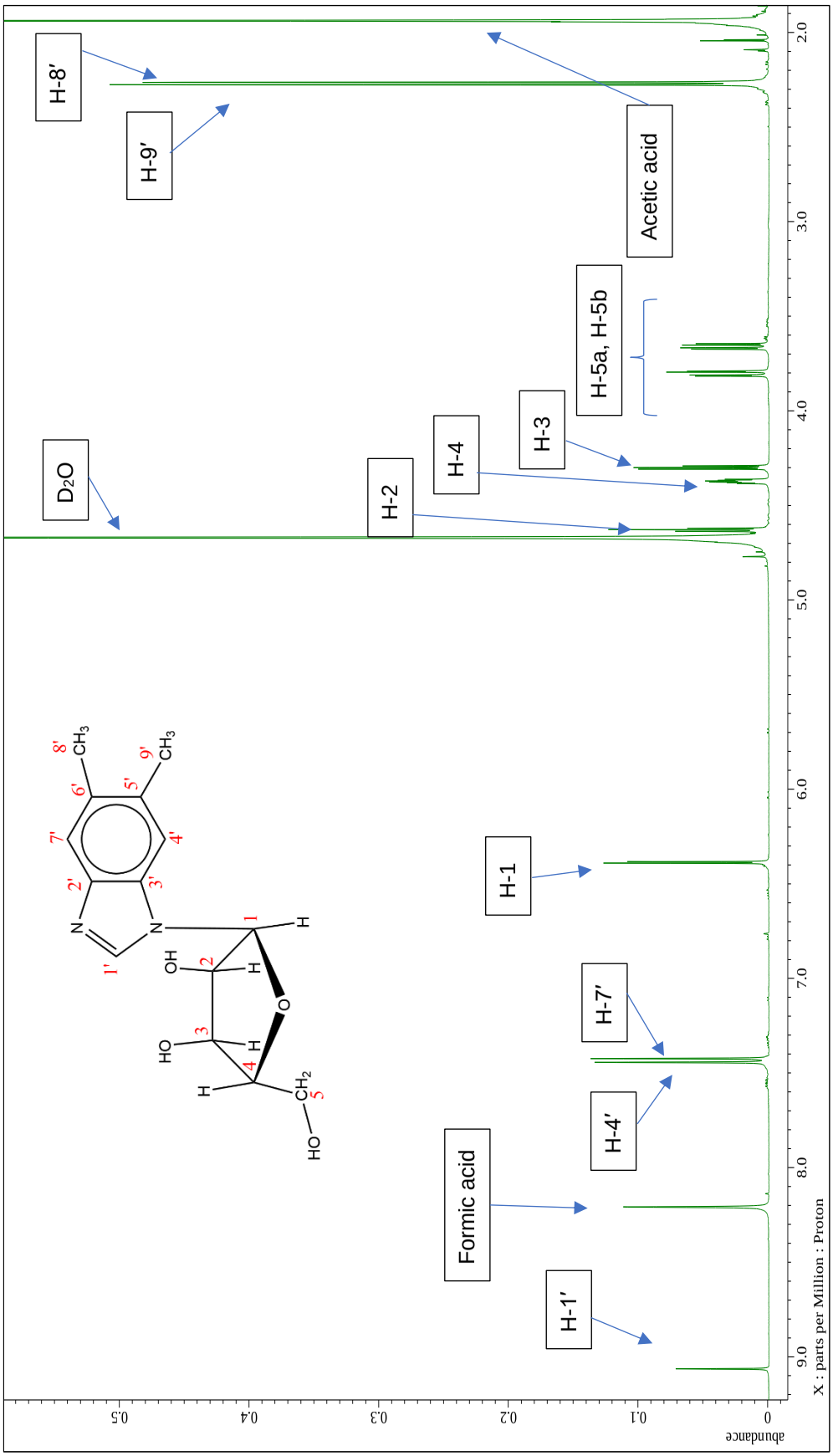


Figure 3.6. ^{13}C NMR spectrum of α-ribazole in 1% acetic acid in D_2O


H-8'
H-9'
Acetic acid
H-5a, H-5b
H-3
H-4
H-2
D2O
H-1
H-7'
H-4'
Formic acid
H-1'
X : parts per Million : Proton
abundance

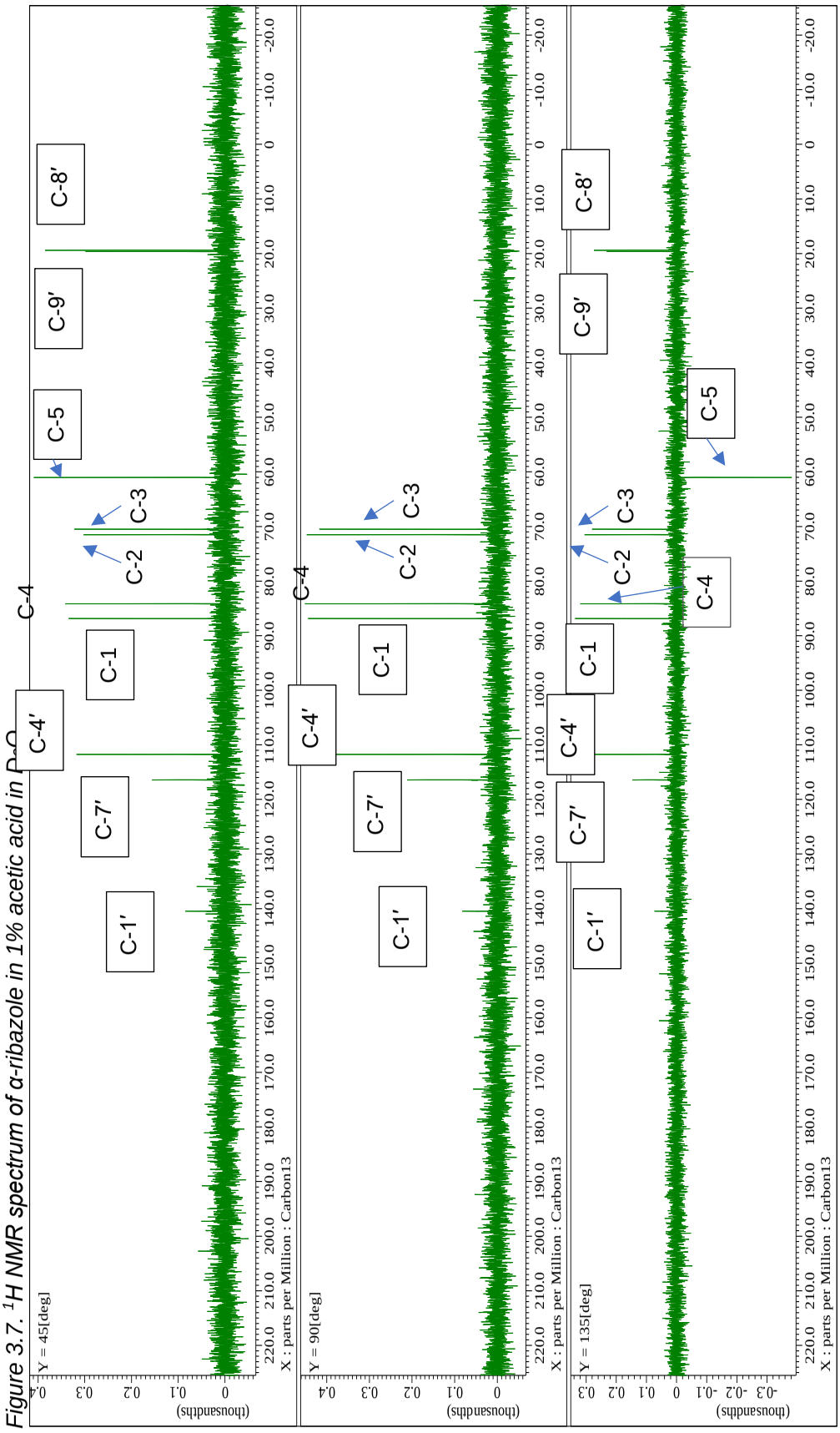


Figure 3.7. ^{1}H NMR spectrum of α-ribazole in 1% acetic acid in D_2O

Figure 3.8. DEPT spectra of α-ribazole in D_2O

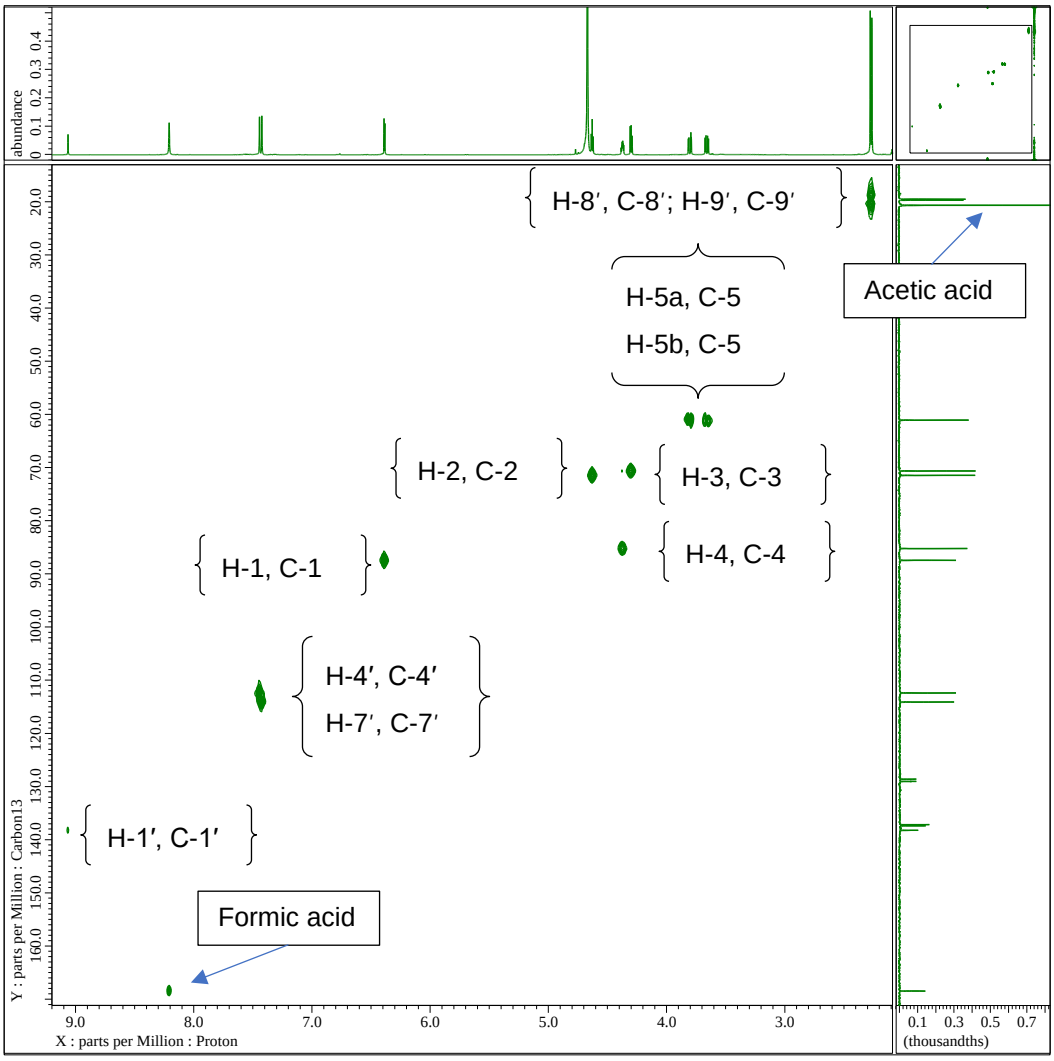


Figure 3.9. HMQC spectrum of α-ribazole in 1% acetic acid in D_2O

Prior to the NMR analysis, the α-ribazole solution was acidified using acetic acid to a similar pH level (~3.0) to that used by Brown and Hakimi (1986) for an appropriate comparison. Acetic acid was favoured for its relatively weaker acidity (pK_a 4.76) and low substitution risk compared to using hydrochloric acid for acidification, therefore it has no effect on the quality of the recovered α-ribazole.

In the ^{13}C spectrum, seventeen signals were found; two (20.67 ppm and 177.22 ppm) belonged to acetic acid, and one was formic acid at 168.36 ppm, which was the residue from the eluent. The chemical shifts for these adulterants were well-matched with the reported values (Babij *et al.*, 2016; Fulmer *et al.*, 2010). The remaining fourteen signals matched the carbon number in the

α-ribazole molecule, with two signals in the methyl region, five in the sugar region and seven in the aromatic region.

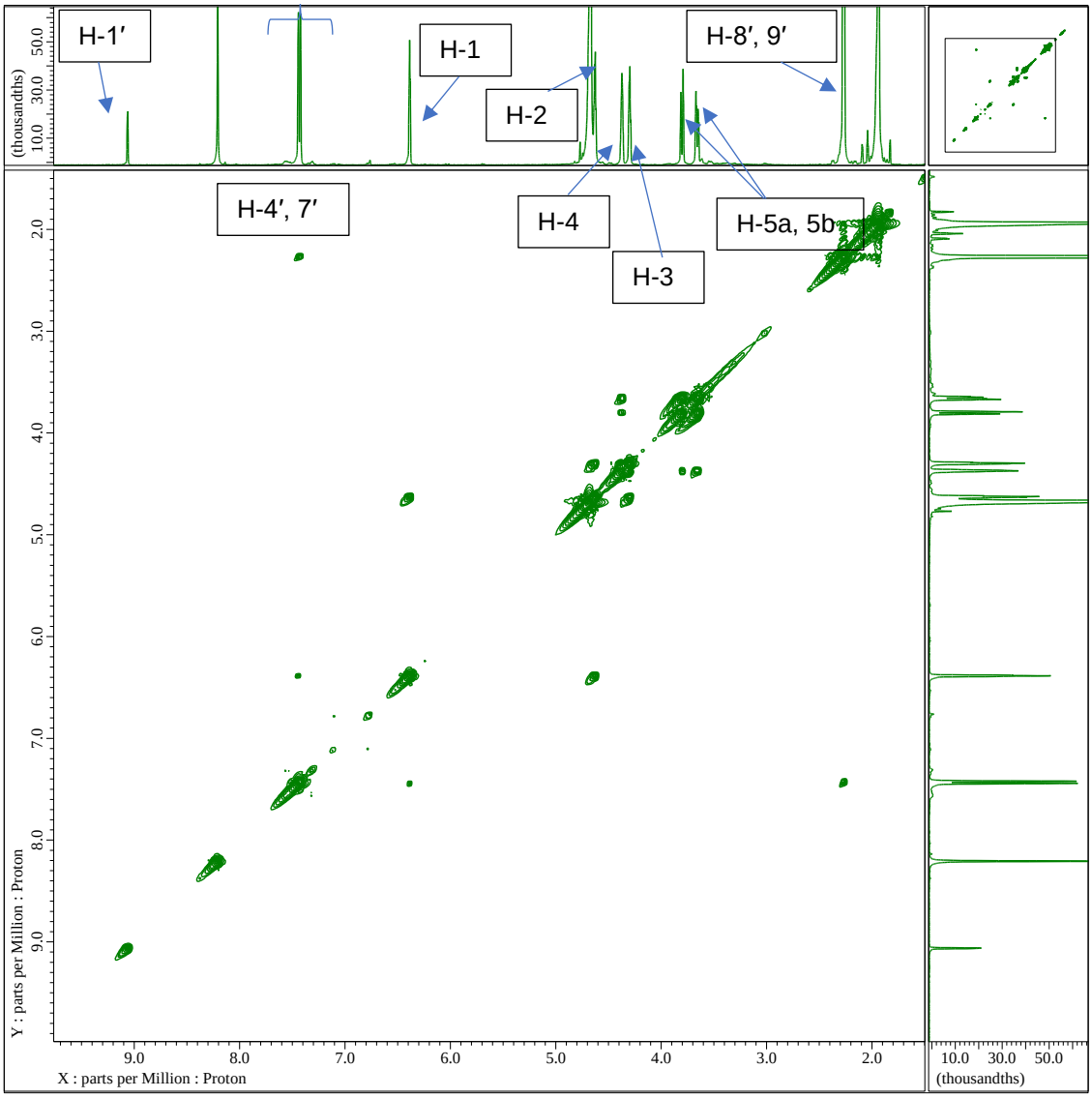


Figure 3.10. ^{1}H-^{1}H COSY spectrum of α-ribazole in 1% acetic acid in D_2O

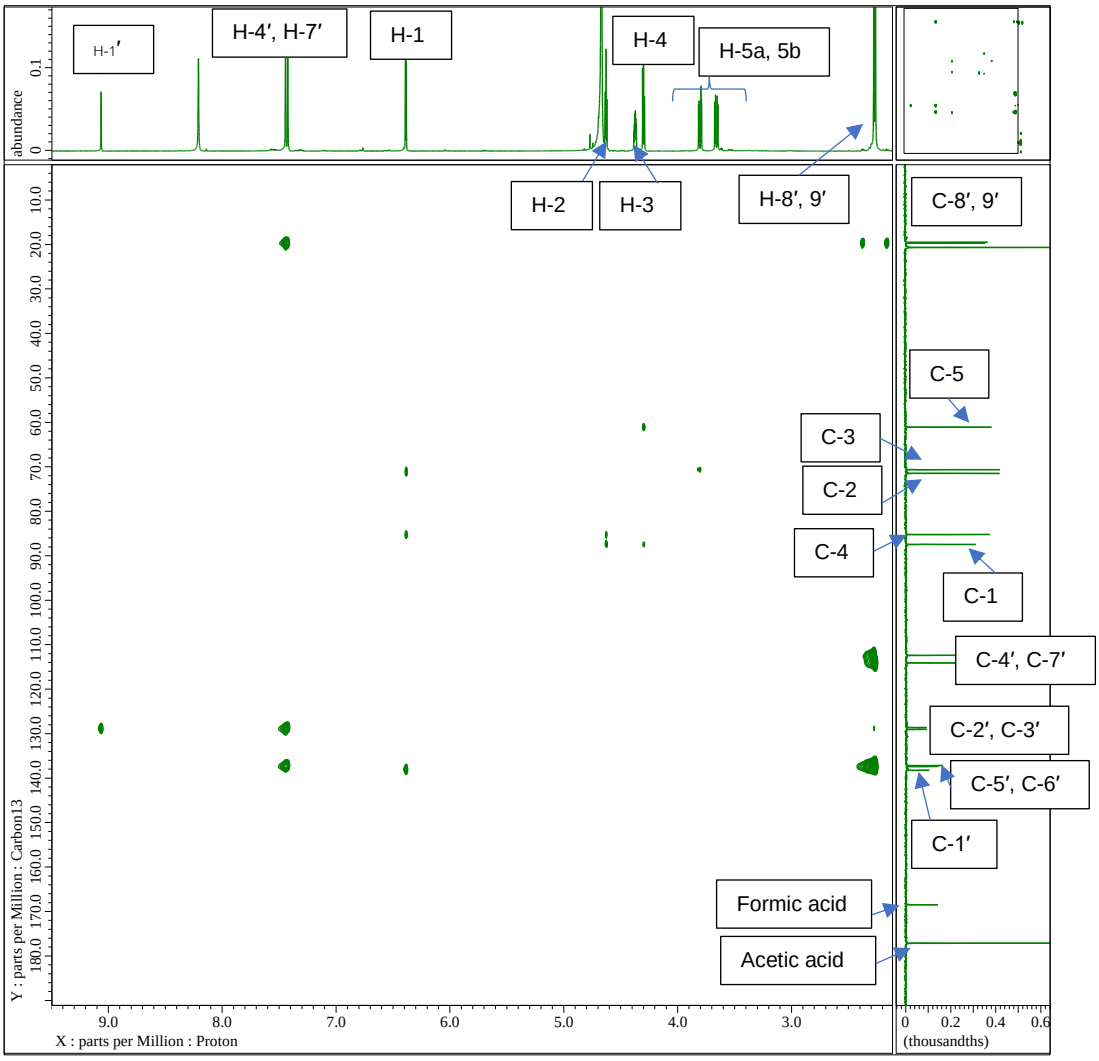


Figure 3.11. ^{13}C-^{1}H HMBC spectrum of α-ribazole in 1% acetic acid in D_2O

In the ^{1}H spectrum, ten signals were observed, excluding the acetic acid (1.94 ppm), formic acid (8.21 ppm) and solvent (4.67 ppm) signals, which were confirmed by the reported chemical shift values (Babij *et al.*, 2016; Berregi *et al.*, 2007; Fulmer *et al.*, 2010), **Figure 3.6**. The DEPT experiments allowed construction of a table of possible assignments, **Table 3.2**.

3.5.1.1 Ribose Moiety Assignment

In **Figure 3.8**, the DEPT-90 experiment confirmed the disappearance of the two carbon signals (19.52 and 19.71 ppm) in the methyl group region C-8′ and C-9′ since only the CH group is shown in the DEPT90 experiment.

Table 3.2. Summary of possible carbon assignment based on the DEPT experiment

^{13}C chemical shift	Presence in DEPT	Group	Possible carbon number
19.52	DEPT-45, 135(+)	CH_3	C-8′, C-9′
19.71	DEPT-45, 135(+)	CH_3	C-8′, C-9′
61.08	DEPT-45, 135(-)	CH_2	C-5
70.51	DEPT-45, 90,135(+)	CH	C-1, C-2, C-3, C-4
71.62	DEPT-45, 90,135(+)	CH	C-1, C-2, C-3, C-4
85.24	DEPT-45, 90,135(+)	CH	C-1, C-2, C-3, C-4
87.45	DEPT-45, 90,135(+)	CH	C-1, C-2, C-3, C-4
112.41	DEPT-45, 90,135(+)	CH	C-4′, C-7′
114.10	DEPT-45, 90,135(+)	CH	C-4″, C-7′
128.69	NA[a]	C	C-2′, C-3′, C-5′, C-6′
129.04	NA	C	C-2′, C-3′, C-5′, C-6′
137.18	NA	C	C-2′, C-3′, C-5′, C-6′
137.39	NA	C	C-2′, C-3′, C-5′, C-6′
138.25	DEPT-45, 90,135(+)	CH	C-1′

[a] NA = carbons without attached protons are not visible in DEPT spectra

Additionally, the DEPT135 experiment allowed the identification of C-5. Since the CH_2 signal is shown in the negative phase, in **Figure 3.12**, at 61.08 ppm this matched with the only CH_2 group at the C-5 position in the ribose. In the HMQC experiment, the C-5 showed $^1J_{CH}$ correlation with two proton signals at 3.66 and 3.80 ppm, **Figure 3.13**, which were also identified by chemical shift and geminal hydroxymethyl protons coupling constant ($^1J_{CH}$ between H-5a and H-5b: 12.4 Hz) in the ^1H spectrum.

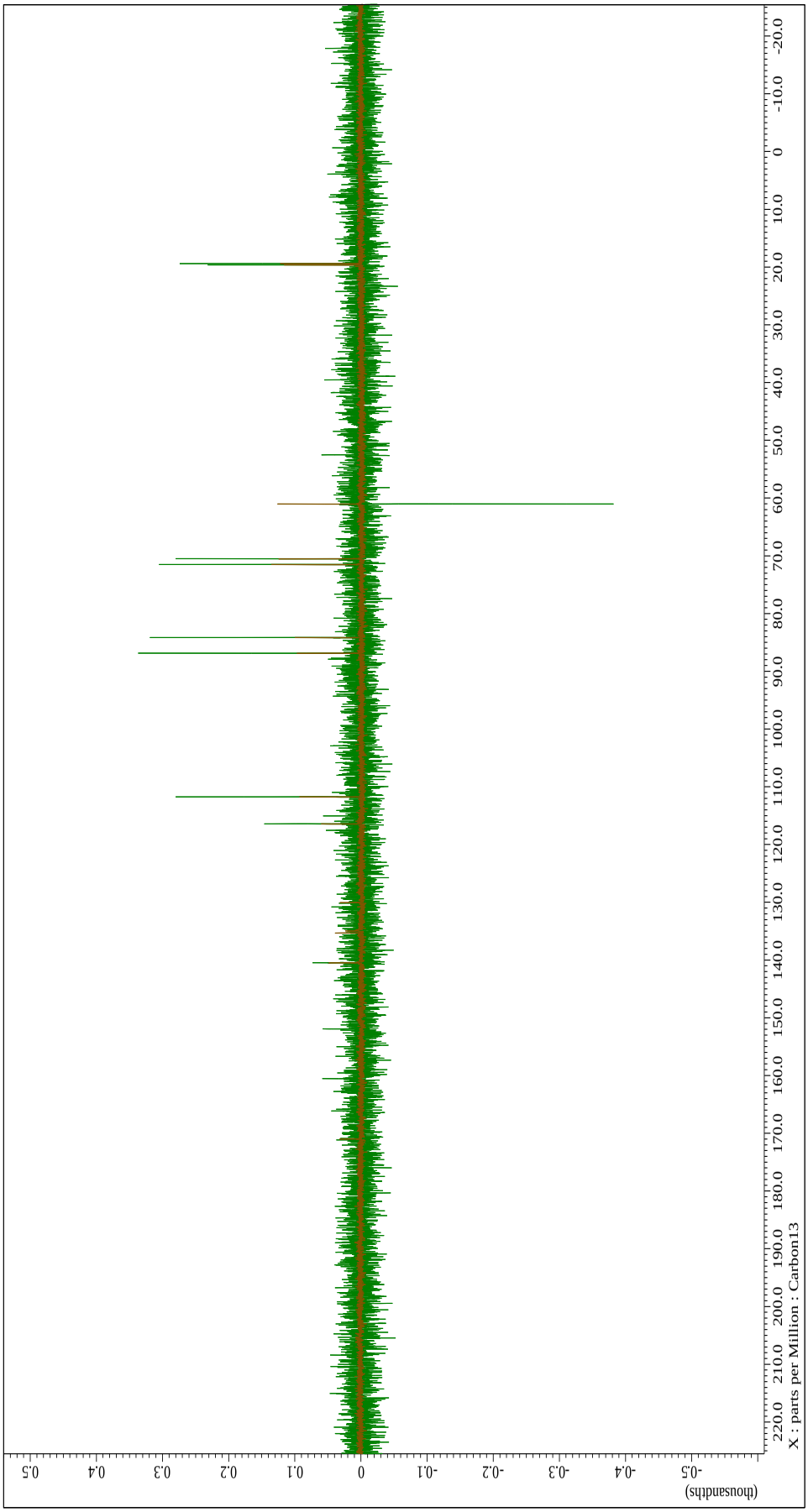


Figure 3.12. DEPT-135 spectrum of α-ribazole overlaid with its ^{13}C spectrum

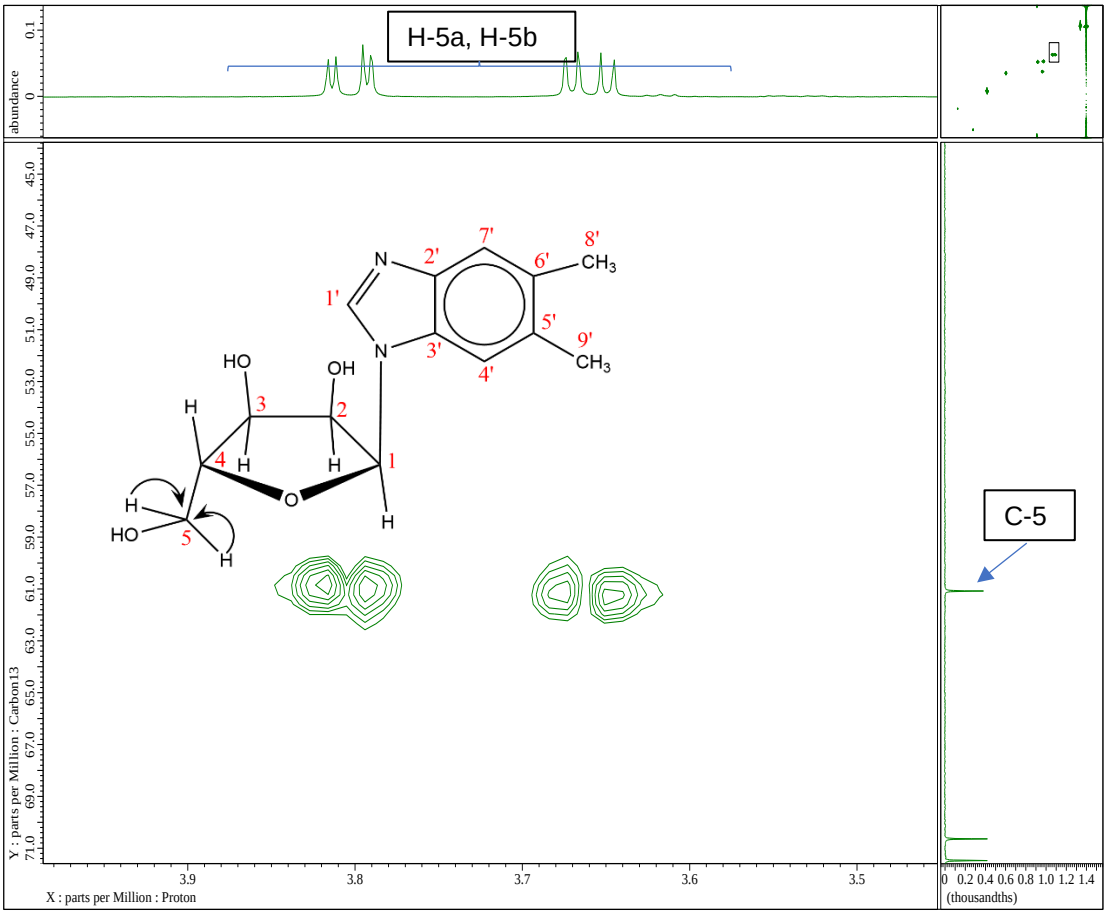
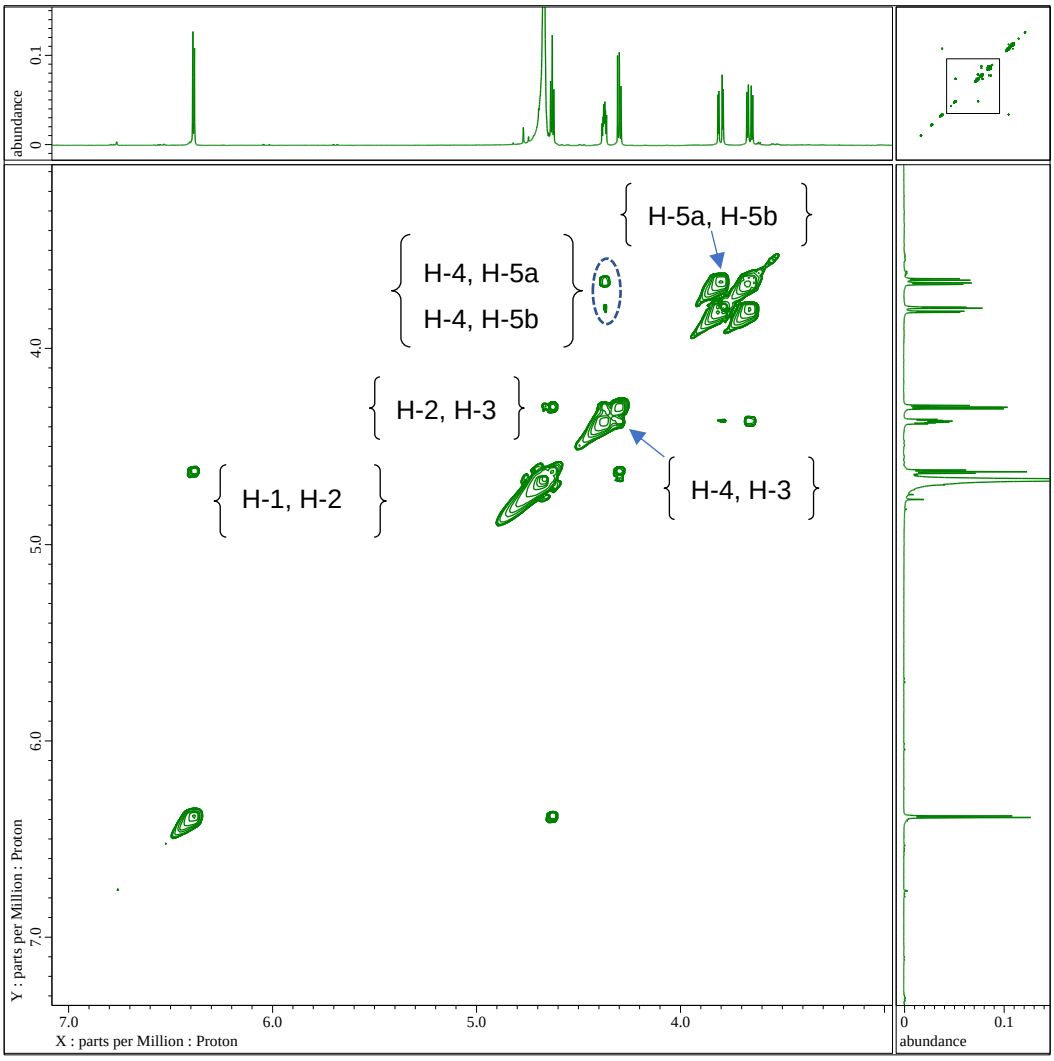


Figure 3.13. Zoom-in HMQC spectrum of α-ribazole, featuring the $^{1}J_{CH}$ correlation between H-5(a, b) and C-5

^{1}H-^{1}H COSY shows the correlation between protons coupled with each other, where $^{2}J_{HH}$and $^{3}J_{HH}$ are observed most easily. Once H-5a and H-5b were identified, H-4, H-3, H-2 and H-1 were confirmed sequentially by using the ^{1}H-^{1}H COSY spectrum, **Figure 3.14**, and expected $^{3}J_{HH}$ couplings for the ribose moiety of α-ribazole, **Table 3.3.**

Table 3.3. ^{1}H, ^{1}H COSY correlation expected for the ribose moiety of α-ribazole

Proton	^{3}J H coupling
H-1	H-2
H-2	H-1, H-3
H-3	H-2, H-4
H-4	H-3, H-5a, b
H-5a, H-5b	H-4



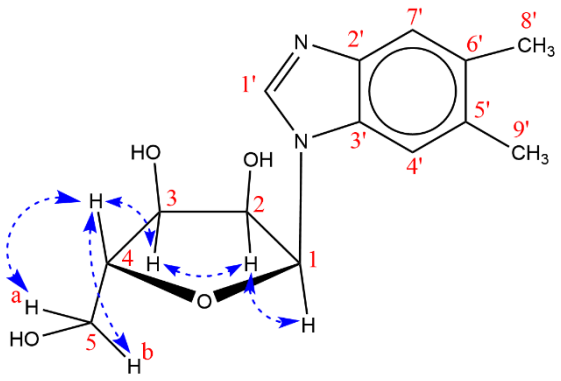


Figure 3.14. Zoom-in ^{1}H-^{1}H COSY spectrum of α-ribazole, featuring the correlations between H-4 and H-5a, b; between H-3 and H-4; between H-2 and H-3; between H-1 and H-2

The HMQC spectrum reveals the $^{1}J_{CH}$ correlation between the proton and its attached carbon. Therefore, based on the assignment of protons of the ribose, the corresponding carbons were confirmed, **Figure 3.15**.

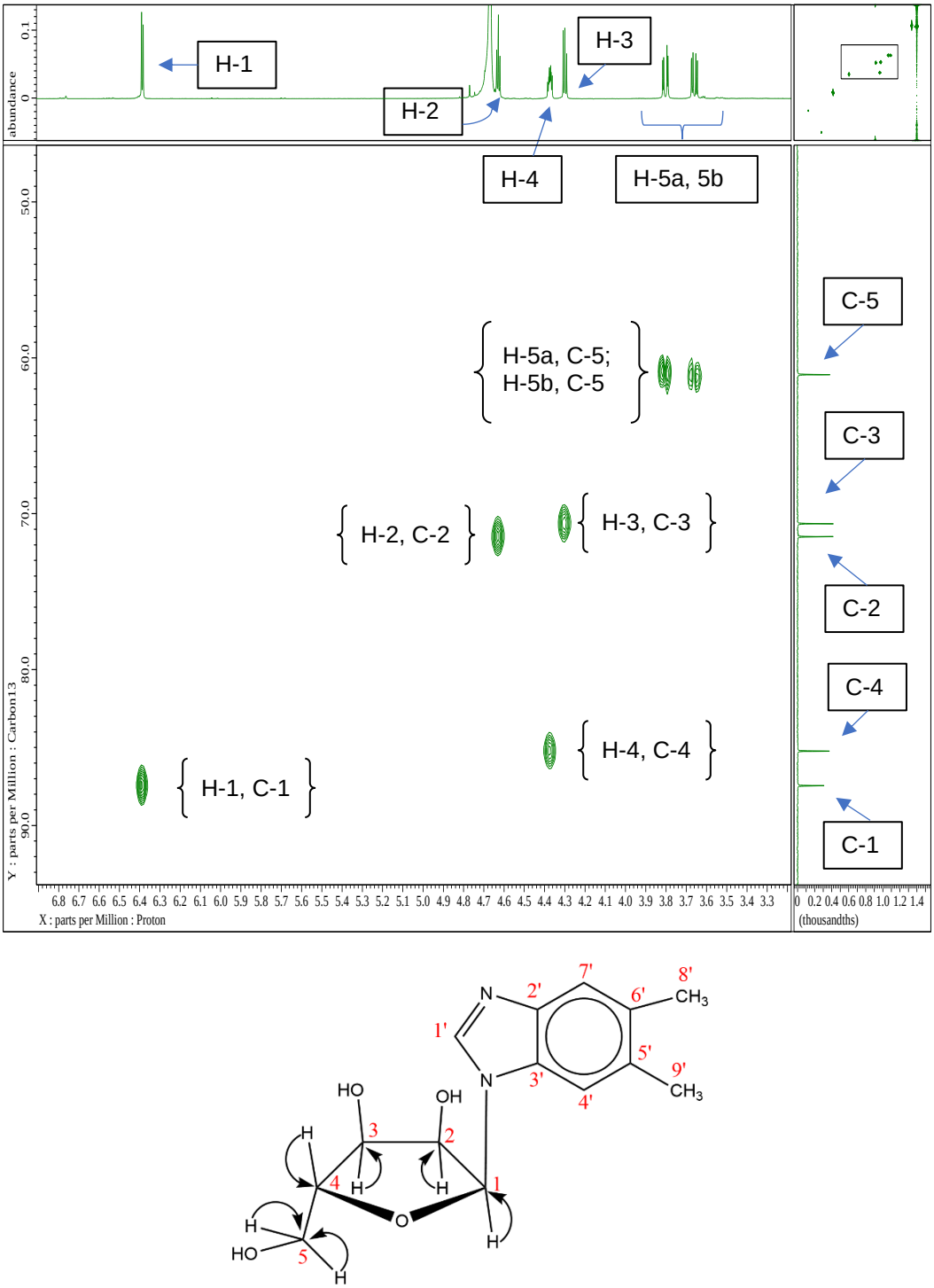


Figure 3.15. Zoom-in HMQC spectrum of α-ribazole, featuring the observed correlations between the ribose carbons and protons

3.5.1.2 DMB Moiety Assignment

The two methyl groups of the α-ribazole structure, the protons H-8′ and H-9′ were assigned by the chemical shift (2.3 ppm for benzylic protons) and

integration (3H) in the ^{1}H spectrum at 2.26 and 2.28 ppm. In the ^{1}H-^{1}H COSY experiment, due to the lack of $2J_{HH}$ or $^{3}J_{HH}$ coupling, H-1′ was also confirmed at 9.06 ppm. In addition, only H-4′ and H-7′ would show $^{4}J_{HH}$ correlations to H-8′ and H-9′, **Figure 3.16**, the former pair was confirmed at 7.42 and 7.44 ppm.

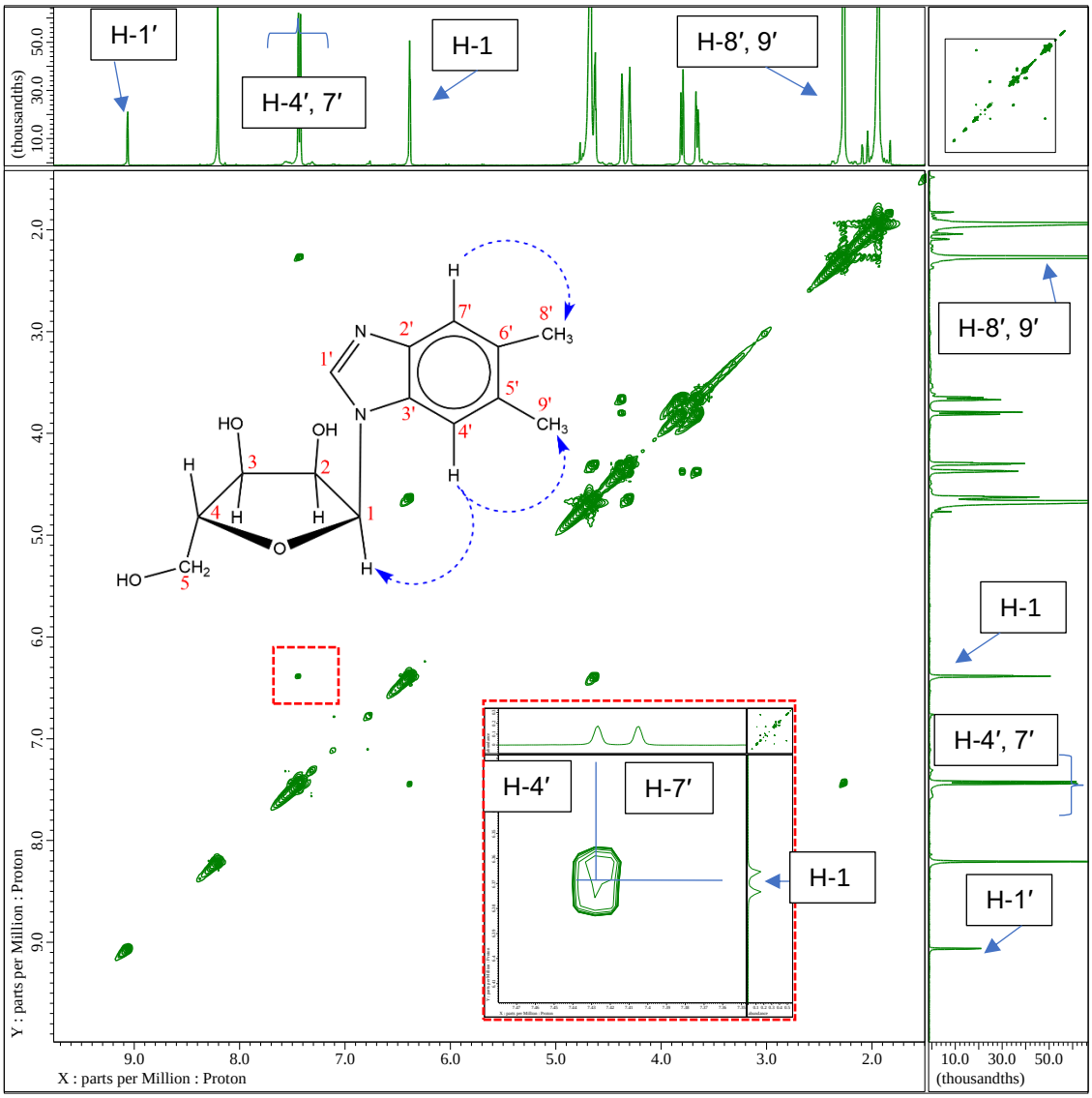


Figure 3.16. Zoom-in ^{1}H-^{1}H COSY spectrum of α-ribazole, featuring H-1′, H-4′ and H-7′, H-8′ and H-9′ signals

The further distinction between H-4′ and H-7′ was achieved by the observation of a $^{5}J_{HH}$ correlation between H-4′ and H-1 as shown in the zoom-in section of **Figure 3.16** since if the cross peak belongs to the $^{6}J_{HH}$ correlation between H-7′ and H-1, a second cross peak of the $^{5}J_{HH}$ correlation between H-4′ and H-1 should be detected as well. Hence, it should only be a $^{5}J_{HH}$ correlation.

However, considering the unusual $^{5}J_{HH}$ long range coupling for a COSY spectrum and the importance of H-4′ and H-7′ for the complete assignment of the DMB moiety, a ^{15}N-^{1}H HMBC experiment was conducted to utilise the two nitrogen atoms in the DMB moiety to distinguish H-4′ and H-7′. In **Figure 3.17**, the observation of cross peaks ($^{2}J_{NH}$ correlations) between H-1 and N-b confirmed the signal of N-b. The presence of two $^{3}J_{NH}$ correlations between H-7′ and N-a, H-4′ and N-b distinguished H-4′ and H-7′ and confirmed their signals at 7.44 ppm and 7.42 ppm, respectively. This assignment matches the results from the ^{1}H-^{1}H COSY spectrum.

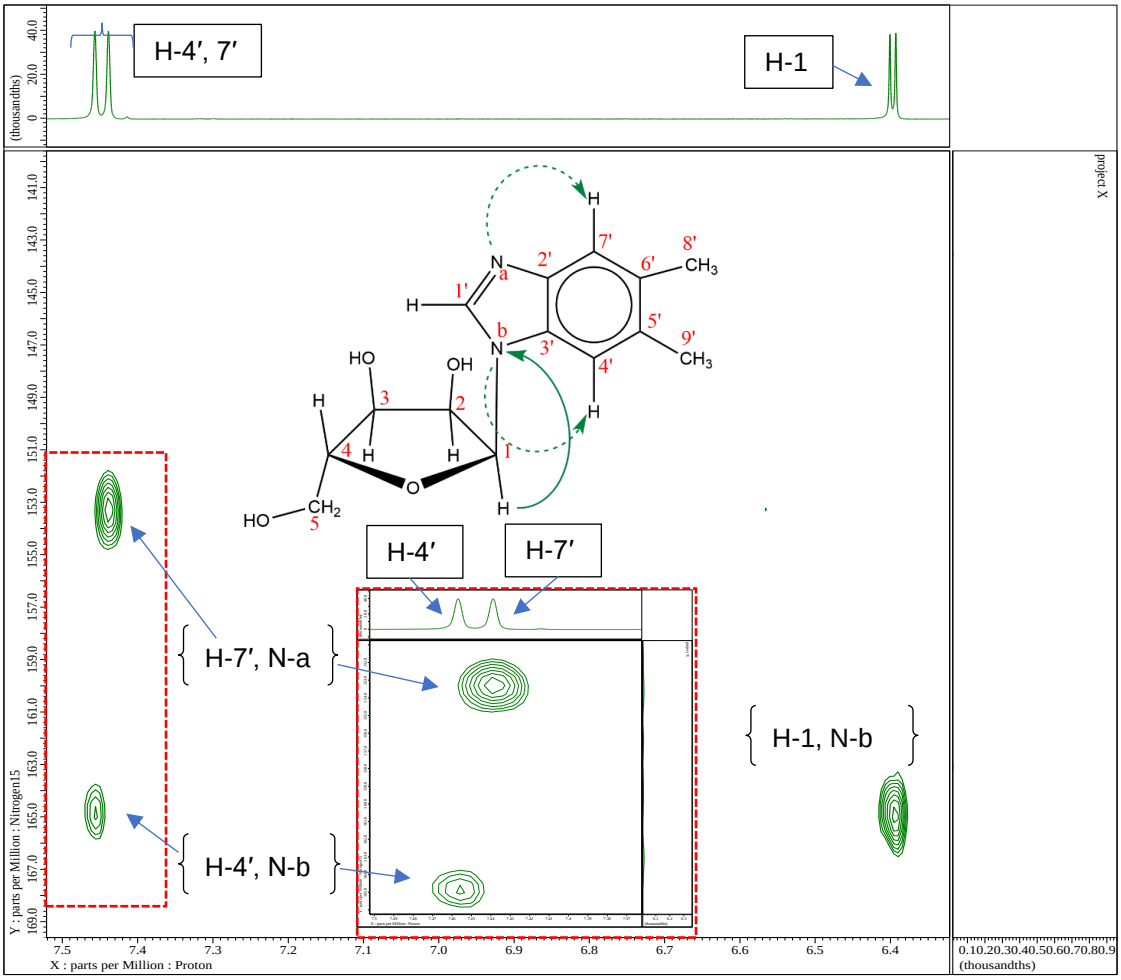


Figure 3.17. Zoom-in ^{15}N-^{1}H HMBC spectrum of α-ribazole, featuring the $^{2}J_{NH}$ correlations between H-1 and N-b and $^{3}J_{NH}$ correlations between H-7′ and N-a and between H-4′ and N-b In the HMQC experiment, based on the assignment of H-1′, H-4′ and H-7′, the corresponding carbons were confirmed: C-1′ (138.25 ppm) and C-4′ (112.4 ppm) and C-7′ (114.10 ppm), as shown in **Figure 3.18.**

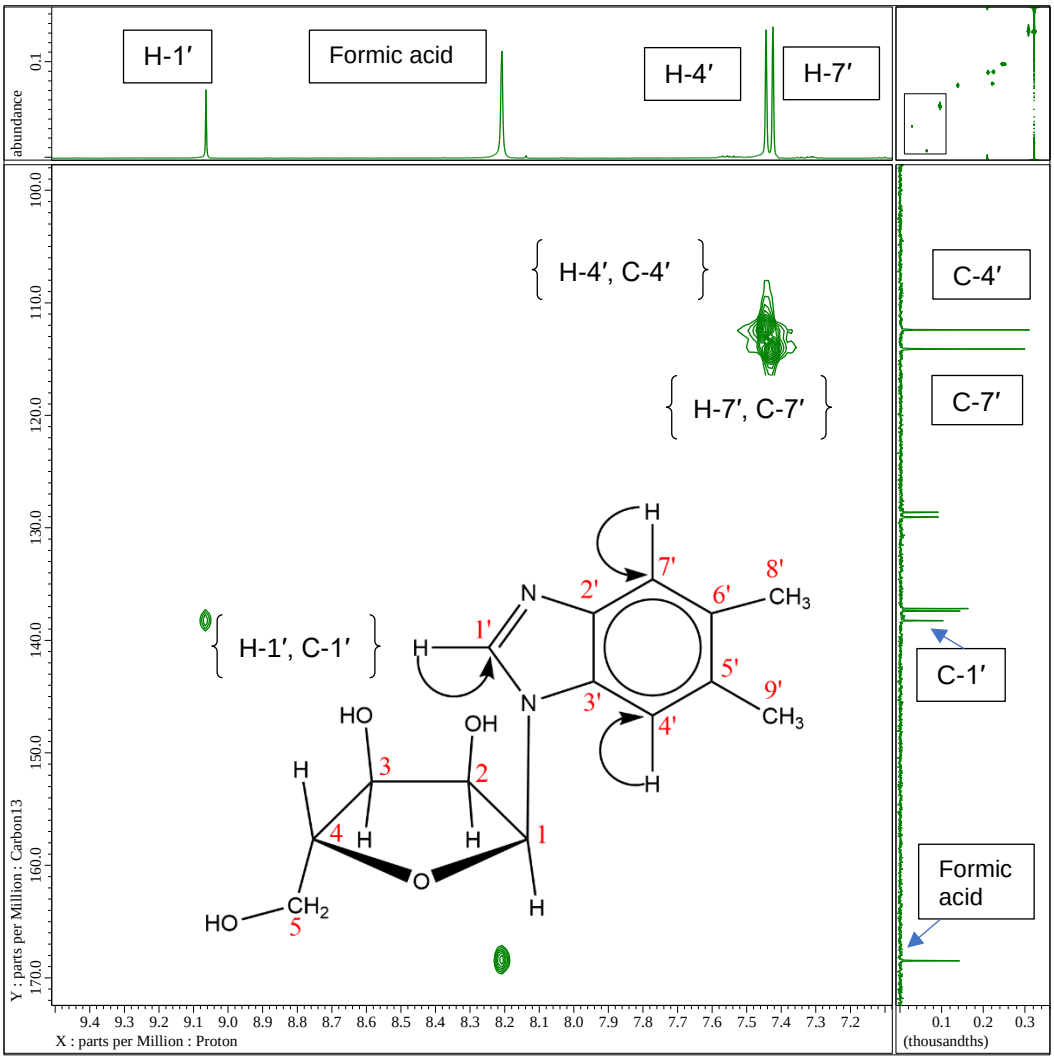


Figure 3.18. Zoom-in HMQC spectrum of α-ribazole, featuring the $^{1}J_{CH}$ correlation between H-1′ and C-1′ and between H-4′, 7′ and C-4′, 7′

In the ^{13}C-^{1}H HMBC spectrum, H-1 showed at least four $^{2}J_{CH}$ and $^{3}J_{CH}$ correlations. From the structure of α-ribazole, H-1 can correlate with C-2, C-3, C-4, C-3′ and C-1′, with the C-2 and C-3 couplings overlapped. Since C-2, C-3, C-4 and C-1′ were assigned earlier, then the correlation of H-1 and C-3′ identified the signal of C-3′ (128.69 ppm), **Figure 3.19**. The signal for C-3′ does not appear in the DEPT spectra as it is a quaternary carbon.

