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Analysis of vitamin B12 in foods - Development and application of stable isotope dilution assays for naturally occurring cobalamins

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Abstract

Vitamin B12 (B12) is the generic term for a group of structurally similar vitamers called cobalamins, amongst which the most predominant forms are adenosylcobalamin (AdoCbl), methylcobalamin (MeCbl), hydroxylcobalamin (OHCbl) and cyanocobalamin (CNCbl). The natural production of B12 is an exclusive attribute of a subset of microorganisms. Humans are not able to synthesize B12 *de novo* and thus rely on external sources to meet the recommended adequate intake of 4 µg/day set by the European Food Safety Authority (EFSA) for adults. In this context, reliable and robust analytical methods are highly required to determine B12 in dietary sources, ideally with the ability to differentiate individual cobalamins. For such a purpose, sensitive and specific LC-MS/MS demonstrates great potential but was largely limited by the issues of matrix effects and low recoveries in previous applications. In this regard, the powerful stable isotope dilution assay (SIDA) reveals particular advantages in the method portfolio for its proven superiority to compensate for losses, discriminations and mass spectrometric interferences. To date, SIDA has not been applied to B12 analysis in real foods.

The poor availability of isotope-labelled internal standards largely restricts further development of SIDA for B12 analysis in foods. To overcome this critical bottleneck, a biosynthetic approach was developed for the preparation of ^{15}N-labelled cobalamins using the bacterium *Propionibacterium freudenreichii*. First, a chemically defined medium (CDM) that could support long-term stable growth of *P. freudenreichii* was developed. The CDM contains ammonium sulphate as a single nitrogen source except three essential vitamins. Together with the cultivation schemes, the CDM was further optimized for *in vivo* production of cobalamins. Applying the optimized CDM in which ammonium sulphate was replaced by ^{15}N-labelled ammonium sulphate and an optimized cultivation scheme, fully ^{15}N-labelled AdoCbl with a yield of 635 ± 102 µg/L and fully ^{15}N-labelled OHCbl with a yield of 312 ± 29 µg/L were obtained in *P. freudenreichii*. Additionally, a second optimized cultivation scheme under anaerobic conditions was employed to produce specifically labelled [^{15}N, $^{14}N_2$]-cobalamins with obtained yields of 990 ± 210 µg/L and 96 ± 18 µg/L, respectively for [^{15}N, $^{14}N_2$]-AdoCbl and [^{15}N, $^{14}N_2$]-OHCbl. The labelled compounds were isolated and purified from cell extracts by solid phase extraction and semi-preparative HPLC. Chemical modifications were performed on isolated [^{15}N]-AdoCbl or [^{15}N]-OHCbl to further generate [^{15}N]-

CNCbl and [^{15}N]-MeCbl. Eventually, ^{15}N-labelled substances were obtained for all four cobalamin vitamers with high HPLC and isotopic purities as well as with anticipated ^{15}N-enrichment and labelling patterns.

Using the biosynthetically prepared ^{15}N-labelled cobalamins as internal standards, the first multiple SIDA was developed for the simultaneous determination of the four cobalamins in meats. The sample preparation was extensively optimized with enzymatic treatment and immunoaffinity purification. The parameters of LC-MS/MS were optimized for the separation and analysis of the cobalamins. The newly developed method was thoroughly validated using soy flour as surrogate blank matrix. The method validation resulted in limits of detection (LOD) ranging from 0.19 to 0.58 ng/g and limits of quantification (LOQ) ranging from 0.68 to 1.73 ng/g. Recoveries between 82% and 121% were determined for all analytes at three spiking levels. Inter-injection, intra-day and inter-day precisions were below 4%, 6% and 11% RSD, respectively. The total B12 content of a reference material determined by the new method demonstrated a variance < 1% from the certified value. The application of LC-MS/MS and SIDA enables unequivocal determination of cobalamins without potential interferences of non-bioactive analogues. Altogether, the developed method demonstrated outstanding sensitivity, specificity, accuracy and reproducibility for its intended use. Subsequently, the developed method was successfully applied to quantitate cobalamins in common categories of meats. The capability of the method to differentiate individual cobalamin forms is clearly a step forward for B12 analysis and will also open further avenues for investigating the biological significance of individual cobalamins in food matrices.

In previous B12 analysis, cyanidation conversion of all cobalamins into CNCbl has been a common practice to determine total B12 contents. However, the completeness of the conversion was not routinely checked leaving the reliability of the obtained results questionable. The last part of the thesis compared the novel method that determines the four native cobalamins and a conversion SIDA method that measures total B12 as CNCbl after cyanidation conversion. The conversion SIDA method was first developed and validated with low LOD (0.09 ng/g) and LOQ (0.29 ng/g), good recoveries (101-111%) and satisfactory precisions (2-4%) obtained for CNCbl. The analysis of the reference material by both the native method and the conversion method resulted in comparable values. During method development, complete conversions have been achieved for the measurements of various meat samples. However, further application of the conversion

SIDA method demonstrated contradictory results of unconverted cobalamins on the LC-MS/MS chromatograms of beef fillet and lamb fillet. This discrepancy and a further obtained conversion rate of 81% revealed that the conversion was affected by various factors including the sample matrices, concentrations of cobalamins and probably also ambient light conditions. These issues cannot be observed in the method validation targeting at the analysis of CNCbl. LC-MS/MS represents the state of the art for identification of cobalamins but might not be sufficient for monitoring conversion in trace levels due to the inevitable fluctuations on sensitivity of the instrument. Considering all uncertainties caused by the cyanidation conversion, the native SIDA method of the present thesis offers a straightforward alternative for B12 analysis, generating reliable quantitative results with additional valuable information of vitamer compositions.

Kurzzusammenfassung

Vitamin B12 (B12) ist ein allgemeiner Begriff für eine Gruppe von strukturell ähnlichen Vitameren, den sogenannten Cobalaminen, unter denen Adenosylcobalamin (AdoCbl), Methylcobalamin (MeCbl), Hydroxylcobalamin (OHCbl) und Cyanocobalamin (CNCbl) die wichtigsten Formen sind. In der Natur kommt B12 ausschließlich in manchen Mikroorganismen vor. Menschen sind nicht in der Lage, B12 *de novo* zu synthetisieren und benötigen externe Quellen, um die ausreichende Zufuhr von 4 µg/Tag zu erreichen, die von der Europäischen Behörde für Lebensmittelsicherheit (EFSA) für Erwachsene empfohlen wird. In diesem Sinne sind sichere und robuste analytische Methoden erforderlich, um B12 in Nahrungsmitteln zu bestimmen. Idealerweise sind auch die einzelnen Cobalamine zu differenzieren. Dafür besitzt die empfindliche und spezifische LC-MS/MS Messtechnik ein großes Potenzial, ist aber in den bisherigen Anwendungen durch Matrixeffekte und niedrige Wiederfindungen stark eingeschränkt. In diesem Fall bietet die Stabilisotopenverdünnungsanalyse (SIVA) besondere Vorteile dank ihrer Fähigkeit, Verluste, Diskriminierungen und massenspektrometrische Interferenzen zu kompensieren. Bis heute ist noch keine SIVA für die B12 Analyse in Lebensmitteln entwickelt.