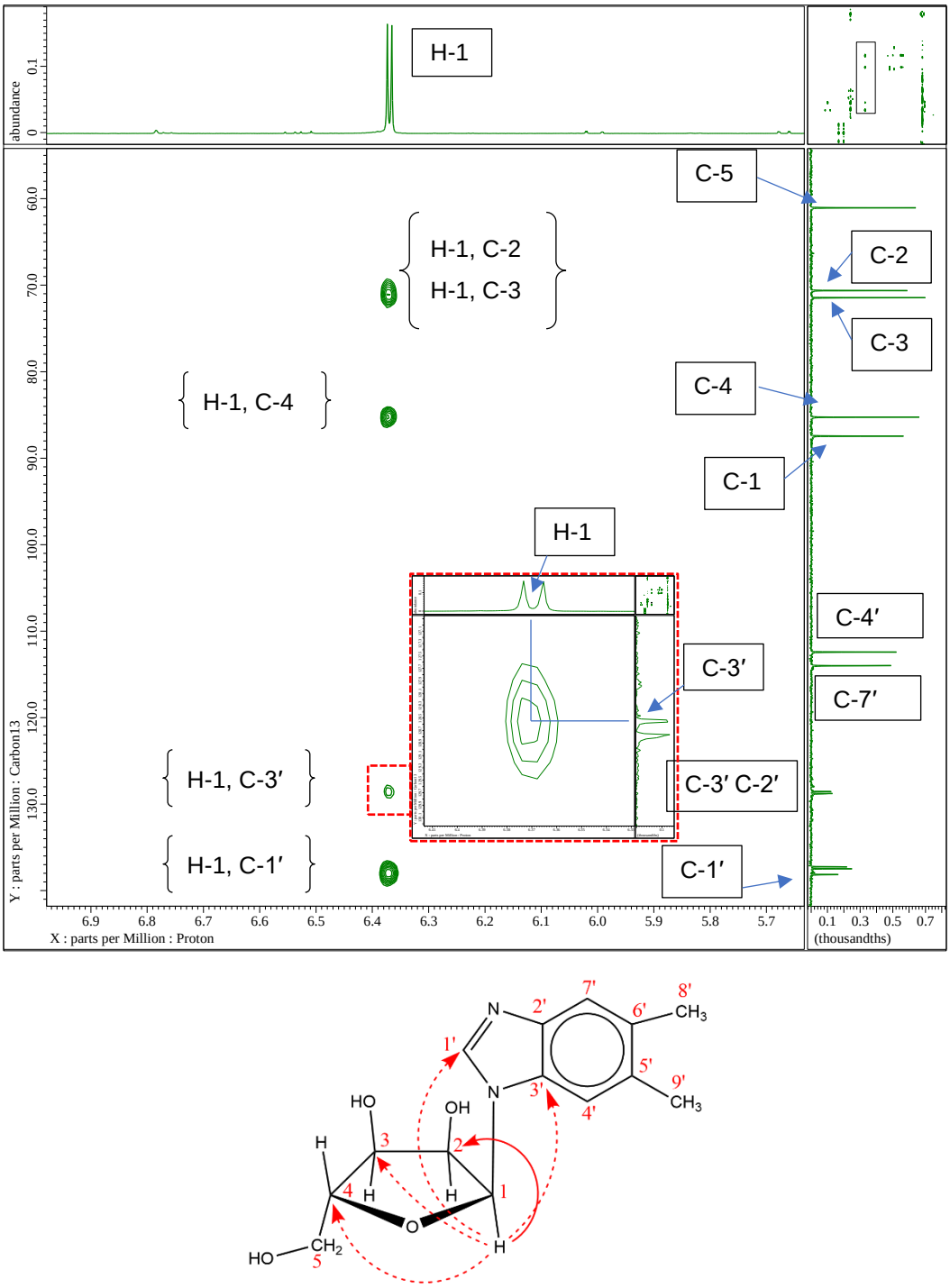


Figure 3.19. Zoom-in ^{13}C-^{1}H HMBC spectrum of α-ribazole, featuring the correlation of H-1 with C-2, C-3, C-4, C-3′ and C-1′

The ^{13}C-^{1}H HMBC spectrum also revealed that H-1′ showed a correlation (highly likely to be two overlapped) with two carbon signals at 128.69 and 129.04 ppm, **Figure 3.20**. Based on the structure, chemical shift region and DEPT experiment results, these two carbons should be C-2′ and C-3′.

Moreover, the C-3′ has been assigned above at 128.69 ppm, further confirming the C-2′ signal at 129.04 ppm.

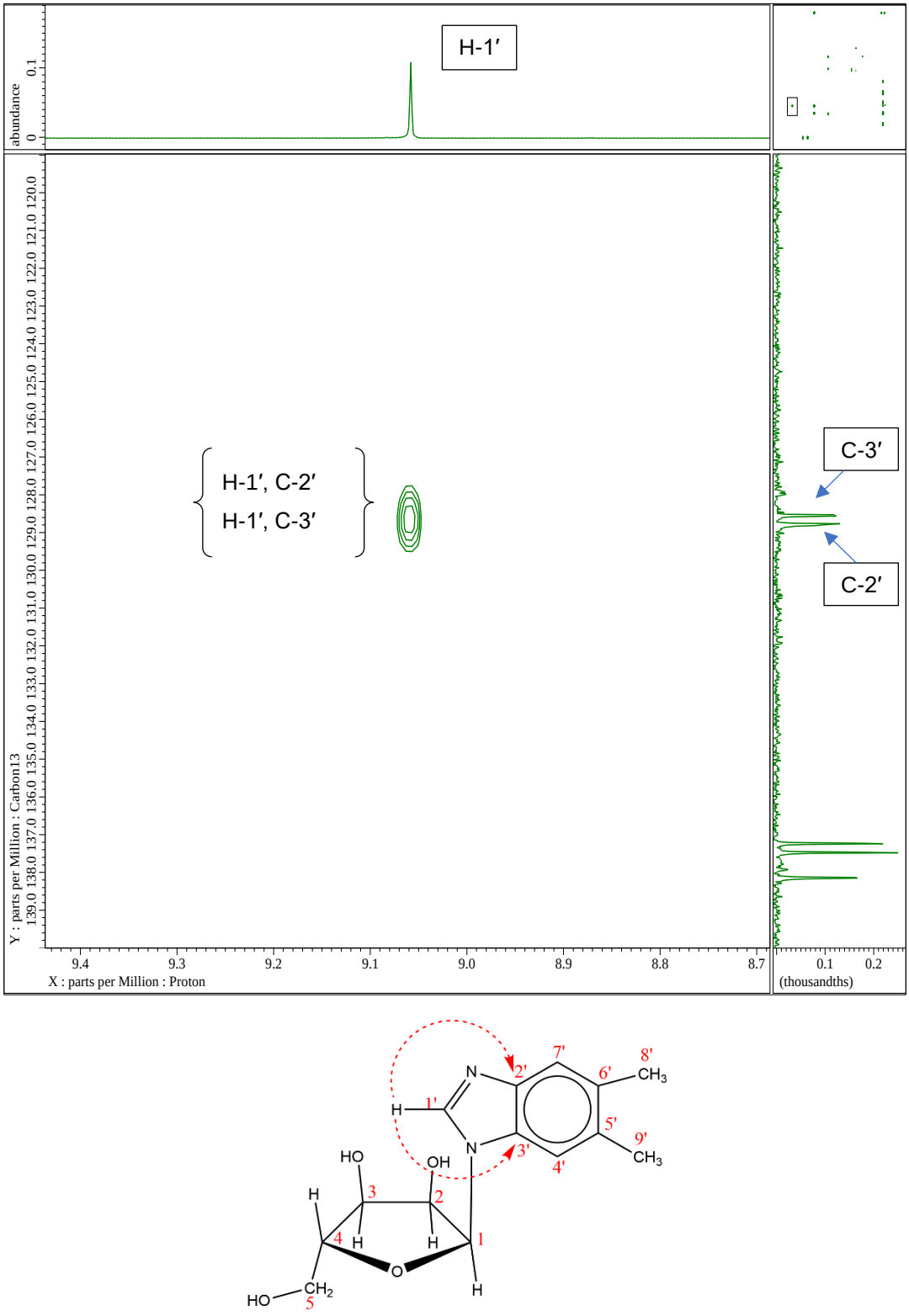


Figure 3.20. Zoom-in ^{13}C-^{1}H HMBC spectrum of α-ribazole, featuring the overlapped correlation of H-1′ with C-2′ and C-3′

From the result of the DEPT experiment, the ^{13}C signals at 137.18 and 137.39 ppm were carbons without protons attached, which matched to C-5′ and C-6′ since other possible candidates: C-2′ and C-3′, had been assigned already. Therefore, the C-5′ and C-6′ were assigned at 137.18 and 137.39 ppm. Extensive effort has been invested into distinguishing between C-5′ and C-6′. Theoretically in the ^{13}C-^{1}H HMBC experiments, the smaller the long-range coupling constant is, the longer bond ($^{3}J_{CH}$,$^{4}J_{CH}$ *even* $^{5}J_{CH}$) correlations would be observed. A series of altered long range coupling constants, (2, 5, 8, 10 and 16 Hz) for the ^{13}C-^{1}H HMBC experiments were conducted for an attempt to reveal the $^{5}J_{CH}$ correlations between H-1 and C-5′. Unfortunately, the outcome was negative as no such cross peak was observed.

A possible inference to distinguish C-5′ and C-6′ can be made on basis of the structural similarity of the pair of C-3′ and C-2′ to C-5′ and C-6′, respectively. In the ^{13}C-^{1}H HMBC spectrum (long rang coupling constant at 2 Hz), $^{3}J_{CH}$ correlations between H-4′ and C-2′, and between H-7′ and C-3′ were observed instead of the $^{2}J_{CH}$ correlations. If the same apply to H-4′, C-6′, and H-7′, C-5′ owing to the structural similarity, their $^{3}J_{CH}$ should be observed as shown in **Figure 3.21**. Therefore, the C-5′ and C-6′ were assigned at 137.18 and 137.39 nm, respectively. Heteronuclear 2 Bond Correlation (H2BC) experiment which designed to exclusively provide $^{2}J_{CH}$ correlations, was conducted attempting the separation of $^{2}J_{CH}$ and $^{3}J_{CH}$ correlations between H-8′, 9′ and C-5′, 6′. However, in H2BC experiment, correlations between protons and non-protonated carbons are absent due to the lack of H-H coupling. Because the quaternary carbon nature of C-5′, 6′, H2BC experiment was not able to offer extra information on the distinguishment of these two carbons and so the assignment had to rely on the inference above.

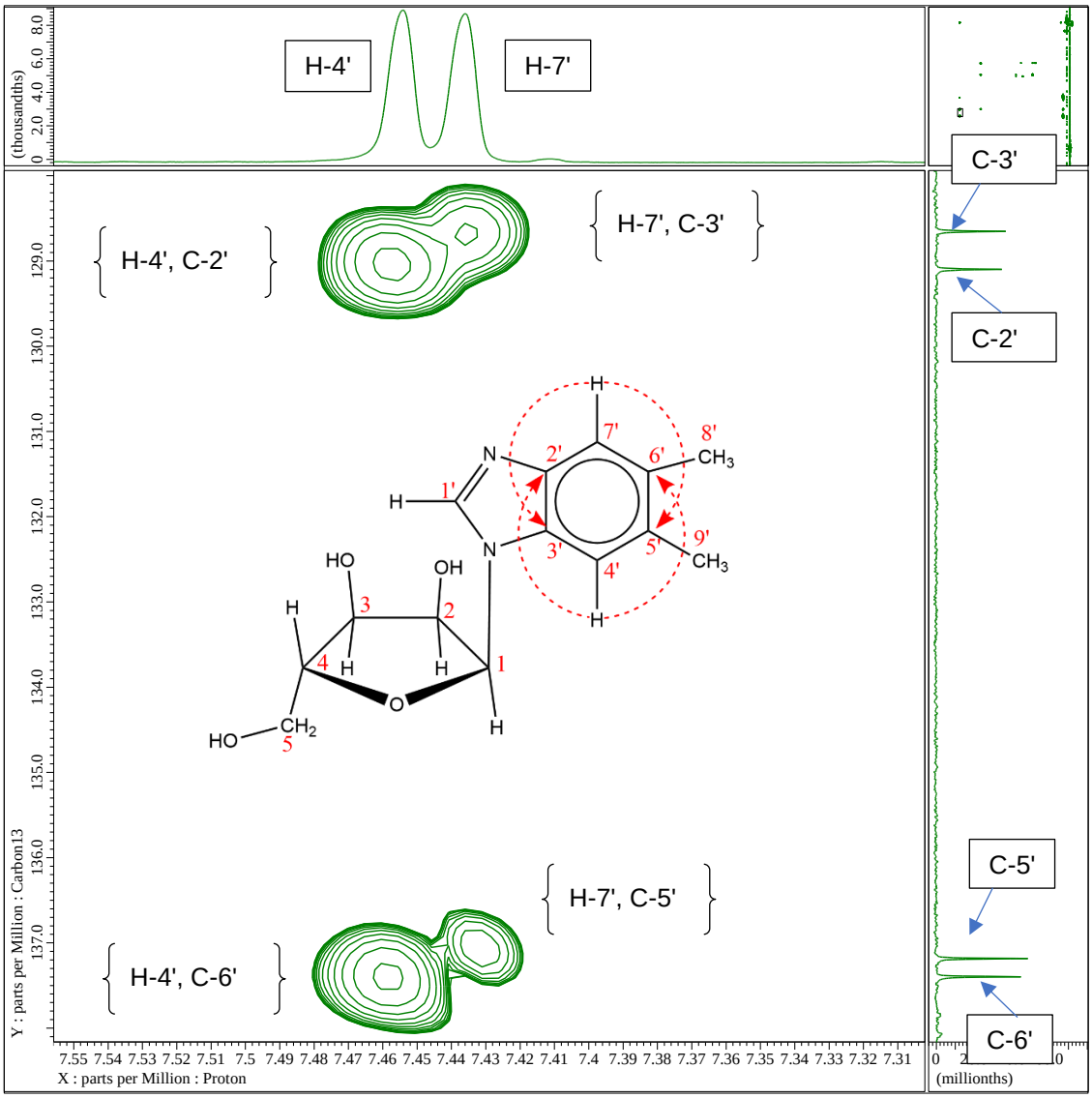


Figure 3.21. Zoom-in ^{13}C-^{1}H HMBC spectrum of α-ribazole, featuring the $^{3}J_{CH}$ correlations of H-4′ to C-2′ and C-6′, and H-7′ to C-3′ and C-5′

In **Figure 3.22**, C-4′ showed $^{3}J_{CH}$ coupling with H-9′ and C-7′ with H-8′, which allowed the distinction between H-8′ (2.26 ppm) and H-9′ (2.28 ppm). Furthermore, as expected, the $^{2}J_{CH}$ and $^{3}J_{CH}$ correlations between H-8′, 9′ and C-5′, 6′ were present.

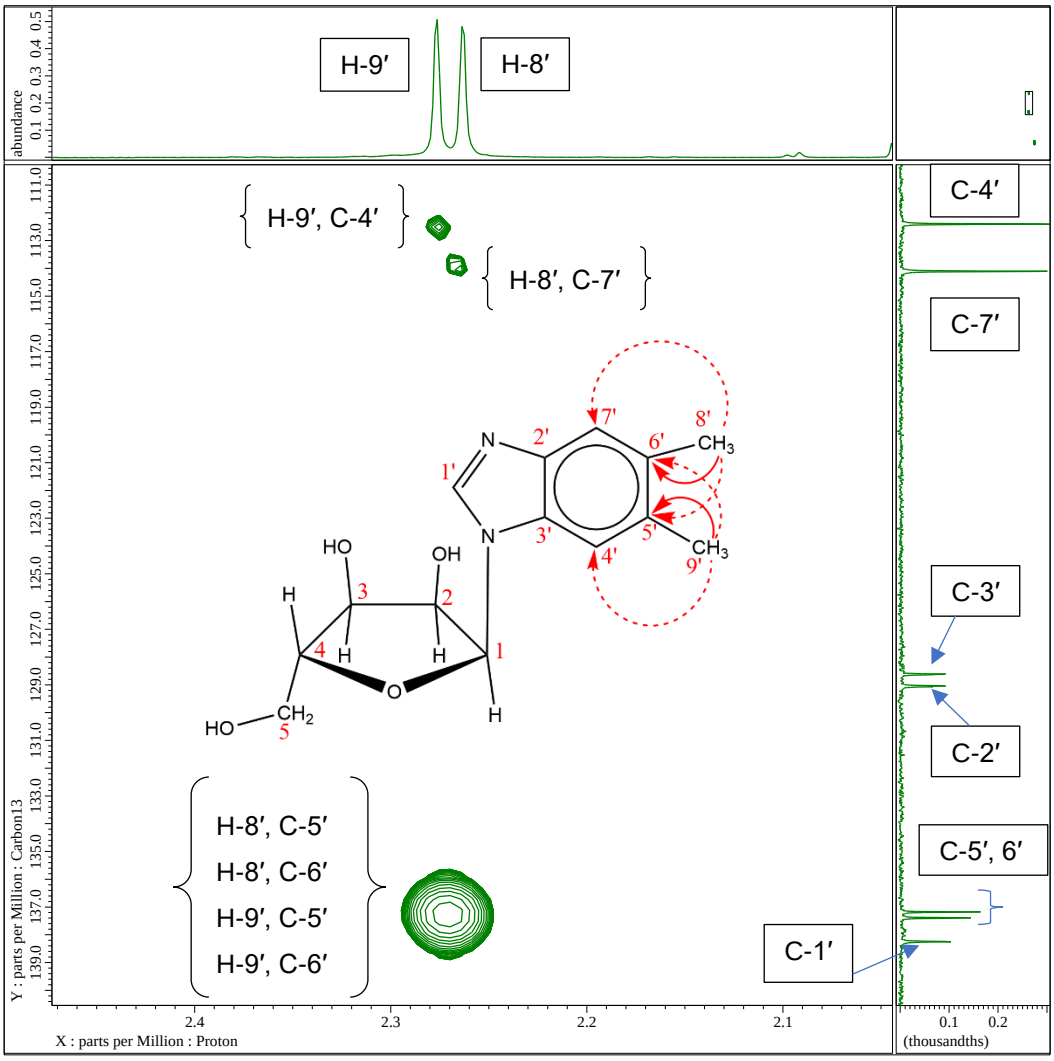


Figure 3.22. Zoom-in ^{13}C-^{1}H HMBC spectrum of α-ribazole, featuring the $^{3}J_{CH}$ correlations of H-8′ and H-9′ with C-7′ and C-4′ respectively and an overlapped $^{2}J_{CH}$ correlations of H-8′ with C-6′ and H-9′ with C-5′; $^{3}J_{CH}$ correlations of H-8′ with C-5′ and H-9′ with C-6′

The distinction between C-8′ and C-9′ could not be achieved using the HMQC spectrum due to strongly overlapped correlations. The chemical shift between the two carbons was close with a 0.19 ppm difference and only 0.02 ppm between H-8′ and H-9′. However, the ^{13}C-^{1}H HMBC spectrum offered the information that allowed the distinction between C-8′ and C-9′. In **Figure 3.23,** two cross peaks were observed between C-8′, C-9′ and H-4′, H-7′, since H-4′ and H-7′ have been assigned earlier, the cross peak under H-4′ should be the $^{3}J_{CH}$ correlation with C-9′ and unlikely to be a $^{4}J_{CH}$ correlation with C-8′. Likewise, the other cross peak was the $^{3}J_{CH}$ correlation between H-7′ and C-8′. Therefore, C-8′ was assigned at 19.52 ppm and C-9′ at 19.71 ppm.

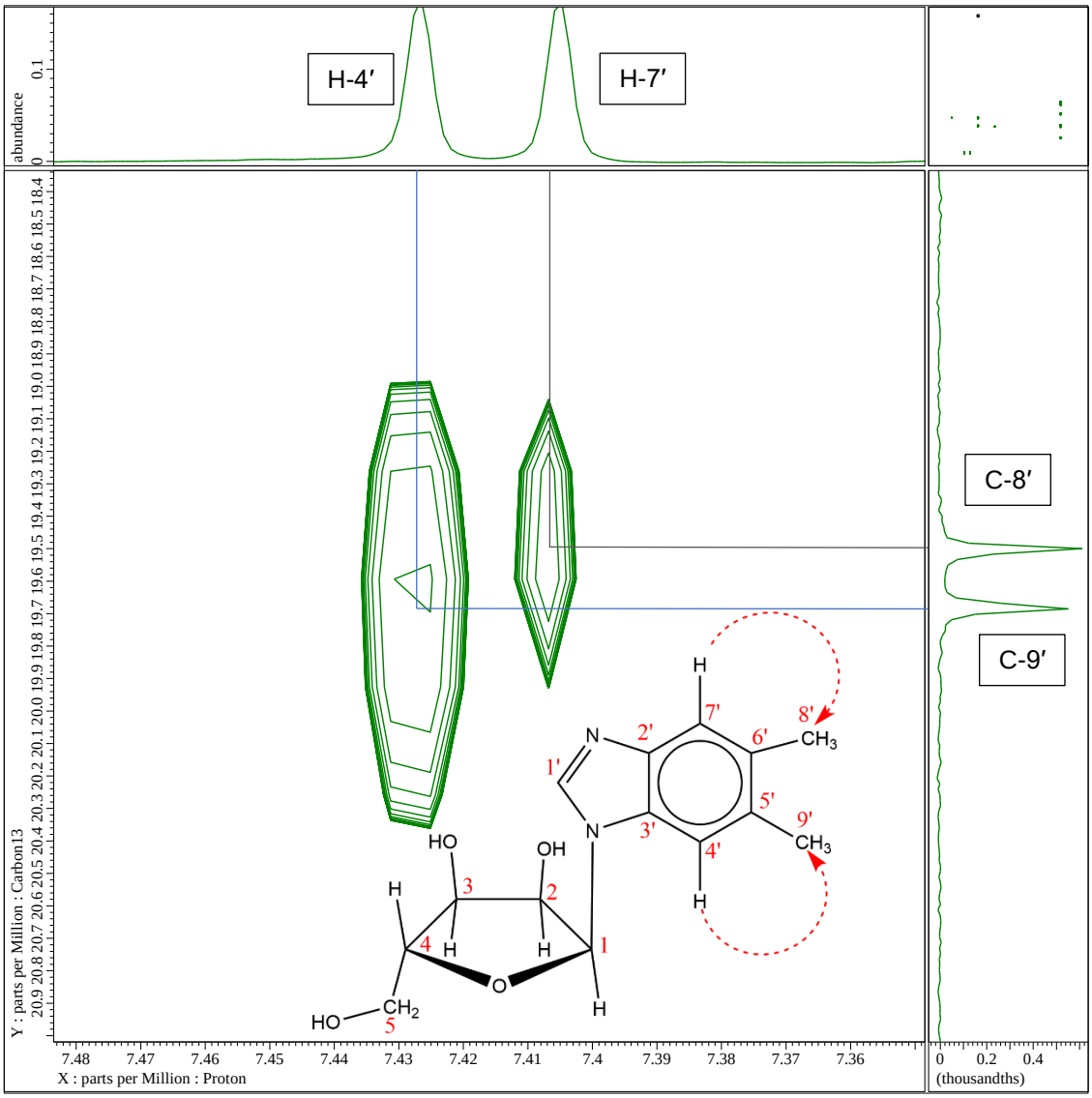


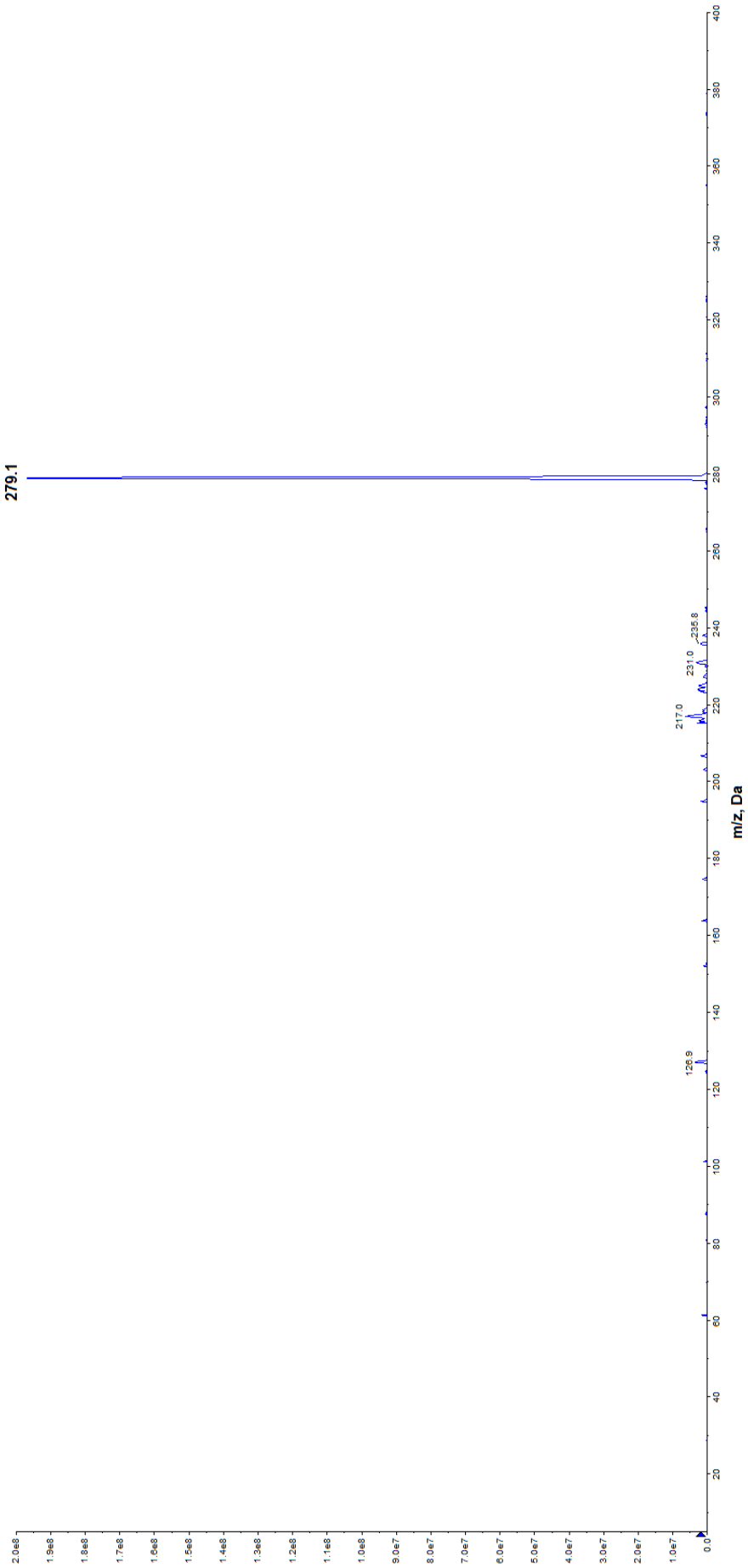
Figure 3.23. Zoom-in ^{13}C-^{1}H HMBC of α-ribazole, featuring the $^{3}J_{CH}$ correlations of H-4′ and H-7′ with C-9′ and C-8′

The NMR evidence described in this chapter is sufficient to prove the identity of the isolated α-ribazole including using ^{15}N-^{1}H HMBC experiments to confirm the presence and position of the nitrogen atoms in the DMB moiety. The full assignment of ^{13}C and ^{1}H NMR spectra was completed and the majority of the assignment matched with the work by Brown and Hakimi (1986) assigning cationic α-ribazole apart from the two methyl groups on the DMB moiety for which the literature reported an opposite assignment. The almost symmetrical structure on the DMB moiety made it very difficult to distinguish these two methyl groups using only 1D NMR. Brown and Hakimi assigned the α-ribazole structure using 1D NMR on a 200 MHz spectrometer employing chemical shift,

relative integrals, multiplicity and decoupling experiments and with only one inconsistency from this work; this was an extraordinary piece of work. Since then, NMR spectroscopy has significantly advanced with a higher magnetic field and more information offered by multiple dimensional NMR. To my best knowledge, the 2D NMR data for α-ribazole presented above is the first reported.

3.5.2 Identification by LC-MS

The isolated α-ribazole standard was subject to mass spectrometry for further confirmation. The existence of the peak of *m/z* = 279.1 in the Q1 scan was consistent with the $[M+H]^+$ ion for α-ribazole (MW = 278.3), **Figure 3.24**; 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, **Figure 3.25**. The absence of ions around *m/z* = 358 in the Q1 scan under positive and negative modes (**Figure 3.26**) indicated that the isolated product did not contain α-ribazole-phosphate, which supports the presence of mass peaks *m/z* = 279.1 and *m/z* = 147.1 resulting from α-ribazole only.


279.1
236.8
231.0
217.0
126.9
m/z, Da

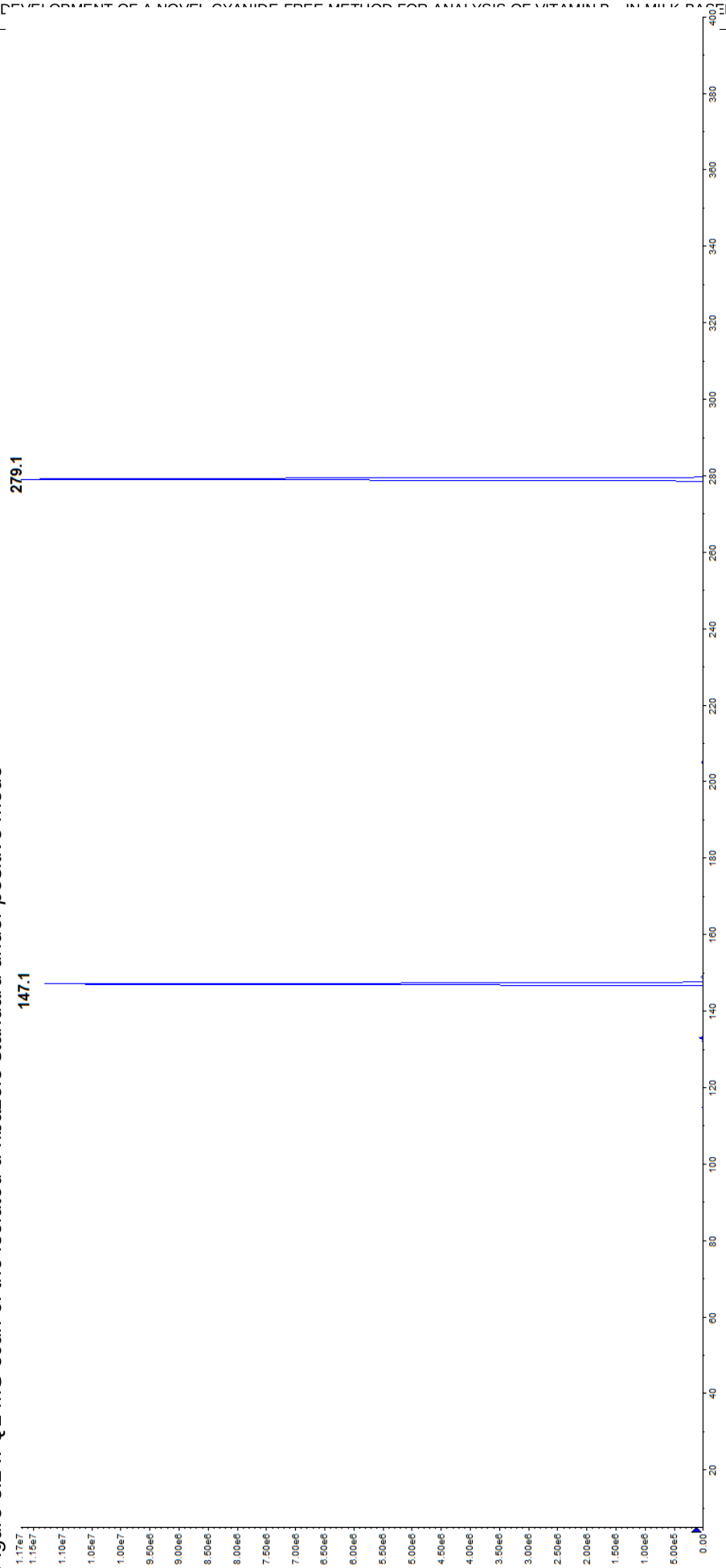


Figure 3.24. Q1 MS scan of the isolated α-ribazole standard under positive mode

Figure 3.25. Product ion scan of the isolated α-ribazole standard under positive mode

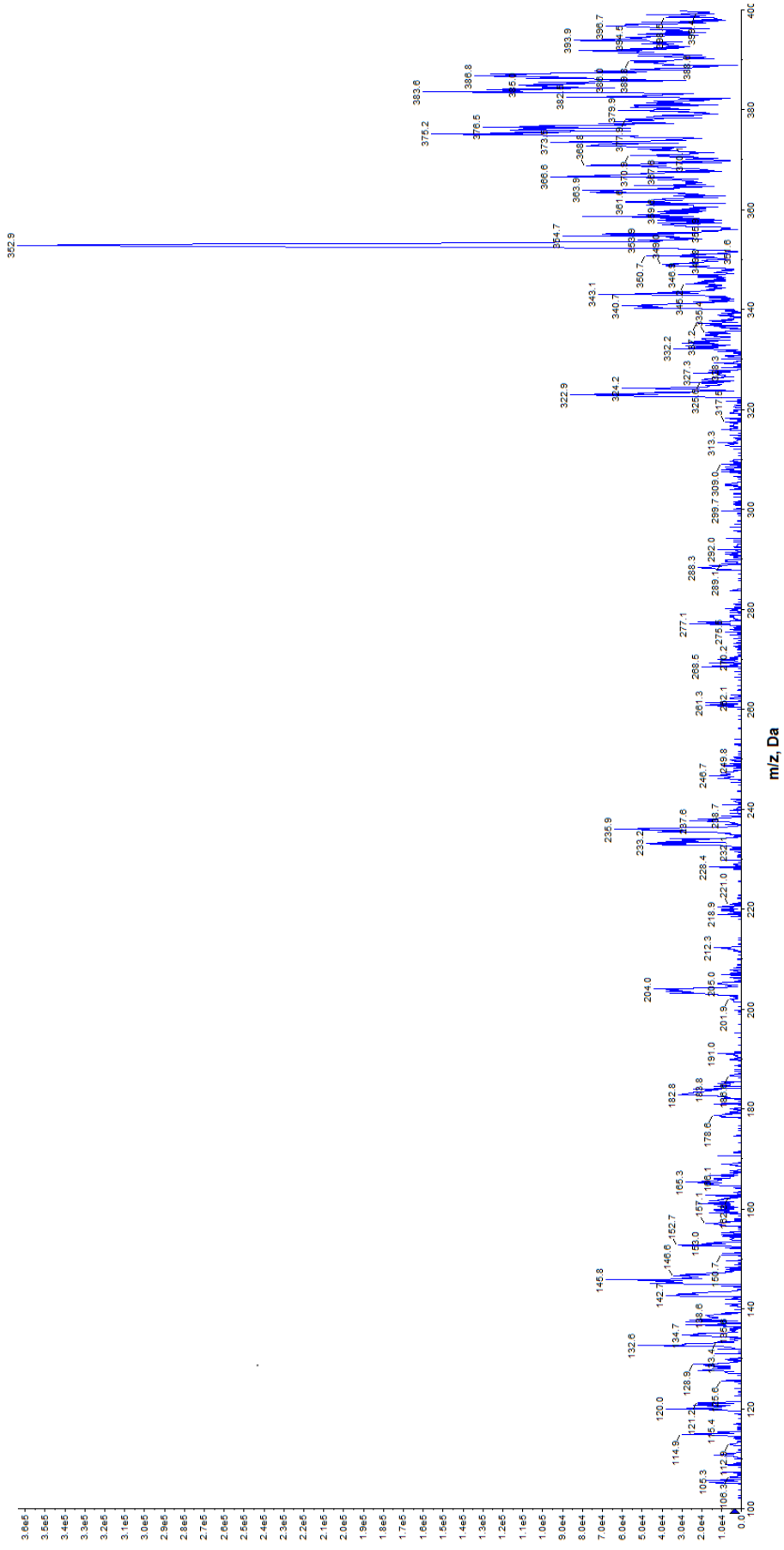


Figure 3.26. Q1 MS scan of the isolated α-ribazole standard under negative mode .

3.5.3 Quantitation by Absorption

The yield of α-ribazole was determined by its concentration in DMSO by Beer-Lambert law. The molar absorption coefficient of DMB at 280 nm (ε_{280}) was calculated from a series of DMB solutions with known concentrations in DMSO. The DMB was a commercially available standard with 99% purity. The average calculated ε_{280} of DMB in DMSO was 6136 mol^{-1} L cm^{-1} while values of 6027 and 6090 mol^{-1} L cm^{-1} have also been reported (Gray and Escalante-Semerena, 2010; Mattes and Escalante-Semerena, 2018).

The stock α-ribazole solution was diluted by a 25-dilution factor and the absorbance measured at 280 nm by a UV/Vis spectrometer. The measurement procedure was repeated after 6 and 18 months to check the stability under the storage condition (−18 °C). The result is shown in **Table 3.4**.

Table 3.4. Yield of α-ribazole by measuring the absorbance

Months	A_{280}	ε (mol^{-1} L cm^{-1})	l (cm)	c (mg mL^{-1})	D.F.	c_o (mg mL^{-1})
0	0.6969			0.0316		0.7902
6	0.7056	6136	1	0.0320	25	0.8001
18	0.6924			0.0314		0.7851
Average c_o (mg mL^{-1})		0.7918		RSD%		0.78

ε: Molar absorption coefficient in DMSO at 280 nm
l: Length of the light path, 1 cm
A280: Absorbance at 280 nm
c: The concentration of the measured α-ribazole solution
DF: Dilution factor
co: The concentration of α-ribazole stock solution

3.6 Summary

Overall, the combination of NMR and MS confirmed that the product of isolation was α-ribazole and is acceptable as a standard for the HPLC analysis method development. The concentration of the yielded α-ribazole standard solution was quantitated by absorption. In conclusion, the isolation of an α-ribazole standard was achieved using a method described in this chapter.

4 Optimisation of the Isolation of α-Ribazole from Cyanocobalamin

The isolation of α-ribazole from cyanocobalamin (CN-Cbl) was modified and optimised from a starting point based on the method of Mattes and Escalante-Semerena (2018). In this chapter, various parameters throughout the isolation process were investigated to determine optimal conditions, including the choice of reagents, concentration, temperature and treatment time in the chemical and enzymatic hydrolysis steps and boronate affinity gel clean-up procedure. As a result, a 76% yield was obtained using the optimal conditions, which improved upon the previously reported yield of 67% (Mattes and Escalante-Semerena, 2018).

4.1 Experimental

4.1.1 Materials and General Methods

All materials, solution preparation and general methods are stated in **Chapter 2.**

4.1.2 RP-HPLC-FLD System

The chromatographic system and fluorescence detector condition were described in **Section 2.4**. The elution was achieved with a gradient procedure, **Table 4.1**. The flow rate was 0.8 mL min^{-1}, and column temperature was 30 °C,

Table 4.1. Gradient procedure of RP-HPLC-FLD staring with 40% of mobile phase B

Time (min)	Mobile phase %A[a]	Mobile phase %B[b]
0	60	40
20	20	80
25	20	80
26	60	40
31	60	40

[a] Mobile phase A = ammonia aqueous solution, 0.3% *v:v*, pH 9.5

[b] Mobile phase B = 100% methanol

4.1.3 Standard Solutions

Cyanocobalamin standard solution and α-ribazole standard solution were prepared as described in **Section 2.5**. An external standard calibration with a linear regression method was used to calculate α-ribazole concentrations in samples.

4.1.4 Chemical Hydrolysis

To isolate the effects of varying conditions during the optimisation experiments, only the chemical hydrolysis step was modified and the conditions in the enzymatic dephosphorylation step remained constant. **Figure 4.1** shows the experimental process involved in the optimisation for chemical hydrolysis step (conducted in duplicates), in which CN-Cbl standard (40 ng)* was used and produced α-ribazole; the latter being measured by RP-HPLC-FLD.

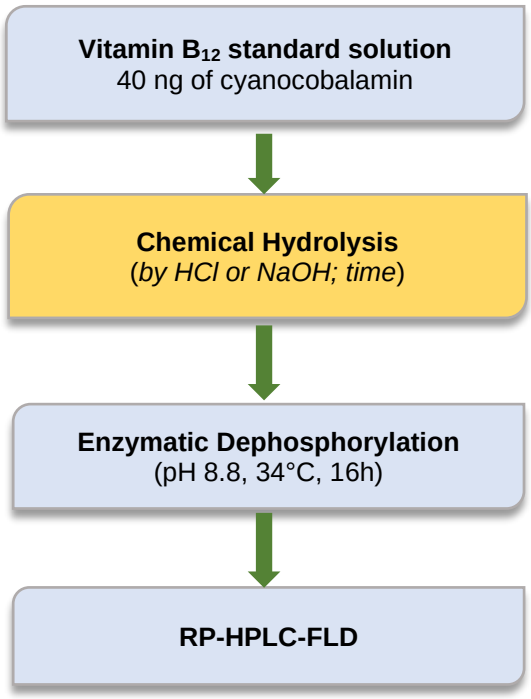


Figure 4.1. *Process chart for the optimisation of the chemical hydrolysis step*

* Since it was not economical to conduct the optimisation experiments at a 50-mg level, the 40-ng level was chosen which matches the vitamin B_{12} level in infant formula samples.

4.1.4.1 UV/Vis Profile of Cyanocobalamin and Hydrolysate

A solution of CN-Cbl (50 mg) fully dissolved in water (5 mL) was prepared. A control solution was prepared by diluting aliquots (5 µL) of the CN-Cbl solution to 5 mL with 3 mM sodium chloride solution, which ensured the same background. The rest of the CN-Cbl underwent chemical hydrolysis by sodium hydroxide (3 M, 5 mL) and neutralisation by hydrochloric acid (3 M, 5 mL), an aliquot (10 µL) of the resultant mixture was taken and diluted with water to 3.33 mL; this dilution factor ensured a similar level of CN-Cbl to the control group. An absorption scan of both solutions was conducted on a Cary 300 UV/Vis spectrometer (Agilent Technologies, Santa Clara, CA) with a wavelength range from 280 nm to 600 nm. Absorbance was measured in quartz cuvettes. Data was processed using Cary WinUV software (version 4.20).

4.1.4.2 Acid and Base Hydrolysis

Vitamin B_{12} standard solution (1.0 µg mL^{-1}, 40 µL) was added into a 15 mL centrifuge tube. Then hydrochloric acid or sodium hydroxide solution (1.0 mL) with various concentrations was added to each tube. The heating treatment was performed at 100 °C for 120 min. After cooling, the hydrolysate was neutralised by adding a corresponding concentration of sodium hydroxide or hydrochloric acid (1.0 mL). Subsequently, incubation buffer (2.0 mL) and alkaline phosphatase (ALP) stock solution (0.05 mL) were added to the mixture, which was incubated (34 °C, 16 hours). The resultant solution was analysed by RP-HPLC-FLD.

4.1.4.3 Optimising the Concentration of Acid

Vitamin B_{12} standard solution (1.0 µg mL^{-1}, 40 µL) was added into a 10 mL glass vial (plastic tubes were not used due to possible degradation under harsh acidic conditions). In addition, hydrochloric acid solution (1.0 mL) with various concentrations from 0.1 M to 4.0 M was added to each vial. The heating treatment was performed at 100 °C for 60 min. After cooling, the hydrolysate was neutralised and buffered to the appropriate pH for the subsequent ALP hydrolysis. The resultant solution was analysed by RP-HPLC-FLD.

4.1.4.4 Optimisation of Heating Duration

Vitamin B_{12} standard solution (1.0 µg mL^{-1}, 40 µL) was added to a 10 mL glass vial. Then hydrochloric acid solution (1.5 M, 1.0 mL) was added. The heating treatment was performed at 100 °C for times between 15 and 120 min. After cooling, the hydrolysate was neutralised and buffered to the appropriate pH for the subsequent ALP hydrolysis. The resultant solution was analysed by RP-HPLC-FLD.

4.1.5 Enzymatic Dephosphorylation

Enzymatic dephosphorylation step was investigated whilst retaining the optimised conditions of chemical hydrolysis step constant. **Figure 4.2** illustrates the process from the starting point (CN-Cbl) to α-ribazole detected by RP-HPLC-FLD.

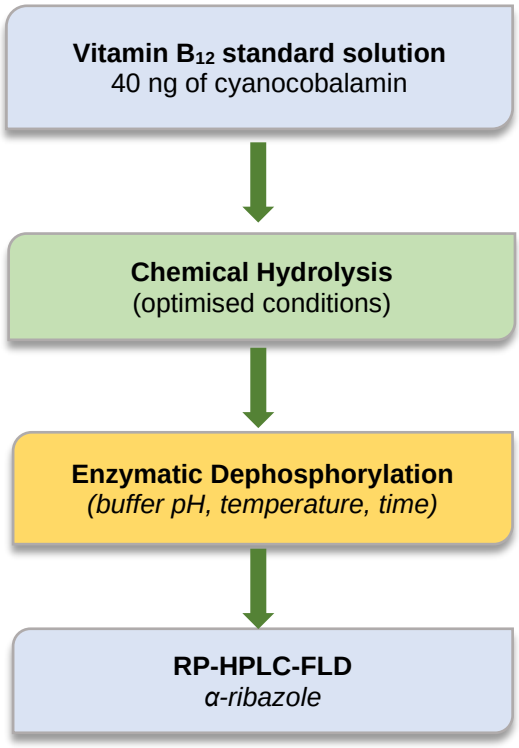


Figure 4.2. Process chart of the investigation experiment to optimise enzymatic dephosphorylation

4.1.5.1 Effect of Incubation Buffer

Vitamin B_{12} standard solution (1.0 µg mL^{-1}, 40 µL) underwent chemical hydrolysis by heating with hydrochloric acid (1.5 M, 1.0 mL) at 100 °C for

45 min, then cooling down in an ice bath for 30 min before being neutralised with sodium hydroxide (1.5 M, 1.0 mL) and buffered by incubating buffer (pH = 8, 8.8 or 9.8). ALP solution (50 µL, > 445 U mL^{-1}) was added to the solution, which was incubated (16 hours, 34 °C). Finally, the resultant solution was filtered using a syringe filter (0.45 µm PTFE membrane) ready for the RP-HPLC-FLD analysis.

4.1.5.2 Effect of Varying Incubation Time

Vitamin B_{12} standard solution (1.0 µg mL^{-1}, 40 µL) underwent acid hydrolysis as described above. ALP solution (50 µL, > 445 U mL^{-1}) was added to the solution, which was incubated for 0.5, 1, 3 and 16 hours at 34 °C. The resultant solutions were filtered using a syringe filter (0.45 µm PTFE membrane) ready for the RP-HPLC-FLD analysis.

4.1.5.3 Effect of Incubation Temperature

Vitamin B_{12} standard solution (1.0 µg mL^{-1}, 40 µL) underwent acid hydrolysis as described above. ALP solution (50 µL, >445 U mL^{-1}) was added to the solution, which was incubated for 16 hours at 34 or 37 °C. Finally, the solution was filtered using a syringe filter (0.45 µm PTFE membrane) ready for the RP-HPLC-FLD analysis.

4.1.6 Separation and Concentration of α-Ribazole

Isolated α-ribazole from **Chapter 3** was used as start material for the optimisation of the separation and concentration step using boronate affinity chromatography (BAC). **Figure 4.3** shows the steps involved in the optimisation experiments.

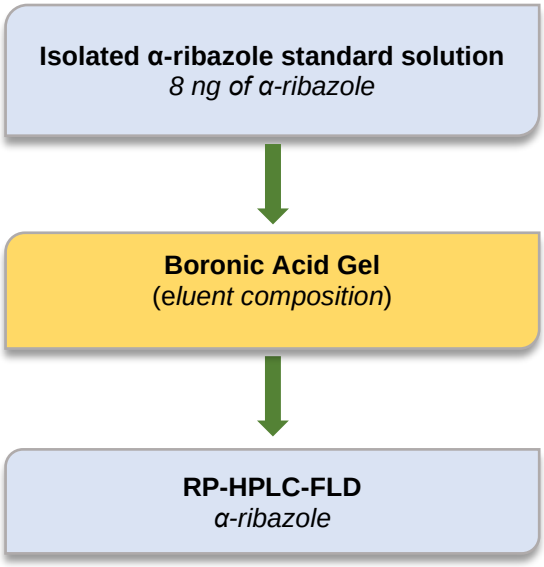


Figure 4.3. Process chart of the optimisation experiment for separation and concentration step using boronate affinity chromatography

4.1.6.1 Effect of Varying the Eluent

A known amount of α-ribazole in incubation buffer (pH 8.8) was loaded onto the boronic acid gel column (0.5 mL) conditioned by the binding buffer (ammonium acetate, 0.3 M, pH 8.8). Subsequently, the column was capped and mixed by hand (30 s) then allowed to stand (15 min) with regular hand-mixing for 30 s every 5 min to ensure complete contact with the gel. The sample was discharged from the column by gravity flow-through, and the gel was washed with binding buffer (3 mL). Finally, the gel was eluted by allowing formic acid (0.1 M or 1.0 M) without or with an organic modifier (methanol or acetonitrile) to flow through the gel.

4.1.7 Statistical Analysis

Analysis of variance (ANOVA) single factor test or *t*-test was performed to examine the existence of significant differences among the data groups at critical value $\alpha = 0.05$. The null hypothesis was that there was no significant difference among the data groups. The statistical analysis was conducted using Microsoft Excel software (version 2303).

4.2 Results and Discussion

Cyanocobalamin underwent the isolation procedure shown in **Figure 4.4**, which highlights the three main steps: chemical hydrolysis, enzymatic dephosphorylation and separation were optimised in this work.

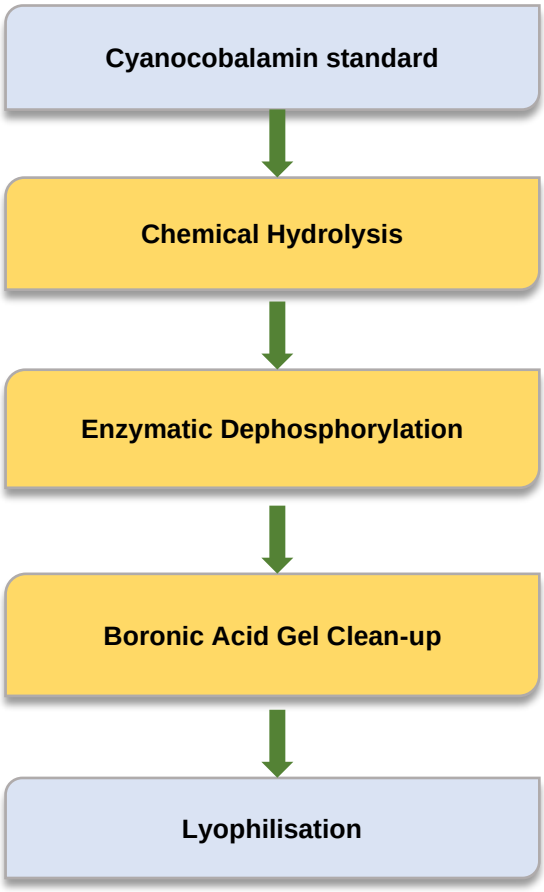


Figure 4.4. Isolation scheme of α-ribazole from cyanocobalamin

4.2.1 RP-HPLC-FLD Analysis

α-Ribazole analysis was conducted using RP-HPLC-FLD; the resultant chromatogram of an α-ribazole standard is shown in **Figure 4.5.**

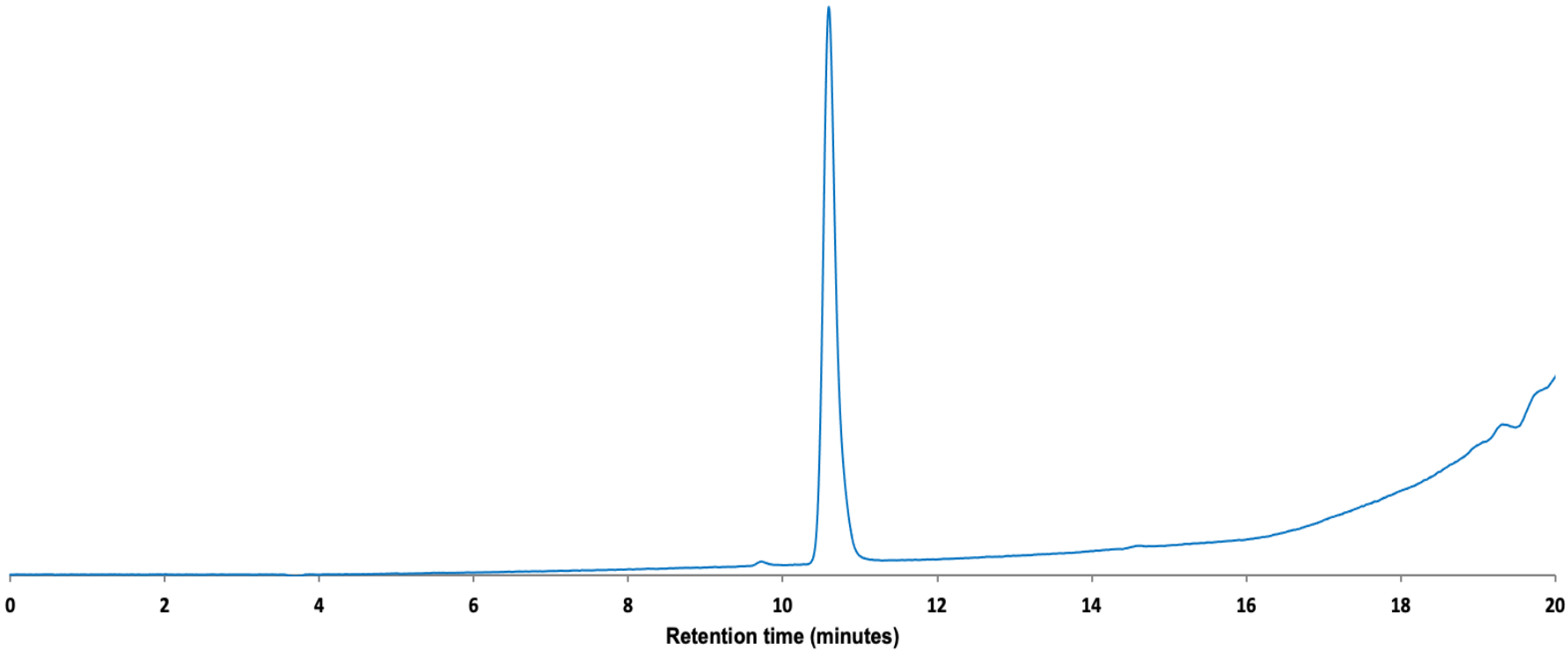


Figure 4.5. RP-HPLC-FLD Chromatogram of α-ribazole standard isolated from cyanocobalamin

4.2.2 Optimisation of Chemical Hydrolysis

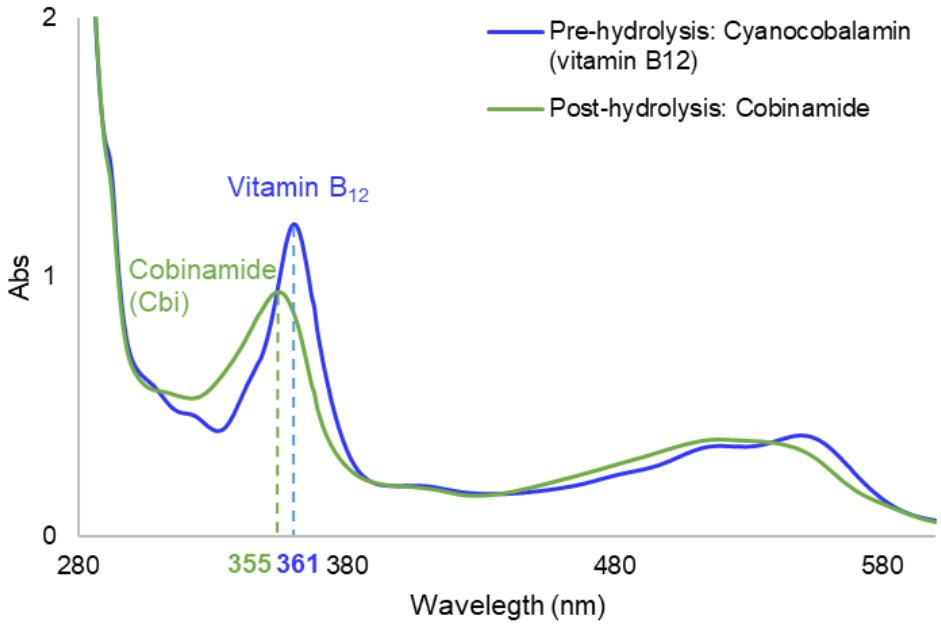
To achieve a maximum α-ribazole yield, acidic hydrolysis using hydrochloric acid was explored; reaction duration and concentration of hydrochloric acid were investigated in this experiment. Several chemical hydrolysis procedures for the CN-Cbl have been reported in the literature. The method has evolved based upon a method developed by Pakin *et al.* (2005), with modifications made to the hydrolysis procedure, including the method by Mattes and Escalante-Semerena (2018). In the original method (Pakin *et al.*, 2005), the heat treatment was conducted at 100 °C for 15 min in sodium hydroxide (2.5 M). Mattes and Escalante-Semerena (2018) employed the same alkaline hydrolysis method using sodium hydroxide (2.5 M) at 80–90 °C for 75 min. **Table 4.2** shows the modifications the latter applied based upon the method by Pakin *el al*. The starting conditions for this study used the parameters from Mattes and Escalante-Semerena (2018).

Table 4.2. Comparison of the chemical hydrolysis procedure between Pakin et al. (2005) and Mattes and Escalante-Semerena (2018)

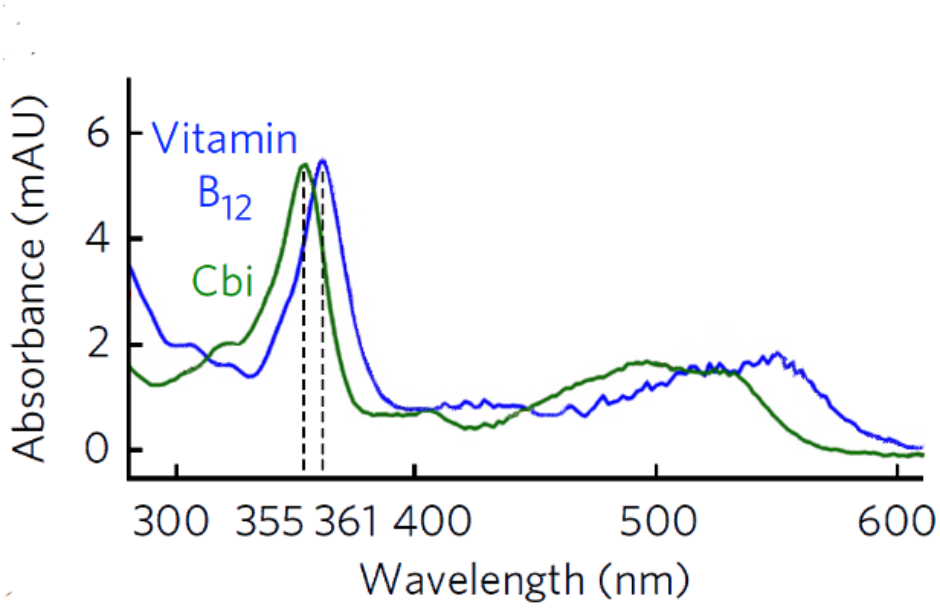
Parameters	Method by Pakin *et al.* (2005)	Method by Mattes and Escalante-Semerena (2018)
Concentration of sodium hydroxide (mol L^{-1})	2.5	2.5
Treatment time (min)	15	70
Temperature (°C)	100	85–90
Vitamin B_{12} amount	50 ng	50 mg

To confirm the effect of the chemical hydrolysis on CN-Cbl, the UV/Vis profiles of the CN-Cbl and post-hydrolysis solution were compared. The post-hydrolysis mixture contained cobinamide (Cbi), the hydrolysed product of CN-Cbl without the α-ribazole-phosphate group. Importantly, Cbi has a different UV/Vis profile from CN-Cbl. **Figure 4.6** shows indirectly that applying chemical hydrolysis to CN-Cbl releases the α-ribazole-phosphate. A hypochromic effect was observed as the λmax of CN-Cbl (361 nm) was shifted to 355 nm due to the decrease of

conjugation in the structure when α-ribazole-phosphate detached from the corrin ring and centred cobalt ion led to Cbi.



(a)



(b)

Figure 4.6. The UV/Vis spectra comparison of vitamin B_{12} and cobinamide from 280–600 nm between this work and literature

4.2.2.1 Comparison of Acid and Alkaline Hydrolysis

The results showed that, with the exception of the 4.0 M concentration, acidic hydrolysis produced more α-ribazole than the alkaline method, under the same conditions of time and temperature**.** The yield for acid hydrolysis reached a maximum (95.5%*) at 1.50 M hydrochloric acid and declined with further addition of acid (**Figure 4.7**). On the other hand, the yield from the alkaline method reached a maximum of 86.7%* at the 2.0 M concentration level and then decreased with higher concentrations, which was not aligned with the conclusions of Pakin *et al.* (2005), who claimed that alkaline hydrolysis produced three-fold higher levels of α-ribazole phosphate than acidic hydrolysis, with identical conditions: 100 °C, 5 min, hydrochloric acid or sodium hydroxide (2.5 M).

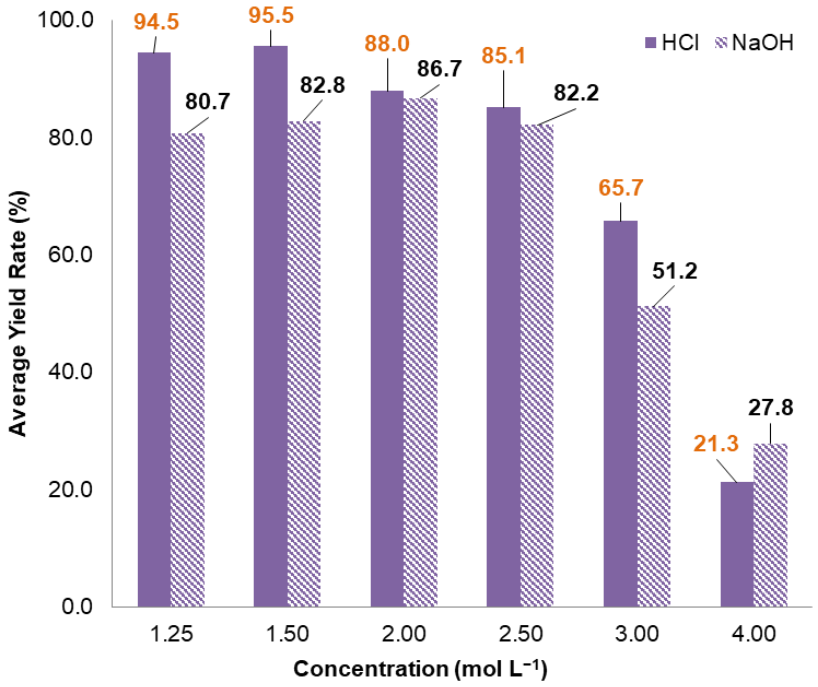


Figure 4.7. Comparison between acidic and alkaline hydrolysis on the yield of α-ribazole

The hydrolysis step of α-ribazole cleavage from the CN-Cbl main structure involves two processes: **a)** the base-off process, that is the breaking of the coordination bond between the *N* of the DMB and cobalt ion (blue line in **Figure**

* The mean value of duplicates.

4.8); and **b)** bond cleavage between the phosphate group of the ribazole moiety and the 1-amino-2-propanol group, which is connected to the corrin ring by an amide group and which is itself subsequently released, although more slowly (Armitage *et al.*, 1953) (red line in **Figure 4.8**).

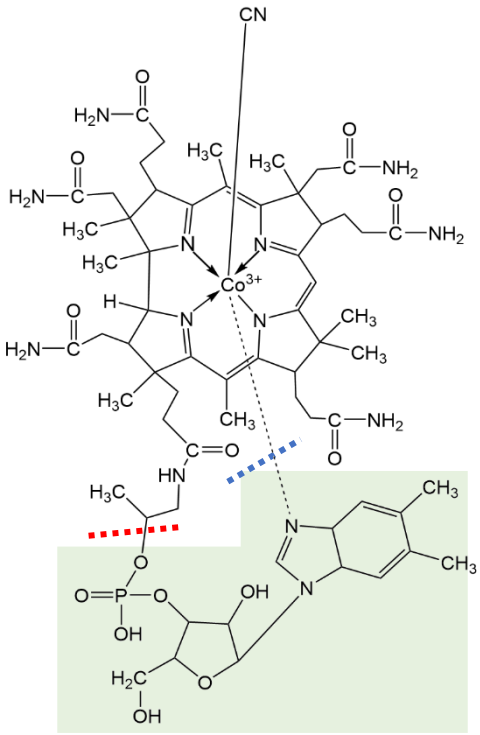
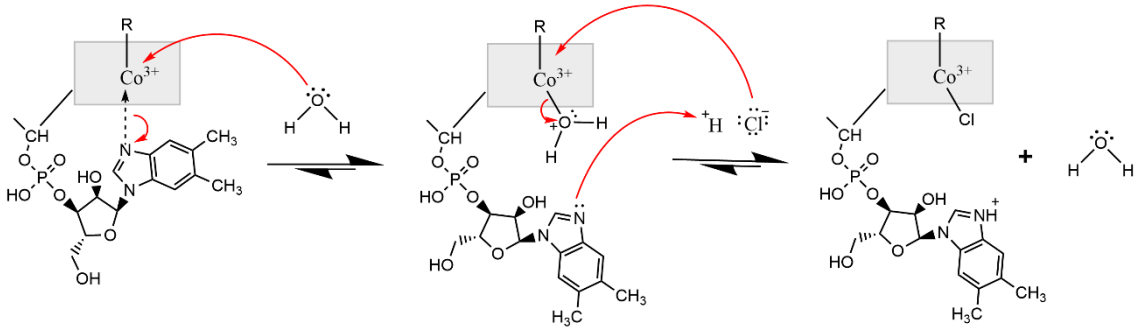


Figure 4.8. The bond cleavage illustration of α-ribazole-phosphate

The cleavage of the coordination bond between cobalt and the DMB can be achieved by both acid and base hydrolysis through different mechanisms. The acid hydrolysis can be conceived as displacement of the base ligand by water followed by the protonation of the DMB portion of the α-ribazole (**Scheme 4.1**). This scheme incorporates the base-on-base-off mechanism for the first step described by Brown *et al.* (1984). This reaction is a pseudo first order reaction as the water concentration is in excess and does not determine the reaction rate.

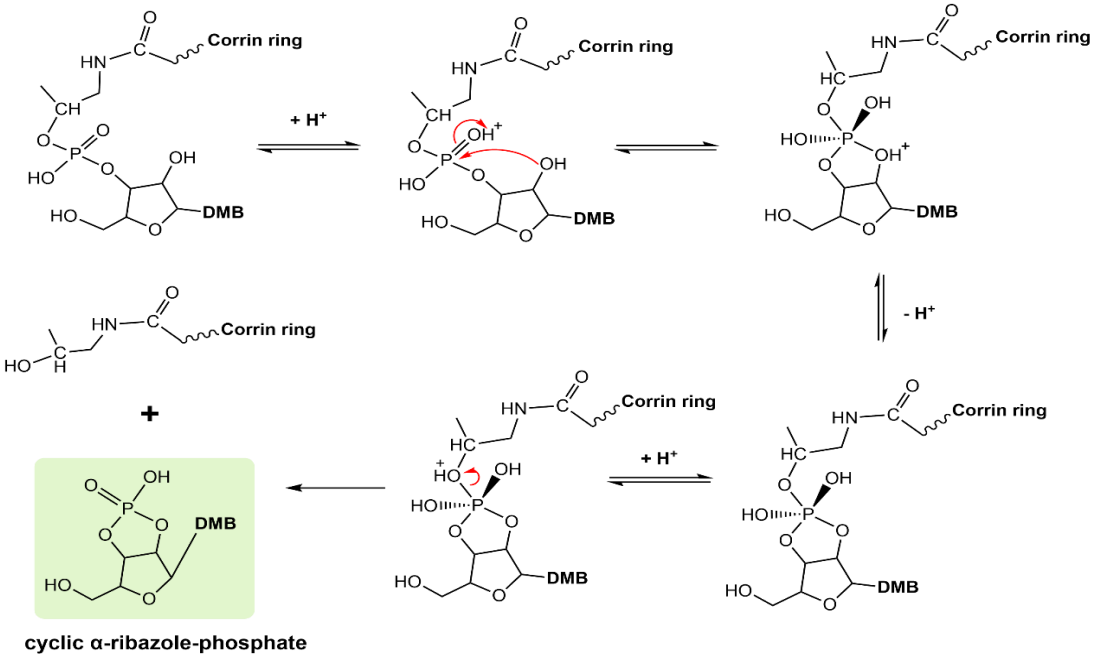


Scheme 4.1. Acid hydrolysis scheme for liberation of 5,6-dimethylbenzimidazole end from CN Cbl

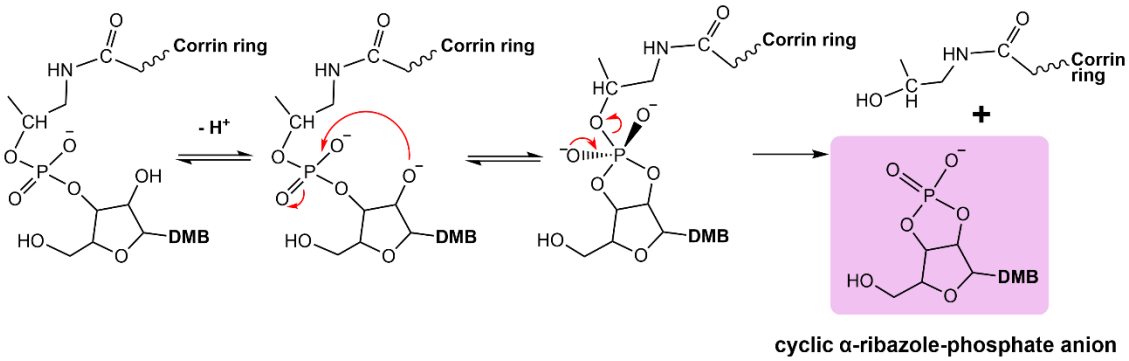
Base hydrolysis of cobalt complexes can occur by an S_N1cB or S_N2 mechanism (Pearson *et al.*, 1960; Roecker, 2008). Despite disagreement about the exact mechanism, base hydrolysis of cobalt complexes often takes place faster than the corresponding acid hydrolysis Baran *et al.* (2002). This lends support to the claim by Pakin *et al.* (2005) that using sodium hydroxide produced larger amounts of α-ribazole than use of hydrochloric acid, since acid hydrolysis is a first order reaction in cobalt complexes whereas base hydrolysis is second order. Thus, the initial reaction rate of the latter is higher. However, the experiment of Pakin *et al.* (2005) was conducted only for 5 min and no data was presented for acid hydrolysis after 5 min, therefore, the higher initial reaction rate might not necessarily suggest a higher total yield, as in the current comparison study, the hydrolyses by both acid and base run for 120 min.

Armitage *et al.* (1953) reported that the scission between the phosphate and aminopropyl group occurred before the liberation of the aminopropanol group from the corrin ring, which indicates that the bond between the phosphate group and aminopropanol is more sensitive to the hydrolysis reaction than the amide linkage within aminopropanol. Clark *et al.* (1954) reported the same finding after comparing the hydrolytic products of vitamin B_{12} and D, L-1-amino-2-propanol *O*-phosphate. The same hydrolysis method cleaves 1-amino-2-propanol from vitamin B_{12} but not from the latter, which suggests breaking of the linkage between phosphate group and aminopropanol in vitamin b_{12} was preferred. Since the linkages between phosphate group and aminopropanol break first, the resulted D, L-1-amino-2-propanol *O*-phosphate will not produce 1-amino-2-propanol as Clark *et al.* (1954) found. In vitamin B_{12}, the 1-amino

2-propanol group is linked to the ribose group through the phosphate by a diester bond. Generally, the phosphodiester is insensitive to hydrolysis compared to its phosphomonoester, for example, a single phosphodiester linkage in DNA has a theoretical half-life of approximately 30 million years at 25 °C (Mikkola *et al.*, 2018). In contrast, the hydrolysis of a phosphodiester bond in RNA is more readily accomplished, thanks to the presence of the 2′-hydroxyl group in the ribose, which acts as an intramolecular nucleophile and allows the occurrence of transesterification leading to a 2′, 3′-cyclic phosphate and cleavage of the 5′-linkage. This process can be catalysed by strong acid and base (Li and Breaker, 1999). The phosphodiester bond in the CN-Cbl resembles that in the RNA, and possible acid and base catalysed hydrolysis process are illustrated in **Scheme 4.2** and **Scheme 4.3**, respectively, based upon the mechanism described for RNA hydrolysis (Jarvinen *et al.*, 1991; Mikkola *et al.*, 2018; Oivanen *et al.*, 1998). The formed cyclic α-ribazole-phosphate would be further hydrolysed to either α-ribazole-2′-phosphate or α-ribazole-3′-phosphate.



Scheme 4.2. Acid hydrolysis process of phosphodiester linkage in cyanocobalamin



Scheme 4.3. Base hydrolysis process of phosphodiester linkage in cyanocobalamin

It is worth noting that there is another potential cyclisation pathway, similar to the enzymatic hydrolysis of adenosine monophosphate (AMP) and guanosine monophosphate (GMP) to form adenosine and guanosine. In this pathway, AMP and GMP undergoes a C3′-C5′ intermediate to form a 3′, 5′-cyclic AMP and GMP, respectively. However, it remains unknown whether CN-Cbl would undergo such a process under highly acidic or basic conditions until structural evidence of the intermediate emerges. For example, using ^{31}P, ^{13}C and ^{1}H NMR spectroscopy to determine the precise cyclisation structure of the intermediate.

Nevertheless, both mechanisms promote the cleavage of the α-ribazole-phosphate by a S_N2i reaction (Li and Breaker, 1999) and no evidence reports whether acid or base catalysis is more rapid. Based on the results in current study, acidic hydrolysis was chosen for further investigation.

4.2.2.2 Effect of Concentration of Hydrochloric Acid

Vitamin B_{12} standard solution (1.0 µg mL^{-1}, 40 µL) was added into a 10 mL glass vial (plastic tubes were not used due to possible degradation under harsh acidic conditions). In addition, hydrochloric acid solution (1.0 mL) with various concentrations from 0.1 M to 4.0 M was added to each vial. The heat treatment was performed at 100 °C for 60 min. After cooling, the hydrolysate was neutralised and buffered to the appropriate pH for the subsequent ALP hydrolysis. The resultant solution was analysed by RP-HPLC-FLD.

When the concentration of hydrochloric acid reached 0.5 M, the yield increased significantly from 14.5% (0.1 M), 67.7% (0.25 M) to 85.0% (**Figure 4.9**). With

the continued increase of concentration, between 1.0 M and 1.5 M hydrochloric acid, the highest yields of α–ribazole were recorded, 89.8%–93.1%, no significant difference was found between 1.0 M and 1.25 M concentrations, 1.5 M showed higher yield results and appeared as the optimal concentration of hydrochloric acid.

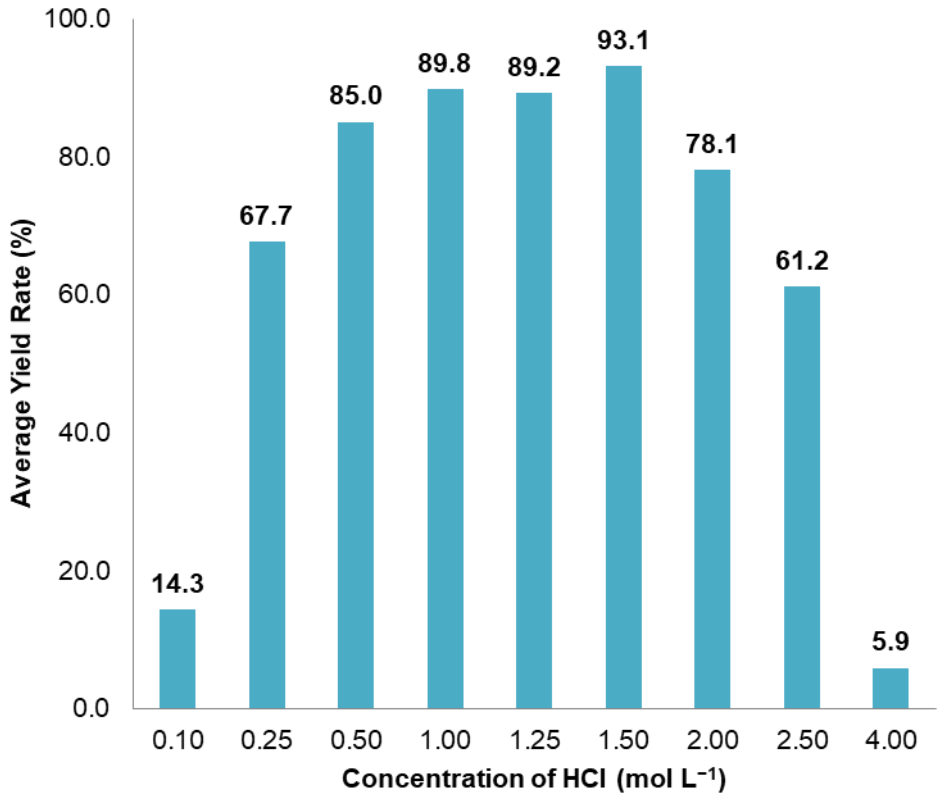


Figure 4.9. The effects of various hydrochloric acid concentrations on the yield of α–ribazole

4.2.2.3 Effect of Heating Duration

The results in **Figure 4.10** showed that even 15 min of heating under such conditions can produce an 85.5% yield. Furthermore, prolonged heating from 30 to 90 min increased the yield of α-ribazole to 91.2–92.3%; when heating for 120 min, a slight decrease to 88.6% was observed.

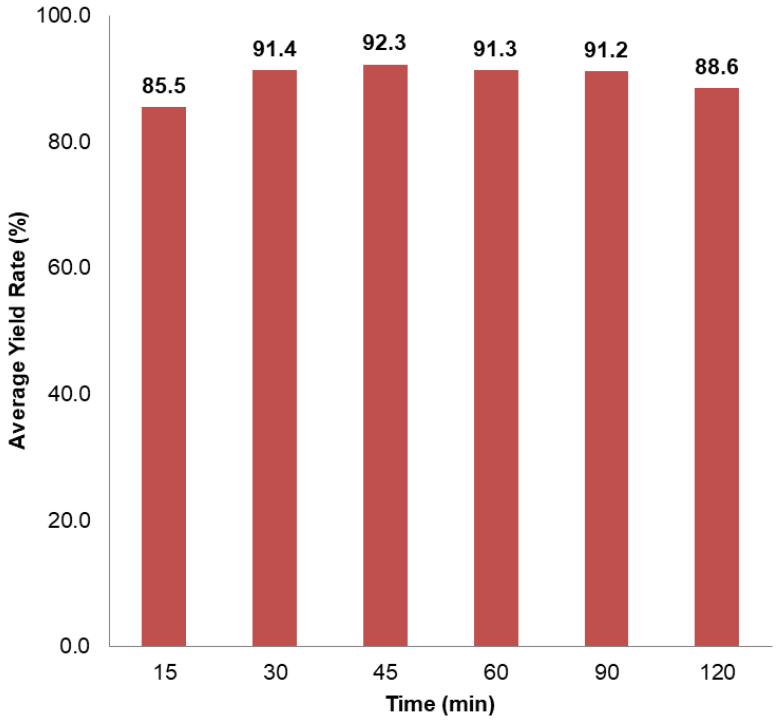


Figure 4.10. The yield of α-ribazole from acidic hydrolysis of vitamin B_{12} by 1.5 M hydrochloric acid for different heating durations.

The results from an ANOVA single factor test (α = 0.05, *p* = 0.42) showed there was no statistical difference in the average yield among heating durations 30 min to 120 min.

In summary, considering the scale-up during the final isolation procedure, heating with hydrochloric acid (1.5 M) for 120 min was chosen for the maximum release of α-ribazole from vitamin B_{12} standard. After the chemical hydrolysis using the optimised parameters and dephosphorylation, the hydrolysate was made up to 50 mL with incubation buffer (ammonium acetate, 0.3 M, pH = 8.8). Then an aliquot of the hydrolysate was diluted by mobile phase A and measured by RP-HPLC-FLD. The amount of α-ribazole released pre-clean-up in this work was compared with the literature method by Mattes and Escalante-Semerena (2018) (**Table 4.3)** and a higher yield was achieved by applying the optimised conditions.

Table 4.3. Comparison of the α-ribazole yield from optimised hydrolysis steps in this work and the literature method

	Theoretical yield of α-ribazole (mg) from CN-Cbl[a]	Actual yield of α-ribazole (mg)	Percentage yield (%)
This work	10.25	9.3	90.7
		9.2	89.8
Literature method[b]	10.4	8.4	80.8
		8.0	77.2

[a] Approximately 50 mg was used by Mattes and Escalante-Semerena (2018)

[b] Method by Mattes and Escalante-Semerena (2018)

4.2.3 Optimisation of Enzymatic Dephosphorylation

The final production of α-ribazole was achieved by applying ALP to the solution resulting from the acidic hydrolysis step. During the investigation, more than 22 units of ALP were used for hydrolysis 40 ng of CN-Cbl. According to the unit definition from the manufacturer, 1 unit will hydrolyse one µmol of 4-nitrophenyl phosphate per minute at pH = 9.8 at 37 °C. 40 ng of CN-Cbl is equivalent to 3×10^{-5} µmol 4-nitrophenyl phosphate, hence, 3×10^{-5} units of ALP required for one minute. Therefore, the quantity of ALP (> 22 units) used was sufficient to dephosphorylate all the CN-Cbl added.

In the final α-ribazole standard production, 50 mg of CN-Cbl is equivalent to 37 µmol of 4-nitrophenyl phosphate. To ensure the sufficiency of ALP, 90 units of ALP was used. In addition, the prolonged incubation time reinforce the dephosphorylation. Mattes and Escalante-Semerena (2018) used more than 1000 units of ALP for same quantity of CN-Cbl and no reason was given for its necessity. Based on the theoretic requirements, the amount of ALP used in this work (> 90 units) is appropriate on both performance and economic level.

4.2.3.1 Effect of pH of the Incubation Buffer

In the literature describing the use of ALP enzyme on α-ribazole-phosphate, the incubation buffer has pH = 8 (Pakin *et al.*, 2005) or pH = 8.8 (Mattes and Escalante-Semerena, 2018), while the manufacturer of the ALP enzyme states

that the activity was determined at pH = 9.8 (Sigma-Aldrich). Therefore, these three levels of pH were chosen for optimisation.

In **Figure 4.11**, the results showed that the highest yield of α-ribazole was obtained under pH = 8.8 condition at 91.6%, and pH = 8.0 produced a close yield of 88.3%, while at pH = 9.8, the yield declined to 64.8%. The incomplete conversion suggested less enzyme activity at pH = 9.8, even though pH = 9.8 was used by the manufacturer to record activity. Therefore, pH = 8.8 was chosen as the optimised pH condition for this work.

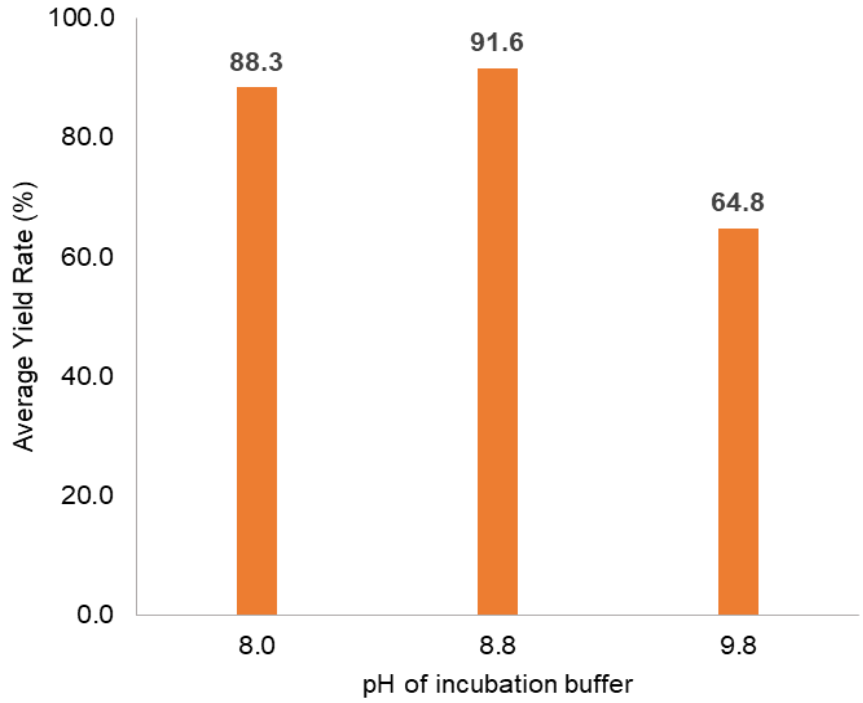


Figure 4.11. The yield of α-ribazole from acidic hydrolysis of vitamin B_{12} by 1.5 M hydrochloric acid at 100 °C for 45 min and incubated overnight at incubation buffer pH = 8.0, 8.8 and 9.8.

4.2.3.2 Effect of Incubation Time

The results of different incubation times showed the average yield reached to 87.1% after 0.5 hours and 92.4% for 16 hours period, **Figure 4.12**. The results from an ANOVA: single factor test indicated that there was no significant difference among the various incubation periods (α = 0.05, *p* = 0.09).

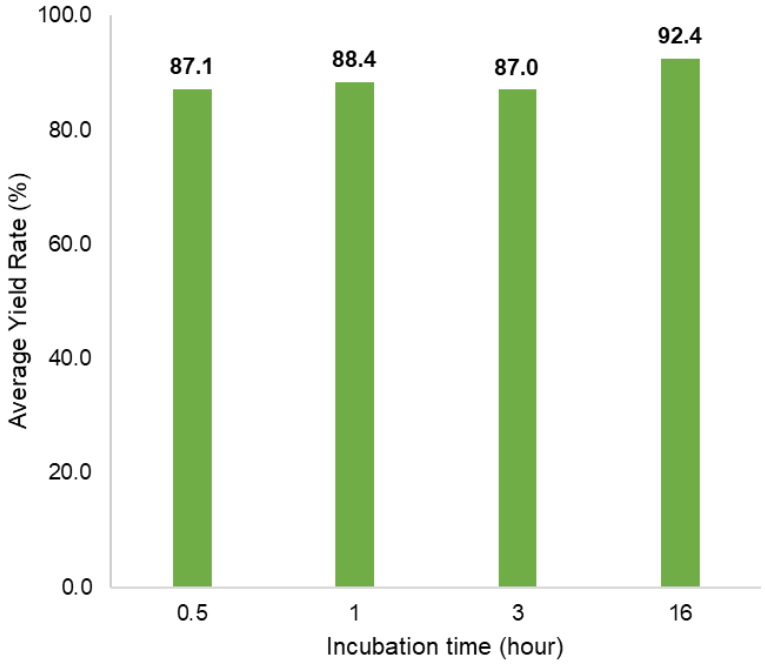


Figure 4.12. The yield of α-ribazole from different alkaline phosphatase incubation time.

The incubation time for ALP from the literature (Mattes and Escalante-Semerena, 2018) was 16 hours (overnight), which is longer than necessary according to the results above. However, for this work, 16 hours was also chosen for practical reasons, as the entire sample preparation requires more than one day to finish, and an overnight incubation is preferred and no loss was observed with a longer time.

4.2.3.3 Effect of Incubation Temperature

According to the manufacturer, the incubation temperature for ALP was chosen at 37 °C. However, there is not a universal temperature labelled as optimal in literature. Common choices include but are not limited to 25, 30 and 37 °C (McComb *et al.*, 1979). Although 37 °C was particularly favoured for its closeness to mammalian body temperature, there is no evidence support such application of ALP from non-mammalian origin. McComb *et al.* (1979) reported that an assay temperature of 37 °C held no special significance. Other temperatures can be used if they can be accurately maintained and cause no denaturation of the enzyme before the rection completes. In this study, a temperature of 34 °C was chosen due to the equipment's availability. To

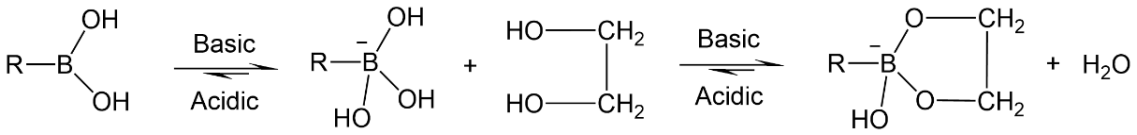
demonstrate this temperature fits the purpose, a comparison test was conducted between temperature of 34 and 37 °C. The average results with two incubation temperatures, 34 and 37 °C are close with 0.5% difference (**Table 4.4**). Therefore, 34 °C could be used without comprising the efficiency.

Table 4.4. Comparison between incubation temperatures

Incubation temperatures (°C)	Duplicate number	Yield of α-ribazole (%)	Mean (%)
34	1	91.1	90.7
	2	90.2	
37	1	88.7	90.2
	2	91.6	

4.2.4 Separation and Concentration of α-Ribazole

Boronic acids as ligands for affinity chromatography has been used to separate nucleoside, nucleotides, nucleic acid, carbohydrates, glycoproteins and other *cis*-diol containing compounds (Liu and Scouten, 2000). This technique was developed based on the interaction between boronic acid ligands and *cis*-diol groups. The interaction, esterification, results in a cyclic diester which is either five-membered (with 1,2 *cis*-diol) or six-membered (with 1,3 *cis*-diol); the 1,2 *cis*-diol groups give the strongest ester bond, whereas the six-membered cyclic esters are less stable (Nishiyabu *et al.*, 2011). As in 1,2 *cis*-diols, the two hydroxyl groups are on adjacent carbon atoms and hence an approximately coplanar configuration forms, which can be rapidly captured by the boronic acid under basic conditions in aqueous solutions. In base, the boronic acid is hydroxylated and transformed from trigonal coplanar to an anion with tetrahedral configuration (Espina-Benitez *et al.*, 2017a; Liu and Scouten, 2000), which can coordinate the *cis*-diol. The process is shown in **Scheme 4.4**. This reaction is reversable by lowering the pH as the neutral cyclic esters that are formed, are not stable and can be hydrolysed in aqueous solution, hence, the retained *cis*-diols can be released from the boronic acids by lowering pH (Liu and Scouten, 2000; Peters, 2014).



Scheme 4.4. The reversible reaction scheme between boronic acid ligand and 1,2-cis diol under basic and acidic condition.

α-Ribazole is a 1,2 *cis*-diol, that is it contains two adjacent hydroxyl groups in the 2′,3′ positions of the ribose ring, which are on the same face of the furanose ring (**Figure 4.13**). Therefore, α-ribazole can form a cyclic ester with the boronic acid ligand under alkaline conditions and consequently be held on the BAC column and separated from contaminants. After the hydrolysis, α-ribazole is the only *cis*-diol compound in the solution, which decreases the chance of impurity in the final product.

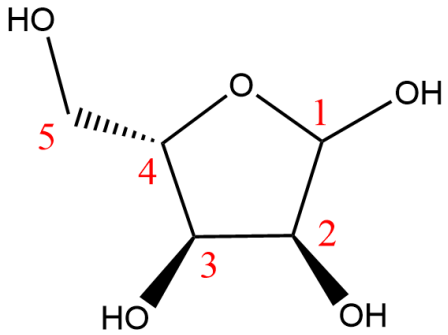


Figure 4.13. Structure of ribofuranose

While the binding between *cis*-diols and boronic acid is the main interaction in the BAC column, there are four secondary interactions: hydrophobic interaction, ionic interaction, hydrogen bonding and coordination interactions, which can affect the retention of the targeted *cis*-diols. A low content of organic modifier, such as methanol or acetonitrile, can be introduced into the BAC column to limit the hydrophobic interactions.

4.2.4.1 Hydrophobic Interactions

Phenyboronic acid is most common ligand employed in BAC. Due to the presence of a phenyl ring, there are potential aromatic π-π interactions involved when the analytes flow through the column as well as hydrophobic interactions with the solid support. These interactions can cause non-specific retention of

undesired compounds. Although there are efforts towards development of novel boronate ligands, in practice, a low ionic strength of the mobile phase (e.g., binding buffer) can limit hydrophobic interactions. A level of 0.05 mol/L is commonly recommended (Liu, 2006). Furthermore, adding a low percentage (maximum 40%) of methanol into the binding solutions can also supress unwanted absorption (Espina-Benitez *et al.*, 2017b).

4.2.4.2 Ionic Interactions

Under alkaline condition, the boronate presents as the anion form and the negative charge can give rise to electrostatic attractions or repulsion with charged analytes. Ionic interactions may cause undesired effects. In such cases, ionic strength of the mobile phase ought to be kept at a high level, which, however, can result in the increase of hydrophobic interaction. Therefore, generally a compromise in the range from 0.05 to 0.5 mol L^{-1} is preferred in order to suppress both interactions (Liu and Scouten, 2000).

4.2.4.3 Hydrogen Bonding

The hydroxyl groups of boronic acid have potential to form hydrogen bonds. Usually this effect is considered negligible, however, under special circumstances it can become the main interaction to cause retention and separation (Liu, 2006). Akparov and Stepanov (1978) found that the hydroxyl groups of boronic acid served as a hydrogen bond acceptor with serine proteinases during the enzyme purification.

4.2.4.4 Coordination Interactions

Boronic acid presents as trigonal configuration under neutral conditions, since the boron atom is sp^2 hybridised and this leaves an empty orbital, which is a potential electron receptor and might lead to coordination interactions. In the presence of electrons donators, such as amines, the boron atom transits into the tetrahedral configuration which is considered as the more active structure and enhances the interaction with *cis*-diols. However, compounds such as tris(hydroxymethyl)aminomethane and ethanolamine have a hydroxyl group adjacent to the amine group and can themselves interact with boronic acid and

reduce the capacity for the retention of *cis*-diols. Therefore, mobile phases containing these compounds should be avoided in BAC (Liu, 2006; Liu and Scouten, 2000).

The primary interaction and secondary interactions mentioned above mean that there are several factors to affect the retention function in BAC: the *cis*-diol configuration of the analytes; other functional groups within the analytes; the pK_a of the boronic acid immobilized on the column; the pH, ionic strength and the other competitive *cis*-diols in the binding buffer (Westmark *et al.*, 1994).

4.2.5 The Capacity of Boronic Acid Gel

According to the manufacturer, the boronic acid gel has 110 μmol adenosine monophosphate (AMP) binding capacity per 1 mL of resin at pH = 8.8. Since there is one *cis*-diol group in the AMP, theoretically, the gel should have the same binding capacity for α-ribazole. 50.0 mg of CN-Cbl can produce 10.3 mg (36.89 μmol) of α-ribazole at 100% recovery. The calculation process is shown in **Equation 4.1** . In the experiment, 1.5 mL of the resin has sufficient capacity (165 μmol) to retain the α-ribazole produced.

$$m_{\alpha\text{-}R} = n \times M_{\alpha\text{-}R} = \frac{m_{CNCbl}}{M_{CNCbl}} \times M_{\alpha\text{-}R} \qquad \textit{Equation 4.1}$$

$$= \frac{\left(\frac{50.0\ mg}{1000}\right) g}{1355.4\ g\ mol^{-1}} \times 278.3\ g\ mol^{-1}$$

$$= 36.89 \times 10^{-6}\ mol \times 278.3\ g\ mol^{-1} = 0.0103\ g = 10.3\ mg$$

$m_{\alpha\text{-}R}$: Mass of α-ribazole

m_{CNCbl}: Mass of CN-Cbl, 50.0 mg

$M_{\alpha\text{-}R}$: The molar mass of α-ribazole, 278.3 g mol^{-1}

M_{CNCbl}: The molar mass of CN-Cbl, 1355.4 g mol^{-1}

n Number of moles of CN-Cbl used, which, as the reaction is 1:1, equates to the number of moles of α-ribazole, 36.89×10^{-6} mol = 36.89 μmol

4.2.5.1 Effect of the pH of Sample

The retention mechanism between a *cis*-diol and boronic acid primarily depends on the pH of the medium. The boronate affinity gel used in this experiment has ligands of 3-aminophenylboronic acid with a pK_a value of 8.8; when the pH is equal to or greater than the pK_a value, binding with *cis*-diols occurs (Liu and He, 2017). According to the manufacturer, the recommended binding buffer was ammonium acetate (0.2 M, pH 8.8) and the sample needed to be adjusted to 8.5–9.0 and diluted with binding buffer at a 1:1 ratio. In the ALP incubation, the pH of the hydrolysate was adjusted by adding the incubating buffer to about pH 8.8, which met the requirements. Although a pH higher than the pK_a (8.8) would possibly result in more binding capacity, it requires an extra adjustment prior to the loading step and when pH = pK_a, at 50% capacity level, it still has sufficiently enough capacity to bind the loaded α-ribazole Therefore, no further change was needed. Moreover, the electrostatic effects of the cations in the binding buffer can stabilise the negatively charged boronic acid complexes. Being the most effective, divalent cations, such as Mg^{2+}, have been used to enhance the retention of a boronate affinity column; magnesium chloride is commonly included in the binding buffer (Tuytten *et al.*, 2007; Uziel *et al.*, 1976). Since the incubating buffer contained magnesium chloride, no further Mg^{2+} was added to the sample.

4.2.5.2 Effect of the Eluent

To optimise the elution step, the acidity of formic acid, the required volume and the choice of organic modifier were investigated to ensure complete recovery of bound α-ribazole from the boronic acid gel.

Formic acid (0.1 M), hydrochloric acid (25 mM) and sorbitol (0.2 M) were recommended by the manufacturer of the boronic acid gel. Formic acid and hydrochloric acid cause the *cis*-diols to elute by lowering the pH and breaking the ring complex formed between the boronic acid ligand and α-ribazole, while sorbitol acts as a competitive *cis*-diol compound and replaces the bound α-ribazole. To ensure the boronic acid gel can be regenerated and reused, lowering the pH is the preferred method than introducing other *cis*-diols such as sorbitol. Moreover, since the eluate would be subject to nitrogen blow-down

evaporation for further concentration, a weaker acid like formic acid was favoured over hydrochloric acid, which was also used by the literature (Mattes and Escalante-Semerena, 2018).

The recovery of α-ribazole using 0.1 M formic acid, 4 mL (the eluent was added sequentially and each eluate portion was analysed individually), was 61.2% (**Figure 4.14**). Lowering the pH with 1.0 M formic acid (4 mL, sequentially applied) increased the recovery to 81.6%. When additional sequential eluent was applied for both concentration levels, no further α-ribazole was detected.