Die mangelnde Verfügbarkeit von isotopenmarkierten internen Standards ist eine starke Einschränkung für die weitere Entwicklung der SIVA Analyse von B12 in Lebensmitteln. Um diesen kritischen Engpass zu überwinden, wurde eine biosynthetische Methode für die Produktion von ^{15}N-markierten Cobalaminen mit Hilfe des Bakteriums *Propionibacterium freudenreichii* entwickelt. Zuerst wurde ein chemisch definiertes Medium (CDM) zusammengestellt, das ein langfristiges und stabiles Wachstum von *P. freudenreichii* gewährleisten kann. Neben drei essentiellen Vitaminen beinhaltete das CDM Ammoniumsulfat als die einzige Stickstoff-Quelle. Zusammen mit den Kultivierungsbedingungen wurde das CDM für die *in vivo* Produktion von Cobalaminen weiter optimiert. In dem optimierten CDM, in welchem unmarkiertes Ammoniumsulfat durch ^{15}N-markiertes Ammoniumsulfat ersetzt wurde, konnten vollständig ^{15}N-markiertes AdoCbl in einer Konzentration von 635 ± 102 µg/L und vollständig ^{15}N-markiertes OHCbl in einer Konzentration von 312 ± 29 µg/L aus *P. freudenreichii* gewonnen werden. Des Weiteren wurde ein zweites optimiertes Inkubationsschema unter anaeroben Bedingungen verwendet und speziell [^{15}N, $^{14}N_2$]-markierte Cobalamine wurden in Konzentrationen von 990 ± 210 µg/L und 96 ± 18 µg/L für [^{15}N, $^{14}N_2$]-AdoCbl

bzw. [^{15}N, $^{14}N_2$]-OHCbl produziert. Die markierten Substanzen wurden isoliert, extrahiert und mittels Festphasenextraktion und semi-präparativer HPLC gereinigt. Chemische Modifikationen wurden an dem isolierten [^{15}N]-AdoCbl oder [^{15}N]-OHCbl durchgeführt, um [^{15}N]-CNCbl und [^{15}N]-MeCbl zu erhalten. Schließlich wurden ^{15}N-markierte Substanzen von allen vier Cobalamin Vitameren mit hoher HPLC- und Isotopen-Reinheit sowie gewünschter ^{15}N-Anreicherung und Markierungsmustern erhalten.

Mit den biosynthetisierten ^{15}N-markierten Cobalaminen als internen Standards wurde die erste multi-SIVA für die parallele Bestimmung der vier Cobalamine in Fleisch entwickelt. Die Probenvorbereitung wurde durch enzymatische Behandlung und Immunaffinitätsreinigung etabliert. Die LC-MS/MS Parameter wurden optimiert, um eine vollständige Trennung und empfindliche Signale zu erhalten. Die neue etablierte Methode wurde mit Sojamehl als analytfreie Leermatrix vollständig validiert. Die Nachweisgrenzen (LOD) lagen zwischen 0,19 und 0,58 ng/g und die Bestimmungsgrenzen (LOQ) zwischen 0,68 und 1,73 ng/g. Die Wiederfindungen zwischen 82% und 121% wurden für alle Analyten bei drei Additions-Niveaus festgestellt. Inter-Injektion, Intra-Day und Inter-Day Präzisionen lagen unter 4%, 6% und 11% RSD. Mit der neuen Methode wurde der Gesamtgehalt an B12 eines Referenzmaterials analysiert und der Wert stimmte mit dem zertifizierten Wert gut überein (Abweichung < 1%). Die Anwendung der LC-MS/MS und SIVA ermöglicht eine eindeutige Bestimmung von Cobalaminen ohne mögliche Störungen durch nicht-bioaktive Analoga. Insgesamt erwies die etablierte Methode eine ausgezeichnete Sensitivität, Spezifizität, Genauigkeit und Wiederholbarkeit. Anschließend wurde die Methode für die Bestimmung von Cobalaminen in den üblichen Fleischsorten erfolgreich angewendet. Die Fähigkeit der Methode, einzelne Cobalamine zu unterscheiden, ist ein wichtiger Fortschritt in der B12 Analyse und eröffnet Wege für die Untersuchung der biologischen Signifikanz einzelner Cobalamine in Lebensmittelmatrices.

In bisherigen B12-Analysen ist eine Cyanidierung, d.h. die Umwandlung von allen Cobalaminen ins CNCbl, eine gängige Prozedur, um den gesamten B12 Gehalt zu bestimmen. Dennoch wurde die Vollständigkeit der Umwandlung bisher nicht routinemäßig geprüft und stellt die Verlässlichkeit der Ergebnisse in Frage. Der letzte Teil dieser Arbeit beinhaltet einen Vergleich zwischen der neuen etablierten Methode zu einzelnen B12 Bestimmungen und einer zusätzlichen SIVA Methode zur Bestimmung

des gesamten B12 durch Cyanidierung. Die zusätzliche SIVA Methode wurde entwickelt und validiert, mit einer niedrigen LOD (0,09 ng/g) und LOQ (0,29 ng/g), zuverlässiger Wiederfindung (101-111%) und hoher Präzision (2-4% RSD). Analysen des Referenzmaterials mit der neuen Methode und der zusätzlichen SIVA Methode ergaben übereinstimmende Ergebnisse. Bei der Methodenentwicklung wurde eine vollständige Umwandlung für die Bestimmung in verschiedenen Fleischproben angenommen. Aber weitere Anwendungen der SIVA Methode zeigten insoweit widersprüchliche Ergebnisse, als bei Rind- und Lammfilet nicht umgewandelte Cobalamine in LC-MS/MS Chromatogrammen beobachtet wurden. Diese Unstimmigkeit und eine daraufhin gemessene Umwandlungsrate von 81% deuteten darauf hin, dass die Umwandlung von verschiedenen Faktoren abhängt, z.B. der Probenmatrix, den B12 Konzentrationen und möglicherweise auch den Licht-Bedingungen. Solche Einflüsse konnten bei der Methodenvalidierung, die auf die Bestimmung von CNCbl fokussiert war, nicht beobachtet werden. Die LC-MS/MS repräsentiert hier den Stand der Technik zur Identifizierung von Cobalaminen. Aufgrund der unvermeidlichen Schwankungen der Empfindlichkeit des Instruments ist dies jedoch möglicherweise nicht immer ausreichend, um die Vollständigkeit der Umwandlung zu überwachen. Im Vergleich mit den Messunsicherheiten, die bei der Cyanidierung auftreten, stellt die hier neuentwickelte SIVA Methode für die nativen B12-Vitamere eine zielführende Alternative für die B12 Analyse dar und erreicht quantitativ zuverlässige Ergebnisse mit zusätzlichen wichtigen Informationen zu den Vitamerzusammensetzungen.

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1 Introduction

Vitamin B12 (hereafter B12), nature's most beautiful yet complex cofactor [1], has been under investigation for more than 160 years. This puzzling yet enchanting vitamin has attracted research of some of the most extraordinary scientists at the time, leading to some important scientific milestones. The earliest encounter of its life-saving effects dates back to 1926 when Minot and Murphy followed the work of Whipple's earlier dog experiments [2] and discovered the effects of raw liver in diets to cure pernicious anaemia [3], a then mysterious and fatal disease first described in 1855 [4]. In 1934, three of them were awarded the first Nobel Prize related to B12 research for their observation. In the following two decades, extensive work has been done to isolate this magic "extrinsic factor" from liver until the final success in purification and crystallization of the reddish needle-like crystals in 1948 [5, 6]. Dorothy Hodgkin, pioneering in X-ray crystallography, then unravelled the intricately complex three-dimensional structure of cyanocobalamin in 1955 after over 8 years of laborious work with estimated 10 million calculations [7, 8]. Partially for this remarkable work, she was awarded the second Nobel Prize related to B12 in 1964. The next legendary story featured the achievement of total chemical synthesis of B12 in 1970s after accumulated work of more than 100 scientists during a period of 11 years under the lead of Eschenmoser and yet another Nobel Prize laureate, Woodward [9, 10].

These are the major landmarks in the fascinating history of B12 research, but they can only represent the tip of the iceberg. Numerous brilliant works have been done in diverse areas stretching widely from chemistry to system biology and covering complex topics which often required intertwined efforts from different expertise. Apart from the well-explored chemical and biochemical properties of the vitamin [11, 12] and its important roles and mechanisms in human physiology [13], many remarkable achievements in the field of microbiology are worth noting. Since Shorb first identified that *Lactobacillus lactis* requires B12-containing liver extract for growth [14, 15], it gradually became clear that microorganisms are the only primary producers of B12 in nature [16]. Subsequently, much attention has been paid to discover the unique and impressive ways of constructing B12 in nature. It was only until 1985 that the first gene sequence associated with B12 biosynthesis was identified [17] and later two distinct biosynthetic pathways were most elucidated by combining strengths of molecular

genetics and various chemical and biochemical techniques [18]. The important discoveries in the microbial field not only largely contributed to deepening our understanding of B12 but also laid a solid foundation for industrial production of the vitamin.

Today, B12-related research is ever emerging in different fields as diverse as from organometallic chemistry to marine eco-system. B12, with its intriguing properties and widespread biological relevance in all domains of life, never fails to astonish scientists with new findings and challenges. A thorough review of B12 in all dimensions is beyond the scope of this chapter. This thesis focuses on the analysis of B12 in foods and this chapter summarizes the most relevant chemical, nutritional, analytical and microbiological backgrounds.

1.1 Structures of B12

B12 is the generic term for a group of structurally similar compounds called cobalamins. Cobalamins are members of the cobamide family. Cobamides and their corrin-containing biosynthetic precursors and degradation products are collectively termed corrinoids [19]. The fundamental core structure of corrinoids is a cobalt-centred tetracyclic corrin ring system (Figure 1.1). Cobamides are "complete" corrinoids that have an upper and a lower ligand. The upper (β-) ligand is covalently bound to the cobalt atom and the lower (α-) ligand is attached to the corrin ring via a nucleotide loop. Cobamides are distinguished from one another based on the structure of the lower ligand. All cobalamins possess 5,6-dimethylbenzimidazole (DMB) as the lower ligand (Figure 1.1-A, boxed), which is an essential structural criterion for the selective binding of the vitamin to intrinsic factor (IF) during absorption in the human body. The other cobamides have alternative chemical variants as the lower ligand other than DMB, which can be purine, phenolic or other benzimidazole compounds [20, 21] (Figure 1.1-C). The cobalamin-like corrinoids are often referred as cobalamin analogues. Despite of their widespread relevance in other domains of life, these analogues are generally considered biologically inactive for humans and should be clearly distinguished from cobalamins from the perspective of human nutrition. The upper ligand of the corrinoids can be various chemical forms and the most predominant ones include adenosyl, methyl, hydroxyl and cyano groups (Figure 1.1-B). The designation of corrinoids was partially determined by the nature of these radicals. Cobalamins containing one of these four upper ligands have

corresponding names of adenosylcobalamin (AdoCbl), methylcobalamin (MeCbl), hydroxocobalamin (OHCbl) and cyanocobalamin (CNCbl), respectively. These four naturally occurring cobalamins are the analytical targets of the present thesis.

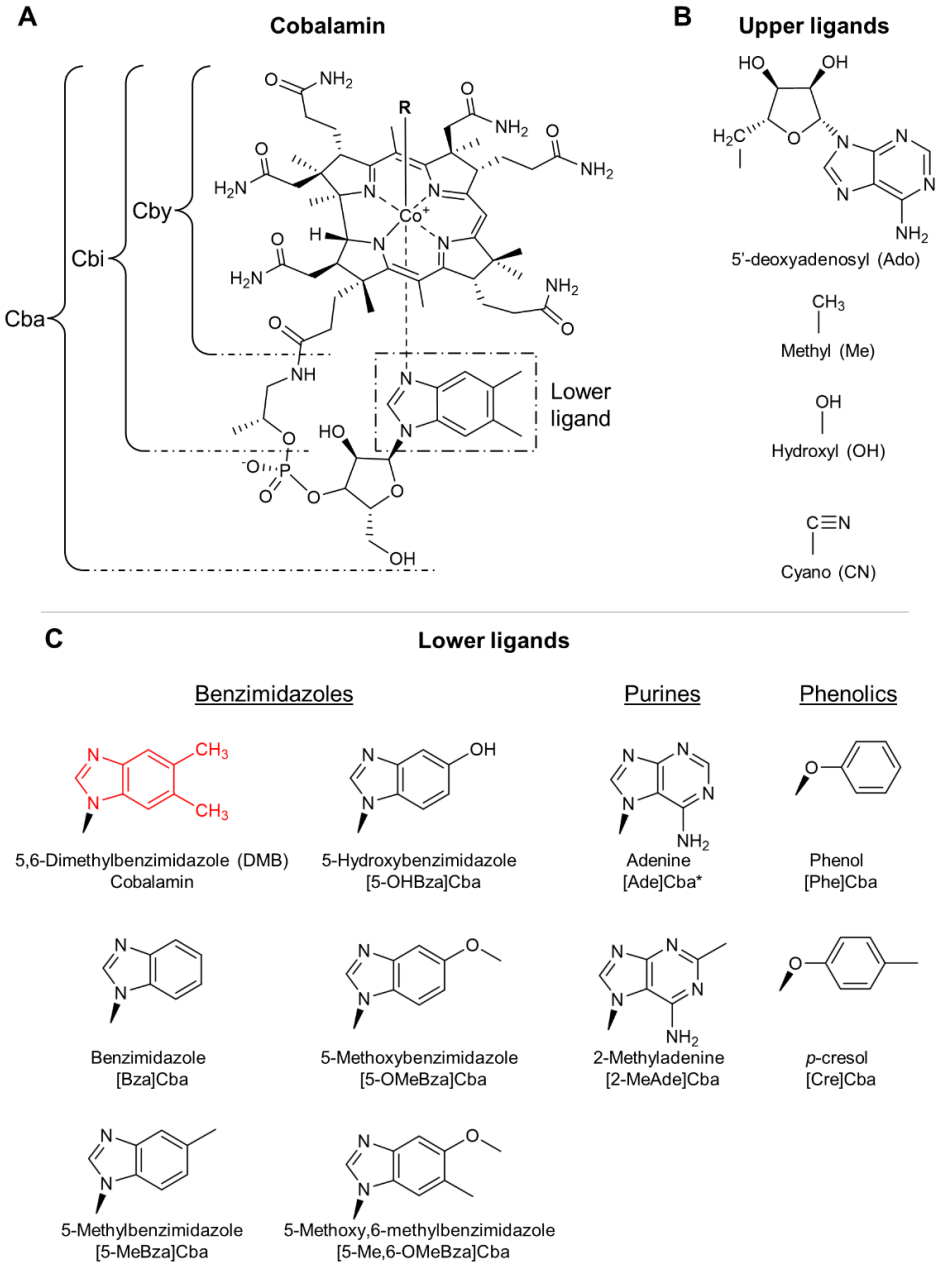


Figure 1.1. Structures of corrinoids. Chemical structures of cobalamin (A) with structural precursors indicated by dashed lines, upper ligands (B) and representative lower ligand bases (C) with abbreviations describing corresponding cobamides given below. R represents upper ligand; Cby, cobyric acid; Cbi, cobinamide; Cba, cobamide. *, with adenine as lower ligand also often referred as pseudovitamin B12. Corrinoids include compounds that contain a corrin ring. Cobamides are corrinoids that have an upper and lower ligand. Cobalamins, the authentic B12 forms, are cobamides possess DMB as the lower ligand.

1.2 B12 and human nutrition

1.2.1 Metabolic functions of cobalamins in humans

B12 is required for the metabolic functions of two essential enzymes in mammalian cells (see Figure 1.2). MeCbl is required as cofactor for cytosolic methionine synthase (E.C. 2.1.1.13) and AdoCbl for mitochondrial methylmalonyl-CoA mutase (E.C. 5.4.99.2) [22, 23]. Methionine synthase plays a vital role in both methionine and folate cycles. It catalyzes the reaction transferring a methyl group from 5-methyl-tetrahydrofolate (5-CH_3-H_4folate) to homocysteine, concurrently generating methionine and tetrahydrofolate (H_4folate). The resulting H_4folate is further converted to 5,10-methylene-tetrahydrofolate (5,10-CH_2-H_4folate), the precursor of thymidine needed for DNA synthesis and repair [24]. Methylmalonyl-CoA mutase catalyzes the conversion of methylmalonyl-CoA to succinyl-CoA, the important intermediate of the TCA cycle. This reaction is also essential for breaking down branched-chain amino acids, odd-chain fatty acids and the side chain of cholesterol via the propionate catabolism pathway [24, 25].