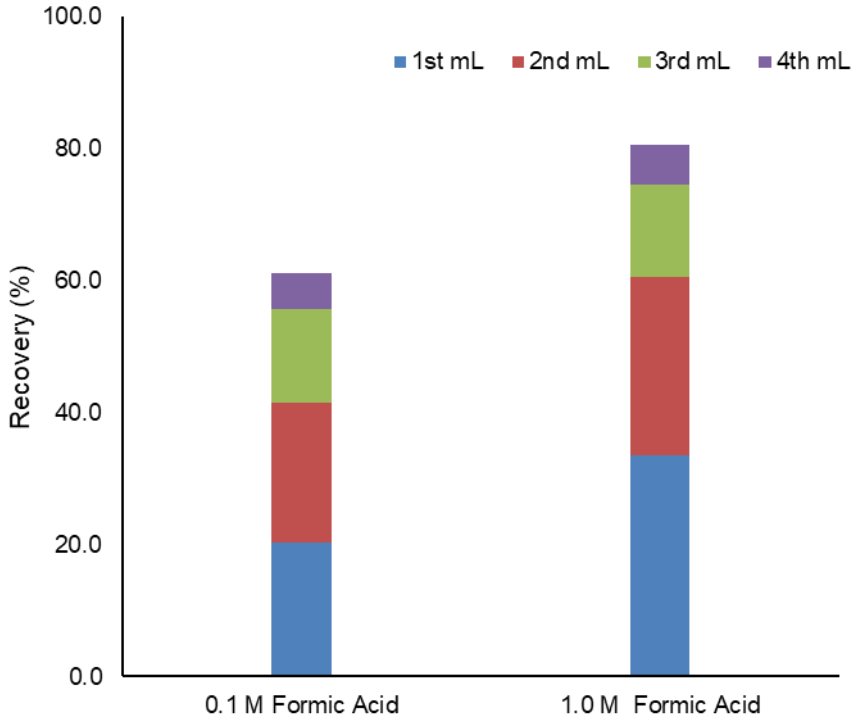


Figure 4.14. The distribution of recovery of α-ribazole from boronic acid gel by formic acid per eluent volume

Incomplete elution might be caused by the secondary binding mechanism, hydrophobic interaction; therefore, introducing an organic modifier into the eluent was necessary. Methanol and acetonitrile are two commonly used solvents in chromatography, and both have been used in boronate chromatography. Up to 40% of methanol has been used to suppress the hydrophobic interactions in the binding step. On the other hand, the affinity was significantly impaired when acetonitrile was added as the organic modifier (Espina-Benitez *et al.*, 2017a; Espina-Benitez *et al.*, 2017b).

The addition of methanol in the formic acid (0.1 M) (20:80 *v:v*) eluent yielded a higher recovery. A higher percentage of methanol (30:70 *v:v* with 0.1 M formic acid) did show a slightly higher recovery when 4 mL of eluent was applied to the gel. In **Figure 4.15**, the total recoveries from both eluents were similar at about 87% after applying the two more additional eluent. No α-ribazole was detected in the further sequential elutions.

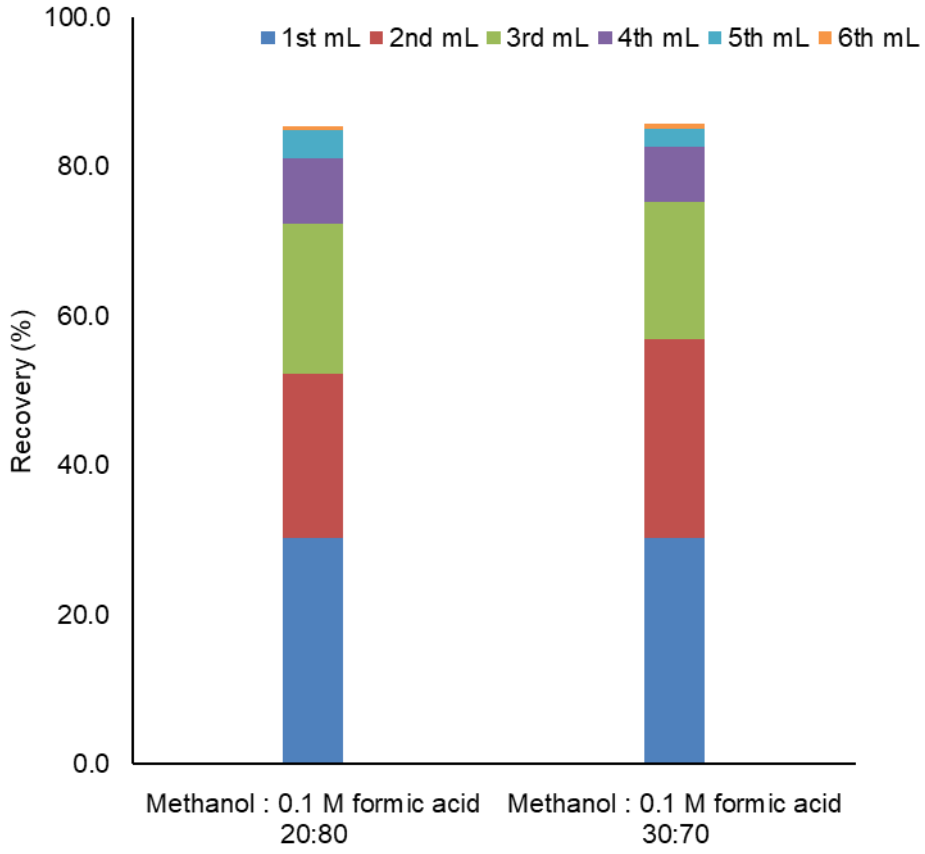


Figure 4.15. The distribution of recovery by formic acid with methanol per eluent volume): methanol: 0.1 M formic acid, 20:80 (v:v) and methanol: 0.1 M formic acid, 30:70 (v:v)

The effect of acetonitrile as the organic modifier was also tested. A 90.7% recovery was reached using 4 mL of acetonitrile: 0.1 M formic acid (20: 80 *v*:*v*), When a higher content of acetonitrile was used (30:70 *v*:*v*, with 0.1 M formic acid), the recovery increased to 93.5%, **Figure 4.16,** all eluent was added sequentially in 1 mL each time. A fifth sequential eluent addition yielded no further increase of the recovery.

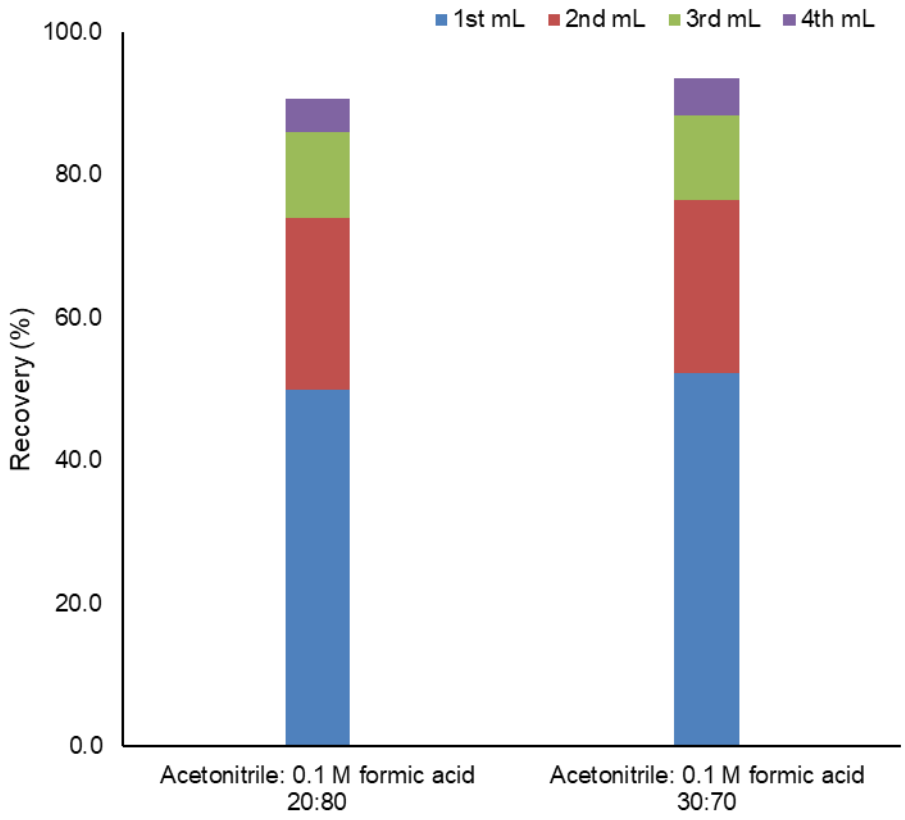


Figure 4.16. The distribution of recoveryα-ribazole from boronic acid gel by formic acid with acetonitrile per eluent volume): acetonitrile: 0.1 M formic acid, 20:80 (v:v) and acetonitrile: 0.1 M formic acid, 30:70 (v:v)

The above tests suggested that formic acid (1.0 M) with acetonitrile as the organic modifier can promote elution efficiency. Therefore, a combined approach using an eluent consisting of 30% acetonitrile and 70% formic acid (1.0 M) was chosen for further investigation. **Figure 4.17** shows the recovery distribution of 4 mL of the sequential elution. No α-ribazole were detected in the additional eluate. The chosen eluent (acetonitrile: 1.0 M formic acid, 30:70 *v*:*v*) resulted in a recovery of 94.2%. The volume of eluent (4 mL) is equivalent to 8 times of column volume (0.5 mL). Therefore, during the α-ribazole standard production, at least 8 times column volume of eluent should be used to recovery the attached α-ribazole.

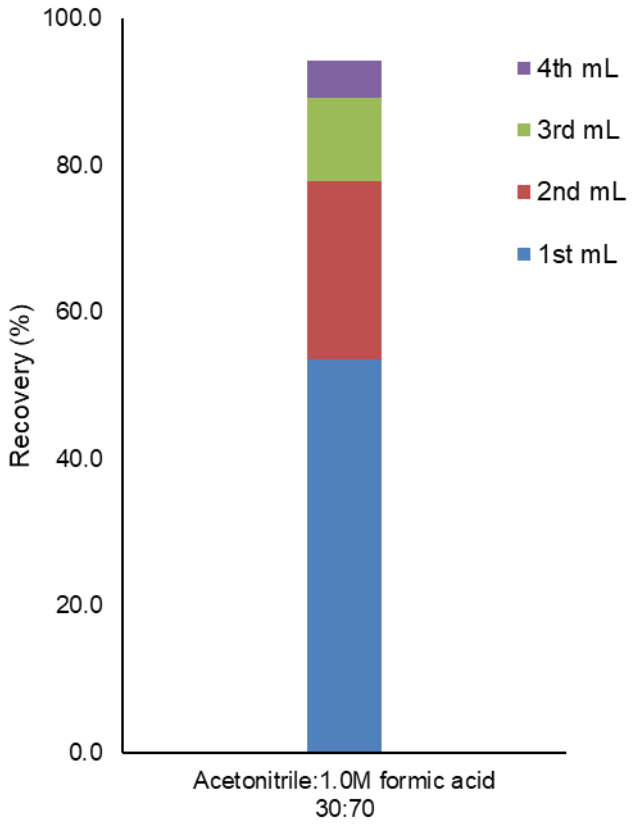


Figure 4.17. The distribution of recovery α-ribazole from boronic acid gel by formic acid with acetonitrile per eluent volume: acetonitrile: 1.0 M formic acid, 30:70 (v:v)

During the production of the standard, *circa* 9.31 mg of α-ribazole resulting from hydrolysis steps was loaded into a 1.5 mL boronate gel column, and acetonitrile: 1.0 M formic acid (30:70 *v:v,* 15 mL,10x column volume) was used for elution. Approximately 85% of the material loaded was recovered. Mattes and Escalante-Semerena (2018) reported a 76% recovery (6.2 mg) when applying 6 mL of formic acid (0.1 M) to elute 8.2 mg loaded α-ribazole from a freshly-packed boronic acid gel column (1.5 mL). According to the above finding, using 4× column volume (4×0.5 mL) was not enough to achieve a maximum recovery. Instead, an eluent with a higher amount (8 × column volume) and higher acidity (1.0 M formic acid) were required as well as the addition of organic modifier (acetonitrile).

4.2.6 Yield and Scaling-up of α-Ribazole

During the optimisation, the two main steps: hydrolysis and boronate affinity gel clean-up, both reached above 90% yield or recovery. However, in the final standard isolation process, the amount of vitamin B_{12} standard used (50 mg) was significantly greater than the usage (40 ng) in the optimisation experiments. Therefore, scaling-up was applied on the heating time length, the amount of hydrochloric acid and sodium hydroxide used, the amount of ALP used and the volume of boronate affinity gel on the final isolation experiment. The details are shown in **Table 4.5.** The comparison summary between the final procedure and the method by Mattes and Escalante-Semerena (2018) is shown in **Table 4.6**; the comparison of yields is summarised in **Table 4.7.** The final procedure produced a 76% yield rate (n = 2) which is an improvement on the 67% reported by (Mattes and Escalante-Semerena, 2018).

Table 4.5. The details of scaling-up of α-ribazole isolation

Parameters	Tested value	Scaled up value
Heating time length (min)	30–120	120
Amount of hydrochloric acid (mol)	0.0015	0.015
Amount of alkaline phosphatase (unit)	≥ 22	≥ 90
Boronate affinity gel (mL)	0.5	1.5

Table 4.6. Comparison of α-ribazole isolation procedure between this work and the literature method

Parameters	Literature method[a]	This work
Chemical hydrolysis reagent and concentration	sodium hydroxide, 2.5 M	hydrochloric acid, 1.5 M
Chemical hydrolysis temperature	85–90 °C	95–100 °C
Treatment duration	75 min	120 min
Amount of alkaline phosphatase	> 1000 U	> 90 U
Incubation temperature	37 °C	34 °C
Eluent of boronate affinity chromatography and volume	Formic acid (0.1 M, 6 mL)	Acetonitrile: formic acid (1.0 M), (30:70 (*v*:*v*), 15 mL)

[a] Method by Mattes and Escalante-Semerena (2018)

Table 4.7. Comparison of the yield of α-ribazole between this work and Mattes and Escalante-Semerena (2018) method

	Expected α-ribazole (mg) from CN-Cbl[a]	Measured α-ribazole (mg) from CN-Cbl	Yield rate (%)
This work	10.38[a]	7.9[b]	76%[b]
Literature	10.3	6.9	67%

[a] 50.53 mg of cyanocobalamin (CN-Cbl) was used in this work. 50 mg for literature method (Mattes and Escalante-Semerena, 2018)

4.3 Summary

Overall, three main steps: chemical hydrolysis, enzymatic hydrolysis and separation and concentration by BAC were optimised. A number of parameters were investigated, including hydrolysis reagent choice, concentration, heating duration, ALP buffer pH, incubation temperature, duration, BAC binding pH and elution solvents. The final isolation procedure was determined by scaling up these parameters and the final isolation method produced a 76% yield rate.

5 Extraction of Cobalamin-derived α-Ribazole from Infant Formulas

A sample preparation procedure was developed using cobalamin-derived α-ribazole for vitamin B_{12} analysis in infant formulas, which was based on the optimisation for the isolation of α-ribazole from cyanocobalamin (CN-Cbl) standard described in **Chapter 3**. The investigation looked into the effects caused by sample matrices. This process removed the requirement for the utilisation of cyanide for conversion of all vitamers to CN-Cbl. Reconstituted infant formula samples were processed through a series of extraction steps including protein denaturation by thermal and acid treatment, sugar removal by SPE, acid hydrolysis, enzymatic dephosphorylation and final clean-up by boronate affinity chromatography (BAC). This chapter describes the sample preparation in detail and discusses how each step is optimised for the final quantitation and the importance of each step.

5.1 Experimental

5.1.1 Materials and General Methods

All materials and general equipment are detailed in **Chapter 2.** Infant formula products were purchased from local supermarkets (Hamilton, New Zealand)*.

An SPE manifold (Supelco, Bellefonte, PA) was used for the solid phase extraction. C_{18} SPE cartridges (Part No. 8B-S001-HCL, 500 mg 6 mL) were purchased from Phenomenex (Torrance, CA).

5.1.2 Sample Preparation

Every sample was analysed twice; first for endogenous α-ribazole (in duplicate) and then for total α-ribazole, also in duplicate.

* The infant formula products used for the method development were from the same batch unless otherwise indicated.

5.1.2.1 Reconstitution of Infant Formula Samples

Infant formula powder (7.0 g, average from label instruction) 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 hour and shaken by hand for 30 s every 20 min to ensure full rehydration.

5.1.2.2 Protein Denaturation

Reconstituted infant formula milk (2.0 g) was weighed into a 10 mL glass vial. Sodium acetate solution (0.1 M, pH = 4.0, 5 mL) was added to the vial which was capped and vortexed for 30 s and then heated at 90 °C in a heating block for 30 min. Subsequently, the vial was cooled in an ice water bath. The extract was centrifuged (30 min, 3900 rpm, 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 unit prior to sugar removal by C_{18} SPE.

5.1.2.3 Removal of Sugars

A C_{18} SPE cartridge was conditioned and equilibrated with methanol (5 mL) followed by sodium acetate solution (0.1 M, pH = 4.0, 5 mL). The filtered extract sample from previous step were applied to the cartridge and allowed to drain by gravity flow. Another 5 mL of sodium acetate solution (0.1 M, 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 sorbent. 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 elutions). The combined methanol fractions were evaporated to dryness under nitrogen (50 °C).

5.1.2.4 Acid Hydrolysis

Endogenous α-Ribazole

To ensure the sample for endogenous α-ribazole had a similar solution condition to the sample for total α-ribazole after hydrolysis, sodium chloride

solution, prepared by mixing hydrochloric acid (1 mL, 1.5 M) and sodium hydroxide (1 mL, 1.5 M), was added to the dried extract. The vial was capped and mixed by aid of vortex (1 min). Since an acid hydrolysis was not required, this solution was ready for dephosphorylation by alkaline phosphatase (ALP).

Total α-Ribazole

Hydrochloric acid solution (1.5 M, 1 mL) was added to the dried extract and the capped vial was vortex mixed for 1 min and heated (100 °C, 60 min). After cooling in an ice bath for 30 min, sodium hydroxide solution (1.5 M, 1 mL) was added to neutralise the hydrochloric acid.

5.1.2.5 Alkaline Phosphatase Enzymatic Hydrolysis

The incubating buffer (ammonium acetate, 0.3 M: magnesium chloride, 2 mM; pH = 8.8, 2 mL) and an ALP stock solution (50 µL, prepared as described in **Section 2.2**) were added to both the acid-hydrolysed sample (total α-ribazole) and the non-hydrolysed sample (endogenous α-ribazole) and mixed well by aid of vortex for 30 s. The resultant solutions were incubated (34 °C, 16 hours minimum).

5.1.2.6 Boronate Affinity Chromatography Clean-up

The α-ribazole was separated from the hydrolysate mixture by passing through a boronate affinity column packed with Immobilised Boronic Acid Gel™ (0.5 mL). Prior to the sample loading, the column was equilibrated with binding buffer ammonium acetate (0.3 M, pH = 8.8, 3 mL). Then 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 to 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. Then the solution was drained by gravity flow and the column was washed with ammonium acetate (0.3 M, pH = 8.8, 3 mL). Finally, acetonitrile: formic acid (1.0 M, 30:70 *v*:*v*, 5 mL) was used to elute the retained α-ribazole. The collected eluate was

evaporated under nitrogen stream (60 °C), re-suspended by adding 1 mL of mobile phase A (ammonia aqueous solution, 0.3% *v*:*v*, pH = 9.5), filtered by 0.45 µm PTFE syringe filter, and analysed by RP-HPLC-FLD. The used column was regenerated by washing with the same eluent (5 mL) and binding buffer (5 mL).

5.1.3 RP-HPLC-FLD System

The details of the chromatography system and FLD are described in **Section 2.4**. Gradient elution was used for chromatographic separation (**Table 5.1**).

Table 5.1. Gradient procedure of RP-HPLC-FLD staring with 10% mobile phase B

Time (min)	Mobile phase %A[a]	Mobile phase %B[b]
0	80	10
25	36	64
30	20	80
35	20	80
37	80	10
47	80	10

[a] Mobile phase A = ammonia aqueous solution, 0.3% *v:v*, pH = 9.5

[b] Mobile phase B = 100% methanol

5.1.4 Standard Solutions

CN-Cbl and α-ribazole standard solutions were prepared as described in **Section 2.5**. An external standard calibration with a linear regression method was used to calculate α-ribazole concentrations in samples.

5.2 Results and Discussion

5.2.1 Infant Formula Reconstitution

For infant formula samples, it was noticed that when water was added to the powder, undissolved deposits formed on the wall of the bottle. It was challenging to obtain a homogenous reconstitution even with vigorous inverted

mixing. Therefore, to ensure that residues did not form, the infant formula powder was always added to the water.

The rehydration time can be reduced by applying strong mechanical agitation and elevated temperature (Richard *et al.*, 2013), promoting a better liquid distribution of solid particles. However, irreproducible α-ribazole results were observed when the infant formula powder reconstitution was poorly conducted. To ensure a homogenous state of the reconstituted infant formula, a warm bath (around 35 °C) with inverted shaking every 20 min for 30 s for 1 hour was used. Any temperature above 37° C should be avoided as the risk of denaturation and aggregation of protein increases, preventing an inhomogeneous sample. Furthermore, the sealed sample bottle was inverted several times before sampling.

5.2.2 Denaturation of Protein

The protein-bound vitamin B_{12} must be released as free forms before further conversion and analysis. It is also necessary to remove protein to limit the matrix effect. An acid and heat combined protein denaturation method was employed since it is the most common way to precipitate protein in milk products. Whey protein can be denatured by heating at a high temperature. In bovine milk, whey protein constitutes about 20% of total protein but in infant formula it occupies ~60% of total protein to match the protein content of human milk. Denaturation that occurs above 70ºC results in irreversible aggregation and eventually gelation, which form insoluble whey protein gel (Wang and Guo, 2019). Different whey proteins show different denaturation behaviour; therefore, the heating block was set to 100 °C to achieve 90 °C of the sample solution, for a complete denaturation. However, casein, another main protein in infant formula, shows more heat resistance and heat-induced denaturation may not be sufficient. At pH = 4–5, which is approximately the isoelectric point, casein is almost insoluble. Acetic acid with sodium acetate is a frequently reported reagent for acid-induced denaturation of casein (AOAC International, 2023a; Joyce *et al.*, 2017; Rowland, 1938). In literature methods for vitamin B_{12} analysis, the concentrations of sodium acetate have ranged from 0.05 M to

0.4 M, and pH = 4.0 was a common choice. Therefore, a sodium acetate buffer with a concentration of 0.1 M with pH = 4.0 was chosen for this method (Campos-Gimenez, 2014; Heudi *et al.*, 2006; Pakin *et al.*, 2005; Perez-Fernandez *et al.*, 2016; Repossi *et al.*, 2017; Schimpf *et al.*, 2012).

To investigate the extraction efficiency for the vitamin B_{12} freed from its bound protein, the reconstituted infant formula sample was spiked with CN-Cbl standard (20 ng) and processed as described in **Section 5.1.2.2**, with single or double extraction by sodium acetate buffer. A spiked procedural blank (vitamin B_{12} standard) was included for comparison. From **Table 5.2**, a 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 and that the less than 100% recovery was caused by other steps rather than the protein denaturation. Therefore, a double step protein denaturation was used for this method.

Table 5.2. Recovery results from different cycles of protein denaturation.

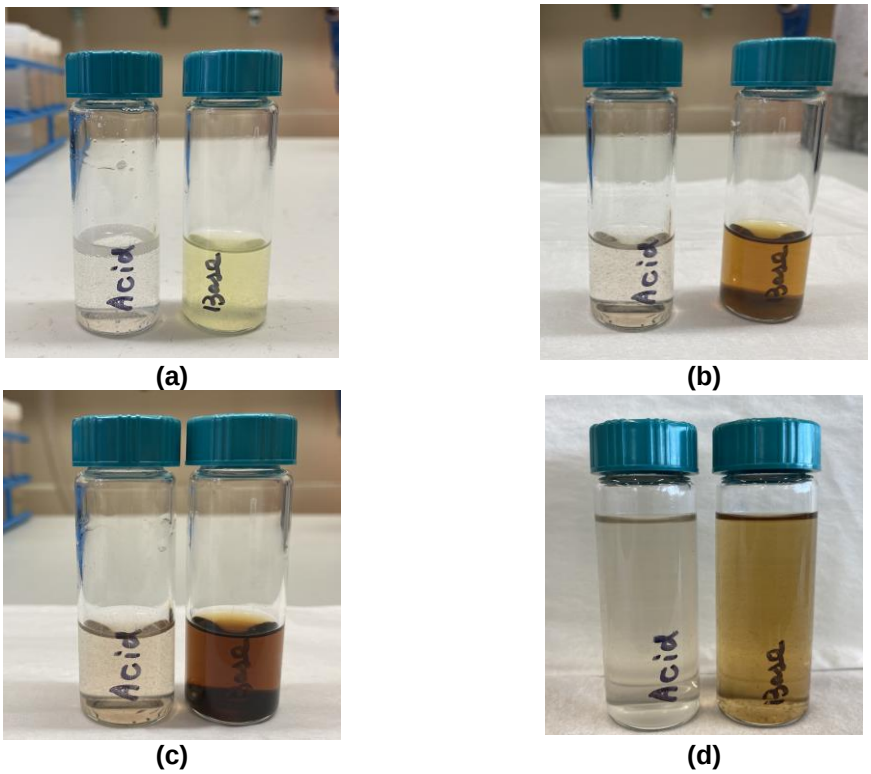
Protein denaturation and Vitamin B_{12} extraction cycles	Recovery ± SD (%)[a] for sample	Recovery ± SD (%)[a] for spiked blank[b]
Single	59.0 ± 2.6	70.2 ± 3.1
Double	73. 9 ± 7.1	69.9 ± 2.4

[a] Mean of two replicates ± standard deviation
[b] Spiked procedural blank = cyanocobalamin standard in water (2.0 g)

5.2.3 Sugar Removal for Preventing Browning

If sugars, primarily lactose, present in the extract, are not removed, discolouration will occur with production of yellow/brown pigment during the chemical hydrolysis stage. There are two main pathways by which this occurs. First, lactose, a reducing sugar, can react with amino acids to undergo the Maillard reaction, during which coloured compounds are produced, including the intense brown compounds called melanoidins in the final stages, especially under basic conditions (Nursten, 2002; Van Boekel, 1998). The second pathway is the caramelisation of sugars at high temperature heating and this

process is catalysed under either acidic or alkaline conditions and eventually produces brown polymers (Corzo-Martínez *et al.*, 2012; Webb, 1935). Discolouration of the extract solution from the protein denaturation step was assessed comparing the effect of acid and base. The samples (the supernatant from 2 g of milk after protein denaturation) were heated with hydrochloric acid (1.5 M) or sodium hydroxide (1.5 M) for 60 min at 100 °C. A series of comparison photos were taken at 1 min (**Figure 5.1.a**), 30 min (**Figure 5.1.b**), 60 min (**Figure 5.1.c**) and post-incubation (overnight, **Figure 5.1.d**) processing point. Under basic conditions yellow colour appeared after only 1 min heating duration and became darker as heating progressed., In contrast, the acidic condition showed only a light yellow/brown colour after 60 min. The colour remained after the ALP incubation with pH adjustment (to 8.8) and coloured material would have been carried on to following steps if no prevention was employed.



(a) **(b)**

(c) **(d)**

*Figure 5.1. Discolouration of infant formula sample extract under acidic and basic hydrolysis without prior removal of sugars alkaline phosphatase*Although an acidic medium can inhibit the Maillard reaction, unfortunately it does not afford complete prevention of discoloration, which can subsequently cause several problems for the later preparation steps. For example, competition for binding sites on the boronic acid gel from the compounds responsible for the

colouration and massive interference in the chromatogram due to the intensive colour and complicated fluorescence of compounds produced during the Maillard reaction (Nursten, 2002; O'Brien, 1997). Therefore, it is necessary to remove the sugar before the chemical hydrolysis.

There are several potential ways to remove sugars from the milk extract exploiting their high hydrophilicity, including liquid-liquid extraction (LLE) and SPE.

Acetonitrile is a common aprotic solvent used in LLE for aqueous samples as it is very easily separated from the water by salting-out or sugaring-out. The QuEChERS method is an example of acetonitrile utilisation (Anastassiades *et al.*, 2019). Lactose, the primary sugar source in milk and infant formula, ranges from 48–70 g L^{-1} of liquid bovine milk or prepared feed (Saxena *et al.*, 2021; Thompkinson and Kharb, 2007). According to the report from Wang *et al.* (2008), disaccharides can trigger the phase separation of an acetonitrile-water solution and about 90% of the acetonitrile was recovered when the sugar concentration is above 50 g L^{-1}. Additionally, lactose is almost insoluble in acetonitrile (Katainen *et al.*, 2005), and the multiple ring structure of vitamin B_{12} indicated that it could be soluble in acetonitrile. Therefore, the addition of acetonitrile into the extract after protein denaturation had the potential to extract vitamin B_{12} and separate lactose.

A trial study was conducted to investigate the efficiency of using acetonitrile in the LLE for sugar removal. Vitamin B_{12} standard (CN-Cbl, 20 ng) was spiked into 5 mL of milk extract from the protein denaturation step. Acetonitrile (10 mL) was added into the extract and it was mixed on an orbital shaker for 30 min. After centrifugation (3900 rpm, 10 min), the supernatant was evaporated under a nitrogen stream (60 °C) until dryness. The dried product was further prepared as described from **Section 5.1.2.4** to **5.1.2.6** and analysed by RP-HPLC-FLD. The recovery was calculated by subtracting the matrix blank (infant formula extract without CN-Cbl spike). However, the results showed low recovery (< 50%, n = 2) of vitamin B_{12} even with repeated extraction cycles (another 10 mL of acetonitrile was added into the residue after the removal of

supernatant). Possibly, vitamin B_{12} was more hydrophilic than initially expected, and its partitioning between the two phases might be in favour of the aqueous solution.

Reversed-phase SPE is another technique which can separate sugar (lactose) from vitamin B_{12} due to their different strength interactions with the stationary phase. Sugars are not strongly retained on RP SPE cartridges due to their high hydrophilicity, whereas vitamin B_{12}'s large molecule size and multiple hydrophobic groups might lead to longer retention. C_{18} SPE has been used and well-studied in vitamin B_{12} analysis until it was replaced by immunoaffinity extraction, which has higher selectivity for CN-Cbl but less selectivity towards other forms of vitamin B_{12}. Heal *et al.* (2014) reported a method which showed that a C_{18} SPE resin could retain all four forms of vitamin B_{12} with 88, 90, and 96% mean recovery for OH-Cbl, Me-Cbl and Ado-Cbl, respectively but with lower recovery of 71% for CN-Cbl. A similar method reported by Okbamichael and Sañudo-Wilhelmy (2004) gave a higher recovery (> 90%) for CN-Cbl. Although both these methods were developed for seawater samples, the mechanism remains the same. Therefore, C_{18} SPE was chosen for the current method. The effect of the pH of the sample on the retention of vitamin B_{12} on a C_{18} SPE cartridge has been reported. However, the results were not conclusive as Iwase and Ono (1997) claimed that the optimal pH for CN-Cbl ranged from 2 to 7.5 with a complete recovery; while Okbamichael and Sañudo-Wilhelmy (2004) reported that maximum recovery of 96–101%, occurred at a pH range of 6.1–6.7 and outside this range, recovery reduced to 53–73%. The former conducted the test with a CN-Cbl standard directly loading onto the SPE cartridges, whilst in the latter paper, the test was applied on seawater. This implies that ionic strength may have some impact. Although both studies used the C_{18} SPE technique, different sorbent, capacity and matrices might vary the optimal pH range. Therefore, was necessary to determine an optimal pH for this method. The sample extract from the previous step was in a solution with pH = 4.0. Therefore, pH = 4.0 and 6.5 were chosen for further tests. The infant formula samples were prepared as described in **Section 5.1.2** with modification during the C_{18} SPE step for the pH 6.5 group: the pH of the sample extract was adjusted to 6.5 by adding sodium hydroxide (3 M), and the SPE cartridge was

equilibrated with water instead of sodium acetate buffer. The recovery results across all steps of the entire preparation are shown in **Table 5.3**. These recoveries are less than those in the reports by Iwase and Ono (1997), Okbamichael and Sañudo-Wilhelmy (2004) and Heal *et al.* (2014), however, they span more steps and were recovered from a more complex matrix. The pH = 4.0 group showed higher recovery which matched with the recovery results from spiked procedural blanks (69.9 ± 2.4%), therefore, pH of 4.0 was used and no additional adjustment was required.

Table 5.3. Comparison of recovery results of pH 4.0 and 6.5 of sample in C_{18} SPE step.

pH of the sample extract		Recovery (%)
4.0	Dup 1	65.7
	Dup 2	71.4
6.5	Dup 1	47.0
	Dup 2	58.2

The eluent used to recover the vitamin B_{12} from the sorbent was either 100% methanol (D'Ulivo *et al.*, 2017; Heal *et al.*, 2014; Okbamichael and Sañudo-Wilhelmy, 2004; Zhu *et al.*, 2011) or water-diluted acetonitrile (Iwase and Ono, 1997; Schimpf *et al.*, 2012). The manufacturer of the C_{18} SPE cartridges, which was used in this work, recommended employing 100% methanol as an eluent. The study by Iwase and Ono (1997) found that 100% acetonitrile failed to elute CN-Cbl from the cartridge. A 50% acetonitrile gave total elution, suggesting that a relative polar solvent was required to elute vitamin B_{12}, which correspond to finding of the low recovery of vitamin B_{12} in the acetonitrile phase after LLE for sugar removal. Considering the eluate has to be evaporated under nitrogen stream, 100% methanol, rather than an aqueous mixture, was preferred due to its lower boiling point (methanol: 65.0 °C and acetonitrile: 81.6 °C) (*CRC Handbook of Chemistry and Physics*, 2023).

5.2.4 Chemical Hydrolysis

The chemical hydrolysis step, to liberate α-ribazole-phosphate from vitamin B_{12}, has been optimised in **Section 4.2.2** and acidic hydrolysis was chosen for its

higher conversion rate and the merit of less colour development since sugar cannot be totally eliminated even with a clean-up by C_{18} SPE. Results from **Section 4.2.2** showed that altering heating duration, over a range of between 30 min and 120 min, was found to show no significant difference from the ANOVA test. A 60 min heating duration was chosen after balancing the time and ensuring maximum hydrolysis was achieved. Heating with hydrochloric acid (1.5 M) at 100 °C for 60 min was applied to derive α-ribazole-phosphate from the vitamin B_{12} in infant formula samples.

5.2.5 Alkaline Phosphatase Enzymatic Hydrolysis

The enzymatic hydrolysis to liberate α-ribazole from α-ribazole-phosphate was optimised in the **Section 4.2.3**. Incubation buffer: ammonium acetate (0.3 M) magnesium chloride (2 mM) with a pH of 8.8 showed higher yield of α-ribazole, hence, it was used for sample preparation. 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 (Ball, 2006; Gille and Schmid, 2015; Greibe and Nexo, 2016). According to the ALP enzyme unit definition: 1 unit will hydrolyse 1 µmol of 4-nitrophenyl phosphate per minute at pH = 9.8 at 37 °C. As there is a 1:1 ratio of α-ribazole to vitamin B_{12}, 50 µL of ALP enzyme stock solution (> 22 units) has enough capacity to catalyse the dephosphorylation of the α-ribazole-phosphate of vitamin B_{12} present in the sample. The overnight (at least 16 hours) incubation period was employed for convenience as the experiment requires two days to complete.

5.2.6 Boronate Affinity Chromatography Clean-up

Boronic acid gel was used during the clean-up step of isolation of α-ribazole from CN-Cbl in **Section 3.4.2.3**. For its high selectivity and reusability, a similar boronate affinity column was applied for the reconstituted infant formula sample clean-up. Boronic acid gel (0.5 mL) was packed in an empty centrifuge column, which 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. As mentioned above, bovine whole milk (1 g) contains 0.6×10^{-3} µmol of

vitamin B_{12}, so 0.5 mL of gel has sufficient capacity to retain all the α-ribazole yield from 2 g of reconstituted infant formula sample in this work. However, there are other *cis*-diol containing compounds present in milk and the infant formulas, for example, ribose containing nucleosides and nucleotides. Gill *et al.* (2012) reported that total potentially available nucleosides in bovine milk was 7.9 μmol 100 mL^{-1}, which is equivalent to approximately 0.1 μmol g^{-1} (assuming milk density is 1.25 g mL^{-1}) and once again less than the binding limit of the boronate affinity column (0.5 mL, 55 μmol) even combined with the vitamin.

The clean-up procedure was the same as used in **Section 4.2.4** but with modification in the washing step, where water was replaced by binding buffer, ammonium acetate (0.3 M, pH = 8.8, 3 mL) in order to reduce the risk of losing bound α-ribazole by lowering pH before elution. Acetonitrile: formic acid (1.0 M, 30:70 *v*:*v*, 5 mL) was used to free the bound α-ribazole.

5.2.7 RP-HPLC-FLD Analysis

To demonstrate the necessity of employing BAC step in the sample preparation, α-ribazole standard and bovine milk-based infant formula with and without BAC were analysed by RP-HPLC-FLD. The excluding the clean-up and concentration step by BAC led to strong noise signal and high baseline (**Figure 5.2 (a)**). The strong signal of other non-targeted compounds was either naturally present in the sample or generated during the preparation process, which reduced the resolution of α-ribazole peak. The chromatogram of the sample using BAC clean-up (**Figure 5.2 (b)**) showed a lower base line and less background noise, where a higher signal response of α-ribazole peak was observed thanks to the concentration effect of using the BAC. The retention time of identified α-ribazole in the sample matched with the standard (**Figure 5.2 (c)**). Therefore, based on the comparison results, the use of BAC to collect α-ribazole and exclude interference were necessary for the sample preparation.

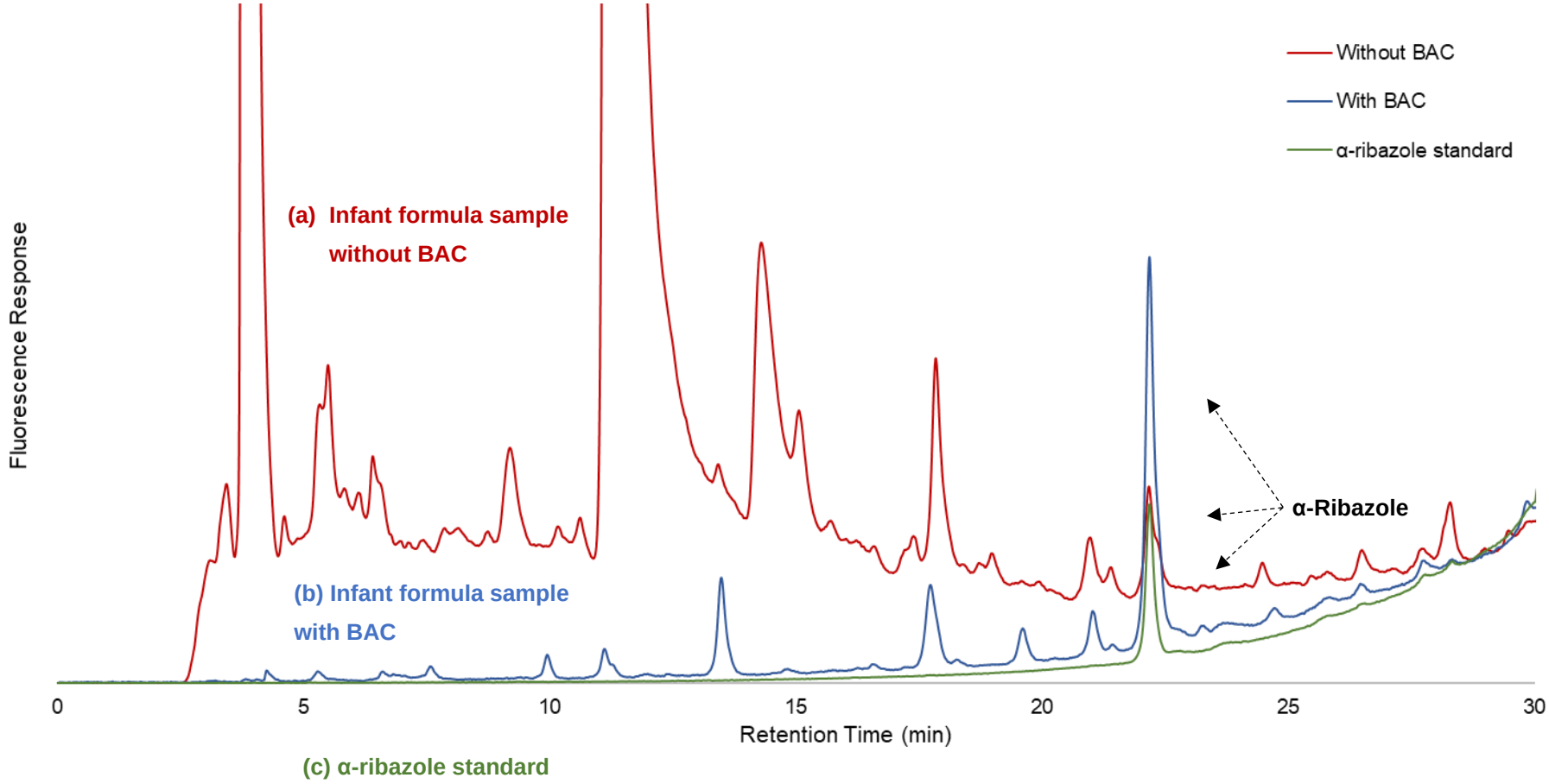


Figure 5.2. RP-HPLC-FLD chromatogram of infant formula sample without boronate affinity chromatography and α-ribazole standard

5.3 Summary

Overall, the developed sample preparation procedure was able to liberate α-ribazole from vitamin B_{12} in the infant formula samples ready for chromatographic analysis. During this process, several issues were resolved by including additional clean-up steps, which unfortunately but unsurprisingly led to a total recovery of 68.8–69.9% for tested infant formula samples. These recovery results are likely due to the number of steps that were applied through the analysis. Nevertheless, the recovery results are sitting at the lower end of the expected range (70–125%) for such a low concentration (AOAC International, 2023c). The loss of recovery might occur across the entire preparation due to the multiple steps including: SPE for sugar removal, acid and enzymatic hydrolysis for α-ribazole liberation and boronate affinity column collection. However, these steps were necessary for producing chromatography-ready samples as demonstrated in this chapter. After the instrumental method was optimised (**Chapter 6**), a full method validation was conducted on infant formula matrices (**Chapter 7**).

6 Instrumental Analysis of Cobalamin-derived α-Ribazole in Infant Formulas

Common chromatography techniques rely on the polarity of the analytes to achieve efficient separation. **Figure 6.1** illustrates the polarity scheme for RP-HPLC and HILIC. Non-polar analytes are retained by RP-HPLC primarily through hydrophobic interactions between the stationary phase and analytes. In contrast, very polar compounds can be retained by HILIC. HILIC is a type of normal-phase separation technique yet employs eluents normally used for reversed-phase separation, for instance, water-acetonitrile and water-methanol. There is a range of overlap between the two modes in the polar region. It has been reported that α-ribazole can be retained by RP-HPLC (Mattes and Escalante-Semerena, 2017; Pakin *et al.*, 2005). As an ionisable species, protonated α-ribazole shows the potential to be retained by HILIC. To identify a suitable separation chromatography mode for α-ribazole in infant formula samples, the samples were analysed using both RP and HILIC modes.

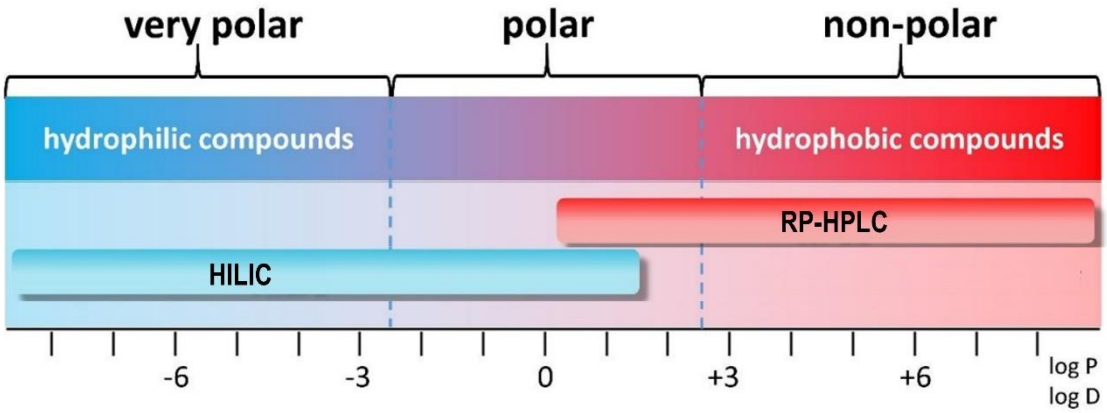


Figure 6.1. The polarity range of RP-HPLC and HILIC separation modes

During the sample analysis trials of this work, a co-eluting peak was observed by RP-HPLC with FLD using a C_{18} column. Therefore, initially this chapter focuses on optimising the chromatographic conditions to increase the resolution on a C_{18} column. A series of chromatographic variables were tested as well as the fluorescence wavelength pair. Changing the detection method to mass spectrometry allowed a list of ions to be identified as the possible interferences by comparing the retention time (RT) and peak shape to its fluorescence

chromatogram. Under HILIC mode, these possible interference ions were absent around the retention time of α-ribazole, and no co-eluting peak was observed with fluorescence. So ultimately, HILIC was used for the chromatographic separation in the analysis of vitamin B_{12}-derived α-ribazole in infant formula samples. Since this work was designed to develop a method that uses a simple detection technique with low maintenance such as fluorescence rather than MS which requires a high skilled operator. Furthermore, fluorescence is more economic and readily accessible, therefore, MS was only used to aid the investigation of the interference in the infant formula sample and fluorescence was still the preferred detection method in the final method.

6.1 Experimental

6.1.1 Materials and General Methods

All materials and general methods are stated in **Chapter 2.** Infant formula products were purchased from local supermarkets.

6.1.2 Sample Preparation

The infant formula samples were processed by the procedure reported in **Section 5.1.2**.

6.1.3 Fluorescence Spectroscopy

The 3D fluorescence scan of α-ribazole was conducted on an Aqualog fluorescence spectrometer (HORIBA, Japan) with wavelength range 220 to 800 nm (Excitation) and 250 to 830 nm (Emission). The sample solution was measured in standard quartz cuvettes (1 cm pathlength). Data was processed using Aqualog software (version 4.2). α-Ribazole solutions were diluted with the solvent used as 100% mobile phase A (ammonia aqueous solution (0.3% *v*:*v*, pH = 9.5) for RP-HPLC or 100% acetonitrile for HILIC. The corresponding solvent was also used as a blank during the measurement.

6.1.4 RP-HPLC-FLD System

The details of the RP-HPLC-FLD system were described in **Section 2.4**.

During the optimisation of the chromatographic separation, eluent strength, stationary phase (different brands of C_{18}), pH of mobile phase, and column temperature were investigated (**Table 6.1)**.

Table 6.1. Optimisation for RP-HPLC chromatographic conditions

RP-HPLC Conditions	Modification approach or values
Solvent strength	Gradient elution with various initial mobile phase compositions and acceleration
pH of mobile phase	8.5
	9.5
Temperature of column (°C)	30
	40
	50

6.1.5 RP-LC-HRMS System

Separation for high resolution mass spectrometry (HRMS) was carried out on an UltiMate 3000 LC system configured with an HPG-3400RS pump, WPS-3000 autosampler and TCC-3000 column oven (Dionex, Waltham, MA). The C_{18} LC column and mobile phase, used for the chromatographic separation were the same as used for RP-HPLC-FLD (**Section 2.4**). A full mass scan was conducted on an Orbitrap Exploris 480 high-resolution mass spectrometer (Thermo Scientific, Waltham, MA) with a heated-electrospray ionisation (H-ESI) ion source. MS parameters are summarised in **Table 6.2**. Data was processed using Freestyle™ software (version 1.6).

Table 6.2. High resolution mass spectrometer parameters summary

Parameters	Value
Positive ion spray voltage (V)	3800
Negative ion spray voltage (V)	3000
Ion transfer tube temperature (°C)	350
Vaporizer temperature (°C)	350
Sheath gas (Arb[a])	40
Sweep gas (Arb[a])	1
Auxiliary gas (Arb[a])	20

[a] Arb = arbitrary units

6.1.6 HILIC-FLD System

The details of the HILIC-FLD system were described in **Section 2.4**. FLD remained the same at 251/303 nm (Excitation/Emission) wavelength.

6.1.7 HILIC-HRMS System

The same LC-MS system described in **Section 6.1.5** with the identical HILIC column, mobile phase and elution conditions from **Section 2.4.2** were used for the HILIC-MS analysis.

6.1.8 Standard Solutions

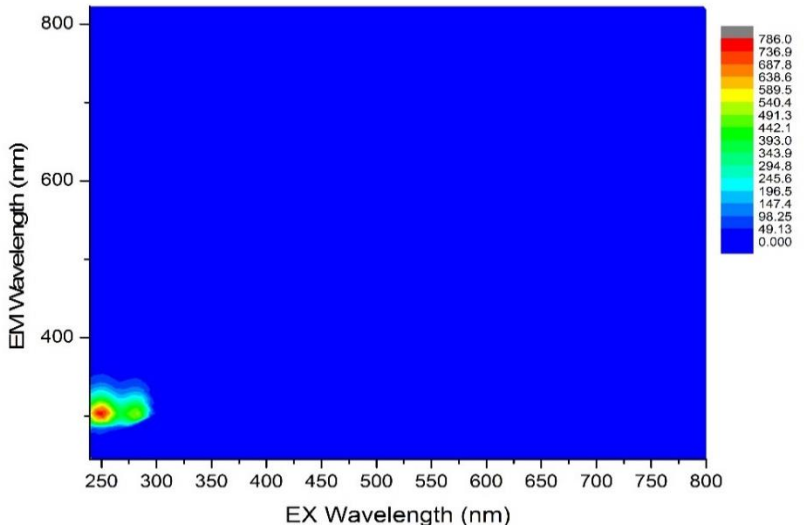
The α-ribazole standard solutions were prepared as described in **Section 2.5** by diluting the stock solution with aqueous ammonium solution (0.3% *v*:*v*, pH = 9.5) to appropriate concentrations for RP-HPLC. For HILIC mode, the standard solutions were diluted with 100% acetonitrile.

6.2 Results and Discussion

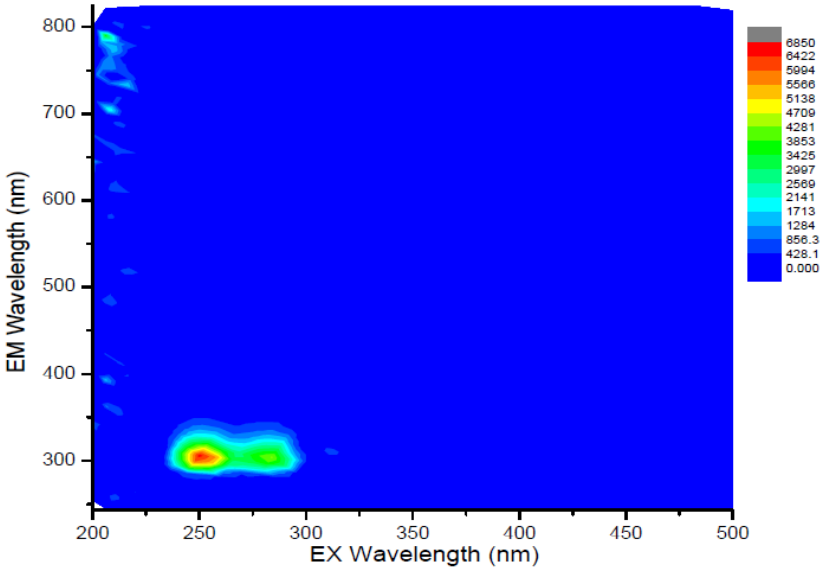
6.2.1 Optimal Excitation and Emission Wavelength

The absorption and emission fluorescence spectra of α-ribazole standard solutions were obtained to determine the maximum excitation and emission wavelength. **Figure 6.2** shows λ_{max} (emission) for α-ribazole was 303 nm when exciting at 251 nm for either diluting solvent (RP and HILIC), which is close to

the wavelength pair (250/312 nm) used by Pakin *et al.* (2005). A second peak occurs at 284/303 nm., however, the maximum is lower than 251/303 nm by approximately 36%.



(a)



(b)

Figure 6.2. Contour plot of α-ribazole's excitation and emission wavelength scan.

6.2.2 Optimisation of RP-HPLC Conditions

Due to the unresolved peak co-eluting with α-ribazole during analysis of infant formula samples, an optimisation of the RP-HPLC method was conducted. According to the Fundamental Resolution Equation (Snyder, 2009), as shown in

Equation 6.1, resolution (R_s) is affected by three elements: efficiency (N), selectivity (α) and retention (k').

$$R_s = \underbrace{\frac{\sqrt{N}}{4}}_{Efficiency} \times \underbrace{\frac{\alpha}{\alpha+1}}_{Selectivity} \times \underbrace{\frac{k'}{k'+1}}_{Retention} \qquad \textit{Equation 6.1}$$

Therefore, the increase of one or more of the three elements theoretically leads to the resolution improvement. **Table 6.3** summarises the common factors that influence these elements.

Table 6.3. Summary of the chromatographic resolution change by varying efficiency, selectivity and retention factors

Factors	Achieved by
Retention k′	Weaker solvent
	Gradient elution started with lower organic solvent content
	Changing mobile phase pH
	Stronger stationary phase
Selectivity α	Altering solvent strength
	Changing mobile phase pH
	Changing stationary phase
	Changing temperature
Efficiency *N*	Increasing column length
	Decreasing particle size
	Changing temperature

Other reversed phases, for example, C_8 and phenyl phases can offer moderate methylene selectivity and additional aromatic interaction, respectively, which affects the resolution and even elution order. However, due to the cost, this work was not able to test other columns with different RP stationary phases. In addition, a high pH stability (pH > 8) was required for analysing α-ribazole on RP-HPLC, which further limited the number of hydrophobic stationary phases which might be tested. The length of the C_{18} column used in this work was 250 mm, which was the longest analytical column available. Therefore, the

optimisation experiments were primarily focused on the parameters related to mobile phase, elution programs and column temperature.

6.2.2.1 Solvent Strength

The most convenient way to improve the resolution in RP-HPLC is to lower the solvent strength by decreasing the organic solvent percentage in the mobile phase. Thus, the non-polar analytes will be retained longer on the hydrophobic stationary phase. The hydrophobicity of α-ribazole at neutral state (logP = 0.7) suggested its retention possibility on a C_{18} column. When the initial methanol proportion in the mobile phase was 20%, 10% and 5% (mixed with ammonia aqueous solution (0.3% *v*:*v*, pH = 9.5)), the retention times of α-ribazole were 18.98, 22.72 and 32.08 min respectively, but the interfering signal was not separated from the α-ribazole peak as shown in **Figure 6.3**. When changing the initial methanol percentage, the elution time was increased to ensure a similar gradient, **Table 6.4**. When elution started from 5% of methanol, the resolution improved such that a second peak became more visible. However, it was far from sufficiently separated.

Table 6.4. Gradient elution procedure for different initial mobile phase B compositions

20% mobile phase B		10% mobile phase B		5% mobile phase B	
Time (min)	B%[a]	Time (min)	B%[a]	Time (min)	B%[a]
0	20	0	10	0	5
22	64	25	64	40	80
25	80	30	80	45	80
30	80	35	80	50	5
31	20	37	10	60	5
41	20	47	10	-	-

[a] Mobile phase A = ammonia aqueous solution (0.3% *v*:*v*, pH = 9.5), Mobile phase B = 100% methanol

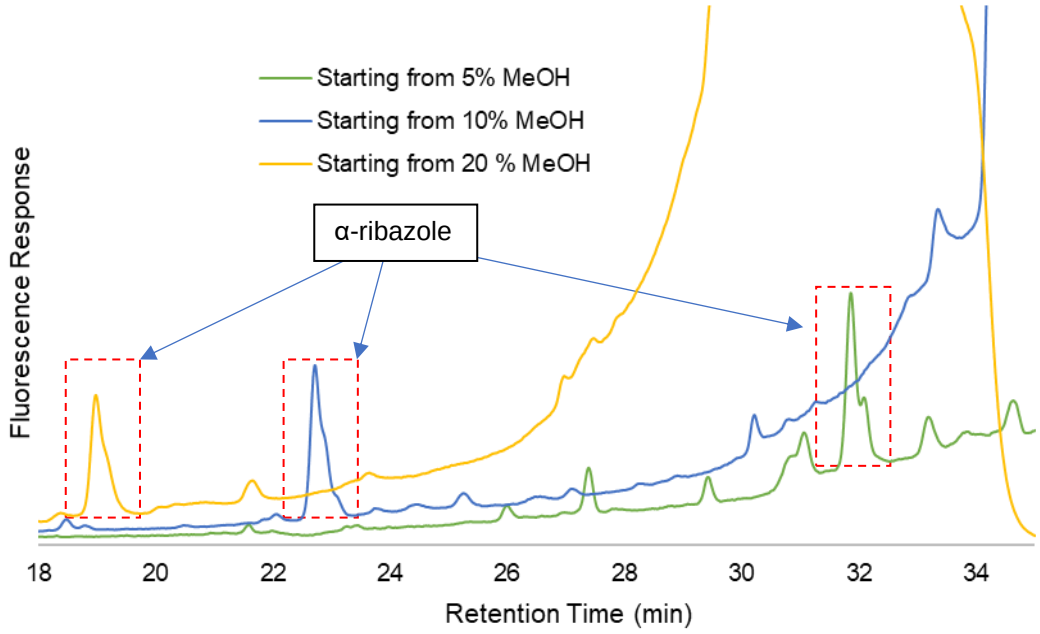


Figure 6.3. The partial chromatograms (18–34 min) of α-ribazole in the infant formula sample by RP-HPLC-FLD with various initial methanol (mobile phase B) compositions for the gradient elution.

Since α-ribazole is eluted from the C_{18} column when the organic modifier composition increases to 57.8–61.6%, slowing down the gradient around the elution time of α-ribazole may alter the mobile phase strength and increase the resolution without further extending the total analysis time. Two different degrees of logarithmic growth were applied to replace linear growth of methanol (mobile phase B). **Figure 6.4** illustrates the gradient elution diagram, where the logarithmic growth stretched the later elution period of the gradient and resulted in a lower acceleration of adding organic solvent than linear growth. The mobile phase B of all three gradients started from 10% and reached to 64% at 25 min.

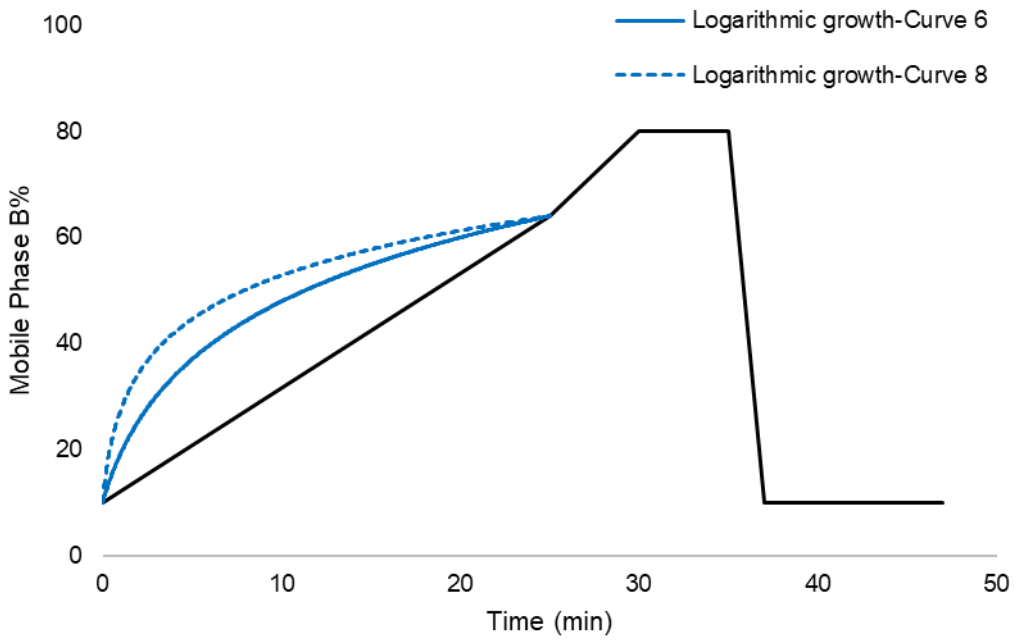


Figure 6.4. The illustration of the two gradient elution modes investigated

In the Chromeleon (v 6.8) software for HPLC gradient setting, the logarithmic gradient mode is achieved by setting curve value from 6 to 9 (curve 5 corresponds to a linear gradient), where the higher the curvature set, the longer the stretched period extends. The chromatograms of two degrees of logarithmic gradient (curve 6 and 8) and linear gradient (curve 5) are shown in the **Figure 6.5**. The elutions with the logarithmic gradient, as expected, gave a longer RT for α-ribazole, where the RT increased from 22.72 min (linear) to 25.88 min and 30.71 min for curves 6 and 8, respectively. For both logarithmic gradients, the co-eluted peak was more resolved than being a peak “shoulder” in the linear gradient elution, especially using curve 8. Unfortunately, even maximum logarithmic acceleration of the methanol in the mobile phase failed to give complete separation.

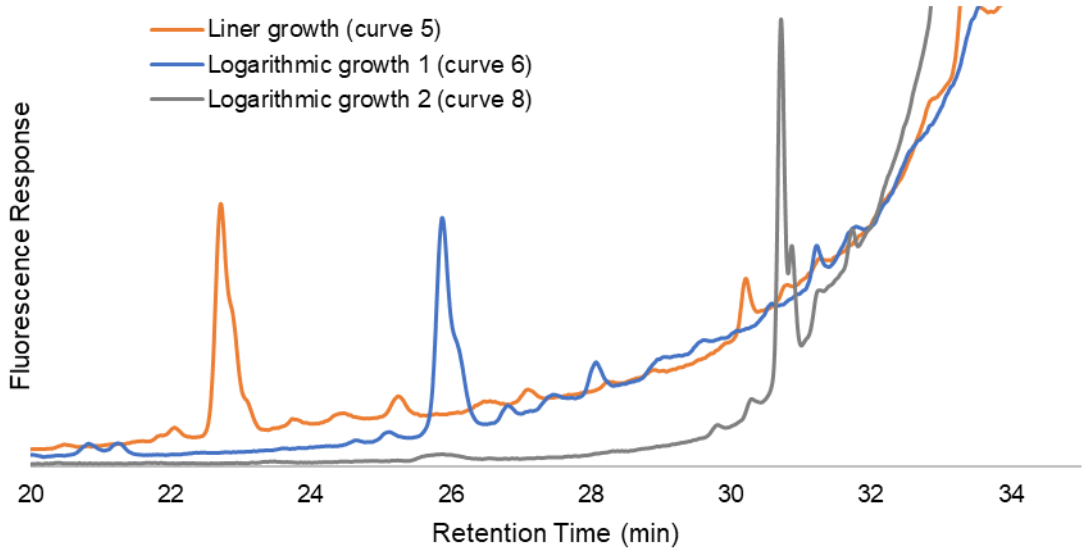


Figure 6.5. Expanded chromatograms(20–35 min) of α-ribazole in infant formula samples with logarithmic and linear gradient from 10% to 64% of mobile phase B (methanol)

Noticeably, the retention time of α-ribazole using curve 8 extended beyond 30 min where the mobile phase was composed of 80% methanol, which did not agree with the earlier assumption that the α-ribazole eluted at 57.8–61.6% of methanol (observed under linear gradient elution). The change of retention time might be caused by effects combined from the change of column pressure during the mobile phase mixing and the association of methanol water mixture.

The change of column pressure during the mobile phase mixing. Operating pressure can impact the retention factor of analytes and both negative and positive effects have been reported (Kempen and Stoll, 2021). Normally, for system using aqueous-organic mixture as mobile phase, the back pressure reduces with the addition the less viscous organic solvent (e.g., acetonitrile) (Shimadzu Corporation). Such phenomenon can be only observed for eluent which has greater than 50% of methanol in the composition. In contrast, when the mobile phase contains less than 50% methanol, the further addition of methanol will increase the back pressure (**Figure 6.6**).

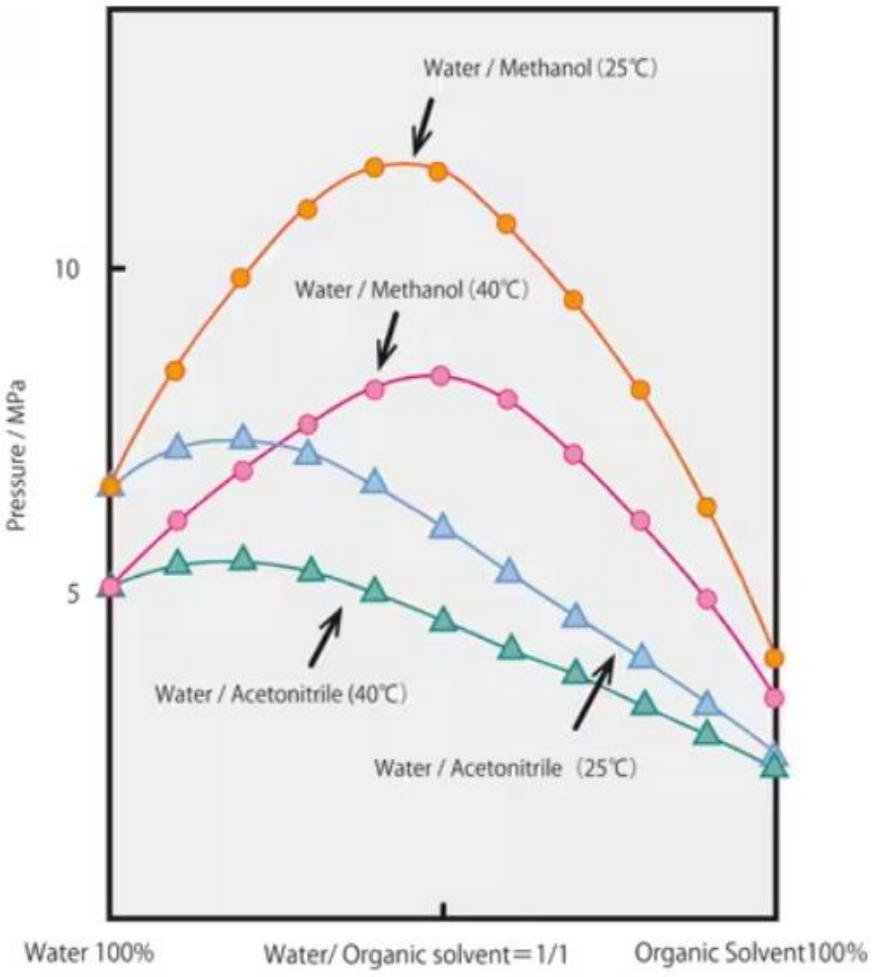


Figure 6.6.Relation between organic solvent (methanol or acetonitrile) - water ratio and column pressure

In this work, the gradient started with 10% methanol, and the increase of backpressure was noticed during the increase of the methanol component up to 50% and dropped when more than 50% of methanol added. The back pressure of the system using the elution mode of curve 6 was higher than the elution mode curve 8 during the investigated gradient period (0–25 min), as showed in **Figure 6.7**. The possible explanation is as the rise of methanol percentage in the mobile phase slows the pressure change becomes less pronounced, thus, the different back pressure profile might cause the extended retention time for curve 8 case.

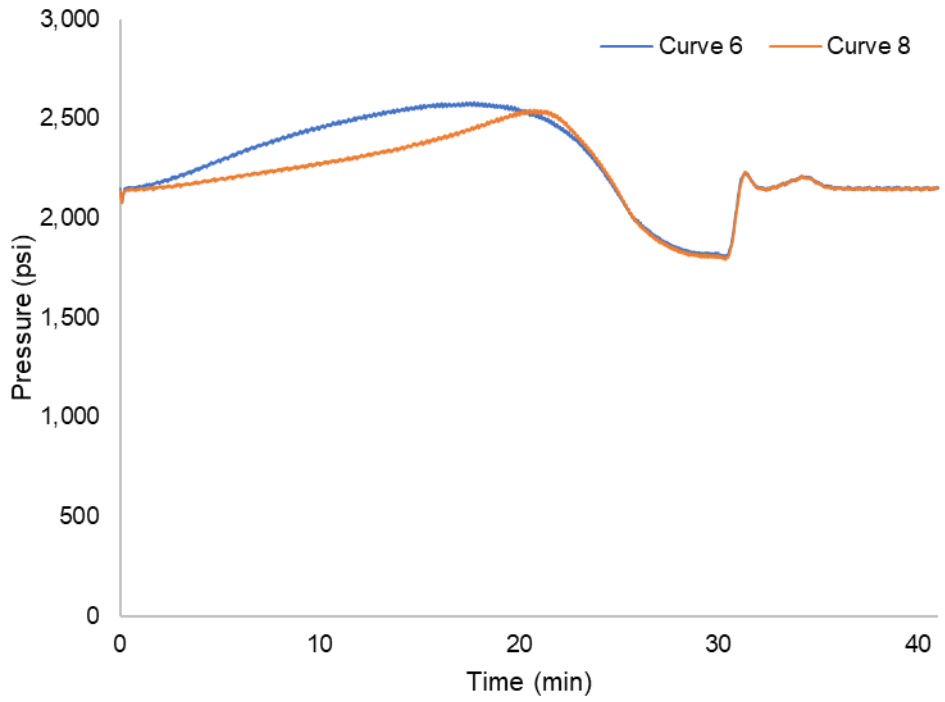


Figure 6.7. Back pressure in RP-HPLC of using different gradient elution

The association of methanol-water mixture, in which three chemical species are present: free methanol, free water and methanol-water associate. In a methanol/water mobile phase system, the distribution of analytes in the mobile phase is related to the concentration of free methanol instead of the total methanol concentration (Katz *et al.*, 1989). **Figure 6.8** illustrates the relationship between the concentration of the three chemical species in the mixture and the original methanol percentage of the mobile phase. The increase of the concentration of free methanol along with the total methanol content of the mobile phase is exponential-like. However, a gradient elution is a continuous changing of mobile phase composition, the online mixing (with different curvature) might also influence the association between the methanol and water and eventually alter the retention of analytes.

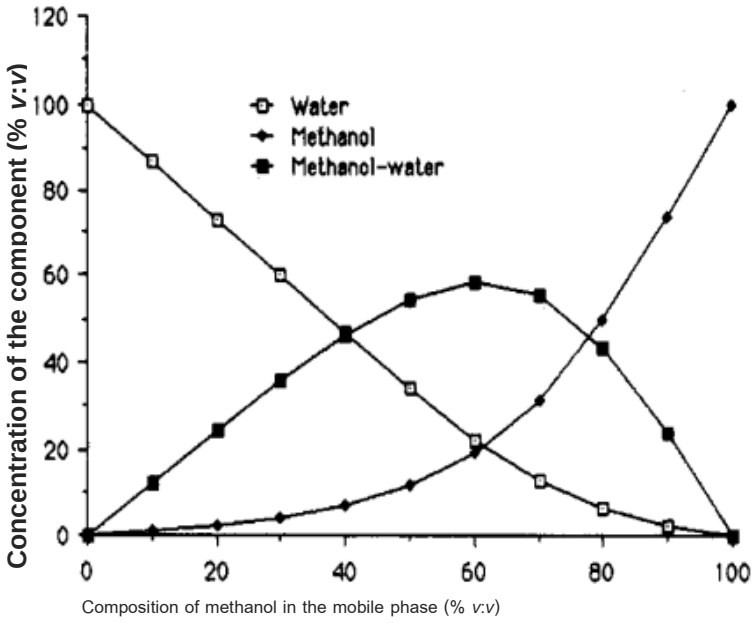


Figure 6.8. Relationship between the composition of methanol in the mobile phase and the concentration of methanol, water and methanol-water associate

Altering solvent strength by changing the methanol content through gradient showed no improvement on the resolution of α-ribazole and the interfering peak. For this case, lowering solvent strength to increase resolution exhausted its potential since methanol is the weakest organic solvent commonly used in RP-HPLC. Hence, investigation of other variables was required.

6.2.2.2 pH of the Mobile Phase

The mobile phase pH can affect the selectivity of a separation by changing the ionisation state of ionisable species under investigation. α-Ribazole is a basic compound with a pKa value (of the conjugate acid) around 6 (5.39–5.92, dependent upon temperature and ionic strength) (Brown *et al.*, 1984).

The dissociation equilibrium of α-ribazole is shown in **Figure 6.9**, which also illustrates the relationship between the ionisation state of a basic analyte and its hydrophobicity. When the pH of the mobile phase is greater than the pK_a value, the analyte has the highest hydrophobicity as it is in a neutral state and has stronger interactions with the stationary phase of a RP column and hence longer RT. At pH = pK_a (~6), 50% of α-ribazole are ionic species and less

retained on a RP column, which will result in shorter retention time and peak asymmetry issues in the chromatogram. More than 99% of α-ribazole should be in its neutral state when the mobile phase is at least two units above its pK_a. To ensure a near complete deprotonation of α-ribazole, the mobile phase pH must be greater than 8.

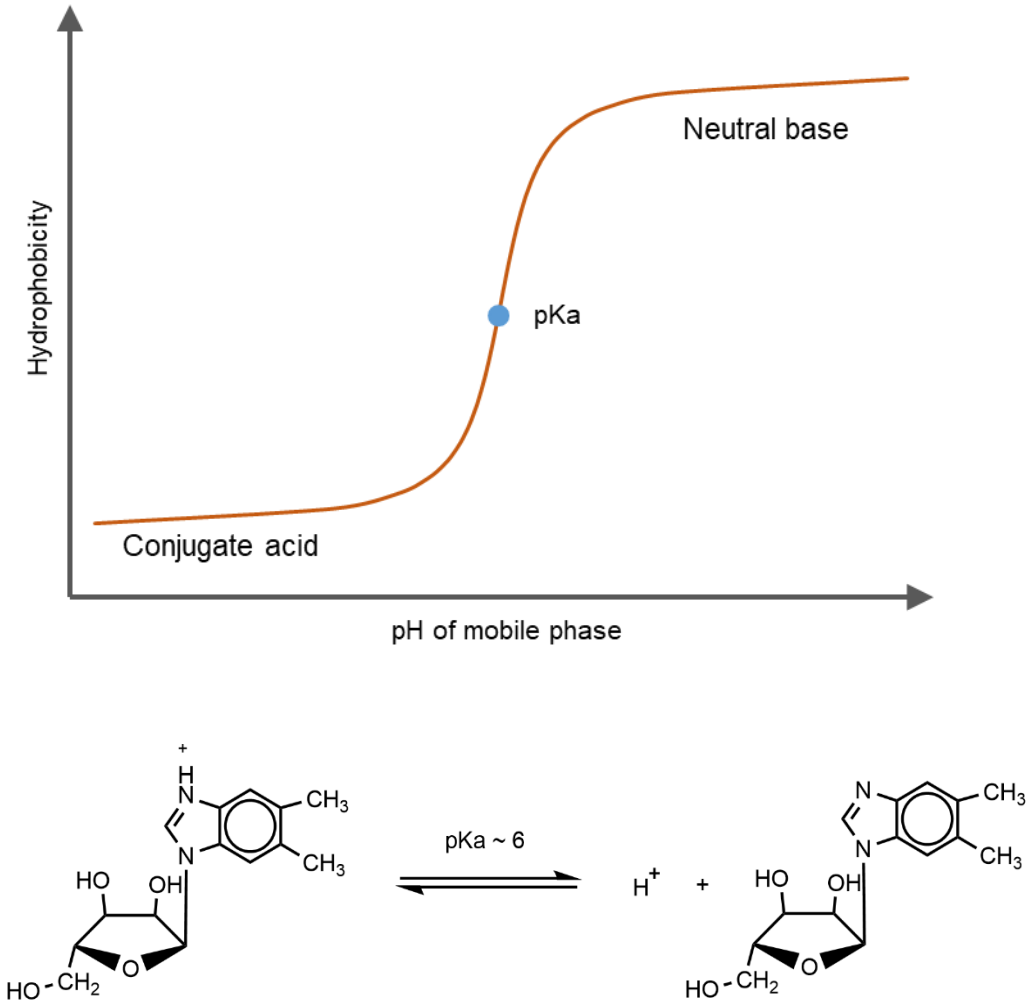


Figure 6.9. The relationship between the hydrophobicity of α-ribazole and its ionisation state relative to the pH of the mobile phase and dissociation equilibrium of α-ribazole cation to α-ribazole

For common silica-based LC columns, the operational pH range is 2–8; as at pH < 2, the bonded phase can be hydrolysed due to the acidity while at pH beyond 8, the silica tends to be more soluble, and the lifespan of the column is diminished. Modern LC columns use higher purity silica which can tolerate high

pH up to 12. The pH range of a Gemini C_{18} column (Phenomenex), used in this work, is 1–12, where the pH above 10 should only be used in gradient elution as recommended by the manufacturer.

The choice of buffer is primarily dependent on the pH required. **Table 6.5** lists a series of typical buffer options for HPLC. The most effective buffering range is calculated as its pK_a value ± one pH units.

Phosphate is a popular buffer choice for HPLC due to its low UV cut-off wavelength (less than 220 nm) and three pK_a values which covers acidic (pH = 1.2–3.2), neutral to basic (6.2–8.2) and very basic range (11.3–13.3). However, the low solubility of phosphate in mobile phase with high organic modifier is a well-known issue. The solubility of potassium phosphate in 80% acetonitrile aqueous solution has been reported as 5 mM (Schellinger and Carr, 2004). The precipitated phosphate salt can lead to column back pressure increase and problems with check valves of the pump. Therefore, the maximum amount of organic modifier in the mobile phase needs be considered depending on the solvent type and buffer concentration. Phosphate tends to have a higher solubility in methanol/water than acetonitrile/water systems.

Table 6.5. Common mobile phase buffers used in HPLC

Buffer	pKa (25 °C)[a]	Effective pH range[b]	MS compatible
Trifluoracetic acid	0.52	< 1.5	Yes
Phosphate	2.16 (pKa_1)	1.2–3.2	No
Formate[c]	3.75	2.8–4.8	Yes
Acetate[d]	4.76	3.8–5.8	Yes
Phosphate	7.21 (pKa_2)	6.2–8.2	No
Ammonia	9.25	8.2–10.2	Yes
Triethylamine	10.75	9.7–11.7	Yes
Phosphate	12.32 (pKa_3)	11.3–13.3	No

[a] From CRC Handbook of Chemistry and Physics (2023)
[b] Most effective pH range using pKa ± 1 (1 decimal)
[c] Compatible with LC-MS as ammonium formate
[d] Compatible with LC-MS as ammonium acetate

In this work, aqueous ammonia was initially chosen for its basic buffer range (8.2–10.2) and high solubility with organic solvents. The pK_a (9.25) exceeds the

targeted pH of 8 to ensure the α-ribazole is neutral, which made it a suitable candidate for controlling the pH for retaining α-ribazole on a C_{18} column. Additionally, ammonia is compatible with LC-MS and hence easier for method transfer to mass spectrometry.

Ammonia aqueous solution (0.3% *v*:*v*) together with acetic acid (for pH adjustment) was prepared with pH values 8.5 and 9.5 in order to identify a pH level which might improve the separation of α-ribazole from interference. **Figure 6.10** shows that using either pH 8.5 or 9.5 failed to improve the resolution as the peak shoulder was present in both to virtually the same extent.

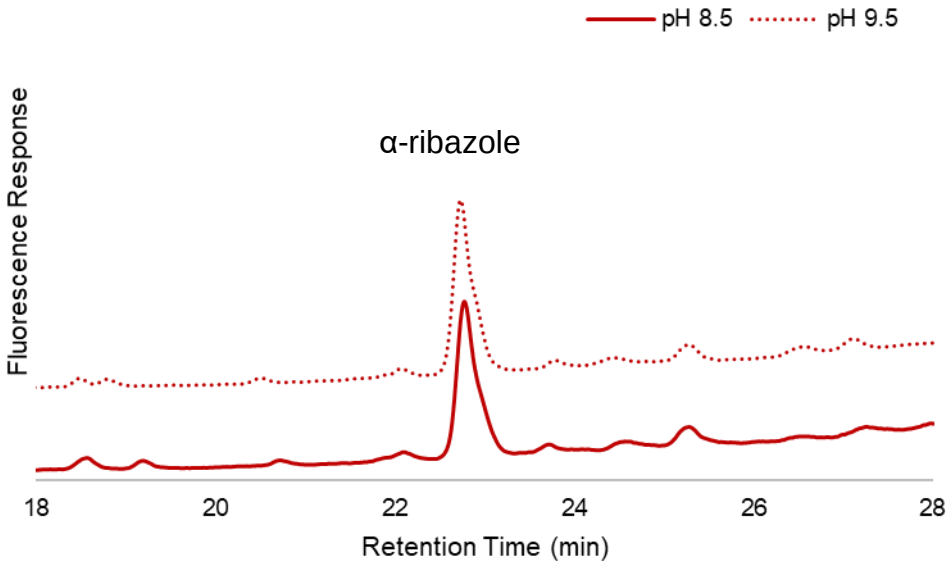


Figure 6.10. Expanded chromatograms of α-ribazole from an infant formula sample by RP-HPLC-FLD with different pH values of mobile phase (18–28 min)

Mobile phase A with a lower pH (7.5) was also tested. Since pH 7.5 is outside of the buffer range of aqueous ammonia solution, a different buffer system was required. Phosphate (pK_{a2} = 7.21) is a suitable buffer for neutral pH range. Potassium monophosphate (10 mM) aqueous solution was prepared with pH of 7.5 to replace the ammonium hydroxide and other chromatographic conditions remained the same. The test was applied to the same infant formula sample. An α-ribazole standard was used for peak identification. The resultant chromatograms are shown in **Figure 6.11**. The RT was 19.16 min shorter than

when using ammonia as buffer. Changing of buffer type and concentration (ionic strength) might contribute to the earlier elution. However, an unresolved peak after α-ribazole was observed in the infant formula sample and the resolution problem remained. Assuming the co-eluted peak was the same as the one present using ammonia buffer and represents only a single compound, it is more clearly resolved than at the higher pH or using ammonia as buffer: however, lowering the pH any further risks problems of insufficient deprotonation of the α-ribazole. Any further pursuit of resolution requires more information regarding the identity of interference.

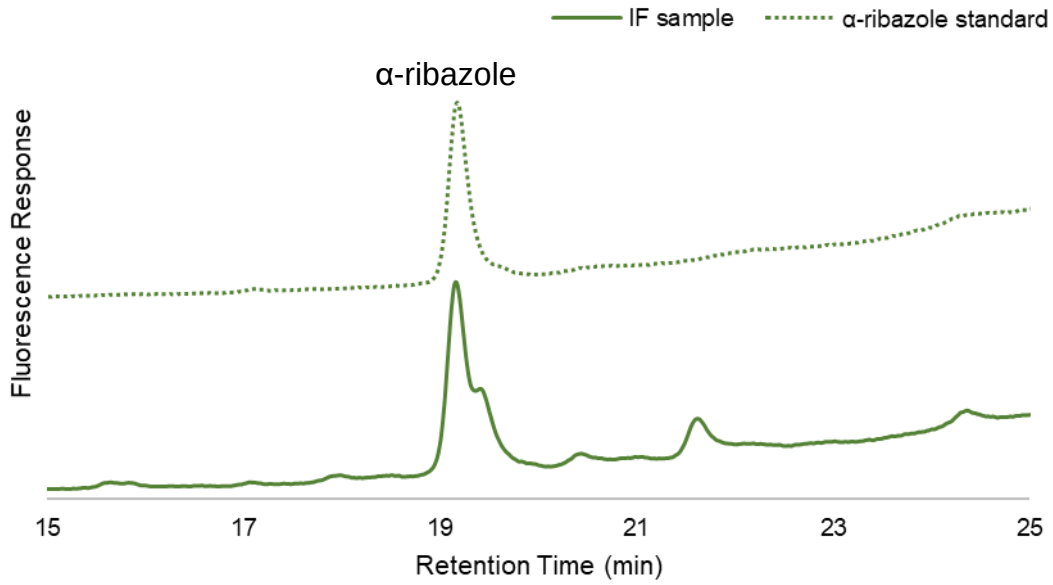


Figure 6.11. Expanded chromatograms of α-ribazole as a standard and from infant formula by RP-HPLC-FLD with pH of 7.5 for mobile phase A: potassium phosphate (10 mM)

During the foregoing investigation, the manipulation of the pH of the mobile phase was achieved by changing the aqueous pH of mobile phase A. However, the addition of organic solvent can affect the final pH of the mobile phase which varies with the concentration of the organic solvent and type. The change of pH also depends on the buffer type, initial concentration, and pH of the aqueous buffer solution (mobile phase A). Subirats *et al.* (2007) developed equations to estimate the pH values of the mobile phase with up to 80% of methanol addition and for the initial buffer concentration ranging from 0.001 to 0.1 mol L^{-1}. According to Subirats *et al.* (2007), when using the ammonia buffer system, the pH of the mobile phase decreases with increasing methanol fraction and can

reduced by up to 0.55 pH units at 80% methanol. The buffer concentration used in this work was 0.043 mol L^{-1} and the maximum methanol fraction was 80% in the mobile phase composition, thus with an initial aqueous buffer pH value of 9.5, the mobile phase pH can decrease to less than 9 over the course of the gradient. However, it is still well above the pK_a of α-ribazole and the neutral state of α-ribazole will be maintained by solely changing the aqueous pH despite the addition of methanol.

6.2.2.3 Column Temperature

Generally, elevated temperature lowers viscosity of solvent and causes compounds to elute faster from the column. The column temperature affects the selectivity, particularly of ionisable compounds since equilibrium constants are temperature dependent. By keeping other conditions unchanged, including gradient elution, mobile phase and flow rate, three different column temperatures, 30, 40 and 50 °C, were tested for their effect on the separation. **Figure 6.12** shows the resultant chromatograms, in each case the α-ribazole was derived from the same sample of infant formula. When the column temperature was 40 or 30 °C, (RT = 21.81 min and 22.82 min, respectively) no improved resolution between α-ribazole and the interference peak was observed. At 50 °C (RT = 20.67 min), the peak shoulder was not visible, however, lack of any additional peak suggested that the interference peak was completely overlaid with the α-ribazole peak. This indicates that raising column temperature worsens rather than improves resolution and operation at a lower temperature is preferable.

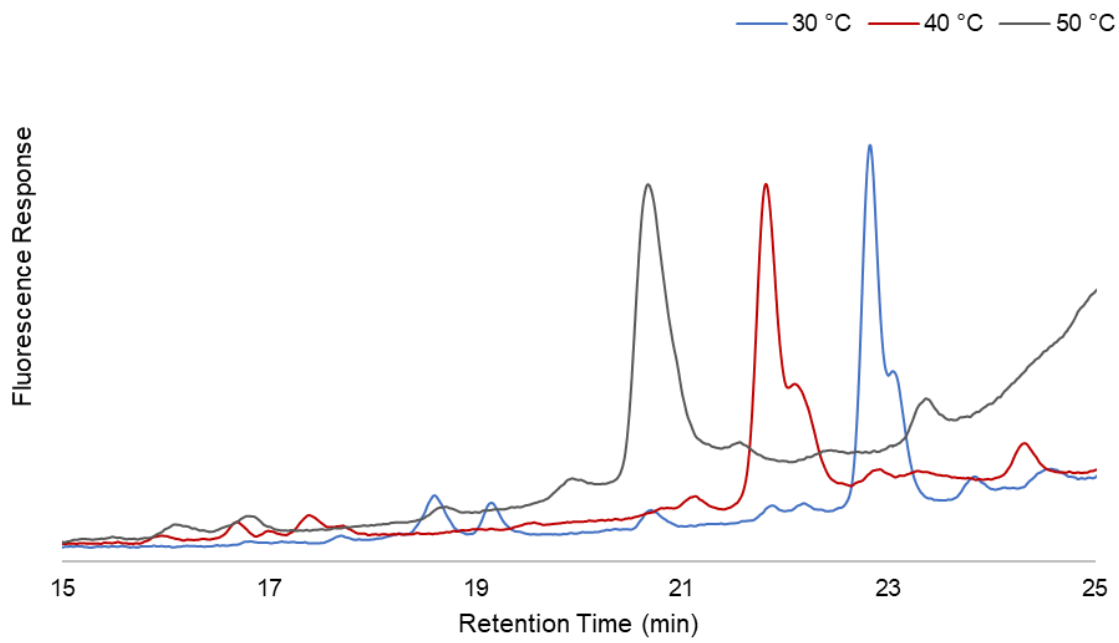


Figure 6.12. Expanded chromatograms of α-ribazole from the same infant formula sample by RP-HPLC-FLD with different column temperatures

6.2.2.4 Summary of Optimisation of RP-HPLC

In summary, the methods of improving the resolution investigated in this section through adjusting solvent strength, pH of mobile phase and column temperature did not show promising outcomes. However, there are more refined approaches which could be followed in the investigation, for example, testing tailored gradient elutions, pH of mobile phase and column temperatures while using acetonitrile as organic modifier. Acetonitrile is a stronger solvent in this system than methanol due to its lower polarity. As an aprotic solvent, acetonitrile might offer a different retention behaviour than using methanol in the mobile phase. Because of low buffer solubility, acetonitrile was not favoured during this method development stage but it does not invalidate the potential of providing better resolution. However, it was felt that some information about the identity of the interference might allow a more targeted approach.

6.2.3 Investigation of the Interfering Compound by RP-LC-HRMS

LC-MS can provide structural information of the analytes through the mass spectra of resulted ions (of molecules or fragments) after ionisation. The

analytes can be identified by an isotopic signature with high-resolution MS (HRMS) or a characteristic fragmentation pattern with MS/MS. Therefore, the same samples derived from infant formula, which were used in **Section 5.1.2** were analysed by a LC-HRMS system with identical chromatographic condition as in RP-HPLC-FLD, including the same C_{18} column for separation. A full scan MS (100–1000 *m/z*) for both polarities was performed on α-ribazole standard and on an infant formula sample on a high resolution Orbitrap-based MS. Comparison of the mass spectra of the α-ribazole standard under both positive and negative mode, indicated that the positive mode showed a clearer response as shown in **Figure 6.13**. The mass spectra were extracted at the retention time of 27.71 min with background subtraction (RT 20.00–21.00 and 23.00–24.00 min).

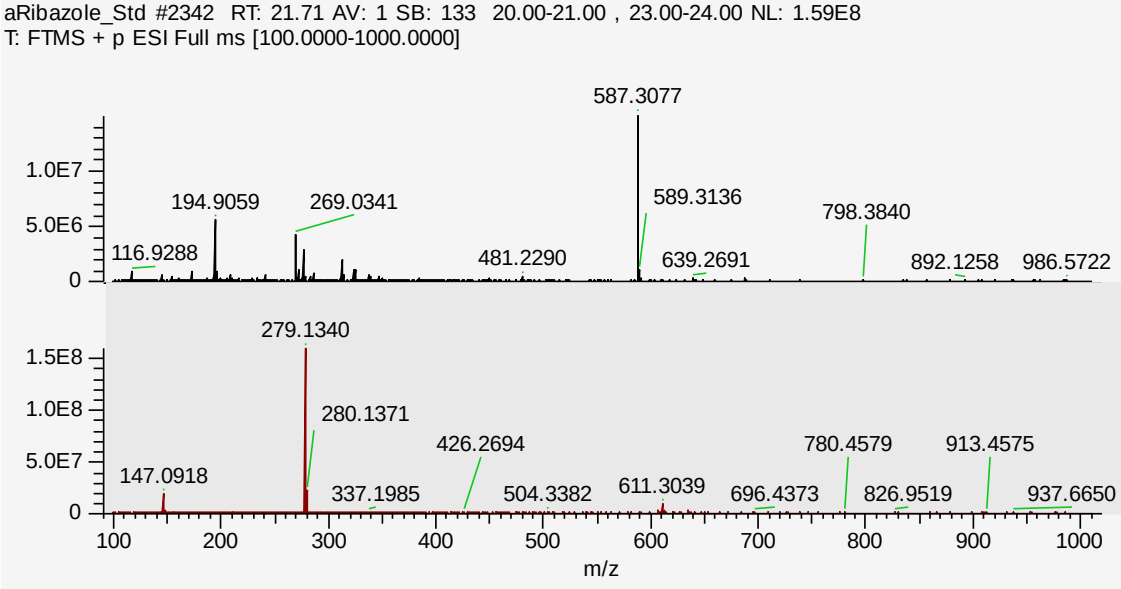


Figure 6.13. Mass spectra of α-ribazole under negative mode and positive mode

An α-ribazole standard was analysed using positive ionisation mode; the ion at *m/z* 279.1339 was extracted (RT = 21.71min) and corresponded to the $[M+H]^+$ ion of α-ribazole, the total-ion chromatogram (TIC) and extracted-ion chromatogram (EIC) of the α-ribazole standard are shown in **Figure 6.14**. Due to the low concentration of α-ribazole and other noise, the peak of α-ribazole ($[M+H]^+$) in the TIC was not visible. The approximately 1-min difference between the RTs of RP-HPLC-FLD (22.72 min) and RP-LC-HRMS were

expected due to variations in the length and inner diameters of the plumbing between the two systems.

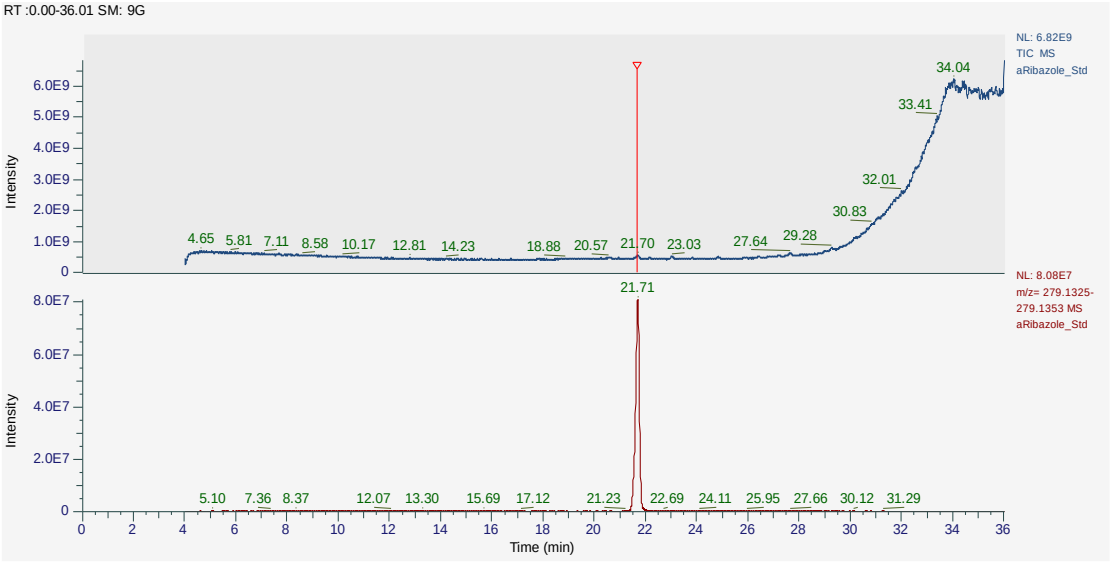


Figure 6.14. Chromatograms of α-ribazole standard by LC-HRMS: total-ion chromatogram and extracted-ion chromatogram of $[M+H]^+$

In the chromatogram of the infant formula sample (**Figure 6.15**) at this RT (21.73 min), the same ion at *m/z* 279.1339 was observed, which confirmed the presence of the α-ribazole. The extracted mass spectrum at this RT (21.73 min) is shown in **Figure 6.16**, where a background subtraction (RT 21.0–21.00 and 23.00–24.00 min) was applied to limit the background noise. The high noise most likely due to the low concentration of α-ribazole in the infant formula sample. Signals of several ions were observed when analysing α-ribazole standard. The ion at *m/z* 696.4372 signal was the most abundant in the mass spectrum of the infant formula sample at RT 21.73 min, the EIC of it showed a peak with a RT of 21.64 min, which did not match with the RT of interference on RP-HPLC based on the RT difference between the α-ribazole and interference.

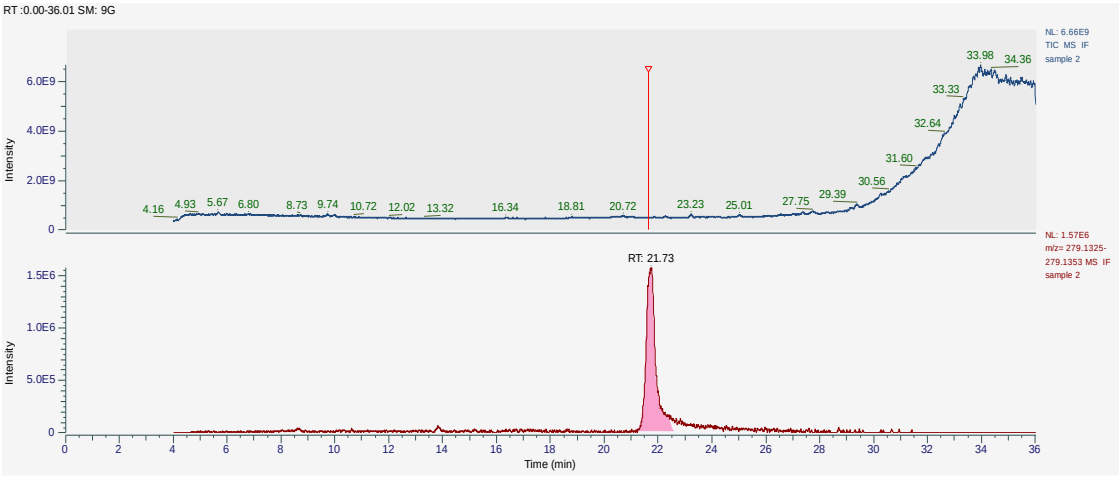


Figure 6.15. Chromatogram of α-ribazole in infant formula sample by LC-HRMS: total-ion chromatogram and extracted-ion chromatogram of $[M+H]^+$ ion at m/z 279.1339

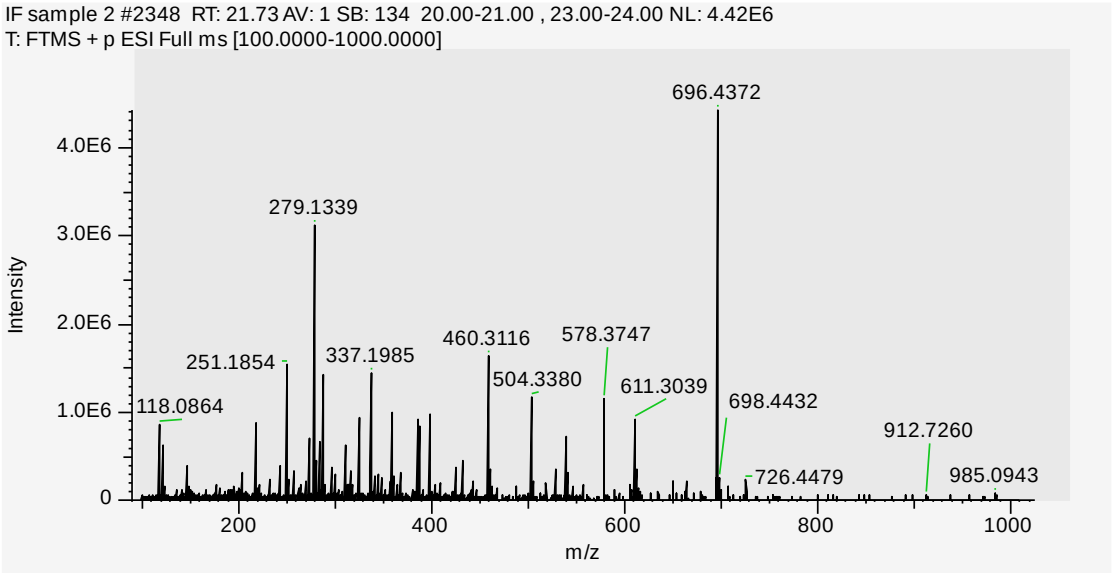


Figure 6.16. Mass spectrum of infant formula sample at RT 21.73 min under positive mode by LC-HRMS with background subtraction

Since no change was made to the chromatographic conditions, the elution order of compounds should remain the same. A mass spectrum, **Figure 6.17,** was extracted at the predicted RT of the interference compound at 21.93 min, which was estimated on the basis of RT difference, 0.2 min, between the α-ribazole and interference in RP-HPLC-FLD. A background spectrum subtraction (RT: 21.0–21.75 min) was also performed to produce a cleaner spectrum.