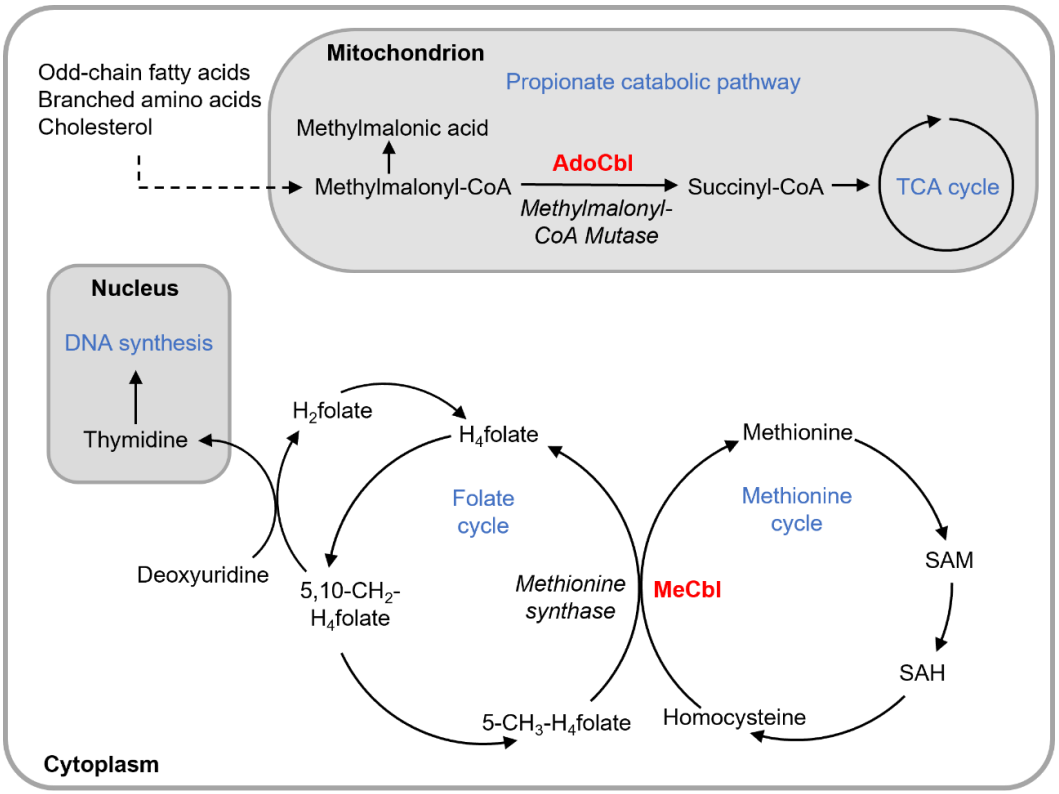


Figure 1.2. Cobalamins in human metabolism [13, 25]. H_2folate, dihydrofolate; H_4-folate, tetrahydrofolate; 5-CH_3-H_4folate, 5-methyl-tetrahydrofolate; 5,10-CH_2-H_4folate, 5,10-methylene-tetrahydrofolate; SAM, *S*-adenosyl-methionine; SAH, *S*-adenosyl-homocysteine.

1.2.2 Transport and absorption of B12 from foods to cells

Humans are not able to synthesize B12 de novo and thus completely depend on external dietary sources of the vitamin. The trafficking of B12 from foods to cells is regulated by a sophisticated combination of carrier proteins, receptors and transporters (Figure 1.3). The following sections summarize key aspects from previous comprehensive reviews [24, 26, 27].

Following intake, B12 is liberated from its food matrix and bound by haptocorrin (HC), a protein which is produced by salivary ligands for protecting B12 from the acidic environment. Upon entry to duodenum, B12 is released from HC and captured by IF, which is secreted by gastric parietal cells. The formed IF-B12 complex is endocytosed by a receptor called cubam in the terminal ileum. In the endosomes, IF-B12 complex is transferred to lysosome, which degrades the IF and releases the B12. The free B12 is transported to the plasma from the basolateral membrane by multiple drug resistance protein 1 and binds to transcobalamin (TC). Carried by the TC, B12 is transported and delivered to tissue cells via blood circulation. The TC-B12 complex from blood is readily taken up by target cells by an endocytic receptor called CD320 protein. Inside the target cells, B12 is released from TC in the lysosome and transported to cytosol. B12 is then stepwise directed to the corresponding enzymes and functions as cofactors after conversion into AdoCbl or MeCbl. The exact mechanisms on the handling of B12 in the cells from the point of lysosomal exit to its usage as cofactors and further to its export from cells are still under investigation. The majority of B12 is stored in liver and some in bile for enterohepatic circulation.

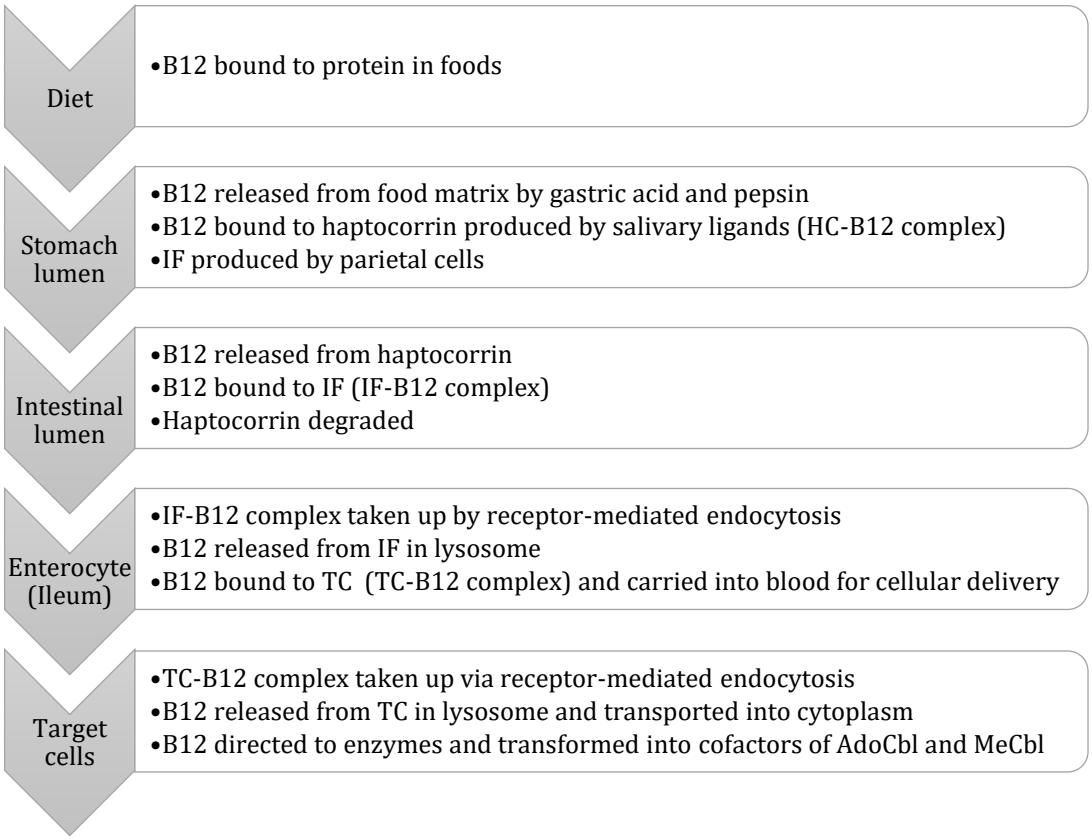


Figure 1.3. Overview of uptake and transport of B12 in human physiology [27]. HC, haptocorrin; IF, intrinsic factor; TC, transcobalamin.

1.2.3 B12 deficiency- symptoms, causes and prevention

B12 deficiency is associated with complications at the level of the whole organism, with diverse symptoms mainly seen in the blood and nervous systems. At the cellular level, the lack of proper coenzymes due to B12 deficiency causes impaired methylation and impaired methylmalonyl-CoA metabolism, resulting in accumulation of homocysteine and methylmalonic acid and reduction of methionine, *S*-adenosyl-methionine (SAM) and succinyl-CoA [13, 28, 29]. Methionine is an essential amino acid for humans and SAM is required as a universal cellular methyl donor for various epigenetic reactions. The accumulation of homocysteine is related to increased cellular stress, apoptosis and homocysteinylation of functional proteins in the blood and tissues [13, 30].

In the blood system, one major consequence of B12 deficiency is the development of megaloblastic anaemia, which results from disruption of normal DNA synthesis [13]. Neurological manifestations caused by B12 deficiency are diverse and can precede or even occur without haematological complications [13, 31]. Typical cases of neuropathy,

such as myeloneuropathy, polyneuropathy, optical neuropathy, and paraesthesia without abnormal symptoms, are observed as complications of B12 deficiency affecting the nervous systems [32, 33]. Moreover, low or deficient B12 status is associated with various neuropsychiatric symptoms such as emotional lability and psychosis [31, 33, 34]. B12 deficiency is also associated with brain atrophy and cognitive impairment, especially in elderly individuals [13, 35, 36].

B12 deficiency is mainly caused by inadequate intake, malabsorption or genetic defects that lead to impairments of the B12 transport system or the intracellular metabolism [13, 37]. To date, thirteen different inherited disorders disrupting B12 transport in blood, cellular uptake or intracellular metabolism of the vitamin have been identified [38]. Acquired cases of B12 deficiency affecting people of all age groups often stem from malnutrition or malabsorption. The classical pernicious anaemia is one of the most prevalent causes for acquired B12 deficiency as the consequence of an auto-immune gastritis that leads to failure of IF production [27]. The lack of IF results in malabsorption of both dietary B12 and recycled biliary B12, eventually leading to B12 deficiency after progressive exhaustion of B12 reserved in the body. Besides pernicious anaemia, various disorders, such as some intestinal diseases, pancreatic insufficiency and certain chronic gastric diseases, also contribute to B12 deficiency due to insufficient absorption [13, 37, 38]. These absorption-related disorders, except pernicious anaemia, are sometimes categorized as food B12 malabsorption syndrome [38].

Another principal cause for B12 deficiency is simply an insufficient dietary intake of the vitamin. This is a major risk factor for developing B12 deficiency in lower income countries, in which individuals have a low intake of animal-derived foods [39]. However, with the currently increasing numbers of vegetarians and vegans worldwide, this risk factor is becoming more and more relevant in a global dimension as the sole source of B12 in conventional diets is animal-derived products. Therefore, particular attention should be paid to address this issue.

Severe clinical B12 deficiency with classical haematological and/or neurological dysfunctions is relatively straightforward for clinicians to diagnose if distinguished from other diseases that show resembling symptoms. However, more prevalent subclinical deficiency marked by low or marginal B12 status without known clinical expression is particularly challenging for clinicians to decide if intervention is necessary [13, 40, 41].

Moreover, the diagnosis of subclinical B12 deficiency depends solely on biochemical biomarkers including total serum B12, serum holo-transcobalamin (holo-TC), serum and urinary methylmalonic acid, and plasma total homocysteine. As all these biomarker tests show generally poor specificity and sensitivity [42, 43], their reliability and suitability in identifying B12-deficient individuals require further investigations.

The development of B12 deficiency can take years before showing overt manifestations. Potential neurological damages in the progress, if not identifiable, pose great health risks as they can not always be reversed by treatments. Due to the complexity in determining true B12 deficiency or the trend towards true B12 deficiency, it is of great importance to guarantee sufficient B12 intakes as prevention. To achieve that, one straightforward approach is to promote the consumption of B12-containing foods in general.

For a sufficient supply of B12 from the diets to the body, the European Food Safety Authority (EFSA) set recommended dietary intakes for different life stage groups (Table 1.1) [44]. Generally, the adequate intake (AI) for adults is 4 µg/day. The proposed AIs for infants and children vary between 1.5 µg/day and 4 µg/day based on the ages. Taken the transfer of B12 to fetal organs and breast milk into consideration, a higher AI of 4.5 µg /day is recommended for women with pregnancy and lactation. In the USA, the recommended dietary allowance (RDA) set by Institute of Medicine (IOM) is 2.4 µg/day for people over 14 years old [45]. The corresponding values for infants and children until 13 years old range from 0.4 µg/day to 1.8 µg/day based on the ages. Additional intakes are as well recommended for women with pregnancy and lactation.

Table 1.1. Adequate intakes (AIs) of B12 according to EFSA.

Life stage group	AI (µg/day)
7–11 months	1.5
1–3 years	1.5
4–6 years	1.5
7–10 years	2.5
11–14 years	3.5
15–17 years	4
≥ 18 years	4
Pregnancy	4.5
Lactation	5

1.3 B12 in foods

In nature, cobalamins are exclusively produced by a subset of bacteria and archaea [46]. Due to natural food-chain enrichment, animal-derived foods are considered to be exclusive sources of cobalamins in conventional diets. Plant-based foods are generally devoid of cobalamins unless contaminated or intentionally biofortified with certain bacteria. Table 1.2 lists B12 contents in a variety of widely consumed foodstuffs of animal origin according to USDA (United States Department of Agriculture) [47]. Among all, animal liver is an outstanding B12 source as it is the organ for long-term storage of the vitamin.

The recommended daily intakes are easily met if sufficient animal-derived products are included in the diets but not when diets are solely and strictly based on plant-derived foods. For example, typical vegan diets provide only trace levels of B12, which are less than 0.25 μg/day [13, 37]. Although it would be most ideal to obtain sufficient B12 from natural B12-containing foods to meet the daily requirements, eating habits of certain groups are not easily changeable due to various restrictions including economic, cultural or social factors. Therefore, alternative solutions are highly needed for these populations who are not able to acquire enough B12 from their common diets.

Supplementation via vitamin pills or intramuscular injections is a straightforward approach. Consuming B12-fortified foods, such as ready-to-eat breakfast cereals and juices, is another feasible way to obtain B12, especially for vegetarians, vegans and elderly populations. In addition, search for natural B12-rich food sources that are not animal-based is ongoing with some identified examples such as certain edible algae [48]. Alternatively, B12 can be introduced into plant-based food matrices via fermentation using specific food-grade bacterial strains [49-51]. However, in the latter two cases, it is of great importance to confirm the presence of authentic cobalamins rather than non-bioactive analogues using reliable and specific analytical methods. A typical example is *Spirulina* supplements, which were considered as an alternative B12 source for vegetarians and vegans until researchers found out that biologically inactive analogues (mainly pseudovitamin B12) are the major cobamides instead of the desired cobalamins [52]. Regarding the biofortified foods, it must be noted that the contents and authenticity of B12 highly depend on adopted bacterial strains and cultivation

conditions. Table 1.3 shows a selection of reported foods of non-animal origin that contain nutritionally relevant amounts of B12.

Table 1.2. B12 contents in commonly consumed foods of animal origin according to USDA database.

Food	**B12 content [a] (µg/100g)**
Meat and meat products	
Beef liver (raw)	59.3
Lamb liver (raw)	90.05
Pork liver (raw)	26
Beef (ground, raw)	1.97 - 2.26
Pork (ground, raw)	0.64 - 0.73
Lamb (ground, raw)	2.31
Chicken (ground, raw)	0.56
Fish and shellfish	
Salmon (raw)	1.3 - 8.52
Trout (raw)	2.77 - 7.79
Herring (raw)	10 - 13.67
Shrimp (mixed species, raw)	1.11
Clam (mixed species, raw)	11.28
Mussels (blue, raw)	12
Oyster (raw)	8.75 - 16.2
Egg (whole, raw, fresh)	0.89*
Milk and dairy products	
Milk (whole, 3.25% milk fat)	0.45
Mozzarella cheese (whole milk)	2.28
Feta cheese	1.69
Swiss cheese	3.06
Yogurt (plain, whole milk)	0.37

Note: [a], values only serve as estimated reference and B12 content of each category can vary widely depending on various factors such as origins, compositions (e.g. fat contents), further cooking methods, and cuts of the animal in the case of meats; *, majority of B12 present in egg yolk.

Table 1.3. B12 contents in a selection of foods of non-animal origin.

Food	B12 content [a] (µg/100g)	Reference
Edible algae		
Chlorella supplements (*Chlorella sp.*)	<0.1 - 415	[53-55]
Purple laver (*Porphyra sp.*)	28 - 134	[56]
Green laver (*Enteromorpha sp.*)	64	[57]
Edible plants *		
Sea buckthorn berries (*Hippophae rhamnoides*)	37	[58]
Sidea couch grass (*Elymus repens*)	26	[58]
Elecampane (*Inula helenium*)	11	[58]
Fermented foods **		
Tempeh (fermented soybean product)	0.7 - 8 [b]	[59]

Note: [a], dry basis unless specified; [b], wet basis; *, symbiosis with B12-synthesizing bacteria; **, contents and authenticity of B12 highly depend on bacterial strains involved in fermentation.