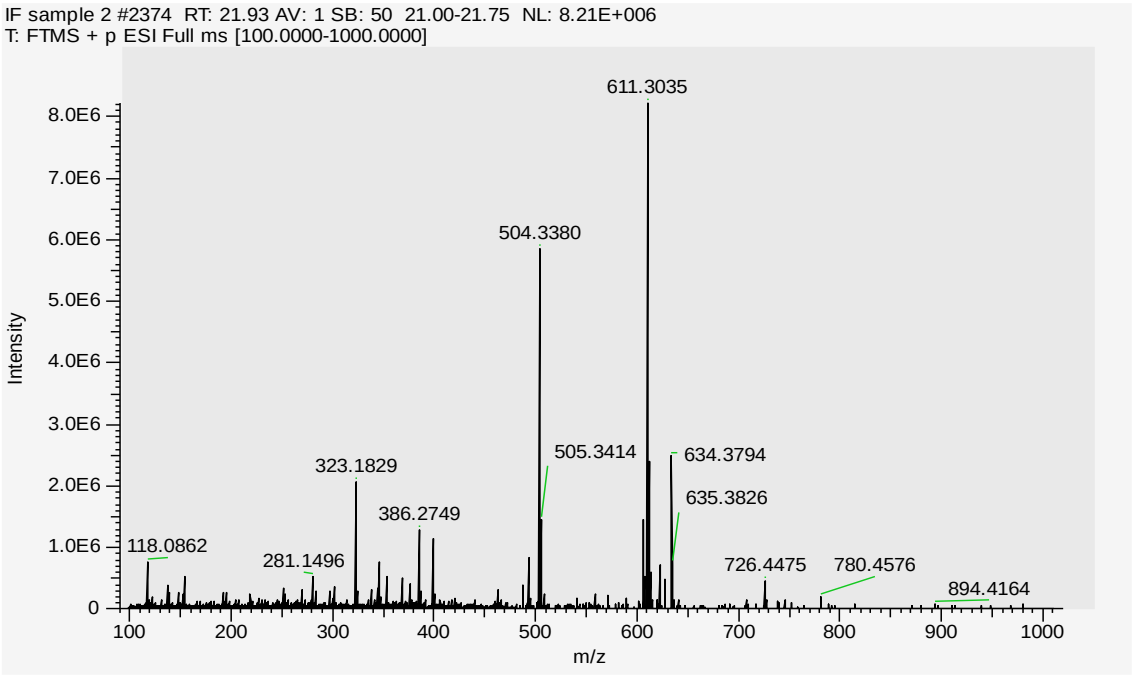


Figure 6.17. Mass spectrum of infant formula sample at RT 21.93 min with background subtraction

When comparing the mass spectrum of the infant formula sample to the α-ribazole standard at the same RT (21.93 min), **Figure 6.18,** several ions stood out as either appearing or significantly increasing in the infant formula sample spectrum. The EICs (**Figure 6.19**) of these ions showed the retention time differences from α-ribazole ranged between 0.13–0.26 min (detailed summary in **Table 6.6**).

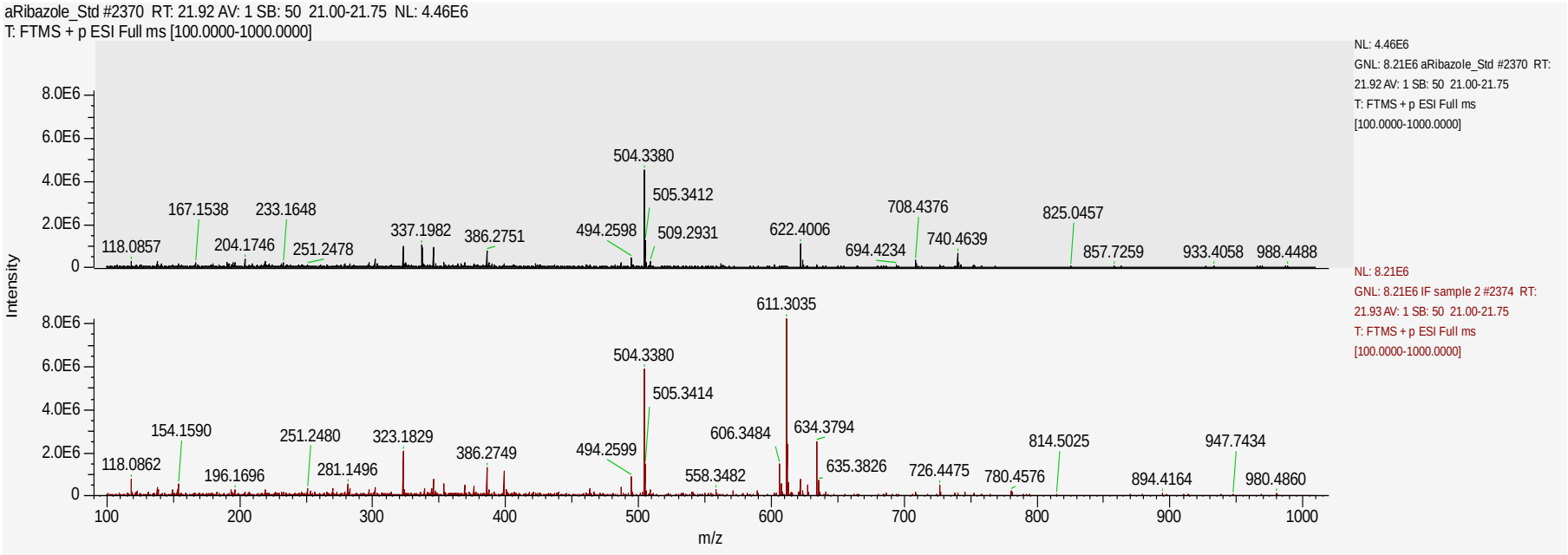


Figure 6.18. Comparison of the mass spectra between α-ribazole standard and infant formula sample at RT 21.93 min.

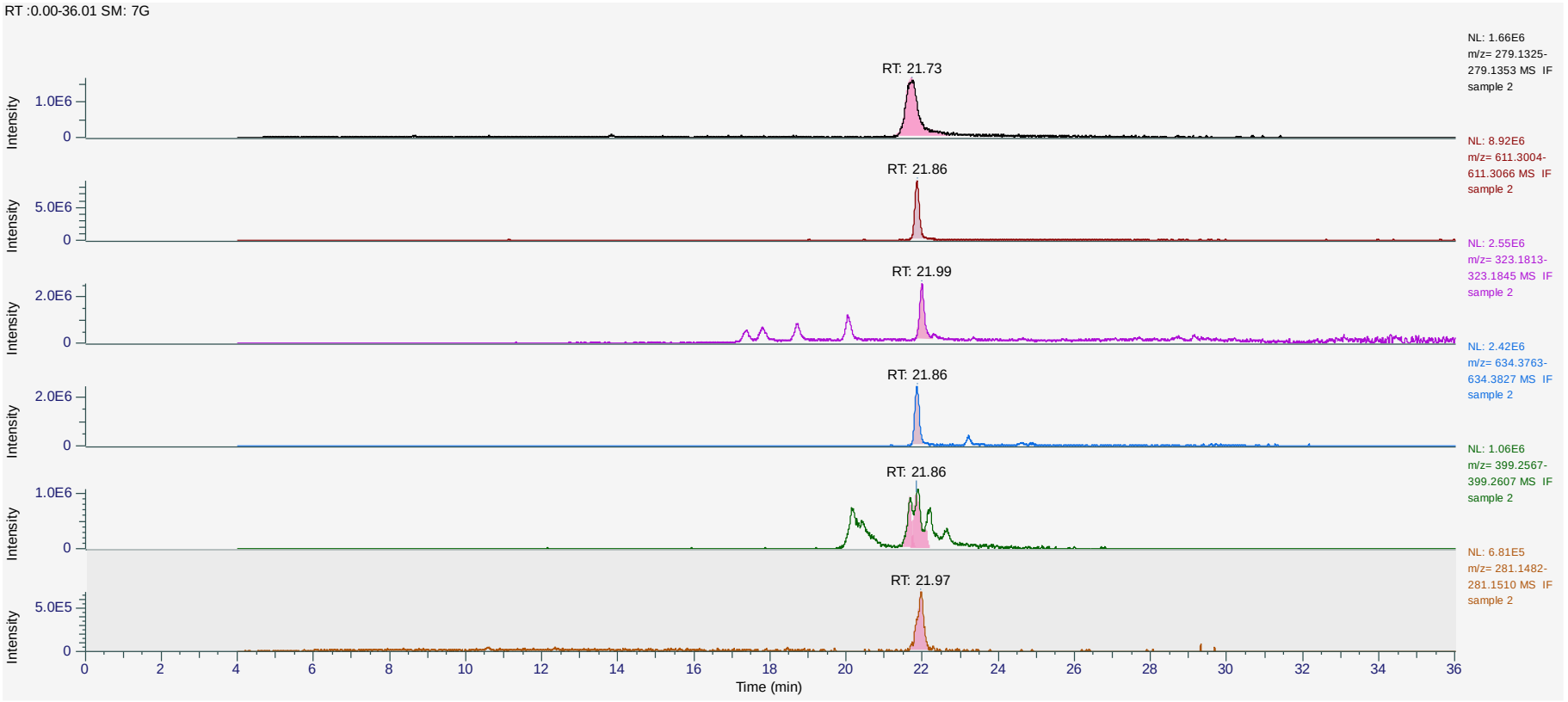


Figure 6.19. Extracted-ion chromatograms of ions which only appeared or significantly increased in the infant formula sample.

Table 6.6. Retention time details of the potential interference ions

Ions (*m/z*)	RT (min)	Δ RT[a] in LC-HRMS (min)	Δ RT[b] in RP-HPLC (min)
611.3035	21.86	0.13	
323.1829	21.99	0.26	
634.3795	21.86	0.13	0.18–0.20
399.2587	21.86	0.13	
281.1496	21.97	0.24	

[a] RT difference between the ion and the $[M+H]^+$ ion of α-ribazole (279.1339 *m/z*) in the LC-HRMS

[b] Retention time difference between co-eluting peak and α-ribazole in the RP-HPLC

Among the five ions listed above, the ion at *m/z* 323.1829 (ΔRT = 0.26 min) and *m/z* 281.1510 (ΔRT = 0.24 min) were the closest to the RT difference on RP-HPLC-FLD (0.18–0.20 min). The ion at *m/z* 611.3035 had the highest signal intensity among the five ions, yet a high signal intensity in MS reflects an easier ionisation degree and not necessary a greater concentration. However, without further fragmentation information regarding these ions, it is almost impossible to identify the compounds. Unfortunately, the access to hiring the LC-HRMS instrument was limited due to the cost and no further investigation on the structural information was conducted. However, the absence of these ions under other chromatographic conditions can be used to show the interference has been eliminated (see below **Section 6.2.5**).

Overall, since there were multiple suspected ions, it is likely that no fine-tuning on the RP-HPLC could cover all targets to improve the resolution. An alternative chromatographic separation rather than reversed-phase should be explored for resolving the co-eluting issue.

6.2.4 Alternative Separation Technique: HILIC-FLD

HILIC is an alternative HPLC mode for separating polar analytes, which can be neutral or charged under buffer control, on a polar surface (stationary phase) with mobile phase usually consisting of water-miscible polar organic solvents, such as acetonitrile and water (at least 3%). In contrast to RP-HPLC, organic solvents are weak eluents in HILIC mode and polar compounds show longer retention times when the mobile phase contains high levels of the organic

solvent. Although there is not an agreement on the separation mechanism, partition theory is the most popular explanation in literature, that is the partitioning phenomenon between the acetonitrile-rich mobile phase and a water layer adsorbed onto the stationary phase causes the separation of compounds with different degrees of polarity (Buszewski and Noga, 2012).

As α-ribazole (conjugate acid) has a pK_a value around 6, it can be converted into the positively-charged protonated form under acidic conditions, hence, promoting hydrophilicity and becoming retainable on a HILIC column. Therefore, a separation of the α-ribazole from an infant formula sample was trialled on a HILIC column with both FLD and MS detection. The infant formula samples were prepared as in **Section 5.1.2** with modification on the final reconstitution solvent using 100% acetonitrile instead of methanol. The chromatograms of infant formula samples and the α-ribazole standard by HILIC-FLD is shown in **Figure 6.20**, in the infant formula sample, a single peak was observed at RT 3.66 min which matched with the RT of α-ribazole standard (RT 3.65 min) with a mobile phase consisting of acetonitrile (95%) and 100 mM ammonium formate (pH 3.5) (5%). There was no visible co-eluting peak or peak shoulder on the α-ribazole signal in the chromatogram of the infant formula sample. Additionally, the retention time was shortened significantly to 3.66 min and the total run time (10 min) became less than a quarter of the time used by RP-HPLC-FLD method (41–60 min, depending on the elution starting point). A further advantage was that the method did not require a gradient to achieve sufficient separation.

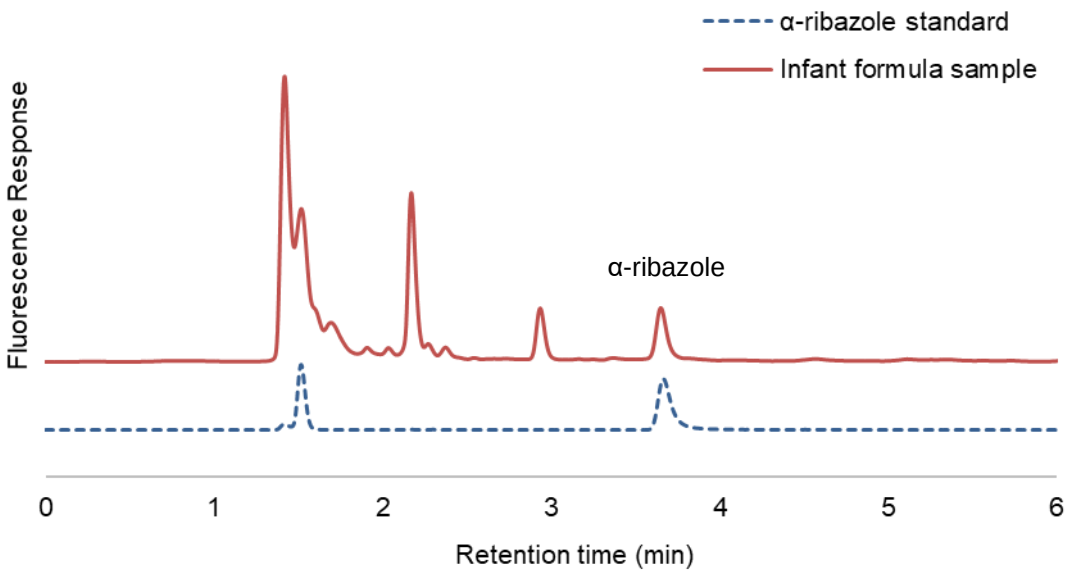


Figure 6.20. Chromatogram of infant formula sample and α-ribazole standard by HILIC-FLD.

6.2.5 Confirmation of Elimination of Interference by HILIC-MS

To demonstrate the absence of the identified possible ions of interference compounds in the infant formula samples after switching to HILIC for separation, the same infant formula samples were analysed by HILIC-HRMS under the same chromatographic conditions including using the same HILIC column.

The EICs for the $[M+H]^+$ ion of an α-ribazole standard (RT 3.41 min) and the α-ribazole from an infant formula sample (RT 3.40 min) are shown in **Figure 6.21**. Both retention times matched with the corresponding RT by HILIC-FLD (3.65 and 3.66 min, respectively). The 0.24–0.26 min difference between the two systems was smaller than the one found when using a RP column. For the RP-HPLC method, a gradient elution was required while in the case of HILIC, it used a simple isocratic elution with more reproducible retention times.

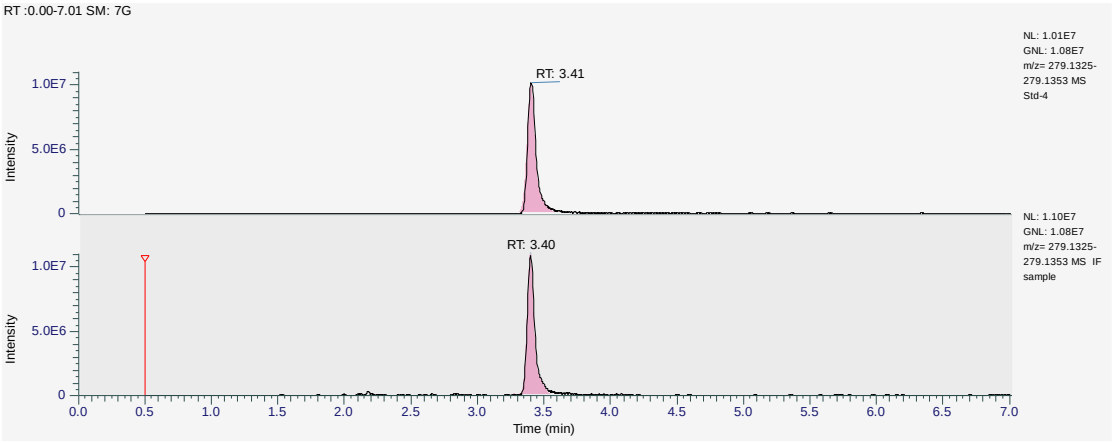


Figure 6.21. Extracted-ion chromatogram of α-ribazole standard and infant formula sample by HILIC-HRMS

The co-eluting ions found in the infant formula sample in **Section 6.2.3**: signals at *m/z* 611.3035, *m/z* 323.1829, *m/z* 634.3795, and *m/z* 281.1496 were not observed around the RT of α-ribazole when comparing their EIC for HILIC-HRMS (**Figure 6.22**) with of RP-LC-HRMS (**Figure 6.19**). The first three ions eluted earlier than α-ribazole at around 2 min and the ion at *m/z* 281.1496 eluted at 6.5 min, which indicated that the separation was achieved.

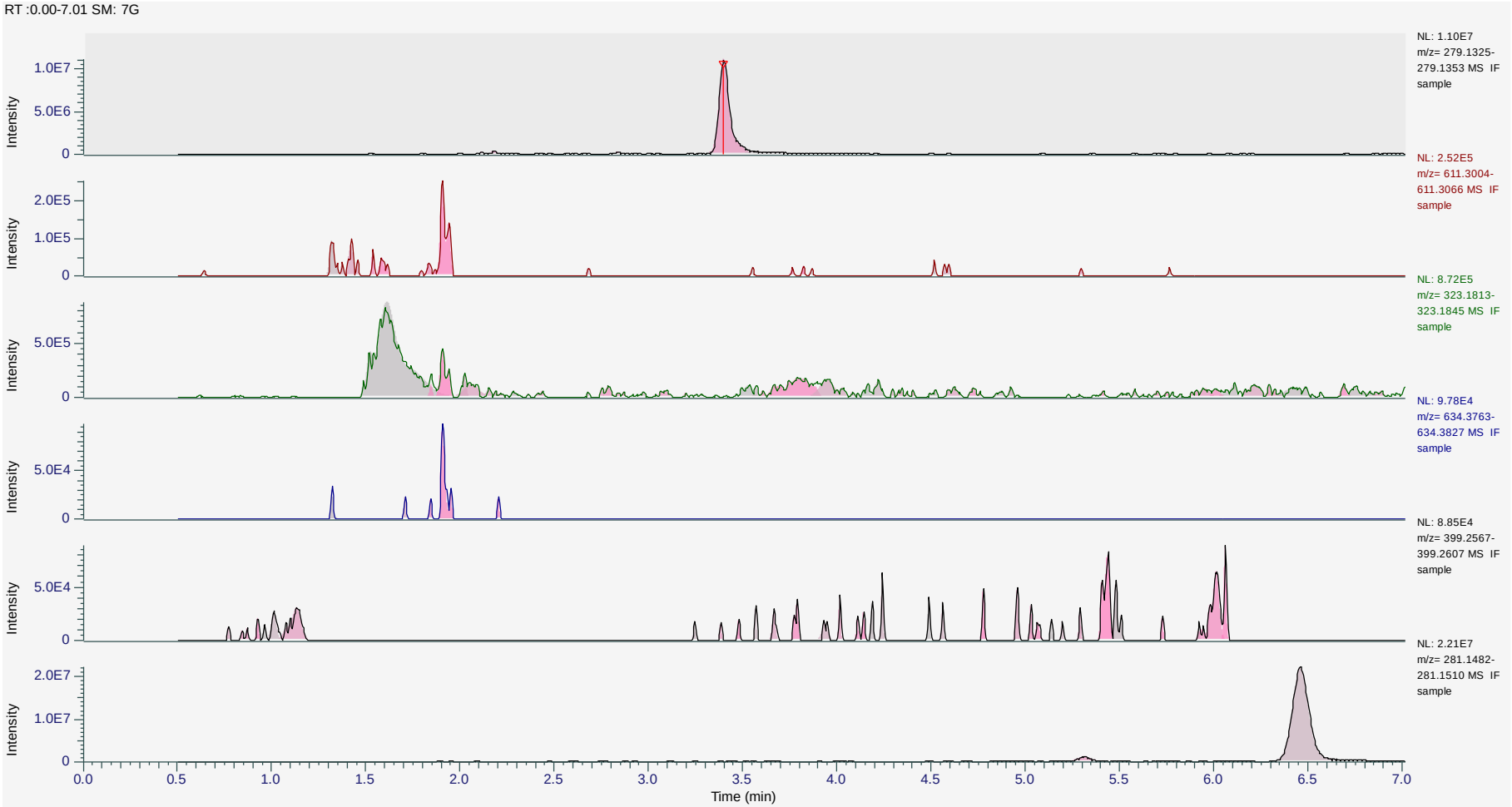


Figure 6.22. Extracted-ion chromatograms of α-ribazole and the interference ions on RP-HPLC in the infant formula sample during HILIC-HRMS

6.3 Summary

RP (C_{18}) columns are commonly used in laboratories, are less expensive than the cost of HILIC columns and have a lower running cost. However, a series of optimisation experiments on the RP-HPLC failed to achieve an acceptable resolution for α-ribazole in the infant formula. There are more approaches that might be explored to achieve separation, for example, investigating column temperatures, elution programs, pH whilst using acetonitrile in the mobile phase, switching to C_{18} columns with different modified surface. However, for all practical purposes it appears that reversed phase is not suitable for this analytical procedure. There is a risk that someone might attempt to use C_{18} to analyse the α-ribazole in infant formulas and might not even notice the presence of interference since RP (C_{18}) columns are commonly found in laboratories.

The issue of the interference signal co-eluting with the α-ribazole peak in the RP-HPLC-FLD chromatogram was solved by changing the separation technique to hydrophilic interaction chromatography. This removed the interference away from the α-ribazole peak and significantly shortened the instrumental analysis time (10 min compared to 41–60 min).

In this research, the decision was made to employ HILIC separation due to the ability to remove the interference from α-ribazole. The full method validation using HILIC-FLD for quantifying vitamin B_{12} in infant formula is presented in the following chapter.

7 Validation of a HILIC-FLD Method to Quantify Vitamin B_{12} in Infant Formulas Using Cobalamin-derived α-Ribazole

A method validation study is required to examine the performance of any newly developed method. In this chapter, required parameters were assessed to determine the system's suitability and a single laboratory validation study was conducted according to the AOAC guidelines (AOAC International, 2023c). A brief literature review of the method validation parameters involved in this work is included in this section. Finally, the candidate method was used to analyse the vitamin B_{12} content in various infant formula products purchased locally. Results were compared with those obtained from the currently used analysis procedure involving cyanide, which was conducted at an external laboratory, to demonstrate the application potential of the candidate method.

7.1 Literature Review of Method Validation

7.1.1 HPLC System Suitability

The HPLC chromatographic system should initially be proved suitable for application in the desired analysis. A desirable chromatographic analysis means the analyte(s) of interest can be optimally separated from other substances over the shortest total time period. There are a number of parameters that can be used to evaluate the chromatographic performance in a system suitability study, including resolution, tailing factor, capacity factor, repeatability of injection, and theoretical plates count (CDER, 1994).

7.1.1.1 Asymmetry Factor

Band broadening in the column results in peak tailing or fronting, which leads to an asymmetric peak. Significant tailing or fronting will compromise the accuracy of quantitation as there is a higher degree of uncertainty in determining the peak area as well as the risk of overlapping with neighbouring peaks.

The asymmetry factor (A_s) is a ratio of peak width at 10% peak height to the twofold distance between the peak front and of the maximum peak height, **Equation 7.1**. An A_s value greater than 2 indicates that the peak is unacceptable. In general, tailing is more common in the HPLC analysis, which can be minimised once the cause is found. There are various causes for severe peak tailing; however, in most cases, tailing is due to either a stronger sample solvent than the eluent or a non-specific interaction occurring on the stationary phase, such as interaction with amines and absorption of acidic compounds (CDER, 1994).

$$A_s = \frac{b}{a} \qquad \textit{Equation 7.1}$$

Where,

a = Front half width of the peak measured at 10% from the baseline of the peak height

b = Back half width of the peak measured at 10% from the baseline of the peak height

7.1.1.2 Capacity Factor

Capacity factor (*k'*), also known as the retention factor, is a calculation of the retention of the peak of interest with respect to the non-retained portion. By definition, *k'* is the ratio of retention time of an analyte on the column to the time of the non-retained portion, **Equation 7.2**. The peak of interest should be well-resolved from other interfering peaks in the void volume; the value of *k´* should be greater than 2 so the analyte has sufficient interaction with stationary phase (CDER, 1994).

$$k' = \frac{(t_R - t_o)}{t_o} \qquad \textit{Equation 7.2}$$

Where,

t_R = Retention time of the analyte

t_o = Elution time of the non-retained portion

7.1.1.3 Repeatability of Injection

The performance of an HPLC system can be affected by several factors, including but not limited to eluent composition, pump condition, injection volume, column temperature, detector condition, and subjective peak integration. Hence, repeatability checks of the injection will assess whether the system can produce the same peak response for the same analyte after replicate injections. Expressed as RSD, a value of less than 1% is preferable (CDER, 1994).

7.1.1.4 Theoretical Plate Number

The efficiency of an HPLC column is expressed as theoretical plate number (*N*). For columns with the same length, the one with a larger value of *N* shows less band broadening, resulting in narrower peaks. In general, an *N* value greater than 2000 is acceptable. However, in the calculation of resolution, because the role of *N* is less important than other factors, such as capacity factor, different theoretical plate numbers may barely show a difference on visual inspection of the chromatogram (CDER, 1994; Fornstedt *et al.*, 2015).

7.1.2 Single Laboratory Validation

Method validation is the process of defining and confirming the validity of an analytical method considering sample preparation and instrument analysis. Therefore, a validation procedure can be used to check the reliability of the method to ensure the quality of the analytical results. There are a number of performance characteristics involved in the procedure; in the practice of single laboratory validation studies, the typical components include selectivity/specificity, linearity, accuracy, precision, the LOD, LOQ and robustness/ruggedness (Deshmukh *et al.*, 2019; Eurachem Guide, 2014; Taverniers *et al.*, 2008).

7.1.2.1 Confirmation of Identity

The term of confirmation of identity is used to reflect the fact that the signal of an analyte of interest is only contributed by the analyte itself and is not due to the presence of other interferences. Since it is impossible to eliminate matrix

inferences completely through sample preparation, a suitable identification test should be conducted. In the chromatographic system, this can be achieved by comparison of the assumed peak in the sample and that of an authentic standard with high purity with reference to retention time (Taverniers *et al.*, 2008).

7.1.2.2 Linearity

Linearity of an analytical procedure is the ability to produce an instrument detector response which is directly proportional to the analyte concentrations in the sample. Often a correlation coefficient (r^2) is used to measure the linearity. However, it is often misused as a large r^2 value does not necessarily indicate a linear relationship between the measurements (Analytical Methods Committee, 1988; Jurado *et al.*, 2017). A visual examination can be used for the evaluation, and an objective approach called the residuals plot is also available for this purpose. Residual values are the difference between the observed signal value and the predicted value from the regression equation for each concentration. Linearity can be confirmed when the calculated residuals are randomly and closely distributed about the regression curve (AOAC International, 2023c; Chan, 2008; Deshmukh *et al.*, 2019; Taverniers *et al.*, 2008).

7.1.2.3 Accuracy

The accuracy of an analytical procedure is defined as the nearness of measured value to the conventional true or accepted reference value, which can be assessed by both a bias study and a recovery study (AOAC International, 2023c; Chan, 2008; Deshmukh *et al.*, 2019).

The analysis of a reference material with a known concentration can be used to evaluate the overall bias of the method. The reference materials can be, for instance, quality control samples and certified reference materials (CRMs). The matrix CRM is considered ideal for its similarity to the sample of interest. In addition, comparison to a reference method can be an alternative way to measure the overall bias in the method. The reference method should be well-characterised; for example, the recommended methods from standard

organisations such as AOAC or routine methods currently used in the laboratory. For the comparison, the sample should be identical for both methods. The existence of any significant difference between methods should be tested at a 95% level of confidence (Chan, 2008; Deshmukh *et al.*, 2019; Taverniers *et al.*, 2008).

A recovery experiment can be performed by comparing the analyte-free matrix with/without a spike of a known amount of analyte. The recovery is expressed as a percentage. If an analyte-free matrix is unavailable, a material of similar composition to the matrix of interest can be used; alternatively, a subtraction is necessary when a matrix containing the analyte is applied. However, analysts face an underlying problem in the recovery study: the introduced analyte may not be as firmly bound to the matrix as the endogenous ones are. Hence, they are more easily extracted and thus, a good recovery may not actually correspond to good accuracy of the method as it is possible for an inaccurate method to return a recovery of 100%. However, recovery should always be established as a necessary part of the evaluation. Acceptable recovery is a function of the analyte concentration, although different guidelines offer various requirements. Generally, when recovery falls outside the acceptable range of a minimum of 60 or 70% to 110%, further investigation on the improvement of extraction efficiency or better chromatographic separation is required (AOAC International, 2023c; Taverniers *et al.*, 2008).

7.1.2.4 Precision

Precision is the closeness of agreement between a series of measurements obtained from multiple analyses of the same samples under designated conditions. Precision of an analytical procedure can be assessed from three levels: repeatability, intermediate precision and reproducibility (Chan, 2008; Deshmukh *et al.*, 2019).

Repeatability represents the precision under the same conditions over a short period of time, in which the same equipment is operated by a single analyst. It is expected to produce the smallest variation among the three levels (Eurachem Guide, 2014).

Intermediate precision is the variation between different analysts, different instruments and different performing days within a single laboratory (Deshmukh *et al.*, 2019).

Reproducibility is defined as the precision measurement between laboratories under possibly different conditions. It is expected to give the largest variation compared to the other two levels (Eurachem Guide, 2014).

Typically, precision measurements are expressed as SD or RSD and should be determined for multiple independent replicates on representative test matrices at different concentration levels (Eurachem Guide, 2014). The chosen matrices should be measured by the assessed method at least seven times (AOAC International, 2023c).

The observed precision results can be compared with existing methods which have already been validated. Alternatively, predicted RSD of reproducibility ($pRSD_R$) can be calculated from the Horwitz equation (**Equation 7.3**), which is a function describing the empirical relationship between the precision of analytical methods and the concentration of the analyte regardless of the nature of the matrix and method used. The predicted repeatability is typically half to two-thirds of the predicted reproducibility (Horwitz and Albert, 2006). AOAC guideline use half of $pRSD_R$ for acceptable predicted repeatability as the best-case scenario (AOAC International, 2023d). The ratio of the RSDs of the candidate method compared to the predicted RSDs value is called Horwitz Ratio (HorRat) values; the acceptable HorRat range is 0.5–2 for reproducibility and 0.3–1.3 for repeatability (AOAC International, 2023c; Deshmukh *et al.*, 2019; Horwitz and Albert, 2006).

$$pRSD_R\ \% = 2 \times c^{-0.15} \qquad \textit{Equation 7.3}$$

Where,

$pRSD_R\%$= Predicted RSD %for reproducibility

c = Concentration, as mass fraction

7.1.2.5 Limits of Detection and Limits of Quantitation

Although several official guidelines have established the significant role of LOD and LOQ in method validation, measurement approaches are varied. In HPLC methods, detection limits are commonly estimated based on a visual evaluation of the S/N ratio (Deshmukh *et al.*, 2019; Vial and Jardy, 1999). An alternative method of measuring the LOD and LOQ is based upon the variability of the blank (AOAC International, 2023d).

Limit of Detection

LOD is the lowest quantity of an analyte in a sample detected with a given confidence level but not necessarily quantified as an exact value. Generally, it is expressed as the concentration of analyte with an S/N ratio of 3:1 (Taverniers *et al.*, 2008; Vial and Jardy, 1999). Alternatively, the blank value plus 3 times the standard deviation of the blank may be used, A sample with low analyte content may be used in the absence of an analyte-free blank (AOAC International, 2023d).

Limit of Quantitation

LOQ is the lowest amount of analyte in a sample that can be quantitatively measured with suitable precision and accuracy. It is sometimes taken as the concentration of analyte with an S/N ratio of 10:1 (Deshmukh *et al.*, 2019; Taverniers *et al.*, 2008) or the blank value plus 10 times the standard deviation of the blank or a sample containing low levels of analyte.

7.2 Experiments

7.2.1 Materials

General materials and equipment are listed in **Chapter 2**. The tested infant formula products were purchased at local supermarkets (details are shown in **Section 2.6**)

Standard Reference Material (SRM) 1869 Infant/Adult Nutritional Formula II was purchased by the National Institute of Standards and Technology (NIST), U.S.

7.2.2 Sample Preparation

The sample preparation was conducted as described in **Section 5.1.2.**

7.2.3 HILIC-FLD System

The HILIC system described in **Section 2.4** was used for the chromatographic separation, and the quantitation was achieved by measuring fluorescence at excitation/emission wavelength pair: 251/303 nm. The standard solutions were prepared as described in **Section 2.5**.

7.2.4 Quantitation

The vitamin B_{12} content of prepared infant formula milk samples were calculated using **Equation 7.4.** The mass of powder for each sample was calculated by **Equation 7.5.**

$$C = \left[\left(\frac{c_t \times v_t}{m_t}\right) - \left(\frac{c_e \times v_e}{m_e}\right)\right] \times \frac{M_{B_{12}}}{M_{\alpha-R}} \times \frac{100}{1000} \qquad \textit{Equation 7.4}$$

Where,

$C=$ Content of vitamin B_{12} (as cyanocobalamin, µg 100 g^{-1})

$m_t =$ The mass of sample that underwent analysis for total α-ribazole (g)

$m_e =$ The mass of sample that underwent analysis for endogenous α-ribazole (g)

$c_t =$ The concentration of total α-ribazole in the sample solution by HILIC-FLD (ng mL^{-1})

$c_e =$ The concentration of endogenous α-ribazole in the sample solution by HILIC-FLD (ng mL^{-1})

v_t , $v_e =$ The volume of the sample solution (total and endogenous) analysed by HILIC-FLD: 1.0 mL

$M_{B_{12}} =$ Molar mass of vitamin B_{12}: 1355.358 g mol^{-1}

$M_{\alpha\text{-R}} =$ Molar mass of α-ribazole: 278.30 g mol^{-1}

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

Equation 7.5

Where,

$m_{powder} =$ The mass of infant formula powder used for reconstitution (g)

$m_{prep} =$ The mass of the sample taken of prepared reconstituted milk (g)

$m_{water} =$ The mass of water used for reconstitution (g)

7.2.5 Method Performance

7.2.5.1 System suitability

An α-ribazole standard was analysed in replicate (*n* = 6). Retention factor, theoretical plates, tailing factor and injection repeatability were calculated.

7.2.5.2 Single Laboratory Validation

Repeatability

Duplicates of each sample were processed on each day and repeated for five days. The samples were prepared by the same analyst and analysed at different times. The standard deviation (SD_r) and RSD_r of repeatability were calculated using **Equation 7.6** (Campos-Giménez *et al.*, 2008).

$$SD_r = \sqrt{\frac{\sum_1^5 (c_{ia} - c_{ib})^2}{n}}$$

Equation 7.6

Where,

SD_r Standard deviation of repeatability (*n* = 10)

c_{ia}, c_{ib} Concentrations (c) of the duplicates, a and b at day i (1–5).

Intermediate Precision

The replicates from the five days (*n* =10) were used for the calculation of intermediate precision by **Equation 7.7** (AOAC International, 2023d).

$$SD_{iR} = \sqrt{\frac{\sum(c_i - \bar{c})^2}{n}}$$

Equation 7.7

Where,

SD_{iR}	Standard deviation of intermediate precision
c_i	Concentrations (c) of replicate
$\bar{c}$	Mean of concentrations (n = 10)

Accuracy

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 onto the sample after accurately weighing the reconstituted infant formula sample then mixed well. The remaining preparation was carried out as described in **Section 5.1.2.** A bias study against the SRM 1869 were conducted in replicates (n = 12) over two days. The SRM 1869 was processed using the procedure outlined in **Section 5.1.2.**

7.2.6 Method Application

Eleven different infant formula products and ten milk products (including five milk powder products and five liquid milk products) were prepared (in duplicate) as described in **Section 5.1.2** and analysed for vitamin B_{12} content by HILIC-FLD as described in **Section 2.4**. The same samples were analysed by an external agency using a cyanide-based HPLC-UV method (Campos-Giménez *et al.*, 2008) for results comparison. A paired *t*-test was used for comparison between the candidate method and the HPLC-UV method (Campos-Giménez *et al.*, 2008) for analysis of vitamin B_{12} in infant formulas. A critical value (α) of 0.05 was chosen and the null hypothesis of the test was no difference between the two sets of results. The statistical analysis was conducted using Microsoft Excel software (version 2303).

7.3 Results and Discussion

7.3.1 System Suitability

The instrumental performance was assessed by analysing an α-ribazole standard (2.59 ng mL^{-1}, equivalent to two times of LOD level) in replicate (n = 6). Results are summarised in **Table 7.1**. Sample chromatogram of the α-ribazole standard and infant formula samples are shown in **Figures 7.1**. All assessed parameters met the guideline requirements (CDER, 1994).

Table 7.1. HILIC-FLD α-ribazole method: system suitability

Parameter	Result[a]	Guideline value[b]
Retention time (min)	3.7 (0.4%)	—
Retention factor, k	2.6 (0.4%)	> 2
Theoretical plates, N	9373 (9.4%)	> 2000
Tailing factor, T_f	1.8 (0.8%)	< 2
Peak area	195.3 (0.8%)	—

[a] Mean (relative standard deviation%) of 6 replicates of α-ribazole standard

[b] The guideline value is based on CDER (1994)

7.3.2 Single Laboratory Validation

Linearity was assessed as part of the single laboratory validation study and results are shown in **Table 7.2**. A calibration curve consisting of six α-ribazole standard solutions across the expected concentration range in samples were analysed (n = 3), **Figure 7.2**. The linear regression produced a squared correlation coefficient R^2 within acceptable level (> 0.99). Residuals were calculated and plotted against the concentrations of standard. In the context of linearity regression, a residual plot without any systematic deviations or discernible trends indicates non-biased linearity. In **Figure 7.3**, a random scatter of points formed around the zero line with sum and mean of residuals close to zero confirms linear fit of the observed data.

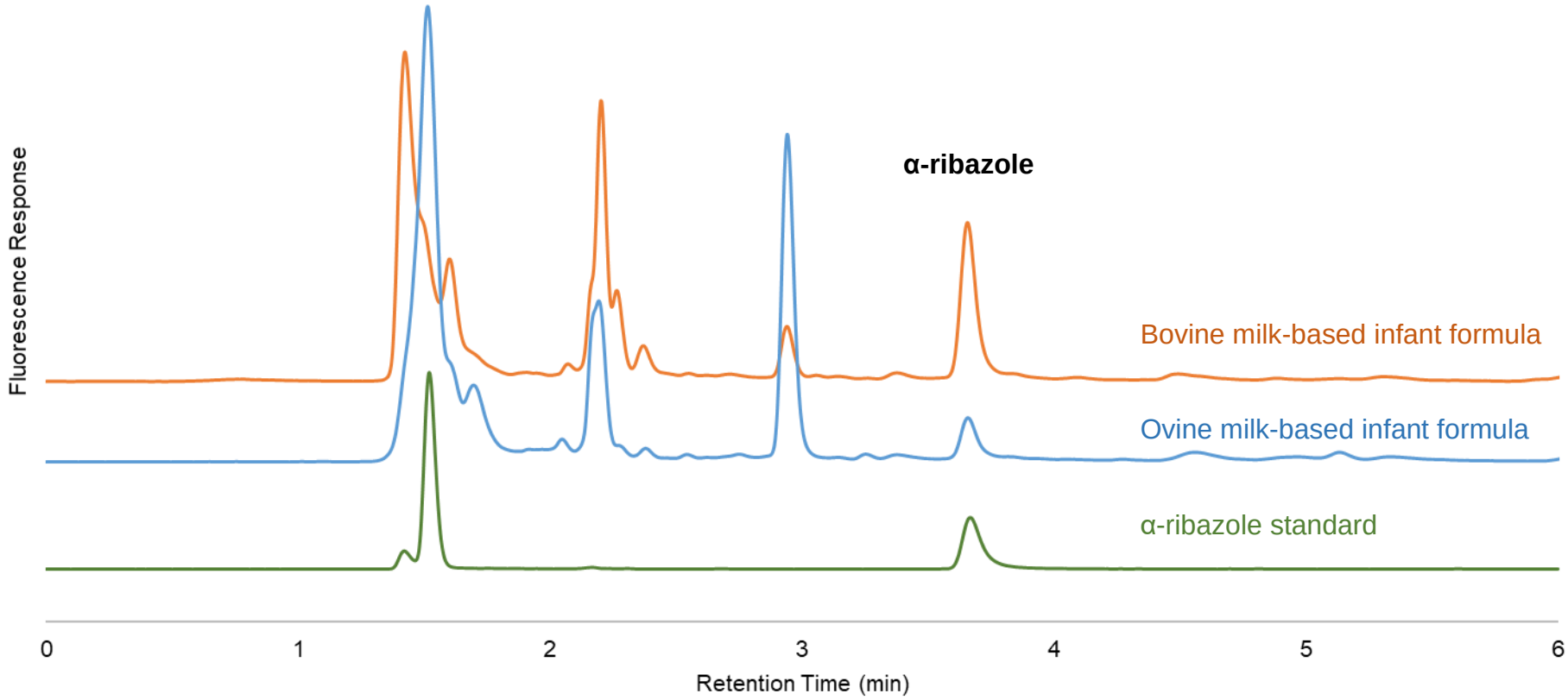


Figure 7.1. Chromatograms (0–6 min) of bovine milk-based, ovine milk-based infant formula and an α-ribazole standard by HILIC-FLD.

Table 7.2. HILIC-FLD α-ribazole method validation summary: linear regression

α-ribazole	Range (ng mL^{-1})	Linear regression equation	r^2
Rep 1	0.65–6.48	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

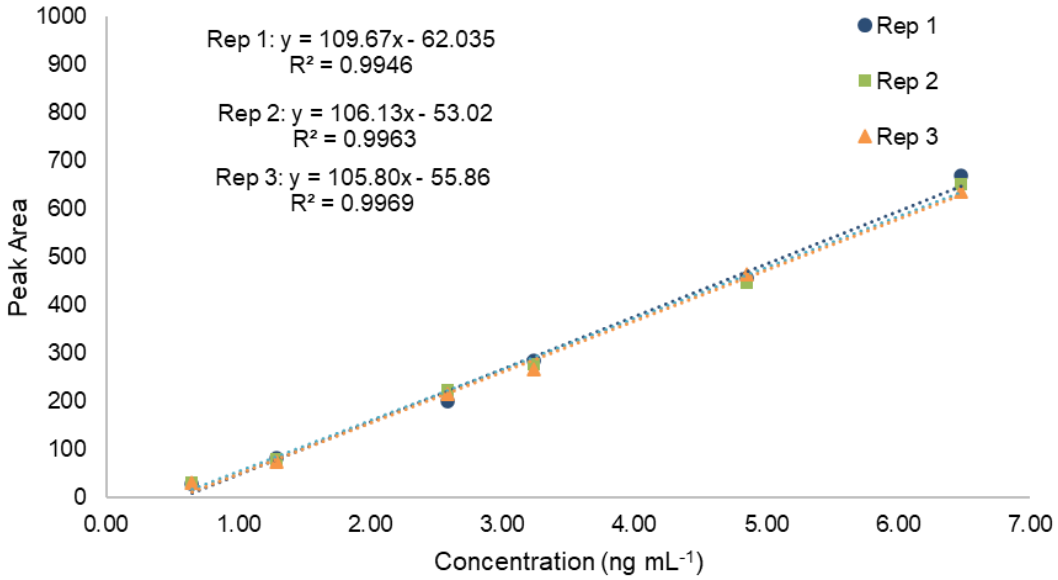


Figure 7.2. α-Ribazole linear regression plot

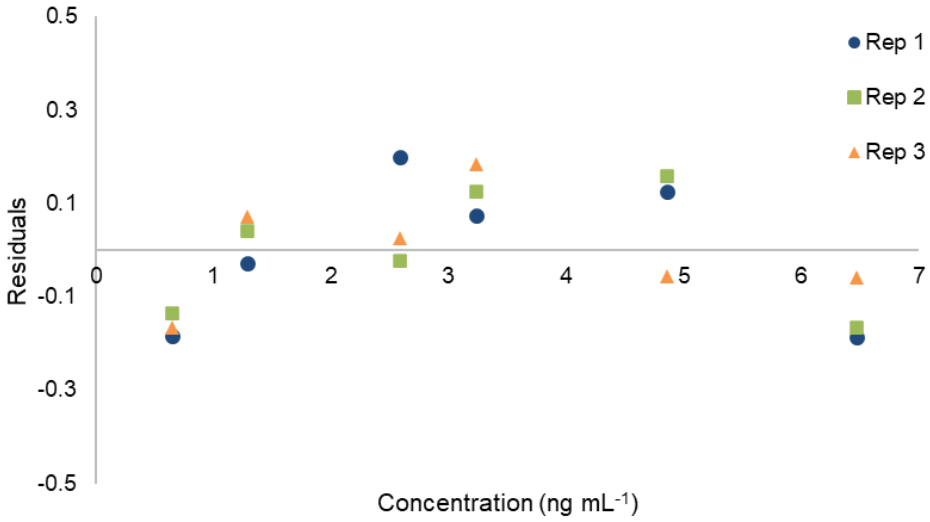


Figure 7.3. α-Ribazole standard residuals plot.

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 from the same sample replicates tested on five different days (*n* = 10) (**Table 7.3**). HorRat values for repeatability were 0.3–0.4 which fall in the typical range of 0.3–1.3 (AOAC International, 2023c). The intermediate precision RSD (RSD_{iR}) values were 3.59–10.0%. which fall between the repeatability and reproducibility (inter-laboratory precision).

Table 7.3. HILIC-FLD α-ribazole method validation summary: precision measurements

Sample type	SD_r[a]	RSD_r (%)[b]	HorRat[c]	RSD_{iR} (%)[d]
Bovine milk based infant formula 1	0.12	4.13	0.3	3.84
Bovine milk based infant formula 2	0.14	3.78	0.3	3.59
Ovine milk-based infant formula	0.08	5.47	0.4	10.0

[a] SD_r = 95% confidence interval of repeatability SD

[b] RSD_r = repeatability RSD% (*n* = 10)

[c] HorRat = $RSD_r/pRSD_r$ ($pRSD_r$ = predicated repeatability RSD), where $pRSD_r$ = 1/2 ($pRSD_R$)

[d] RSD_{iR} = intermediate precision RSD% ((*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, and calculating standard deviation, **Equation 7.8**. The sample used contained vitamin B_{12} greater than LOQ but less than ten times the final calculated LOQ.

$$LOD = \frac{10}{3} \times \sigma$$
$$LOQ = 10 \times \sigma$$
$$LOQ < \bar{x} < 10 \times LOQ$$

Equation 7.8

Where,

$\bar{x}$ = Mean of analysed low content vitamin B_{12} samples (*n* =10)

σ = Standard deviation (*n* = 10)

The LOD and LOQ were calculated as 0.4 and 1.2 µg 100 g^{-1} of dry weight respectively. The LOQ of the HPLC-UV method reported by Campos-Giménez *et al.* (2008) for analysis of vitamin B_{12} in infant formula was 0.3 µg 100 g^{-1}. The difference between the two methods might be partially due

to the fact that Campos-Giménez *et al.* (2008) used 10.0 g of infant formula powder (dry weight), which is equivalent to 30.0 ng of absolute vitamin B_{12} mass at the LOQ, level (calculated using **Equation 7.9**). In contrast, this new method used only 0.25 g of dry weight, which is equivalent to 3.0 ng of absolute vitamin B_{12} mass is at the LOQ level, which is 90% less than the HPLC-UV method.

$$Absolute\ vitamin\ B_{12}(ng) = \frac{sample\ quantity\ (g) \times LOQ(\mu g\ 100\ g^{-1}) \times 1000}{100}$$

Equation 7.9

An improved UHPLC-UV method was reported by Campos-Gimenez (2014) with LOQ of 0.013 µg 100 g^{-1} of reconstituted infant formula product (25 g + 200 g of water). This is the equivalent of 0.117 µg 100 g^{-1} of dry weight for the LOQ, which results are lower than those of the earlier reported method (0.3 µg 100 g^{-1}). Considering the sample amount, 60 g of reconstituted product (6.7 g of dry weight) used in the latter study, the absolute LOQ level was at 7.8 ng of vitamin B_{12}, which again was higher than the LOQ of the candidate method. **Table 7.4** summarises the comparison of the LOD and LOQ between the candidate methods and other literature methods.

The RP-HPLC-FLD method reported by Pakin *et al.* (2005) has LOQ of 3 µg 100 g^{-1} which was 130% higher than this work, in addition, it used at least 800% more sample. Thus, the new method showed higher sensitivity while the required sample quantity dropped significantly. It is worth noting that all listed literature methods used different approaches to calculate the LOD and LOQ.