1.4 Analysis of B12 in foods

The analysis of B12 in foods is rather challenging mainly due to the following reasons:

i) the low natural concentrations of cobalamins in non-fortified foods,
ii) the occurrence of different vitamers with even lower individual concentrations,
iii) the chemical instability of cobalamins and inter-conversion between forms,
iv) the possibility of other corrinoids and non-corrinoid compounds present as interferences.

Over the past decades, various analytical methods have been developed for the analysis of B12 in foods using different techniques. Traditionally, microbiological assays (MBAs) are routinely used for B12 analysis in foods and biological materials. MBA is sensitive but lacks necessary specificity and selectivity, which largely hampers the reliability of the obtained results. With the advancement of modern separation technologies, methods using (ultra-) high performance liquid chromatography ((U)HPLC) in combination with appropriate detection systems are emerging. Among all, (U)HPLC-UV based methods are considered state-of-the-art for B12 quantification in foods. However, the performances of current (U)HPLC-UV methods are far from satisfactory; the sensitivity is low even with highly selective immunoaffinity purification due to a lack of adequate chromophores in the structure of cobalamins and the specificity is still limited by the inherent principles of UV detection despite of chromatographic separation. Therefore, there is a trend in developing methods applying more specific and sensitive detection techniques such as mass spectrometry (MS), in order to achieve unequivocal determination of B12 in trace levels. Apart from the applied analytical systems, sample preparation also plays a vital role in B12 analysis. Purification and concentration of the analytes during sample treatment contribute largely to the final sensitivity of the developed methods.

In the following sections, details about MBAs and hyphenated chromatographic methods are given, as well as the current progress on sample preparation. In addition to the MBAs and chromatographic methods, there are some other methods relying on alternative techniques such as atomic absorption spectroscopy, chemiluminescence analysis, protein-binding assays and radio isotope dilution assay. Nevertheless, these methods are generally restricted by their poor specificity and selectivity for analyzing

B12 in foods and thus being replaced by methods applying modern analytical techniques nowadays. Detailed reviews about these methods can be found elsewhere [60].

1.4.1 Microbiological assay (MBA)

The use of MBA for B12 analysis has a history of more than 70 years [14]. Starting originally as "tube" assay, MBA is nowadays performed in microtiter plates after simplified by Kelleher and Broin [61]. As the name already suggests, MBA is based on the growth of microorganisms (e.g., *Lactobacillus delbrueckii* ATCC 7830) that require exogenous supply of B12 and the growth is measured based on turbidimetric methods.

Despite of its long history and widespread use, MBA suffers from an inherent drawback of poor specificity; the test organisms are not able to distinguish cobalamins from other microbiologically active analogues. Moreover, when dealing with complex matrices like foods, some non-corrinoid substances such as nucleic acids could also interfere with the growth of the test organisms [62]. Ideally, the growth of test organism is proportional to the amounts of cobalamins in the test sample extracts, assuming a lack of interfering compounds. However, this ideal scenario is hardly achievable since cobalamins and other corrinoids often co-exist in natural materials as they ultimately origin from microbial activities. In general, analogues could account for about 5-30% of the reported B12 contents determined by MBAs in foods [62]. Heudi et al. [63] compared B12 contents of a selection of food products determined by MBA with values of the same samples analyzed by HPLC-UV. Inconsistencies were observed between the two sets of results. Similarly, Guggisberg et al. [64] reported higher B12 contents (up to 2.2-fold) of a range of meat products from MBA than from HPLC-UV. Chamlagain et al. [65] further demonstrated a sample-specific difference on B12 contents obtained from MBA and from UHPLC-UV when analysing fermented cereal matrices.

The poor specificity of the MBA, in an unavoidable way, hampers the quality of the obtained results. When using MBA for foods or biological materials that are known for containing authentic cobalamins, the poor specificity of MBA is less problematic, although quantitative results will always remain questionable. But in other cases, a false result can be critical. Clearly, MBAs are not suitable for analyzing fermented foods when identities of the produced corrinoids are critical. Furthermore, MBA often exhibits bad

precision and is prone to contamination and errors due to the nature of biological assays.

It has to be noted that MBA has certain advantages. To date, MBA is still one of the most sensitive methods for B12 analysis with limit of quantification (LOQ) approaching 1.0 pg/mL of assay growth media [66]. This sensitivity enables the quantification of low concentrations of B12 in foods (< 0.5 μg/100g) and thus most of B12 contents in food databases have been obtained using MBAs [67]. It is also recognized by the Association of Official Agricultural Chemists (AOAC) as an official method for analyzing B12 in infant and adult nutritional formulas [68]. In addition, despite that MBA requires long incubation time for the test organisms to grow, it is relatively less labour-intensive and more economically viable compared to other more specific instrumental methods which require vigorous and costly sample clean-up. Moreover, this type of assay is not restricted by the availability of specific analytical instruments. These are the major reasons why MBA has been popular for B12 analysis for a long period of time. In scenarios that cobalamins are confirmed to be the majority of the present corrinoids or when measuring total corrinoids without the need of differentiation, MBA may still be a useful tool to provide acceptable results.

1.4.2 (U)HPLC-UV

Methods using (U)HPLC-UV are becoming the mainstream for B12 analysis in foods for their superior selectivity and specificity compared to MBAs. To date, (U)HPLC-UV based methods have been frequently adopted for measuring B12 in infant formulas, dietary supplements, fortified foods, fermented foods, and occasionally in non-fortified natural foodstuffs containing relatively high levels of B12 such as meat products [63-65, 68-73].

Typically, the (U)HPLC systems are operated in reverse phase conditions using C18-based columns for chromatographic separation of CNCbl [66, 74]. With optimized mobile phases and gradients, baseline separation of the four major B12 vitamers has also been demonstrated [75-78]. The detection is frequently performed at a wavelength of 361 nm (λ_{max} of CNCbl) or covers a desired wavelength range if diode array detection (DAD) is employed; the latter provides additional spectroscopic information of the analytes and thus further enhances specificity of the detection.

For (U)HPLC-UV methods, sample preparation is a critical index for determining the final sensitivity and selectivity, especially when handling complex food matrices where interfering components are often present in considerable amounts. In order to improve sensitivity, most of the currently applied (U)HPLC-UV methods target at solely analyzing CNCbl for total B12 contents. For such a purpose, all natural vitamers are converted into CNCbl during extraction. Furthermore, immunoaffinity chromatography (IAC), which has high affinity and selectivity towards CNCbl, is widely used for B12 enrichment from food samples. The application of IAC improves the sensitivity and specificity to the next level, rendering the quantification of B12 in non-fortified foods by (U)HPLC-UV a possible task. Nevertheless, the improvement on sensitivity via sample preparation is rather limited; the developed (U)HPLC-UV methods require large sample amounts for analysis even with cyanidation conversion and immunoaffinity purification. Sensitivity remains one of the major issues in analyzing trace levels of B12 in natural foodstuffs, especially when quantification of naturally occurring B12 vitamers is the aim of the analysis.

Due to the inherent properties of UV detection, the improvement on specificity is also limited for (U)HPLC-UV methods. Despite that chromatographic separation between pseudovitamin B12 and CNCbl has been achieved [65], the widespread presence of a large variety of structurally similar analogues (see Figure 1.1) always poses potential questions concerning the reliability of the (U)HPLC-UV methods. These analogues can be easily confused with target analytes due to the similar absorption patterns if they are not resolved from the cobalamins.