Table 7.4. Limits of detection and quantitation comparison between this work and literature methods using LC for analysis of vitamin B_{12} in infant formula

Analysis method	LOD[a] (μg 100 g^{-1})	LOQ[a] (μg 100 g^{-1})	Determining method	Sample quantity[b] (g)	Absolute vitamin B_{12} (ng)	References
HILIC-FLD	0.4	1.2	Low vitamin B_{12} content sample[c]	0.25	3.0	This work
RP-HPLC-FLD	—[d]	3	Unknown	2–8	6–24	Pakin *et al.* (2005)
	—[d]	2.5	S/N ratio[e]	5–25	12.5–62.5	Heudi *et al.* (2006)
RP-HPLC-UV	0.1	0.3	Spiked infant cereal[f]	10.0	30.0	Campos-Giménez *et al.* (2008)
	0.072	0.117	Overdiluted liquid sample[g]	6.7	7.8	Campos-Gimenez (2014)

[a] All results were reported or converted as dry weight powder (μg 100 g^{-1})

[b] Sample quantity was converted as dry weight powder

[c] LOD = (10/3)×σ, LOQ = 10×σ, where σ = standard deviation of a low vitamin B_{12} content infant formula (n = 10)

[d] No data available

[e] LOQ equivalent S/N =10 of a diluted standard solution

[f] Estimated by accessing the method performance of vitamin B_{12} spiking experiment on a non-fortified infant cereal

[g] LOD = Mean+3×SD, LOQ = Mean+10×SD, where SD = standard deviation of an overdiluted non-fortified liquid sample (sample nature was not specified)

A recovery study was conducted on three different infant formula products and the results are summarised in **Table 7.5**. 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 68.7–80.0%. Due to the impossibility of obtaining an analyte-free infant formula matrix, the recovery was calculated using **Equation 7.10.** Both spiked and unspiked sample were analysed by the same method. The recovery results were within the acceptable limits of 60–115% at the 10 µg kg^{-1} concentration level stated in Guidelines for *Standard Method Performance Requirements* (AOAC International, 2023d), whilst slightly lower than the expected limits, 70–125% at the same concentration level from Guidelines for Dietary Supplements and Botanicals (AOAC International, 2023c). The multiple clean-up steps contribute to the reduced recovery since each step has its own recovery and more steps often means lower overall recovery.

Table 7.5. HILIC-FLD α-ribazole method validation summary: recovery

Sample Types	Recovery (%)[a]	
	50% level	150% level
Bovine milk based infant formula 1	68.9 (4.6)[b]	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 spiked at 50% and 150% of typical concentrations of vitamin B_{12} (cyanocobalamin) in infant formula
[b] Mean (standard deviation) of six replicates

$$Recovery\ \% = \frac{(c_s - c_u)}{c_t} \times 100 \qquad \textit{Equation 7.10}$$

Where,

c_s = Concentration of the spiked sample

c_u = Concentration of the unspiked sample, the mean of unspiked sample was used for the recovery calculation (*n* = 6)

c_t = Theoretical concentration of vitamin B_{12} added to the sample

A bias study was conducted on SRM 1869 by replicate (*n* = 12) tests and the results are shown in **Table 7.6**. In addition, a *t*-test (at a 95% level of confidence) showed that there is no significant difference between the SRM

certified value and results by the candidate method. The no bias result shows that the candidate method to be accurate.

Table 7.6. HILIC-FLD α-ribazole method summary: bias study vs SRM 1869

		Vitamin B_{12} (µg 100 g^{-1})
Reference material	SRM 1869 value[a]	4.47 ± 0.49
Candidate method	Mean	4.00
	Standard deviation	0.59
	p-value (α = 0.05, *n* = 12, *df* = 11)[b]	0.12
	Confidence interval 95%	3.63–4.48

[a] α = level of significance, n = number of replicates, df = degrees of freedom

[b] Value is expressed as certified value ± expanded uncertainty of the certified value with 95% confidence

7.3.3 Method Application

The candidate method was applied on various infant formula products from three popular milk origins: ovine, bovine and caprine at different stages of infant development. The vitamin B_{12} content results are shown in **Table 7.7** together with label values and results obtained by an external laboratory using an HPLC-UV method (using cyanide to convert all vitamin B_{12} forms to cyanocobalamin) based upon the method described by Campos-Giménez *et al.* (2008).

Table 7.7. Comparison of vitamin B_{12} content of infant formulas from product label, HPLC-UV method and this work

Sample types	Vitamin B_{12} content (µg 100 g^{-1})		
	Stated concentration[a]	External laboratory (HPLC-UV)[b]	This work (HILIC-FLD)[c]
Ovine milk-based follow-on formula 1	1.68	1.77	1.47
Bovine milk-based infant formula 1	1.50	3.94	3.37
Bovine milk-based infant formula 2	1.60	1.81	1.86
Bovine milk-based follow-on formula 1	2.69	2.93	3.06
Bovine milk-based follow-on formula 2	2.58	2.78	2.95
Bovine milk-based toddler-milk formula 1	1.52	1.24	1.46
Bovine milk-based toddler-milk formula 2	1.28	1.18	0.98
Caprine milk-based infant formula 1	1.38	1.74	1.67
Caprine milk-based infant formula 2	1.49	2.09	2.04
Caprine milk-based follow-on formula 1	1.28	0.99	1.18
Caprine milk-based follow-on formula 2	2.59	4.73	4.47

[a] Extracted from the nutrition information table of each product

[b] Adapted from Campos-Giménez *et al.* (2008); All results are in singlicate

[c] Mean of duplicates

The label value from the nutritional information table is a typical content, hence, observed vitamin B_{12} content might be greater than labelled since manufacturers tend to err on the side of caution. For example, in caprine milk-based follow-on formula 2, results of vitamin B_{12} content using both methods (HPLC-UV method and this work) were a much higher amount than the label.

A paired *t*-test was performed on the vitamin B_{12} content of infant formula products (n = 11) using this work and HPLC-UV method. 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 HPLC-UV 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.

Although the target matrix of this method was infant formula products, it might be used for analysing vitamin B_{12} in other milk products. Hence, to demonstrate this potential, four non-vitamin B_{12}-fortified powdered milk products including whole milk and trim milk (known as skim milk), one vitamin B_{12}-fortified nutritional milk powder, four bovine liquid milk and one caprine liquid milk were tested using this new HILIC-FLD method. The results are summarised in **Table 7.8.** The vitamin B_{12} content found in the tested liquid milk, 0.33–0.43 µg 100 g^{-1} are within the range of literature data 0.2–0.7 µg 100 g^{-1} (see **Table 1.1**). The vitamin B_{12} content in the milk powder products were also analysed by the external laboratory using the HPLC-UV method (see **Table 7.8)**. The results by the candidate method were lower than using HPLC-UV method apart from the sample bovine milk-based whole milk powder 1. However, it should be noted that the HPLC-UV method used in the external laboratory is only validated for infant formula products and only single replicates are analysed and so the confidence of results on other non-infant formula milk products are unknown. Furthermore, the low level of vitamin B_{12} content might also be a factor causing the large difference between the two sets of data.

Table 7.8. Vitamin B_{12} contents for various milk samples by HILIC-FLD method

Sample types[a]	Result (µg 100 g^{-1})	
	HILIC-FLD method[b]	HPLC-UV method[c]
Bovine milk-based whole milk powder 1	2.37	2.33
Bovine milk-based whole milk powder 2	1.01	2.14
Bovine milk-based Trim milk powder 1	1.49	2.57
Bovine milk-based Trim milk powder 2	1.00	2.53
Nutritional milk powder[d]	1.03	2.62
Bovine whole milk 1	0.33	N/A[e]
Bovine whole milk 2	0.38	N/A
Bovine Trim milk 1	0.43	N/A
Bovine Trim milk 2	0.42	N/A
Caprine whole milk	0.37	N/A

[a] All products were purchased from local supermarket
[b] Mean of duplicates
[c] Singlicate results
[d] The vitamin B_{12} content from the product label is 1.6 µg 100 g^{-1}
[e] Not applicable. The liquid milks were not analysed by the HPLC-UV method

7.4 Summary

Overall, the method performance was assessed as suitable for analysing vitamin B_{12} for infant formula products using cobalamin-derived α-ribazole. The LOD and LOQ (0.4 and 1.2 µg 100 g^{-1}) showed some improvement compared with other literature methods. The recovery results (68.7–80.0%) of three tested infant formula samples reasonably matched the recommended range (70–115%) by the AOAC guideline at the corresponding concentration level. The repeatability (3.78–5.47%) and intermediate precision (3.59–10.0%) showed excellent precision performance. No significant bias was found against either SRM or the routine vitamin B_{12} analysis method, which indicates the candidate method is accurate. This method achieved the requirement of vitamin B_{12} analysis for infant formula without using cyanide during the sample preparation.

8 Final Conclusions and Recommendations for Future Research

8.1 Final Conclusions

Vitamin B_{12} is an essential micronutrient and is involved in multiple metabolic processes in the human body. For infants and toddlers, infant formula is a major vitamin B_{12} source beside breast milk. Therefore, the quantitation of vitamin B_{12} in infant formula is necessary to ensure the appropriate amount of vitamin B_{12} is available. Not only is the extremely low concentration an analytical challenge, but the current methods also use cyanide for converting unstable vitamers to the stable cyanocobalamin (CN-Cbl). Thus, routine analysis in the workplace laboratory has attendant health and safety issues. This project has developed and validated an indirect quantitation approach to analyse vitamin B_{12} in the milk-based infant formulas which precludes the need for cyanide.

The quantitative analysis of vitamin B_{12} in infant formula was achieved by detecting cobalamin-derived α-ribazole by 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. 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. This work demonstrates that quantitation of α-ribazole is fit for purpose as an indirect method for analysing vitamin B_{12}.

Since a commercial α-ribazole standard is not available, a cobalamin-derived α-ribazole standard was isolated from the CN-Cbl standard material with modification from a method reported by Mattes and Escalante-Semerena (2018) and characterised by NMR and LC-MS (**Chapter 3**).

The isolation procedure was optimised for a higher yield of α-ribazole. Various conditions using acid hydrolysis, alkaline phosphatase (ALP) dephosphorylation, and boronate affinity chromatography (BAC) clean-up were

investigated. Several major modifications from the literature method were made, including using acid hydrolysis rather than base to release α-ribazole-phosphate (precursor of α-ribazole), and using acetonitrile: 1.0 M formic acid (30:70 *v*:*v*) to elute the retained α-ribazole from the boronate affinity column. The final procedure showed a higher yield than the literature (**Chapter 3**).

The work described in **Chapters 5** and **6** focused on the development of a sample preparation method and analysis of α-ribazole. The procedure derived α-ribazole from infant formula through protein denaturation, solid phase extraction, acidic hydrolysis, ALP dephosphorylation, and collection with BAC. The final sample preparation process is illustrated in **Figure 8.1**. The chromatographic conditions and FLD wavelength for α-ribazole were optimised for suitable performance. Details of the final method are summarised in **Table 8.1**. The analysis of α-ribazole was carried out with HILIC-FLD due to an unresolved interference observed when using RP-HPLC-FLD. The HILIC-FLD method removed the interference and also significantly reduced the analysis time to 10 min.

Table 8.1 Final chromatographic conditions and fluorescence detection parameters for analysing cobalamin-derived α-ribazole as a proxy for vitamin B_{12} in infant formulas

HILIC-FLD[a]		Parameter details
Chromatographic	LC Column	HILIC 150 mm × 4.6 mm 2.6 µm
	Mobile phase	A: ammonium formate, 100 mM, pH = 3.5 B: 100% acetonitrile
	Elution mode	Isocratic A: 5% and B: 95%
	Flow rate	1.0 mL min^{-1}
	Column temperature	30 °C
	Analysis time	10 min
Fluorescence	Excitation wavelength	251 nm
	Emission wavelength	303 nm
	Flow cell temperature	40 °C

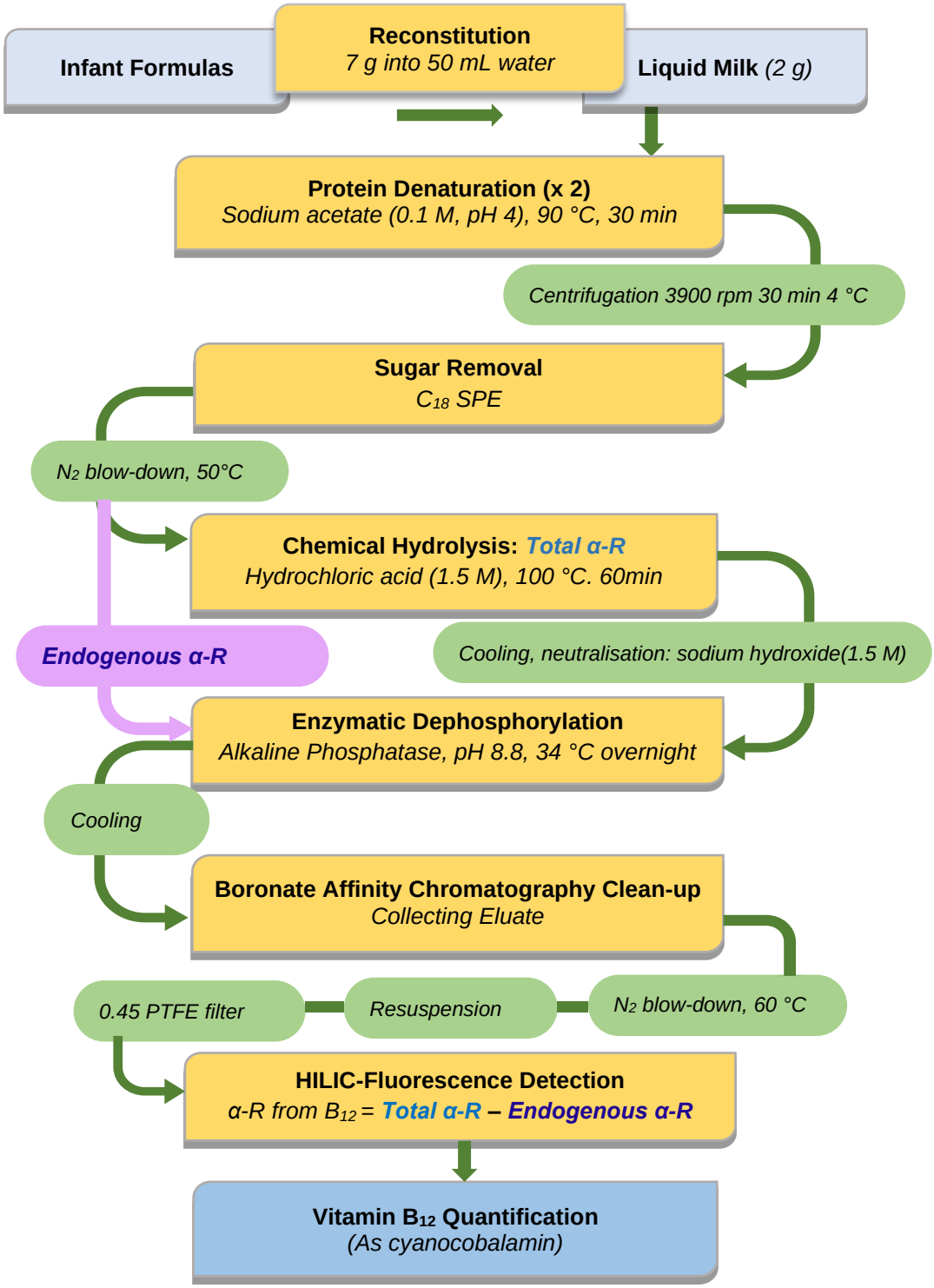


Figure 8.1. Sample preparation for analysis of vitamin B_{12} in infant formulas for detection by HILIC-FLD

Using the developed method, a single validation study was conducted on infant formula products from bovine and ovine milk origins to demonstrate the suitable method performance of this work (**Chapter 7**). The external calibration curve of the α-ribazole standard showed good linearity at the appropriate concentration

range. A satisfactory precision was achieved through repeatability and intermediate precision. A bias study against SRM 1869 showed that the developed method is accurate.

The final method was applied to eleven infant formula products (six of bovine, four of caprine and one of ovine milk basis) and compared to the results from an external laboratory, which routinely uses the AOAC method by Campos-Giménez *et al.* (2008), that is, conversion to CN-Cbl and analysis by HPLC-UV. A paired *t*-test (at 95% confidence level) revealed that there was no significant difference between the results obtained using the developed HILIC-FLD method and the AOAC method. In conclusion, the developed cyanide free method for quantitation of vitamin B_{12} in infant formulas using α-ribazole for detection by HILIC-FLD is fit for purpose with acceptable performance.

8.2 Time Breakdown and Cost Analysis

To illustrate the potential of this method for application in commercial laboratories, a time breakdown and cost analysis were conducted for both a single sample and a batch sample consisting of 12; this number constrained by the number of positions on the SPE manifold.

8.2.1 Time Breakdown

The complete method includes four main stages: reagent/solution preparation, sample preparation, instrumental analysis, and results processing. The reagents and solutions which need to be ready prior to sample processing are summarised in **Table 8.2** with corresponding preparation time. A total of 90 minutes is required for preparing all the reagents and solutions involved in the method. The sample preparation can be divided into seven main steps. A time breakdown of the sample preparation and analysis per sample and per batch (12 samples) is shown in **Table 8.3**. Active and passive time was separated for each step. For a single sample, the time required from start to obtaining a result is 23.4 hours, however, the active working time is only 1.7 hours, while the rest of the time (21.7 hours) occurs during waiting periods

of heating, centrifugation, cooling, incubation, nitrogen stream concentration and HILIC-FLD instrument running. If a batch of 12 samples were to be processed, the active time is only increased to 5.7 hours and most of the passive time remained the same, apart from additional instrumental analysis (11 × 10 min). This new method requires two working days to finish the sample preparation, due to the 16-hour incubation period. However, 76% of the time is passive which the analyst can use for other tasks.

Table 8.2. Required time for the reagents and solutions preparation for vitamin B_{12} analysis using α-ribazole by HILIC-FLD

Reagents/Solutions	Time per 1 L volume (min)	Volume (mL) per sample
Sodium acetate (0.1 M, pH = 4)	10	20
Hydrochloric acid (1.5 M)	5	1
Sodium hydroxide (1.5 M)	5	1
Alkaline phosphatase incubating buffer: ammonium acetate (0.3 M), magnesium chloride (2 mM) (pH = 8.8)	10[a]	0.05
Boronate affinity chromatography (BAC) binding buffer: ammonium acetate (0.3 M, pH = 8.8)	10	2
BAC eluent: formic acid (1.0 M): acetonitrile 30:70, (*v*:*v*)	10	12
Mobile phase A: ammonium formate (0.1 M, pH = 3.5)	10	6
α-Ribazole stock, working standard solutions cyanocobalamin stock and spike standard solutions	30[b]	—

[a] The time for making 10 mL of alkaline phosphatase incubating buffer
[b] The time for total preparation of standard solutions of appropriate volume

For the HPLC-UV method, according to the external laboratory, a batch of 12 samples requires total 8 hours of sample preparation (including passive time). For the candidate method, the total sample preparation time for a batch of 12 samples is 11.2 hours plus an overnight ALP incubation period (16 hours). Although the candidate method requires a longer time for sample preparation, there are other factors that should be considered when comparing the two methods, for example, the cost of consumables and the fact that cyanide was excluded from the candidate method.

Table 8.3 Time breakdown of method steps for vitamin B_{12} analysis using α-ribazole by HILIC-FLD with comparison to HPLC-UV method

Method Steps	Time per sample (min)		Time per 12 samples (min)	
	Active	Passive[a]	Active	Passive[a]
HILIC-FLD				
Reconstitution and sampling	5	20	60	20
Protein denaturation (twice)	2	120	24	120
C_{18} SPE sugar removal	35	30	120	30
Acidic hydrolysis	1	70	12	70
Alkaline phosphatase hydrolysis	1	960	12	960
Boronate affinity chromatography collection	57	90	90	90
HILIC-FLD analysis	2	10	24	120
Total time (hour)	1.7	21.7	5.7	23.5
	23.4		29.2	
HPLC-UV				
Overall sample preparation time (hour)[b]	–		8	

[a] Passive time includes waiting periods occurring during heating, centrifugation, cooling, incubation, nitrogen stream blow-down and instrumental analysis

[b] Total sample preparation including passive time

8.2.2 Cost Analysis

Instrumentation and equipment are the major one-off costs for a commercial laboratory. The instrument and equipment used in the cyanide-free method are common laboratory facilities, hence, no extra capital investment, other than the possible purchase of a fluorescence detector, would be required to adopt the new method for commercial application. This section is primarily focused on the cost of consumables and reagents used during the sample preparation and instrumental analysis by HILIC-FLD, which are detailed in **Table 8.4**. The cost for common reusable labware including glassware and water was not included, nor was labour cost. The cost varies depending on supplier and wage structures respectively, but decreases when a larger volume of samples are being processed.

Table 8.4. Cost analysis for the HILIC-FLD method of vitamin B_{12} analysis of one sample and a batch of 12 samples with comparison to the HPLC-UV method

	Consumable/reagents	Number or amount per batch of 12 sample	Cost per 1 sample (NZ$)[b]	Cost per 1 batch (12 samples, NZ$)
HILIC-FLD	***Consumable***			
	Pipette tips (10 mL)	12	0.88	10.56
	Pipette tips (1 mL)	24	0.02	0.24
	Pipette tips (200 µL)	12	0.01	0.12
	Pasteur pipettes	12	0.03	0.36
	C_{18} SPE cartridge	12	4.53	54.37
	CA 0.45 µm filter unit	12	1.20	14.4
	PTFE 0.45 µm filter unit	12	0.60	7.2
	Syringe (10 mL)	12	0.21	2.52
	Syringe (1 mL)	12	0.21	2.52
	Boronic acid resin	12	3.35	40.14
	HPLC vial, cap, septa	12	0.42	5.04
	Reagent			
	Sodium acetate	3.3 g	0.01	0.17
	Methanol	180.0 mL	0.19	2.30
	Hydrochloric acid (36%)	1.5 mL	0.001	0.01
	Sodium hydroxide	0.7 g	0.002	0.03
	Ammonium acetate	3.9 g	0.04	0.47
	Magnesium chloride	4.6 µg	1.7×10^{-5}	2.0×10^{-4}
	Alkaline phosphatase	0.1 µL	0.22	2.61
	Formic acid	0.8 mL	0.01	0.14
	Acetonitrile	164.4 mL	0.33	4.00
	Ammonium formate	0.04 g	0.001	0.01
	Ammonia (28–30%)	3.1 mL	0.004	0.05
	Glacial acetic acid	5.3 mL	0.01	0.11
	Total	**–**	**12.3**	**147.4**
HPLC-UV	***Consumable***			
	Immunoaffinity cartridge	12	25[c]	300

[a] The prices listed here are calculated based on the price information provided by the suppliers at the time of research. These may change depending on the market or chosen suppliers.

[b] The cost for one sample = cost per unit × units required for analysis, where the cost per unit = bulk price provided by the suppliers/unit volume or mass

[c] General pricing that might vary on regional level

The major contribution to the cost of analysing one sample is the C_{18} SPE cartridge (NZ$ 4.53 per sample) and the boronic acid resin (NZ$ 3.35 per sample), which accounts for 64% of the total cost. Theoretically, the boronic acid resin can be regenerated and reused multiple times (the calculation here was based on 10-fold regeneration) which will reduce the cost further. C_{18} SPE

cartridges are commonly used in laboratories for cleaning up sample matrices and there are multiple suppliers which helps to keep the pricing competitive. The currently used AOAC method requires a single-use immunoaffinity cartridge which costs approximately NZ$25 per cartridge per sample, making it a more expensive method than the newly developed method

Furthermore, the newly developed method eliminates the cost associated with handling, storing and deposing of cyanides, which allows an easier transfer and integration into a commercial laboratory. According to regulations under the Health and Safety at Work Act 2015 and industry guidelines, cyanides should be securely stored in a locked area and separated from incompatible substance, particularly acids, and access should be restricted to authorised personnel only. All personnel on site where cyanide compounds are used or stored should be properly trained in handling procedure, personal protective equipment, and related emergency first aid measures. A proper hazard management policy for tracking cyanides should be in place, throughout the lifecycle from entry to disposal. In laboratories, hazardous waste is normally collected and disposed of by a specialised company, which further increases the cost associated with the test. In the external laboratory where the HPLC-UV method was used, a fume hood is dedicated to the sample preparation only to ensure the hazard is identified in one area. These types of costs would be avoidable by switching to the candidate method.

In conclusion, the exclusion of using cyanide makes the HILIC-FLD method more economical and safer (cyanide-free), hence, a better alternative to be applied in a commercial laboratory for analysing vitamin B_{12} in infant formula products.

8.3 Future Research Recommendation

This work primarily focused on proving the possibility of developing a cyanide-free method for vitamin B_{12} analysis. The author acknowledges that there is further research and investigation that can be undertaken and recommendations for additional development of the method are detailed here.

This method requires two consecutive hydrolysis steps, acidic hydrolysis and ALP dephosphorylation, to cleave α-ribazole from vitamin B_{12}. A single step would be favourable as it would reduce the process time as well as the risk of sample loss during transfer; in addition, if the release of α-ribazole-phosphate can be achieved by a targeted method such as enzymatic hydrolysis, the acidic hydrolysis, which occurs under heat, can be replaced. Thus, the sugars would not undergo browning and their removal by SPE would be unnecessary. Further work could focus on discovering an enzyme that can complete this step.

The recovery results of this method are close to the lower end of the expected range set by AOAC; this is likely caused by the multiple preparation steps, including SPE and BAC. A sample preparation with less steps would be beneficial to the recovery. Future work could be conducted on developing an extraction technique which is specific to all forms of vitamin B_{12} rather than just CN-Cbl as occurs with the currently-used immunoaffinity column. Therefore, less, or possibly none, of the clean-up steps would be required.

Although FLD shows high sensitivity, it is not as selective as mass spectrometry. By using LC-MS, advantage can be taken of the high selectivity using extracted ions or multiple reaction monitoring to solve the interference issue observed in C_{18} RP-HPLC. Mass spectrometry might allow the analysis of a sample with less clean-up and possibly the sugar removal by SPE step and potentially the collection step by BAC could be excluded. If cost-effective stable isotope labelled vitamin B_{12} standard (labelled on DMB moiety) were available as internal standard, LC-MS could be used throughout to correct the recovery for the multiple step preparation. However, the high cost of the current commercial stable isotope labelled standard (~US$ 3000 per µg) made this approach prohibitive. More research work could be invested into the LC-MS method development.

The disadvantage of LC-MS from the viewpoint of a commercial laboratory is the initial cost of purchase and ongoing maintenance. Developing an LC-MS method might not be challenge-free; for example, matrix effects could bring

signal suppression or enhancement. Additionally, a highly trained technician is required to correctly operate and maintain a mass spectrometer.

This work investigated extensively on resolving the interference from α-ribazole observed on the RP (C_{18}) column and decided to use HILIC instead when RP failed to offer acceptable resolution under the tested conditions. However, considering the popularity and wide accessibility of C_{18} columns in laboratories, it is worth further exploration of possible approaches to achieve a desired resolution. There are more means that can be explored such as, using C_{18} columns with different degrees of carbon load and using acetonitrile as an organic modifier with fine-tuned column temperature, elution programs and pH additives.

Due to practical reasons, this work did not extend the investigation on the identity of the interference, which fluoresced and eluted on RP (C_{18}) in a similar manner to α-ribazole. Further work could gather more information of the possible identity of the inference which would offer more guidance on developing the chromatographic conditions for a better separation.

The LOD and LOQ of the work can be further lowered if a larger amount of sample is used. However, such change will increase the interference from the matrix and cause possible break-through on the SPE and BAC cartridges. Therefore, future work could focus on determining an appropriate sample quantity without compromising the effectiveness of clean-up methods.

The final method has been applied to eleven animal milk based infant formula products. A larger number of samples could be analysed to further assess the method performance of the newly developed method. Furthermore, an inclusion of samples from multiple production batches could facilitate exploration of the variability in vitamin B_{12} content across these batches. So far, this validation is conducted within a single laboratory; an inter-laboratory validation is required to provide the data for reproducibility assessment.

Although milk-based infant formulas were the initial target matrix, there is potential that the matrix type could be extended to other dairy products, such as liquid milk, cream, and cheeses. This would require further refinement of the sample preparation method, especially in the case of solid matrices.

REFERENCE

Ahmad, I.; Hussain, W. Stability of cyanocobalamin in parenteral preparations. *Pakistan Journal of Pharmaceutical Sciences* **1993**, *6*, 53-59.

Akcaboy, M.; Malbora, B.; Zorlu, P.; Altinel, E.; Oguz, M. M.; Senel, S. Vitamin B_{12} deficiency in infants. *Indian Journal of Pediatrics* **2015**, *82*, 619-624.

Akparov, V. K.; Stepanov, V. M. Phenylboronic acid as a ligand for biospecific chromatography of serine proteinases. *Journal of Chromatography A* **1978**, *155*, 329-336.

Albala-Hurtado, S.; Veciana-Nogues, M. T.; Izquierdo-Pulido, M.; Marine-Font, A. Determination of water-soluble vitamins in infant milk by high-performance liquid chromatography. *Journal of Chromatography A* **1997**, *778*, 247-253.

Allen, L. H. Bioavailability of vitamin B_{12}. *International Journal for Vitamin and Nutrition Research* **2010**, *80*, 330-335.

Allen, L. H. Multiple micronutrients in pregnancy and lactation: an overview. *The American Journal of Clinical Nutrition* **2005**, *81*, 1206S-1212S.

Analytical Methods Committee Uses (proper and improper) of correlation coefficients. *Analyst* **1988**, *113*, 1469-1471.

Anastassiades, M.; Lehotay, S. J.; Štajnbaher, D.; Schenck, F. J. Fast and easy multiresidue method employing acetonitrile extraction/partitioning and “Dispersive Solid-Phase Extraction” for the determination of pesticide residues in produce. *Journal of AOAC International* **2019**, *86*, 412-431.

Armitage, J. B.; Cannon, J. R.; Johnson, A. W.; Parker, L. F. J.; Smith, E. L.; Stafford, W. H.; Todd, A. R. Chemistry of the vitamin B_{12} group. III. The course of hydrolytic degradations. *Journal of the Chemical Society* **1953**, 3849-3864.

Australia New Zealand Food Standards Code Australia New Zealand Food Standards Code. 2018.

Aytekin, N.; Mileva, K. N.; Cunliffe, A. D. Selected B vitamin and their possible link to the aetiology of age-related sarcopenia: relevance of UK dietary recommendations. *Nutrition Rsearch Reviews* **2018**, *31*, 204-224.

Babij, N. R.; McCusker, E. O.; Whiteker, G. T.; Canturk, B.; Choy, N.; Creemer, L. C.; Amicis, C. V. D.; Hewlett, N. M.; Johnson, P. L.; Knobelsdorf, J. A.;

Li, F.; Lorsbach, B. A.; Nugent, B. M.; Ryan, S. J.; Smith, M. R.; Yang, Q. NMR chemical shifts of trace impurities: Industrially preferred solvents used in process and green chemistry. *Organic Process Research & Development* **2016**, *20*, 661-667.

Ball, G. F. M. *Vitamins in Foods Analysis, Bioavailability, and Stability* Taylor & Francis Group: Boca Raton, FL, USA, 2006.

Baran, Y.; Comba, P.; Lawrance, G. A. Trends in Base Hydrolysis Rates of Chlorocobalt(III) Complexes of a Series of Related Pentaamine Ligands. *Inorganic Reaction Mechanisms* **2002**, *4*, 31-47.

Barsacchi, D.; Cappiello, M.; Tozzi, M. G.; Del Corso, A.; Peccatori, M.; Camici, M.; Ipata, P. L.; Mura, U. Purine nucleoside phosphorylase from bovine lens: purification and properties. *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology* **1992**, *1160*, 163-170.

Beaven, G. R.; Holiday, E. R.; Johnson, E. A.; Ellis, B.; Mamalis, P.; Petrow, V.; Sturgeon, B. The chemistry of anti-pernicious anaemia factors. *Journal of Pharmacy and Pharmacology* **1949**, *1*.

Bennink, M. R.; Ono, K. Vitamin B_{12}, E and D content of raw and cooked beef. *Journal of Food Science* **1982**, *47*, 1786-1792.

Berregi, I.; del Campo, G.; Caracena, R.; Miranda, J. I. Quantitative determination of formic acid in apple juices by ^{1}H NMR spectrometry. *Talanta* **2007**, *72*, 1049-1053.

Bezie, A. The effect of different heat treatment on the nutritional value of milk and milk products and shelf-life of milk products. A review. *Journal of Dairy & Veterinary Sciences* **2019**.

Bito, T.; Bito, M.; Asai, Y.; Takenaka, S.; Yabuta, Y.; Tago, K.; Ohnishi, M.; Mizoguchi, T.; Watanabe, F. Characterization and quantitation of vitamin B_{12} compounds in various chlorella supplements. *Journal of Agricultural and Food Chemistry* **2016**, *64*, 8516-8524.

Bonnett, R.; Raleigh, J. A.; Redman, D. G. The hydrolysis of vitamin B_{12}. Studies with model amides. *Journal of the American Chemical Society* **1964**, *87*, 1600-1607.

Brink, N. G.; Folkers, K. Vitamin B_{12}. X. 5,6-dimethylbenzimidazole, a degradation product of vitamin B_{12}. *Journal of the American Chemical Society* **1950**, *72*, 4442-4443.

Brown, K. L.; Hakimi, J. M. Heteronuclear NMR studies of cobalamins. 4. α-Ribazole-3'-phosphate and the nucleotide loop of base-on cobalamins. *Journal of the American Chemical Society* **1986**, *108*, 496-503.

Brown, K. L.; Hakimi, J. M.; Nuss, D. M.; Montejano, Y. D.; Jacobsen, D. W. Acid-base properties of α-ribazole and the thermodynamics of dimethylbenzimidazole association in alkylcobalamins. *Inorganic Chemistry* **1984**, *23*, 1463-1471.

Buchanan, J. G.; Johnson, A. W.; Mills, J. A.; Todd, A. R. Chemistry of the vitamin B_{12} group. I. Acid hydrolysis studies. Isolation of a phosphorus-containing degradation product. *Journal of the Chemical Society* **1950**, 2845-2855.

Buszewski, B.; Noga, S. Hydrophilic interaction liquid chromatography (HILIC)-a powerful separation technique. *Analytical and Bioanalytical Chemistry* **2012**, *402*, 231-247.

Bzowska, A.; Kulikowska, E.; Shugar, D. Properties of purine nucleoside phosphorylase (PNP) of mammalian and bacterial origin. *Zeitschrift für Naturforschung C* **1990**, *45*, 59-70.

Bzowska, A.; Kulikowska, E.; Shugar, D. Purine nucleoside phosphorylases: properties, functions, and clinical aspects. *Pharmacology & Therapeutics* **2000**, *88*, 349-425.

Cacciapuoti, G.; Gorassini, S.; Mazzeo, M. F.; Siciliano, R. A.; Carbone, V.; Zappia, V.; Porcelli, M. Biochemical and structural characterization of mammalian-like purine nucleoside phosphorylase from the Archaeon *Pyrococcus furiosus*. *The FEBS Journal* **2007**, *274*, 2482-2495.

Campos-Gimenez, E. Improved AOAC first action 2011.08 for the analysis of vitamin B_{12} in infant formula and adult/pediatric formulas: First action 2014.02. *Journal of AOAC International* **2014**, *97*, 1397-1402.

Campos-Giménez, E.; Fontannaz, P.; Trisconi, M.; Kilinc, T.; Gimenez, C. Determination of vitamin B_{12} in food products by liquid chromatography/UV detection with immunoaffinity extraction: single-laboratory validation. *Journal of AOAC International* **2008**, *91*, 786-793.

Campos-Gimenez, E.; Fontannaz, P.; Trisconi, M.; Kilinc, T.; Gimenez, C.; Andrieux, P. Determination of vitamin B_{12} in infant formula and adult nutritionals by liquid chromatography/UV detection with immunoaffinity

extraction: first action 2011.08. *Journal of AOAC International* **2012**, *95*, 307-312.

Casein nigrogen conten of milk. AOAC offical method **998.06**. In *Official Methods of Analysis of AOAC International 22nd Edition*; G. W. Latimer, Jr., Ed.;Online Edn; Oxford University Press: New York, NY, USA, 2023a.

CDER Reviewer Guidance: Validation of Chromatographic Methods. Food and Drug Administration: Rockville, MD, 1994.

Chamlagain, B.; Edelmann, M.; Kariluoto, S.; Ollilainen, V.; Piironen, V. Ultra-high performance liquid chromatographic and mass spectrometric analysis of active vitamin B_{12} in cells of *Propionibacterium* and fermented cereal matrices. *Food Chemistry* **2015**, *166*, 630-638.

Chan, C. C. Analytical method validation: Principles and pracitces. In *Pharmaceutical Manufacturing Handbook Regulations Quality*; S. C. Gad, Ed.; John Wiley & Sons, Incorporated: Hoboken, NJ, 2008.

Chapman, H. R.; Ford, J. E.; Kon, S. K.; Thompson, S. Y.; Rowland, S. J.; Crossley, E. L.; Rothwell, J. 670. Further studies of the effect of processing on some vitamins of the B complex in milk. *Journal of Dairy Research* **1957**, *24*, 191-197.

Cheong, C.-G.; Escalante-Semerena, J. C.; Rayment, I. Structural investigation of the biosynthesis of alternative lower ligands for cobamides by nicotinate mononucleotide: 5,6-dimethylbenzimidazole phosphoribosyltransferase from *Salmonella enterica*. *Journal of Biological Chemistry* **2001**, *276*, 37612-37620.

Clark, R. L.; Jones, W. H.; Raich, W. J.; Folkers, K. Vitamin B12. XXIII. Resolution of DL-1-Amino-2-propanol. *Journal of the American Chemical Society* **1954**, *76*, 3995-3997.

Cobalamin (vitamin B_{12} activity) in milk-based infant formula. Turbidimetric method. AOAC Offical Method **986.23**. In AOAC Offical Methods of Analysis 22nd Edition; G. W. Latimer, Jr., Ed.;Online Edn; Oxford University Press: New York, NY, USA, 2023b.

Coleman, J. E.; Gettins, P. Alkaline phosphatase, solution structure, and mechanism. In *Advances in Enzymology and Related Areas of Molecular Biology*, 1983; pp 381-452.

Collins, R. A.; Harper, A. E.; Schreiber, M.; Elvehjem, C. A. The folic acid and vitamin B_{12} content of the milk of various species. *The Journal of Nutrition* **1951**, *43*, 313-321.

Combs, G. F., Jr. *The vitamins: Fundamental aspects in nutrition and health*, 2nd ed. Academic Press: London, UK, 1998.

Combs, G. F.; McClung, J. P. Chapter 18 - Vitamin B_{12}. In *The vitamins: Fundamental aspects in nutrition and health*; G. F. Combs and J. P. McClung, Eds.; Academic Press: London, UK, 2017; pp 431-452.

Corzo-Martínez, M.; Corzo, N.; Villamiel, M.; del Castillo, M. D. Browning reactions. In *Food Biochemistry and Food Processing*, 2012; pp 56-83.

CRC Handbook of Chemistry and Physics, 103rd ed.;John R. Rumble, Ed. CRC Press: Boca Raton, FL, 2023.

D'Ulivo, L.; Yang, L.; Ding, J. F.; Pagliano, E.; Leek, D. M.; Thibeault, M. P.; Mester, Z. Determination of cyanocobalamin by isotope dilution LC-MS/MS. *Analytica Chimica Acta* **2017**, *990*, 103-109.

De Keukeleire, S.; Marian, P.; Annick, W.; Sofie, T.; Stefan, V.; Michel, L. Stability of vitamin B_{12} - A preanalytical view. *Clinica Chimica Acta* **2015**, *448*, 58-59.

Deshmukh, S.; Chavan, G.; Vanjari, S.; Patil, R. A review on analytical method development and validation by high performance liquid chromatography technique. *Journal of Pharmaceutical Sciences and Research* **2019**, *11*, 3599-3605.

Diaz, F. J. Determination of cyanocobalamin by atomic-absorption spectrophotometry with a premix air-acetylene flame. *Analytica Chimica Acta* **1972**, *58*, 455-458.

Dietary reference intakes. *Nutrition Reviews* **1997**, *55*, 319-326.

Dror, D. K.; Allen, L. H. Effect of vitamin B_{12} deficiency on neurodevelopment in infants: current knowledge and possible mechanisms. *Nutrition Reviews* **2008**, *66*, 250-255.

Duplessis, M.; Pellerin, D.; Cue, R. I.; Girard, C. L. Short communication: Factors affecting vitamin B_{12} concentration in milk of commercial dairy herds: An exploratory study. *Journal of Dairy Science* **2016**, *99*, 4886-4892.

European Food Safety Authority. Outcome of a public consultation on the draft scientific opinion of the EFSA panel on dietetic products, nutrition and allergies (NDA) on dietary reference values for cobalamin (vitamin B_{12}). *EFSA Journal* **2015**, *13*, 1-13.

Espina-Benitez, M. B.; Randon, J.; Demesmay, C.; Dugas, V. Back to BAC: Insights into boronate affinity chromatography interaction mechanisms. *Separation & Purification Reviews* **2017a**, *47*, 214-228.

Espina-Benitez, M. B.; Randon, J.; Demesmay, C.; Dugas, V. Development and application of a new in-line coupling of a miniaturized boronate affinity monolithic column with capillary zone electrophoresis for the selective enrichment and analysis of cis-diol-containing compounds. *Journal of Chromatography A* **2017b**, *1494*, 65-76.

Eto, I.; Krumdieck, C. L. Role of vitamin B_{12} and folate deficiencies in carcinogenesis. *Advances in Experimental Medicine and Biology* **1986**, *206*, 313-330.

Eurachem Guide The Fitness for Purpose of Analytical Methods: A Laboratory Guide to Method Validation and Related Topics. B. Magnusson and U. Örnemark, Eds., 2014.

Fenech, M. The role of folic acid and vitamin B_{12} in genomic stability of human cells. *Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis* **2001**, *475*, 57-67.

Food and Agriculture Organization of the United Nations; World Health Organization “Human vitamin and mineral requirements. Report of a joint FAO/WHO expert consultation,” 2001.

Fornstedt, T.; Forssén, P.; Westerlund, D. Basic HPLC theory and definitions: retention, thermodynamics, selectivity, zone spreading, kinetics, and resolution. In *Analytical Separation Science*; Jared L. Anderson and A. Berthod, Eds.; Wiley-VCH Verlag GmbH & Co. KGaA, 2015.

Fox, B.; Bellini, G.; Pellegrini, L. Chapter 14 - Drying. In *Fermentation and Biochemical Engineering Handbook (Third Edition)*; H. C. Vogel and C. M. Todaro, Eds.; William Andrew Publishing: Boston, 2014; pp 283-305.

Fulmer, G. R.; Miller, A. J. M.; Sherden, N. H.; Gottlieb, H. E.; Nudelman, A.; Stoltz, B. M.; Bercaw, J. E.; Goldberg, K. I. NMR chemical shifts of trace impurities: Common laboratory solvents, organics, and gases in

deuterated solvents relevant to the organometallic chemist. *Organometallics* **2010**, *29*, 2176-2179.

Gill, B. D.; Indyk, H. E.; Manley-Harris, M. Determination of total potentially available nucleosides in bovine, caprine, and ovine milk. *International Dairy Journal* **2012**, *24*, 40-43.

Gille, D.; Schmid, A. Vitamin B_{12} in meat and dairy products. *Nutrition Reviews* **2015**, *73*, 106-115.

Glantz, M. D.; Lewis, A. S. Purine nucleotide phosphorylase from rabbit liver. In *Methods in Enzymology*; Academic Press, 1978; pp 524-530.

Goldstein, S.; Duca, A. Contributions to the analytical chemistry of vitamin B_{12}. The thermal stability of cyanocobalamin, hydroxocobalamin and cobinamide in the solid state. *Thermochimica Acta* **1982**, *59*, 211-220.

Graulet, B.; Girard, C. L. Chapter 15 - B Vitamins in Cow Milk: Their Relevance to Human Health. In *Dairy in Human Health and Disease Across the Lifespan*; R. R. Watson; R. J. Collier and V. R. Preedy, Eds.; Academic Press, 2017; pp 211-224.

Gray, M. J.; Escalante-Semerena, J. C. A new pathway for the synthesis of alpha-ribazole-phosphate in *Listeria innocua*. *Molecular Microbiology* **2010**, *77*, 1429-1438.

Great Britain: Panel on Dietary Reference Values *Dietary reference values for food energy and nutrients for the United Kingdom: Report of the panel on dietary reference values of the committee on medical aspects of food policy* H.M. Stationery Office, 1991.

Greibe, E.; Nexo, E. Forms and amounts of vitamin B_{12} in infant formula: A pilot study. *PLoS One* **2016**, *11*.

Gueant, J. L.; Alpers, D. H. Vitamin B_{12}, a fascinating micronutrient, which influences human health in the very early and later stages of life. *Biochimie* **2013**, *95*, 967-969.

Guidelines for dietary supplements and botanicals. In *Official Methods of Analysis of AOAC International 22nd Edition* G. W. Latimer, Jr., Ed.;Online Edn; Oxford University Press: New York, NY, USA, 2023c.

Guidelines for standard method performance requirements. In *Official Methods of Analysis of AOAC International 22nd Edition*; G. W. Latimer, Jr., Ed.;Online Edn; Oxford University Press: New York, NY, USA, 2023d.

Hale, S. E.; Neumann, M.; Schliebner, I.; Schulze, J.; Averbeck, F. S.; Castell-Exner, C.; Collard, M.; Drmač, D.; Hartmann, J.; Hofman-Caris, R.; Hollender, J.; de Jonge, M.; Kullick, T.; Lennquist, A.; Letzel, T.; Nödler, K.; Pawlowski, S.; Reineke, N.; Rorije, E.; Scheurer, M.; Sigmund, G.; Timmer, H.; Trier, X.; Verbruggen, E.; Arp, H. P. H. Getting in control of persistent, mobile and toxic (PMT) and very persistent and very mobile (vPvM) substances to protect water resources: strategies from diverse perspectives. *Environmental Sciences Europe* **2022**, *34*, 22.

Heal, K. R.; Carlson, L. T.; Devol, A. H.; Armbrust, E. V.; Moffett, J. W.; Stahl, D. A.; Ingalls, A. E. Determination of four forms of vitamin B_{12} and other B vitamins in seawater by liquid chromatography/tandem mass spectrometry. *Rapid Communications in Mass Spectrometry* **2014**, *28*, 2398-2404.

Herbert, V. Vitamin B_{12}: plant sources, requirements, and assay. *The American Journal of Clinical Nutrition* **1988**, *48*, 852-858.

Heudi, O.; Kilinc, T.; Fontannaz, P.; Marley, E. Determination of vitamin B_{12} in food products and in premixes by reversed-phase high performance liquid chromatography and immunoaffinity extraction. *Journal of Chromatography A* **2006**, *1101*, 63-68.

Hodgkin, D. C.; Pickworth, J.; Robertson, J. H.; Trueblood, K. N.; Prosen, R. J.; White, J. G. Structure of vitamin B_{12} : The crystal structure of the hexacarboxylic acid derived from B_{12} and the molecular structure of the vitamin. *Nature* **1955**, *176*, 325-328.

Horwitz, W.; Albert, R. The Horwitz ratio (HorRat): A useful index of method performance with respect to precision. *Journal of AOAC International* **2006**, *89*, 1095-1109.

Indyk, H. E.; Persson, B. S.; Caselunghe, M. C. B.; Moberg, A.; Filonzi, E.; Woollard, D. C. Determination of vitamin B_{12} in milk products and selected foods by optical biosensor protein-binding assay: Method comparison. *Journal of AOAC International* **2002**, *85*, 72-81.

Iwase, H.; Ono, I. Determination of cyanocobalamin in foods by high-performance liquid chromatography with visible detection after solid-phase extraction and membrane filtration for the precolumn separation of lipophilic species. *Journal of Chromatography A* **1997**, *771*, 127-134.

Jarvinen, P.; Oivanen, M.; Lonnberg, H. Interconversion and phosphoester hydrolysis of 2',5'- and 3',5'-dinucleoside monophosphates: kinetics and mechanisms. *The Journal of Organic Chemistry* **1991**, *56*, 5396-5401.

Jiang, Y. J.; Guo, M. 8 - Processing technology for infant formula. In *Human Milk Biochemistry and Infant Formula Manufacturing Technology*; M. Guo, Ed.; Woodhead Publishing, 2014; pp 211-229.

Joyce, A. M.; Brodkorb, A.; Kelly, A. L.; O'Mahony, J. A. Separation of the effects of denaturation and aggregation on whey-casein protein interactions during the manufacture of a model infant formula. *Dairy Science & Technology* **2017**, *96*, 787-806.

Jurado, J. M.; Alcázar, A.; Muñiz-Valencia, R.; Ceballos-Magaña, S. G.; Raposo, F. Some practical considerations for linearity assessment of calibration curves as function of concentration levels according to the fitness-for-purpose approach. *Talanta* **2017**, *172*, 221-229.

Juzeniene, A.; Nizauskaite, Z. Photodegradation of cobalamins in aqueous solutions and in human blood. *Journal of Photochemistry and Photobiology B* **2013**, *122*, 7-14.

Kakitani, A.; Inoue, T.; Matsumoto, K.; Watanabe, J.; Nagatomi, Y.; Mochizuki, N. Simultaneous determination of water-soluble vitamins in beverages and dietary supplements by LC-MS/MS. *Food Additives & Contaminants: Part A* **2014**, *31*, 1939-1948.

Kamel, S.; Thiele, I.; Neubauer, P.; Wagner, A. Thermophilic nucleoside phosphorylases: Their properties, characteristics and applications. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics* **2020**, *1868*, 140304.

Kasalová, E.; Aufartová, J.; Krčmová, L. K.; Solichová, D.; Solich, P. Recent trends in the analysis of vitamin D and its metabolites in milk – A review. *Food Chemistry* **2015**, *171*, 177-190.

Katainen, E.; Niemelä, P.; Harjunen, P.; Suhonen, J.; Järvinen, K. Evaluation of the amorphous content of lactose by solution calorimetry and Raman spectroscopy. *Talanta* **2005**, *68*, 1-5.

Katz, E. D.; Lochmuller, C. H.; Scott, R. P. W. Methanol-water association and its effect on solute retention in liquid chromatography. *Analytical Chemistry* **1989**, *61*, 349-355.

Kempen, T.; Stoll, D. R. Retention factor is independent of pressure in LC, right? *LCGC North America* **2021**, *39*, 471-475.

Keresztesy, J. C. The water-soluble vitamins. *Annual Review of Biochemistry* **1944**, *13*, 367-410.

Knipschildt, M. E.; Andersen, G. G. Drying of Milk and Milk Products. In *Robinson: Modern Dairy Technology: Volume 1 Advances in Milk Processing*; R. K. Robinson, Ed.; Springer US: Boston, MA, 1994; pp 159-254.

Knyazev, A. V.; Smirnova, N. N.; Plesovskikh, A. S.; Shushunov, A. N.; Knyazeva, S. S. Low-temperature heat capacity and thermodynamic functions of vitamin B_{12}. *Thermochimica Acta* **2014**, *582*, 35-39.

Kolhouse, J. F.; Kondo, H.; Allen, N. C.; Podell, E.; Allen, R. H. Cobalamin analogs are present in human-plasma and can mask cobalamin deficiency because current radioisotope dilution assays are not specific for true cobalamin. *New England Journal of Medicine* **1978**, *299*, 785-792.

Kumar, S. S.; Chouhan, R. S.; Thakur, M. S. Trends in analysis of vitamin B_{12}. *Analytical Biochemistry* **2010**, *398*, 139-149.

Lee, J.; Filosa, S.; Bonvin, J.; Guyon, S.; Aponte, R. A.; Turnbull, J. L. Expression, purification, and characterization of recombinant purine nucleoside phosphorylase from *Escherichia coli*. *Protein Expression and Purification* **2001**, *22*, 180-188.

Lee, J. H.; Shin, J. H.; Park, J. M.; Kim, H. J.; Ahn, J. H.; Kwak, B. M.; Kim, J. M. Analytical determination of vitamin B_{12} content in infant and toddler milk formulas by liquid chromatography tandem mass spectrometry (LC-MS/MS). *Food Science of Animal Resources* **2015**, *35*, 765-771.

Li, H.-B.; Chen, F.; Jiang, Y. Determination of vitamin B_{12} in multivitamin tablets and fermentation medium by high-performance liquid chromatography with fluorescence detection. *Journal of Chromatography A* **2000**, *891*, 243-247.

Li, X.; Jiang, X.; Li, H.; Ren, D. Purine nucleoside phosphorylase from *Pseudoalteromonas* sp. Bsi590: molecular cloning, gene expression and characterization of the recombinant protein. *Extremophiles* **2008**, *12*, 325-333.

Li, Y.; Breaker, R. R. Kinetics of RNA Degradation by Specific Base Catalysis of Transesterification Involving the 2'-Hydroxyl Group. *Journal of the American Chemical Society* **1999**, *121*, 5364-5372.

Li, Y.; Gill, B. D.; Grainger, M. N. C.; Manley-Harris, M. The analysis of vitamin B_{12} in milk and infant formula: A review. *International Dairy Journal* **2019**, *99*, 104543.

Lindenbaum, J.; Rosenberg, I. H.; Wilson, P. W. F.; Stabler, S. P.; Allen, R. H. Prevalence of cobalamin deficiency in the Framingham elderly population. *American Journal of Clinical Nutrition* **1994**, *60*, 2-11.

Liu, X.-C. Boronic acids as ligands for affinity chromatography. *Chinese Journal of Chromatography* **2006**, *24*, 73-80.

Liu, X.-C.; Scouten, W. H. Boronate Affinity Chromatography. In *Affinity Chromatography: Methods and Protocols*; P. Bailon; G. K. Ehrlich; W.-J. Fung and W. Berthold, Eds.; Humana Press: Totowa, NJ, 2000; pp 119-128.

Liu, Z.; He, H. Synthesis and applications of boronate affinity materials: From class selectivity to biomimetic specificity. *Accounts of Chemical Research* **2017**, *50*, 2185-2193.

Martin, F.; Gimenez, E. C.; Konings, E. New methods for the analysis of water-soluble vitamins in infant formula and adult/pediatric nutritionals. *Journal of AOAC International* **2016**, *99*, 19-25.

Matte, J. J.; Guay, F.; Girard, C. L. Bioavailability of vitamin B_{12} in cows' milk. *British Journal of Nutrition* **2012**, *107*, 61-66.

Mattes, T. A.; Escalante-Semerena, J. C. Facile isolation of alpha-ribazole from vitamin B_{12} hydrolysates using boronate affinity chromatography. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences* **2018**, *1090*, 52-55.

McComb, R. B.; George N. Bowers, J.; Posen, S. *Alkaline Phosphatase* Springer: New York, 1979.

Mikkola, S.; Lönnberg, T.; Lönnberg, H. Phosphodiester models for cleavage of nucleic acids. *Beilstein Journal of Organic Chemistry* **2018**, *14*, 803-837.

Mosekilde, L.; Lildballe, D. L.; Nexo, E.; Rejnmark, L.; Vestergaard, P.; Streym, S.; Greibe, E. Cobalamin and haptocorrin in human milk and cobalamin-

related variables in mother and child: A 9-month longitudinal study. *The American Journal of Clinical Nutrition* **2013**, *98*, 389-395.

Muthayya, S.; Kurpad, A. V.; Duggan, C. P.; Bosch, R. J.; Dwarkanath, P.; Mhaskar, A.; Mhaskar, R.; Thomas, A.; Vaz, M.; Bhat, S.; Fawzi, W. W. Low maternal vitamin B_{12} status is associated with intrauterine growth retardation in urban South Indians. *European Journal of Clinical Nutrition* **2006**, *60*, 791-801.

Nakos, M.; Pepelanova, I.; Beutel, S.; Krings, U.; Berger, R. G.; Scheper, T. Isolation and analysis of vitamin B_{12} from plant samples. *Food Chemistry* **2017**, *216*, 301-308.

National Health and Medical Research Council. Australian Government Department of Health and Ageing. New Zealand Ministry of Health. Nutrient reference values for Australia and New Zealand.; National Health and Medical Research Council: Canberra, 2006.

Nishiyabu, R.; Kubo, Y.; James, T. D.; Fossey, J. S. Boronic acid building blocks: tools for sensing and separation. *Chemical Communications* **2011**, *47*, 1106-1123.

Nohr, D.; Biesalski, H. K.; Back, E. I. Vitamins | Vitamin B_{12}. In *Encyclopedia of Dairy Sciences (Second Edition)*; J. W. Fuquay, Ed.; Academic Press: San Diego, 2011; pp 675-677.

Nursten, H. Maillard Reaction. In *Encyclopedia of Dairy Sciences*; H. Roginski, Ed.; Elsevier: Oxford, 2002; pp 1657-1672.

O'Brien, J. Reaction chemistry of lactose: Non-enzymatic degradation pathways and their significance in dairy products. In *Advanced Dairy Chemistry Volume 3: Lactose, water, salts and vitamins*; P. F. Fox, Ed.; Springer US: Boston, MA, 1997; pp 155-231.

Oivanen, M.; Kuusela, S.; Lönnberg, H. Kinetics and Mechanisms for the Cleavage and Isomerization of the Phosphodiester Bonds of RNA by Brønsted Acids and Bases. *Chemical Reviews* **1998**, *98*, 961-990.

Okbamichael, M.; Sañudo-Wilhelmy, S. A. A new method for the determination of Vitamin B_{12} in seawater. *Analytica Chimica Acta* **2004**, *517*, 33-38.

Oprean, L.; Iancu, R. M.; Radu, G. L.; Litescu, S. C.; Truica, G. I. HPLC analysis of vitamin B_{12} in fodder and goat milk. *Acta Universitatis Cibiniensis. Series E: Food Technology* **2011**, *15*, 43-50.

Pakin, C.; Bergaentzlé, M.; Aoudé-Werner, D.; Hasselmann, C. α-Ribazole, a fluorescent marker for the liquid chromatographic determination of vitamin B_{12} in foodstuffs. *Journal of Chromatography A* **2005**, *1081*, 182-189.

Parvin, M. H.; Azizi, E.; Arjomandi, J.; Lee, J. Y. Highly sensitive and selective electrochemical sensor for detection of vitamin B_{12} using an Au/PPy/FMNPs@TD-modified electrode. *Sensors and Actuators B-Chemical* **2018**, *261*, 335-344.

Pearson, R. G.; Schmidtke, H. H.; Basolo, F. The mechanism of base hydrolysis and base catalyzed reactions of some cobalt(iii) amine complexes. *Journal of the American Chemical Society* **1960**, *82*, 4434-4435.

Perez-Fernandez, V.; Gentili, A.; Martinelli, A.; Caretti, F.; Curini, R. 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. *Journal of Chromatography A* **2016**, *1428*, 255-266.

Peters, J. A. Interactions between boric acid derivatives and saccharides in aqueous media: Structures and stabilities of resulting esters. *Coordination Chemistry Reviews* **2014**, *268*, 1-22.

Piñón-Balderrama, C. I.; Leyva-Porras, C.; Terán-Figueroa, Y.; Espinosa-Solís, V.; Álvarez-Salas, C.; Saavedra-Leos, M. Z. Encapsulation of active ingredients in food industry by spray-drying and nano spray-drying technologies. *Processes* **2020**, *8*, 889.

Prokopowicz, M.; Cieśla, J.; Kierdaszuk, B. A synergistic effect of phosphate, pH and Phe159 substitution on the formycin A association to the *E. coli* purine nucleoside phosphorylase. *Biochimie* **2018**, *148*, 80-86.

Quadros, E. V.; Regec, A. L.; Khan, K. M. F.; Quadros, E.; Rothenberg, S. P. Transcobalamin II synthesized in the intestinal villi facilitates transfer of cobalamin to the portal blood. *American Journal of Physiology* **1999**, *277*, G161-G166.

Raju, C. S. K.; Yu, L. L.; Schiel, J. E.; Long, S. A simple and sensitive LC-ICP-MS method for the accurate determination of vitamin B_{12} in fortified breakfast cereals and multivitamin tabletst. *The Royal Society of Chemistry* **2013**, *28*, 901-907.

Ramos, J. J.; Saez, T.; Bueso, J. P.; Sanz, M. C.; Fernandez, A. Vitamin B_{12} levels in ewe colostrum and milk and in lamb serum. *Veterinary Research* **1994**, *25*, 405-409.

Ray, J. G.; Blom, H. J. Vitamin B_{12} insufficiency and the risk of fetal neural tube defects. *QJM: An International Journal of Medicine* **2003**, *96*, 289-295.

Raynal-Ljutovac, K.; Lagriffoul, G.; Paccard, P.; Guillet, I.; Chilliard, Y. Composition of goat and sheep milk products: An update. *Small Ruminant Research* **2008**, *79*, 57-72.

Repossi, A.; Zironi, E.; Gazzotti, T.; Serraino, A.; Pagliuca, G. 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. *Italian Journal of Food Safety* **2017**, *6*, 6795.

Richard, B.; Le Page, J.-F.; Schuck, P.; André, C.; Jeantet, R.; Delaplace, G. Towards a better control of dairy powder rehydration processes. *International Dairy Journal* **2013**, *31*, 18-28.

Roecker, L. Synthesis and Base Hydrolysis of a Cobalt(III) Complex Coordinated by a Thioether Ligand. *Journal of Chemical Education* **2008**, *85*, 1562.

Rowland, S. J. 175. The precipitation of the proteins in milk. I. Casein. II. Total proteins. III. Globulin. IV. Albumin and proteose-peptone. *Journal of Dairy Research* **1938**, *9*, 30-41.

Saxena, J.; Adhikari, B.; Brkljaca, R.; Huppertz, T.; Zisu, B.; Chandrapala, J. Influence of lactose on the physicochemical properties and stability of infant formula powders: A review. *Food Reviews International* **2021**, 1-15.

Schellinger, A. P.; Carr, P. W. Solubility of buffers in aqueous–organic eluents for reversed-phase liquid chromatography. *LCGC North America* **2004**, *22*, 544-548.

Schimpf, K.; Spiegel, R.; Thompson, L.; Dowell, D. Determination of vitamin B_{12} in infant formula and adult nutritionals by HPLC: first action 2011.10. *Journal of AOAC International* **2012**, *95*, 313-318.

Scott, J. M. Bioavailability of vitamin B_{12}. *European Journal of Clinical Nutrition* **1997**, *51 Suppl 1*, S49-53.

Sharma, U.; Pal, D.; Prasad, R. Alkaline phosphatase: an overview. *Indian Journal of Clinical Biochemistry* **2014**, *29*, 269-278.

Shimadzu Corporation. 7 Key Differences in the Use of Methanol and Acetonitrile. https://www.shimadzu.com/an/service-support/technical-support/lib/methanol-acetonitrile.html (accessed 4 May 2023)

Shorb, M. S. Proceedings of local branches, Society of American Bacteriologists. *Journal of Bacteriology* **1947**, *53*, 669.

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)

Smith, A. D.; Warren, M. J.; Refsum, H. Chapter Six - Vitamin B_{12}. In *Advances in Food and Nutrition Research*; N. A. M. Eskin, Ed.; Academic Press, 2018; pp 215-279.

Snyder, L. R., Kirkland, Joseph J, Dolan, John W, Basic Concepts and the Control of Separation. In *Introduction to Modern Liquid Chromatography*; John Wiley & Sons, Inc: Hoboken, NJ, USA, 2009; pp 19-86.

Stabler, S. P. Clinical practice. Vitamin B_{12} deficiency. *The New England Journal of Medicine* **2013**, *368*, 149-160.

Stevens, J. D.; Ness, R. K.; Fletcher, H. G., Jr. Syntheses with partially benzylated sugars. XI. Studies on the synthesis of the anomeric 5,6-dimethyl-1-D-ribofuranosylbenzimidazoles (ribazoles). Comparison of the condensation of 2,3,5-tri-*O*)-benzoyl-D-ribofuranosyl bromide and 2,3,5-tri-*O*-benzyl-D-ribofuranosyl chloride with 5,6-dimethylbenzimidazole. *The Journal of Organic Chemistry* **1968**, *33*, 1806-1810.

Subirats, X.; Bosch, E.; Rosés, M. Retention of ionisable compounds on high-performance liquid chromatography XVII: Estimation of the pH variation of aqueous buffers with the change of the methanol fraction of the mobile phase. *Journal of Chromatography A* **2007**, *1138*, 203-215.

Sundin, D. P.; Allen, R. H. Analysis of the nucleoside moiety of cobalamin and cobalamin analogs using gas chromatography-mass spectrometry. *Archives of Biochemistry and Biophysics* **1992**, *298*, 658-666.

Suzuki, M.; Hayashi, K.; Wacker, W. E. C. Determination of cobalt in biological materials by atomic absorption spectrometry. *Analytica Chimica Acta* **1979**, *104*, 389-392.

Taverniers, I.; Bockstaele, E. V.; Loose, M. D. Analytical method validation and quality assurance. In *Pharmaceutical Manufacturing Handbook: Regulations and Quality*; S. C. Gad, Ed.; John Wiley & Sons, Incorporated: Hoboken, NJ, 2008.

Thompkinson, D. K.; Kharb, S. Aspects of Infant Food Formulation. *Comprehensive Reviews in Food Science and Food Safety* **2007**, *6*, 79-102.

Toh, B. H.; van, D. I. R.; Gleeson, P. A. Pernicious anemia. *The New England Journal of Medicine* **1997**, *337*, 1441-1448.

Truswell, A. S. Vitamin B_{12}. *Nutrition & Dietetics* **2007**, *64*, S120-S125.

Tuytten, R.; Lemière, F.; Esmans, E. L.; Herrebout, W. A.; van der Veken, B. J.; Maes, B. U. W.; Witters, E.; Newton, R. P.; Dudley, E. Role of nitrogen Lewis basicity in boronate affinity chromatography of nucleosides. *Analytical Chemistry* **2007**, *79*, 6662-6669.

Uziel, M.; Smith, L. H.; Taylor, S. A. Modified nucleosides in urine: selective removal and analysis. *Clinical Chemistry* **1976**, *22*, 1451-1455.

Vaid, F. H. M.; Zahid, S.; Faiyaz, A.; Qadeer, K.; Gul, W.; Anwar, Z.; Ahmad, I. Photolysis of methylcobalamin in aqueous solution: A kinetic study. *Journal of Photochemistry and Photobiology A: Chemistry* **2018**, *362*, 40-48.

Van Boekel, M. A. J. S. Effect of heating on maillard reactions in milk. *Food Chemistry* **1998**, *62*, 403-414.

Van Wyk, J.; Britz, T. J. A rapid HPLC method for the extraction and quantification of vitamin B_{12} in dairy products and cultures of *Propionibacterium freudenreichii*. *Dairy Science & Technology* **2010**, *90*, 509-520.

Vial, J.; Jardy, A. Experimental comparison of the different approaches to estimate LOD and LOQ of an HPLC method. *Analytical Chemistry* **1999**, *71*, 2672-2677.

Vogiatzoglou, A.; Smith, A. D.; Nurk, E.; Berstad, P.; Drevon, C. A.; Ueland, P. M.; Vollset, S. E.; Tell, G. S.; Refsum, H. Dietary sources of vitamin B-12

and their association with plasma vitamin B-12 concentrations in the general population: the Hordaland Homocysteine Study. *American Journal of Clinical Nutrition* **2009**, *89*, 1078-1087.

Wang, B.; Feng, H.; Ezeji, T.; Blaschek, H. Sugaring-out separation of acetonitrile from its aqueous solution. *Chemical Engineering & Technology* **2008**, *31*, 1869-1874.

Wang, C.; Guo, M. Whey protein structure and denaturation and interactions with other food components. In *Whey Protein Production, Chemistry, Functionality, and Applications*, 2019; pp 67-101.

Wang, H.; Shou, Y.; Zhu, X.; Xu, Y.; Shi, L.; Xiang, S.; Feng, X.; Han, J. Stability of vitamin B_{12} with the protection of whey proteins and their effects on the gut microbiome. *Food Chemistry* **2019**, *276*, 298-306.

Watanabe, F. Vitamin B_{12} sources and bioavailability. *Experimental Biology and Medicine (Maywood)* **2007**, *232*, 1266-1274.

Watanabe, F.; Abe, K.; Takenaka, S.; Fujita, T.; Nakano, Y. Method for quantitation of total vitamin B_{12} in foods using a highly fluorescent vitamin B_{12} derivative. *Journal of Agricultural and Food Chemistry* **1997**, *45*, 4661-4663.

Watanabe, F.; Bito, T. Determination of cobalamin and related compounds in foods. *Journal of AOAC International* **2018**, *101*, 1308-1313.

Webb, B. H. Color development in lactose solutions during heating with special reference to the color of evaporated milk. *Journal of Dairy Science* **1935**, *18*, 81-96.

Wen, Q.-H.; Wang, L.-H.; Zeng, X.-A.; Niu, D.-B.; Wang, M.-S. Hydroxyl-related differences for three dietary flavonoids as inhibitors of human purine nucleoside phosphorylase. *International Journal of Biological Macromolecules* **2018**, *118*, 588-598.

Westmark, P. R.; Valencia, L. S.; Smith, B. D. Influence of eluent anions in boronate affinity chromatography. *Journal of Chromatography A* **1994**, *664*, 123-128.

Wigzell, M. Does processing effect the nurtritional value of the milk you drink? © Copyright 2003. https://www.anchordairy.com/me/en/nutrition-stories/does-processing-affect-the-nutrition-value-of-the-milk-you-drink.html (accessed 18 April 2023).

World Health Organization “Nutritional anaemias. Report of a WHO scientific group,” World Health Organization, 1968.

Yaikhomba, T.; Poswal, L.; Goyal, S. Assessment of iron, folate and vitamin B_{12} status in severe acute malnutrition. *Indian Journal of Pediatrics* **2015**, *82*, 511-514.

Yan, J.; Bi, M.; Bourdon, A. K.; Farmer, A. T.; Wang, P.-H.; Molenda, O.; Quaile, A. T.; Jiang, N.; Yang, Y.; Yin, Y.; Şimşir, B.; Campagna, S. R.; Edwards, E. A.; Löffler, F. E. Purinyl-cobamide is a native prosthetic group of reductive dehalogenases. *Nature Chemical Biology* **2018**, *14*, 8-14.

Zhu, Q.; Aller, R. C.; Kaushik, A. Analysis of vitamin B_{12} in seawater and marine sediment porewater using ELISA. *Limnology and Ocenography: Methods* **2011**, *9*, 515-523.

Zhu, S.; Song, D.; Gong, C.; Tang, P.; Li, X.; Wang, J.; Zheng, G. Biosynthesis of nucleoside analogues via thermostable nucleoside phosphorylase. *Applied Microbiology and Biotechnology* **2013**, *97*, 6769-6778.

Zimmerman, T. P.; Gersten, N. B.; Ross, A. F.; Miech, R. P. Adenine as substrate for purine nucleoside phosphorylase. *Canadian Journal of Biochemistry* **1971**, *49*, 1050-1054.

Zingg, J.-M.; Jones, P. A. Genetic and epigenetic aspects of DNA methylation on genome expression, evolution, mutation and carcinogenesis. *Carcinogenesis* **1997**, *18*, 869-882.

Zironi, E.; Gazzotti, T.; Barbarossa, A.; Devicienti, C.; Scardilli, M.; Pagliuca, G. Technical note: development and validation of a method using ultra performance liquid chromatography coupled with tandem mass spectrometry for determination of vitamin B_{12} concentrations in milk and dairy products. *Journal of Dairy Science* **2013**, *96*, 2832-2836.

Appendix A Publication and Co-Authorship Form

The analysis of vitamin B_{12} in milk and infant formula: A review

Li, Y.; Gill, B. D.; Grainger, M. N. C.; Manley-Harris, M. The analysis of vitamin B_{12} in milk and infant formula: A review. *International Dairy Journal* **2019**, *99*, 104543.

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Certification by Co-Authors

The undersigned hereby certify that:

- the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and

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Review

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.

Contents

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

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(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).

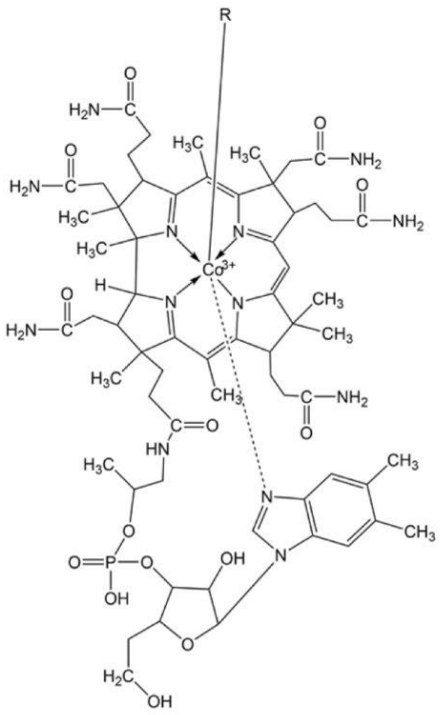

Fig. 1. Chemical structure of vitamin B_{12}. The nature of the R group defines the four vitamin B_{12} forms: methylcobalamin (R = CH_3; Me-Cbl), hydroxocobalamin (R = OH; OH-Cbl), adenosylcobalamin (R = 5′ deoxyadenosyl; Ado-Cbl) and cyanocobalamin (R = CN; CN-Cbl).

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 μg 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 μg L^{-1} (Gille & Schmid, 2015). The vitamin B_{12} content of skim milk in New Zealand was reported as 2–8 μg 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 μg per day for adults, a slightly higher intake for pregnancy at 2.6 μg per day and during lactation at 2.8 μg per day. The daily RDIs of vitamin B_{12} for infants, children and teenagers are even lower than the adult RDI of 2.4 μg (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 μg 100 kJ^{-1} (~0.7 μg L^{-1} prepared feed) with an upper guidance limit of 0.36 μg 100 kJ^{-1} (~9.8 μg 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 μg 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-Giménez, 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-Giménez et al., 2008; Heudi, Kilinc, Fontannaz, & Marley, 2006; Nakos et al., 2017; Watanabe & Bito, 2018). Pakin, Bergaentzlé, Aoudé-Werner, and Hasselmann (2005) used proteolysis, but without further heating or addition of potassium cyanide (Pakin et al., 2005), however, the exclusion of cyanide does risk incomplete vitamin B_{12} extraction when immunoaffinity extraction is applied (Campos-Giménez 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-Giménez et al., 2008; Chamlagain et al., 2015; Heudi et al., 2006; Martin, Gimenez, & Konings, 2016; Nakos et al., 2017; Pakin et al., 2005). The

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

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% NaCN 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% KCN solution	n/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% NaCN 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% KCN solution	n/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	n/c	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 3 h; heated for 35 min at 100 °C	1 mL 1% NaCN solution	94–100	Heudi et al. (2006)
Acidic precipitation					
Milk	4 mL	6 mL 50 mM sodium acetate buffer, pH 4.6	n/c	80–93	Perez-Fernandez et al. (2016)

[a] Abbreviations are: n/a, no data available; n/c, no cyanide applied.

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

SPE cartridge type (Bed weight; volume)	Sample filtrate 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 H_2O	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; n/a)	20–80 mL	20 mL MeCN	10 mL H_2O	5 mL H_2O	4.4 mL 25% or 30% MeCN in H_2O	n/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] Abbreviation: n/a, no data available.

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-Giménez 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-Giménez 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, flow rate, 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 acid-trimethylamine 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 μL 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-Giménez et al. (2008) modified this method utilising the same columns but with a large injection volume (100 μL) 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 mL min^{-1} 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 μL. 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 timesaving 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

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

C_{18} LC column (length × diameter; particle size)	Flow rate (mL min^{-1})	Temperature	Mobile phase	Wavelength	References
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)
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)
150 × 3.0 mm; n/a	0.25	n/a	A: 0.025% TFA in H_2O (pH 2.6) B: MeCN	UV 361 nm	Heudi et al. (2006)
150 or 125 × 3.0 mm; n/a	0.25	n/a	A: 0.025% TFA in H_2O B: 0.025% TFA in MeCN	UV 361 nm	Campos-Giménez et al. (2008)
100 × 2.1 mm; 1.7 μm	0.4	n/a	A: 0.025% TFA in H_2O B: 0.025% TFA in MeCN	UV 361 nm	Campos-Gimenez (2014)
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)
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)
100 × 4.6 mm; 3 μm	1	n/a	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] Abbreviations are: RT, room temperature; n/a, no data available.

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 mm × 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

Table 4
Summary of limit of detection (LOD), limit of quantitation (LOQ), recovery and precision of HPLC-UV/Vis methods in the reviewed literature.[a]

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

[a] Abbreviations are: n/a, no data available; RSD, relative standard deviation. LOD and LOQ are expressed as either instrumental limit (ng mL^{-1}) or as overall method limit (ng g^{-1}). Where there are two discrete values for repeatability, the first is RSD of method precision in powdered milk, the second is for liquid milk; the range presented is that for different samples.

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 STAN 234-1999 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 $^{43}Ca^{16}O$, $Ca^{16}O^1H$, $^{36}Ar^{23}Na$, $^{40}Ar^{18}O^1H$, $^{24}Mg^{35}Cl$ 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.

References

Ahmad, I., & Hussain, W. (1993). Stability of cyanocobalamin in parenteral preparations. *Pakistan Journal of Pharmaceutical Sciences, 6*, 53–59.

Albala-Hurtado, S., Veciana-Nogues, M. T., Izquierdo-Pulido, M., & Marine-Font, A. (1997). Determination of water-soluble vitamins in infant milk by high-performance liquid chromatography. *Journal of Chromatography A, 778*, 247–253.

Alcock, S. C., Finglas, P. M., & Morgan, M. R. A. (1992). Production and purification of an R-protein-enzyme conjugate for use in a microtitration plate protein-binding assay for vitamin B_{12} in fortified food. *Food Chemistry, 45*, 199–203.

Baker, S. A., & Miller-Ihli, N. J. (2000). Determination of cobalamins using capillary electrophoresis inductively coupled plasma mass spectrometry. *Spectrochimica Acta Part B: Atomic Spectroscopy, 55*, 1823–1832.

Ball, G. F. M. (1994). Microbiological methods for the determination of the B-group vitamins. In G. F. M. Ball (Ed.), *Water-soluble vitamin assays in human nutrition* (pp. 317–364). Boston, MA, USA: Springer US.

Ball, G. F. M. (2006). *Vitamins in foods analysis, bioavailability, and stability*. Boca Raton, FL, USA: Taylor & Francis Group.

Berman, D., Yacowitz, H., & Weiser, H. H. (1956). A differential microbiological assay for vitamin B_{12} and pseudovitamin B_{12}. *Applied Microbiology, 4*, 49–52.

Brown, K. L. (2005). Chemistry and enzymology of vitamin B_{12}. *Chemical Reviews, 105*, 2075–2149.

Campos-Gimenez, E. (2014). Improved AOAC first action 2011.08 for the analysis of vitamin B_{12} in infant formula and adult/pediatric formulas: First action 2014.02. *Journal of AOAC International, 97*, 1397–1402.

Campos-Giménez, E., Fontannaz, P., Trisconi, M., Kilinc, T., & Gimenez, C. (2008). Determination of vitamin B_{12} in food products by liquid chromatography/UV detection with immunoaffinity extraction: Single-laboratory validation. *Journal of AOAC International, 91*, 786–793.

Campos-Gimenez, E., Fontannaz, P., Trisconi, M., Kilinc, T., Gimenez, C., & Andrieux, P. (2012). Determination of vitamin B_{12} in infant formula and adult nutritionals by liquid chromatography/UV detection with immunoaffinity extraction: First action 2011.08. *Journal of AOAC International, 95*, 307–312.

Chamlagain, B., Edelmann, M., Kariluoto, S., Ollilainen, V., & Piironen, V. (2015). Ultra-high performance liquid chromatographic and mass spectrometric analysis of active vitamin B_{12} in cells of *Propionibacterium* and fermented cereal matrices. *Food Chemistry, 166*, 630–638.

Codex Alimentarius Commission. (1981). *Codex STAN 72-1981: Standard for infant formula and formulas for special medical purposes intended for infants*. Rome, Italy: Food and Agriculture Organization of the United Nations.

Codex Alimentarius Commission. (1999). *Codex STAN 234-1999: Recommended methods of analysis and sampling*. Rome, Italy: Food and Agriculture Organization of the United Nations.

Combs, G. F., & McClung, J. P. (2017). Vitamin B_{12}. In G. F. Combs, & J. P. McClung (Eds.), *The vitamins: Fundamental aspects in nutrition and health* (5th ed., pp. 431–452). London, UK: Academic Press.

Cook, E. A., & Ellis, L. N. (1968). Variations in the growth response of four different vitamin B_{12} assay microorganisms to the same tissue and standard preparations. *Applied and Environmental Microbiology, 16*, 1831–1840.

D'Ulivo, L., Yang, L., Ding, J. F., Pagliano, E., Leek, D. M., Thibeault, M. P., et al. (2017). Determination of cyanocobalamin by isotope dilution LC-MS/MS. *Analytica Chimica Acta, 990*, 103–109.

Duplessis, M., Pellerin, D., Cue, R. I., & Girard, C. L. (2016). Short communication: Factors affecting vitamin B_{12} concentration in milk of commercial dairy herds: An exploratory study. *Journal of Dairy Science, 99*, 4886–4892.

Fedosov, S. N., Petersen, T. E., & Nexø, E. (1996). Transcobalamin from cow milk: Isolation and physico-chemical properties. *Biochimica et Biophysica Acta – Protein Structure and Molecular Enzymology, 1292*, 113–119.

Gille, D., & Schmid, A. (2015). Vitamin B_{12} in meat and dairy products. *Nutrition Reviews, 73*, 106–115.

Greibe, E., & Nexo, E. (2016). Forms and amounts of vitamin B_{12} in infant formula: A pilot study. *PLoS One, 11*. Article 165458.

Gueant, J. L., & Alpers, D. H. (2013). Vitamin B_{12}, a fascinating micronutrient, which influences human health in the very early and later stages of life. *Biochimie, 95*, 967–969.

Heudi, O., Kilinc, T., Fontannaz, P., & Marley, E. (2006). Determination of vitamin B_{12} in food products and in premixes by reversed-phase high performance liquid chromatography and immunoaffinity extraction. *Journal of Chromatography A, 1101*, 63–68.

Hodgkin, D. C., Pickworth, J., Robertson, J. H., Trueblood, K. N., Prosen, R. J., & White, J. G. (1955). Structure of vitamin B_{12} : The crystal structure of the hexacarboxylic acid derived from B_{12} and the molecular structure of the vitamin. *Nature, 176*, 325–328.

Indyk, H. E., Persson, B. S., Caselunghe, M. C. B., Moberg, A., Filonzi, E., & Woollard, D. C. (2002). Determination of vitamin B_{12} in milk products and selected foods by optical biosensor protein-binding assay: Method comparison. *Journal of AOAC International, 85*, 72–81.

Iwase, H., & Ono, I. (1997). Determination of cyanocobalamin in foods by high-performance liquid chromatography with visible detection after solid-phase extraction and membrane filtration for the precolumn separation of lipophilic species. *Journal of Chromatography A, 771*, 127–134.

Jensen, R. G. (1995). Water-soluble vitamins in bovine milk. In R. G. Jensen (Ed.), *Handbook of milk composition* (pp. 688–692). San Diego, CA, USA: Academic Press.

Juzeniene, A., & Nizauskaite, Z. (2013). Photodegradation of cobalamins in aqueous solutions and in human blood. *Journal of Photochemistry and Photobiology B, 122*, 7–14.

Kakitani, A., Inoue, T., Matsumoto, K., Watanabe, J., Nagatomi, Y., & Mochizuki, N. (2014). Simultaneous determination of water-soluble vitamins in beverages and dietary supplements by LC-MS/MS. *Food Additives & Contaminants: Part A, 31*, 1939–1948.

Karmi, O., Zayed, A., Baraghethi, S., Qadi, M., & Ghanem, R. (2011). Measurement of vitamin B_{12} concentration: A review on available methods. *IIOAB Journal, 2*, 23–32.

Kumar, S. S., Chouhan, R. S., & Thakur, M. S. (2010). Trends in analysis of vitamin B_{12}. *Analytical Biochemistry, 398*, 139–149.

Martin, F., Gimenez, E. C., & Konings, E. (2016). New methods for the analysis of water-soluble vitamins in infant formula and adult/pediatric nutritionals. *Journal of AOAC International, 99*, 19–25.

Matte, J. J., Guay, F., & Girard, C. L. (2012). Bioavailability of vitamin B_{12} in cows' milk. *British Journal of Nutrition, 107*, 61–66.

Minot, G. R., & Murphy, W. P. (1983). Landmark article (JAMA 1926). Treatment of pernicious anemia by a special diet. By George R. Minot and William P. Murphy. *Journal of the American Medical Association, 250*, 3328–3335.

Muhammad, K., Briggs, D., & Jones, G. (1993). The appropriateness of using cyanocobalamin as calibration standards in *Lactobacillus leichmannii* ATCC 7830 assay of vitamin B_{12}. *Food Chemistry, 48*, 427–429.

Nakos, M., Pepelanova, I., Beutel, S., Krings, U., Berger, R. G., & Scheper, T. (2017). Isolation and analysis of vitamin B_{12} from plant samples. *Food Chemistry, 216*, 301–308.

Oprean, L., Iancu, R. M., Radu, G. L., Litescu, S. C., & Truica, G. I. (2011). HPLC analysis of vitamin B_{12} in fodder and goat milk. *Acta Universitatis Cibiniensis. Series E Food Technology, 15*, 43–50.

Pakin, C., Bergaentzlé, M., Aoudé-Werner, D., & Hasselmann, C. (2005). α-Ribazole, a fluorescent marker for the liquid chromatographic determination of vitamin B_{12} in foodstuffs. *Journal of Chromatography A, 1081*, 182–189.

Parvin, M. H., Azizi, E., Arjomandi, J., & Lee, J. Y. (2018). Highly sensitive and selective electrochemical sensor for detection of vitamin B_{12} using an Au/PPy/FMNPs@TD-modified electrode. *Sensors and Actuators B Chemical, 261*, 335–344.

Perez-Fernandez, V., Gentili, A., Martinelli, A., Caretti, F., & Curini, R. (2016). 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. *Journal of Chromatography A, 1428*, 255–266.

Raju, C. S. K., Yu, L. L., Schiel, J. E., & Long, S. (2013). A simple and sensitive LC-ICP-MS method for the accurate determination of vitamin B_{12} in fortified breakfast cereals and multivitamin tabletst. *Royal Society of Chemistry, 28*, 901–907.

Repossi, A., Zironi, E., Gazzotti, T., Serraino, A., & Pagliuca, G. (2017). 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. *Italian Journal of Food Safety, 6*. Article 6795.

Schimpf, K., Spiegel, R., Thompson, L., & Dowell, D. (2012). Determination of vitamin B_{12} in infant formula and adult nutritionals by HPLC: First action 2011.10. *Journal of AOAC International, 95*, 313–318.

Sharma, R., Rajput, Y. S., Dogra, G., & Tomar, S. K. (2007). Estimation of vitamin B_{12} by ELISA and its status in milk. *Milchwissenschaft, 62*, 127–130.

Shorb, M. S. (1947). Proceedings of local branches, society of American bacteriologists. *Journal of Bacteriology, 53*, 669.

Truswell, A. S. (2007). Vitamin B_{12}. *Nutrition and Dietetics, 64*, S120–S125.

Vaid, F. H. M., Zahid, S., Faiyaz, A., Qadeer, K., Gul, W., Anwar, Z., et al. (2018). Photolysis of methylcobalamin in aqueous solution: A kinetic study. *Journal of Photochemistry and Photobiology A Chemistry, 362*, 40–48.

Vogiatzoglou, A., Smith, A. D., Nurk, E., Berstad, P., Drevon, C. A., Ueland, P. M., et al. (2009). Dietary sources of vitamin B-12 and their association with plasma vitamin B-12 concentrations in the general population: The hordaland homocysteine study. *American Journal of Clinical Nutrition, 89*, 1078–1087.

Walsh, H. (2014). Infant formula analysis. In M. Guo (Ed.), *Human milk biochemistry and infant formula manufacturing technology* (pp. 311–344). Chichester, UK: Woodhead Publishing.

Watanabe, F. (2007). Vitamin B_{12} sources and bioavailability. *Experimental Biology and Medicine (Maywood), 232*, 1266–1274.

Watanabe, F., Abe, K., Takenaka, S., Fujita, T., & Nakano, Y. (1997). Method for quantitation of total vitamin B_{12} in foods using a highly fluorescent vitamin B_{12} derivative. *Journal of Agricultural and Food Chemistry, 45*, 4661–4663.

Watanabe, F., & Bito, T. (2018). Determination of cobalamin and related compounds in foods. *Journal of AOAC International, 101*, 1308–1313.

Waters. (2014). *Oasis HLB cartridges and 96-well plates care and use manual.* https://www.waters.com/waters/support.htm?locale=en_US&cid=513209&lid=10076779. Last accessed 22nd May, 2019.

Zhu, Q., Aller, R. C., & Kaushik, A. (2011). Analysis of vitamin B_{12} in seawater and marine sediment porewater using ELISA. *Limnology and Ocenography: Methods, 9*, 515–523.

Zironi, E., Gazzotti, T., Barbarossa, A., Devicienti, C., Scardilli, M., & Pagliuca, G. (2013). Technical note: Development and validation of a method using ultra performance liquid chromatography coupled with tandem mass spectrometry for determination of vitamin B_{12} concentrations in milk and dairy products. *Journal of Dairy Science, 96*, 2832–2836.

Appendix B Copyright Authorisation

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5	**Figure 1.2**. Common molecular structure of Vitamin B_{12} Mattes, T. A.; Escalante-Semerena, J. C. Facile isolation of alpha-ribazole from vitamin B_{12} hydrolysates using boronate affinity chromatography. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences* **2018**, *1090*, 52-55. Permission provided by *Elsevier.*	27 04 2023	Y 27 04 2023
19	**Figure 1.3**. Manufacturing flow chart for powdered infant formula Jiang, Y. J.; Guo, M. 8 - Processing technology for infant formula. In *Human Milk Biochemistry and Infant Formula Manufacturing Technology*; M. Guo, Ed.; Woodhead Publishing, 2014; pp 211-229. Permission provided by *Elsevier*	09 06 2023	Y 09 06 2023
98	**Figure 4.6 (b).** The UV/Vis spectra of vitamin B_{12} and cobinamide (Cbi) from 280–600 nm Yan, J.; Bi, M.; Bourdon, A. K.; Farmer, A. T.; Wang, P.-H.; Molenda, O.; Quaile, A. T.; Jiang, N.; Yang, Y.; Yin, Y.; Şimşir, B.; Campagna, S. R.; Edwards, E. A.; Löffler, F. E. Purinyl-cobamide is a native prosthetic group of reductive dehalogenases. *Nature Chemical Biology* **2018**, *14*, 8-14. Permission provided by *Springer Nature*	27 04 2023	Y 27 04 2023
144	**Figure 6.6**.Relation between organic solvent (methanol or acetonitrile) water ratio and column pressure Shimadzu Corporation. 7 Key Differences in the Use of Methanol and Acetonitrile. https://www.shimadzu.com/an/service-support/technical-support/lib/methanol-acetonitrile.html (accessed 4 May 2023) Permisson provided by *Shimadzu Corporation*	04 05 2023	Y 12 05 2023
146	**Figure 6.8**. Relationship between the composition of methanol (%, v:v) in the mobile phase and the concentration of methanol, water and methanol water associate (%, v:v) (Katz *et al.*, 1989) Katz, E. D.; Lochmuller, C. H.; Scott, R. P. W. Methanol-water association and its effect on solute retention in liquid chromatography. *Analytical Chemistry* **1989**, *61*, 349-355 Permission provided by *American Chemical Society*	26 04 2023	Y 26 04 2023

Appendix C α-Ribazole Isolation Raw Data

Table C.1. UV absorbance of 5,6-dimethylbenzimidazole standard solutions at 280 nm

DMB concentrations (mg mL^{-1})	$A_{280\ nm}$
0.001	0.0439
0.002	0.0838
0.005	0.2019
0.008	0.3358
0.010	0.4235

Table C.2. Effect of acidic hydrolysis on the yield of α-ribazole

Concentration of hydrochloric acid (mol L^{-1})	Yield of α-ribazole (ng)a	
	Dup 1	Dup 2
1.25	7.65	7.85
1.50	7.74	7.94
2.00	7.20	7.24
2.50	7.04	6.93
3.00	5.45	5.34
4.00	1.65	1.84

[a] 40 ng of vitamin B_{12} = 8.21 ng theoretical yield of α-ribazole

Table C.3. Effect of alkaline hydrolysis on the yield of α-ribazole

Concentration of sodium hydroxide (mol L^{-1})	Yield of α-ribazole (ng)[a]	
	Dup 1	Dup 2
1.25	6.56	6.69
1.50	6.91	6.68
2.00	7.16	7.07
2.50	6.86	6.63
3.00	4.30	4.10
4.00	2.36	2.21

[a] 40 ng of vitamin B_{12} = 8.21 ng theoretical yield of α-ribazole

Table C.4. Effect of hydrochloric acid concentrations (0.10–4.00 M) on the yield of α-ribazole

Concentration of hydrochloric acid (mol L^{-1})	Yield of α ribazole (ng)[a]	
	Dup 1	Dup 2
0.10	1.11	1.24
0.25	5.52	5.60
0.50	6.91	7.05
1.00	7.34	7.40
1.25	7.29	7.35
1.50	7.59	7.70
2.00	6.40	6.41
2.50	5.03	5.01
4.00	0.38	0.58

[a] 40 ng of vitamin B_{12} = 8.21 ng theoretical yield of α ribazole

Table C.5. Effect of heating period of hydrochloric acid (1.5 M) on the yield of α-ribazole

Heating period	Yield of α ribazole (ng)a	
	Dup 1	Dup 2
15	7.06	6.98
30	7.30	7.69
45	7.52	7.63
60	7.62	7.37
90	7.48	7.50
120	7.31	7.23

[a] 40 ng of vitamin B_{12} = 8.21 ng theoretical yield of α ribazole

Table C.6. Effect of the incubation buffer pH on the yield of α-ribazole

pH of incubation buffer	Yield of α ribazole (ng)a	
	Dup 1	Dup 2
8.0	7.28	7.21
8.8	7.50	7.53
9.8	5.34	5.29

[a] 40 ng of vitamin B_{12} = 8.21 ng theoretical yield of α ribazole

Table C.7. Effect of the incubation time on the yield of α-ribazole

Incubation time (hour)	Yield of α ribazole (ng)[a]	
	Dup 1	Dup 2
0.5	7.21	7.10
1	7.22	7.29
3	7.30	6.99
16	7.69	7.48

[a] 40 ng of vitamin B_{12} = 8.21 ng theoretical yield of α ribazole

Table C.8. Effect of the incubation temperature 34 and 37 °C on the yield of α-ribazole

Incubation temperature	Yield of α ribazole (ng)[a]	
	Dup 1	Dup 2
34	7.41	7.48
37	7.28	7.52

[a] 40 ng of vitamin B_{12} = 8.21 ng theoretical yield of α ribazole

Table C.9. Effect of eluent on the recovery of α-ribazole from boronate affinity column

Eluent contents	Sequential elution volume (mL)	Recovered of α ribazole (ng)[a]	
		Dup 1	Dup 2
0.1 M formic acid	1st	1.336	1.283
	2nd	1.433	1.330
	3rd	0.932	0.909
	4th	0.356	0.345
1.0 M formic acid	1st	2.334	1.998
	2nd	1.673	1.826
	3rd	0.884	0.929
	4th	0.393	0.393
methanol: 0.1 M formic acid (20: 80 *v:v*)	1st	1.910	2.014
	2nd	1.437	1.282
	3rd	1.287	1.301
	4th	0.563	0.576
	5th	0.253	0.227
	6th	0.039	0.038
methanol: 0.1 M formic acid (30: 70 *v:v*)	1st	1.871	2.056
	2nd	1.761	1.677
	3rd	1.172	1.191
	4th	0.421	0.453
	5th	0.178	0.136
acetonitrile: 0.1 M formic acid (20: 80 *v:v*)	1st	3.357	3.117
	2nd	1.624	1.484
	3rd	0.786	0.760
	4th	0.308	0.308
acetonitrile: 0.1 M formic acid (30: 70 *v:v*)	1st	3.419	3.335
	2nd	1.556	1.571
	3rd	0.781	0.768
	4th	0.329	0.330

[a] 6.48 ng of α-ribazole was loaded into the BAC column

Appendix D HILIC-FLD Method Raw Data

Table D.1. HILIC-FLD method: retention factor

α-ribazole Standard	Results
Rep-1	2.606
Rep-2	2.601
Rep-3	2.625
Rep-4	2.611
Rep-5	2.599
Rep-6	2.596

Table D.2. HILIC-FLD method: theoretical plate number

α-ribazole Standard	Results
Rep-1	9591
Rep-2	9484
Rep-3	7677
Rep-4	9024
Rep-5	10027
Rep-6	10437

Table D.3. HILIC-FLD method: asymmetry factor

α-ribazole Standard	Results
Rep-1	1.84
Rep-2	1.85
Rep-3	1.85
Rep-4	1.85
Rep-5	1.83
Rep-6	1.81

Table D.4. HILIC-FLD method: retention time

α-ribazole Standard	Results
Rep-1	3.700
Rep-2	3.693
Rep-3	3.727
Rep-4	3.707
Rep-5	3.690
Rep-6	3.687

Table D.5. HILIC-FLD method: injection repeatability

α-ribazole Standard	Peak area
Rep-1	194.250
Rep-2	198.167
Rep-3	195.292
Rep-4	196.520
Rep-5	193.363
Rep-6	194.480

Table D.6. HILIC-FLD method: linearity

Standard Number	Concentration (ng mL^{-1})	Peak area			
		Expt-1	Expt-2	Expt-3	Mean
1	0.65	29.187	30.047	30.290	29.841
2	1.30	83.053	79.983	73.640	78.892
3	2.59	200.167	224.363	215.480	213.337
4	3.24	284.797	277.257	267.190	276.414
5	4.86	456.787	445.648	463.787	455.407
6	6.48	668.577	651.875	635.342	651.931

Table D.7. HILIC-FLD method: precision

	Day Tested	Vitamin B_{12} Result (μg 100 g^{-1})
Bovine milk-based infant formula-1 dup-1	1	2.871
Bovine milk-based infant formula-1 dup-2	1	3.152
Bovine milk-based infant formula-1 dup-1	2	3.070
Bovine milk-based infant formula-1 dup-2	2	3.246
Bovine milk-based infant formula-1 dup-1	3	2.978
Bovine milk-based infant formula-1 dup-2	3	3.078
Bovine milk-based infant formula-1 dup-1	4	3.104
Bovine milk-based infant formula-1 dup-2	4	2.983
Bovine milk-based infant formula-1 dup-1	5	2.848
Bovine milk-based infant formula-1 dup-2	5	2.995
Bovine milk-based infant formula-2 dup-1	1	3.799
Bovine milk-based infant formula-2 dup-2	1	3.826
Bovine milk-based infant formula-2 dup-1	2	3.837
Bovine milk-based infant formula-2 dup-2	2	3.462
Bovine milk-based infant formula-2 dup-1	3	3.611
Bovine milk-based infant formula-2 dup-2	3	3.587
Bovine milk-based infant formula-2 dup-1	4	3.662
Bovine milk-based infant formula-2 dup-2	4	3.466
Bovine milk-based infant formula-2 dup-1	5	3.764
Bovine milk-based infant formula-2 dup-2	5	3.655
Ovine milk-based infant formula dup-1	1	1.251
Ovine milk-based infant formula dup-2	1	1.403
Ovine milk-based infant formula dup-1	2	1.382
Ovine milk-based infant formula dup-2	2	1.458
Ovine milk-based infant formula dup-1	3	1.735
Ovine milk-based infant formula dup-2	3	1.743
Ovine milk-based infant formula dup-1	4	1.481
Ovine milk-based infant formula dup-2	4	1.541
Ovine milk-based infant formula dup-1	5	1.548
Ovine milk-based infant formula dup-2	5	1.364

Table D.8. HILIC-FLD method: limits of detection and quantitation

Replicates	Results (μg 100 g^{-1})
1	1.978
2	1.904
3	1.870
4	1.876
5	1.679
6	1.597
7	1.681
8	1.654
9	1.870
10	1.876

Table D.9. HILIC-FLD method: recovery

Samples		Vitamin B_{12} Results (µg 100 g^{-1})
Bovine milk-based infant formula-1	Level 1-dup1	5.203
	Level 1-dup2	5.078
	Level 1-dup3	5.069
	Level 1-dup4	5.070
	Level 1-dup5	4.766
	Level 1-dup6	4.889
	Level 2-dup1	8.025
	Level 2-dup2	7.881
	Level 2-dup3	7.815
	Level 2-dup4	7.860
	Level 2-dup5	7.437
	Level 2-dup6	7.385
Bovine milk-based infant formula-2	Level 1-dup1	6.990
	Level 1-dup2	6.947
	Level 1-dup3	7.248
	Level 1-dup4	7.101
	Level 1-dup5	6.752
	Level 1-dup6	6.637
	Level 2-dup1	10.321
	Level 2-dup2	10.484
	Level 2-dup3	9.888
	Level 2-dup4	10.022
	Level 2-dup5	10.113
	Level 2-dup6	10.240
Ovine milk-based infant formula	Level 1-dup1	3.762
	Level 1-dup2	3.327
	Level 1-dup3	3.478
	Level 1-dup4	3.349
	Level 1-dup5	4.035
	Level 1-dup6	4.249
	Level 2-dup1	7.225
	Level 2-dup2	6.825
	Level 2-dup3	6.419
	Level 2-dup4	6.797
	Level 2-dup5	6.961
	Level 2-dup6	6.817

Table D.10. HILIC-FLD method: bias against SRM 1869

Replicate Number	Day Tested	Vitamin B_{12} Results (μg 100 g^{-1})
1	1	3.22
2	1	4.19
3	1	3.75
4	1	4.66
5	1	3.20
6	1	3.92
7	2	3.68
8	2	4.67
9	2	3.38
10	2	4.61
11	2	3.89
12	2	4.86