1.4.3 LC-ICP-MS

First introduced in the 1980s for trace element quantification, inductively coupled plasma mass spectrometry (ICP-MS) takes advantages of the efficient sample ionization by a high temperature atomizing ion source (the ICP technology) and the sensitive and accurate detection of a mass analyzer. The ICP is a “hard” ionization technique that uses noble gas as plasma gas in combination with extremely high temperature (c.a. 5500 °C) to completely atomize most molecules in the sample [79, 80]. This type of ionization generates much higher analyte ion density and thus higher sensitivity when compared to other soft ionization techniques such as electrospray ionization (ESI) [81]. The “harsh” conditions of ionization also make the ICP ion source more tolerant towards salts and

less prone to contamination [79]. However, the advantages of ICP ion source come at a heavy expense of losing information on molecular structures as all bonds are broken regardless of their chemical bonding in the high-temperature plasma during the ionization [79]. Moreover, the detection of ICP-MS is non-species-specific, which means different chemical forms ("species") containing the same element are not distinguishable if they reach the ion source at the same time. To acquire species-specific information, ICP-MS hyphenated with different separation techniques can be used to separate targeted analytes before detection. LC coupled with ICP-MS (LC-ICP-MS) is one of the most commonly used hyphenated techniques for the detection and quantification of metals, metalloids, and heteroelements in liquid samples [82].

The structures of cobalamins possess a characteristic cobalt atom in the corrin ring, which allows for the analysis using ICP-MS by monitoring the cobalt. A few publications [83-91] have been reported using hyphenated ICP-MS techniques for analysing naturally occurring cobalamins in foods ever since Chassaigne and Lobinski [92] first applied the LC-ICP-MS for determination of cobalamins in pharmaceutical preparations. It is clearly observed that the outstanding sensitivity of ICP-MS enables the detection of low levels of cobalamins in their native forms without pre-concentrating by cyanidation, and HPLC or other functionally similar separation techniques such as capillary electrophoresis or size exclusion chromatography could realize necessary separation between different cobalamins for cobalt speciation. However, determined by the inherent detection principle of ICP-MS, the high specificity and sensitivity of this technique is established on the cobalt atom rather than the intact structures. When analyzing cobalamins in complex food matrices, any cobalt-containing non-cobalamin compounds could cause interference if co-eluted with the targeted analytes. In some cases, it might be possible to separate free cobalt and obvious cobalt-containing interferences which are not cobalamin-alike by optimizing the chromatographic conditions and by using immunoaffinity purification for additional selectivity. Nevertheless, the existence of diverse cobalamin analogues and their potential presence in food matrices largely complicate the analysis of cobalamins using LC-ICP-MS. For this reason, other analytical techniques such as LC-ESI-MS(/MS) have to be applied in addition to LC-ICP-MS to provide complementary structural information if analyte identification and confirmation is needed. The direct use of hyphenated ICP-MS

techniques, therefore, is more suited for analyzing cobalamins in more defined matrices when high sensitivity is required and potential interferences can be monitored.

1.4.4 LC-ESI-MS(/MS)

Combining high sensitivity and specificity, LC methods incorporating MS or tandem MS (MS/MS) with ESI are gaining popularity for B12 determination. ESI, the soft ionization technique, is known for producing molecular or pseudo-molecular ions retaining the intact structure to largest extent during ionization that can be measured by MS unequivocally. Additional structural information can be further obtained via measuring fragmentation patterns using MS/MS if available. In the recent years, LC-ESI-MS(/MS) has revealed its powerful use in structural identification of corrinoids in biological materials and food samples with the ability to clearly differentiate individual structures [21, 65, 93-104]. In most cases, the highly sensitive triple quadrupole mass spectrometer is more suitable for trace level detection, and occasionally a time-of-flight (TOF) mass spectrometer is applied when higher resolution is necessary to acquire more accurate *m/z* data for structural interpretation.

Despite of the obvious advantages of LC-ESI-MS(/MS), there is a rather limited number of publications describing B12 quantification in foods using such a versatile and reliable system. Luo et al. [105] tried to quantify CNCbl in multivitamin-multimineral tablets and infant formulas using LC-ESI-MS with ginsenoside Re as internal standard. Lu et al. [106] described a multi-method for simultaneous determination of four water-soluble vitamins including CNCbl in fortified infant foods by LC-ESI-MS/MS using methotrexate as internal standard. Similarly, the total B12 contents of raw milk and dairy products were quantified by Zironi et al. [107] using LC-ESI-MS/MS also with methotrexate as the internal standard. In addition, the use of LC-ESI-MS to determine cobalamin vitamers in their naturally occurring forms was reported by Szterk et al. [108] for meat samples without the application of internal standards.

These proposed methods, on the one hand, reflect the great potential of LC-ESI-MS(/MS) for B12 quantification in natural food matrices but, on the other hand, reveal several critical shortcomings of the current methods. Firstly, the employment of internal standards that are not structurally related to the analytes being determined are not ideal for LC-ESI-MS(/MS) applications as they often fail to compensate for analyte loss during the sample preparation and for analyte discrimination during ionization of the MS.

Moreover, the reported methods have not been vigorously validated on food matrices according to well-established criteria [109] and thus the proclaimed performances lack credibility. In addition, most of the applications make use of conversion of natural B12 vitamers into CNCbl during sample preparation to ease the analytical difficulties at the cost of losing the valuable information on natural distribution of different vitamers, especially the important native contents of the two co-enzyme forms (i.e., AdoCbl and MeCbl). The only publication aiming at analyzing native cobalamins in meats demonstrated rather unsatisfactory performance of the developed LC-MS method without using internal standards; the recoveries were too low for quantifying OHCbl, AdoCbl and MeCbl (68.3%, 60.7%, and 52.3%, respectively) and the sensitivity was not adequate to detect low levels of MeCbl and CNCbl in beef sirloin [108].

Despite being one of the most selective and sensitive detection techniques in the field of LC methods, MS often suffers from matrix effects when analyzing real food samples. Often, the accuracy and sensitivity are compromised by interferences from the matrix components. In this respect, the powerful stable isotope dilution assay (SIDA) is well recognized as the perfect tool for quantitative LC-MS/MS applications. SIDA utilizes stable-isotope labelled analogues of the analytes as internal standards, which completely compensate for losses and discriminations of analytes during sample preparation, chromatographic separation, and mass spectrometric detection. In addition, SIDA enhances the specificity of the determination as mass traces are assigned for both the analytes and the corresponding co-eluting internal standards [110]. When necessary, the intensity ratios between different mass transitions of the analyte are checked to further ensure the correctness of the assignment as an additional criterion of quality control. To date, SIDA has not been applied to quantitate cobalamins in real foods and, in particular, the native contents of the individual vitamers.

1.4.5 Sample extraction and purification

Different procedures have been applied to extract, isolate and concentrate cobalamins from foods depending on the targeted matrices and applied analytical techniques. For simple matrices such as multi-vitamin pills, simple aqueous extraction with buffers (e.g., sodium acetate buffer at pH = 4) at elevated temperatures (e. g., 121 °C) is generally adequate for cobalamins existing as free forms in relatively high concentrations [105]. On the contrary, enzymatic treatment, most commonly with α-amylase and pepsin, are used

to release protein-bound cobalamins when handling natural food samples with more complicated matrices [111]. Adding diluted cyanide solution during the extraction is a common practice to convert all cobalamin variants into the more stable CNCbl. This step intends to minimize the analyte degradation during extraction by improving the analyte stability and to pre-concentrate analytes for later analysis. The conversion is generally necessary for analytical systems (e.g., (U)HPLC-UV) that are not sensitive enough to detect low levels of individual cobalamins naturally present in foods. For MBAs, the cyanide conversion is also recommended as there are reported discrepancies between growth responses of the microorganisms towards different cobalamins [112]. Since differentiation between vitamers is not possible by MBAs, quantification can only rely on single standard for calculation. In most cases, CNCbl was used as the reference standard.

For the sensitive MBAs, the sample extract without further purification is directly used for performing the assays. For instrumental analysis including (U)HPLC-UV, LC-ICP-MS and LC-ESI-MS(/MS), sample solutions are usually cleaned up by solid phase extraction (SPE) to further concentrate cobalamins while removing matrix components. In the beginning, conventional SPE columns were employed for treating less complex matrices such as multi-vitamin formulations [74]. However, they showed a lack of efficacy in selecting B12 from more complex food matrices [63, 64, 69, 113]. The novel use of IAC was first proposed by Pakin et al. [113] to purify the trace B12 in sample solutions, taking advantage of the highly specific antigen-antibody interaction. The application of IAC largely improved the recovery of CNCbl in pork liver to 98% and enabled the quantification of cobalamins in foodstuffs at trace levels as low as about 3 ng/g. In the following publications [63, 64, 69], optimizations on IAC procedures have been carried out and the enhanced sensitivity and accuracy achieved by such a purification process have been further confirmed. Ever since the commercialization of the immunoaffinity column (R-Biopharm, Glasgow, UK), it has been routinely used in analyzing B12 in foodstuffs due to its undoubted superior performance compared to other clean-up processes. Some researchers tried to develop other more cost-effective materials to purify cobalamins from foodstuffs [114, 115]. However, none of the tested approaches has shown nearly as good performance as the IAC in terms of sensitivity, specificity and selectivity.

It has to be mentioned that IAC has several limitations. Firstly, the commercial immunoaffinity column is so far only tailor-made for CNCbl and might have different affinities towards other cobalamins [69]. Further investigation on the binding is required when targeted analytes include other cobalamins, cobamides and corrinoids. Secondly, the commercial immunoaffinity column (EASI-Extract Vitamin B12, R-Biopharm, Glasgow, UK) has a limited loading capacity of 1 µg of CNCbl and a recommend loading range of 0.01-0.5 µg of CNCbl. Loading sample amounts containing B12 out of the recommended range might lead to unsatisfactory results. In addition, the high cost of IAC is hardly affordable by many analytical labs for large-scale routine analysis. Despite of the shortcomings, IAC still reflects the state of the art for B12 purification in the field of food analysis.

1.5 Production of B12

The lack of isotope-labelled internal standards largely restricts further development of SIDA for B12 analysis in foods. The complexity of cobalamin structure renders the preparation of such a standard an extremely challenging task. In this chapter, a brief overview of the complex chemical synthesis and biosynthetic pathways of cobalamins is given, with references to detailed reviews. The microbial production of cobalamins with highlights on the use of *Propionibacterium freudenreichii* is described. The final part outlines previous reported strategies in preparing isotope-labelled corrinoid compounds.

1.5.1 Chemical synthesis of B12

Total synthesis of CNCbl, with more than 90 steps, was achieved in the 1970s by combined efforts of two groups from Harvard University (R. B. Woodward) and ETH Zürich (A. Eschenmoser) [9, 10]. Being one of the most challenging tasks of synthetic chemistry, the major challenge of this extremely complicated project lays in the chemical synthesis of the corrin core structure, cobyric acid. The cobalt-centred corrin ring has 13 chiral carbon centres, making its chemical synthesis a total nightmare.

Two synthetic strategies were developed, which differ in the way how the ring components were stepwise connected [10] (Figure 1.4). From cobyric acid to B12 (namely CNCbl), straightforward incorporation between the corrin ring and the nucleotide-containing lower ligand has been accomplished since 1960 [116]. Until now, synthetic chemists are still working on alternative routes for a more efficient synthesis of the cobyric acid but so far no other total synthesis has been successfully achieved [117]. The chemical synthesis of B12, therefore, remains extremely complicated and expensive if starting from scratch.

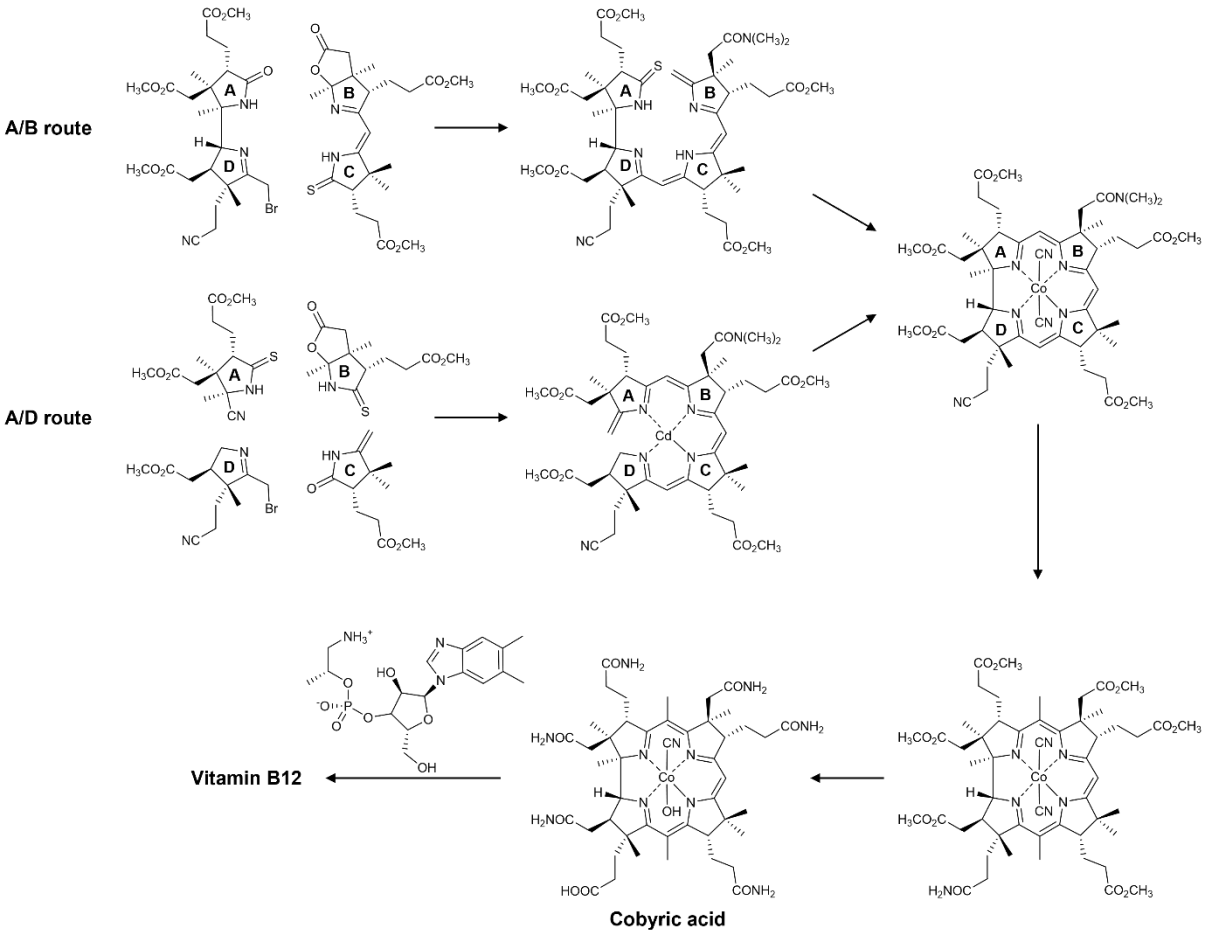


Figure 1.4. Two reported synthetic strategy to cobyric acid [10].

1.5.2 General biosynthesis of B12

1.5.2.1 De novo pathway

De novo cobalamin biosynthesis occurs in some bacteria and archaea via two alternative routes, commonly referred as anaerobic and aerobic pathways. The major differences between these two routes are the timing of cobalt insertion and the mechanism of ring contraction [17]. In the anaerobic pathway, the ring contraction is mediated by a chelated cobalt ion inserted at an early stage, whereas the aerobic pathway, marked by a late cobalt insertion, incorporates molecular oxygen into the macrocycle for the ring contraction [17]. The locus tag prefix *cob* is generally given to genes for cobalamin biosynthesis and prefix *cbi* is given to genes for the anaerobic pathway in contrast to the aerobic pathway [118]. The aerobic pathway has been extensively elucidated in *Pseudomonas denitrificans* [119], and the anaerobic pathway has been characterized in *Salmonella enterica* [120], *Bacillus megaterium* [121, 122] and *P. freudenreichii* [123, 124].

The two pathways are briefly described here as details have been extensively reviewed in previous literature [17, 125-128]. A schematic overview of the two pathways is shown in Figure 1.5. Despite the differences between the two routes, the complete cobalamin biosynthesis may be divided into following stages: 1) formation of 5-aminolevulonic acid (5-ALA) and its conversion to the porphinoid template, uroporphyrinogen III (UroIII), 2) corrin ring formation, and 3) lower ligand formation, activation and attachment.

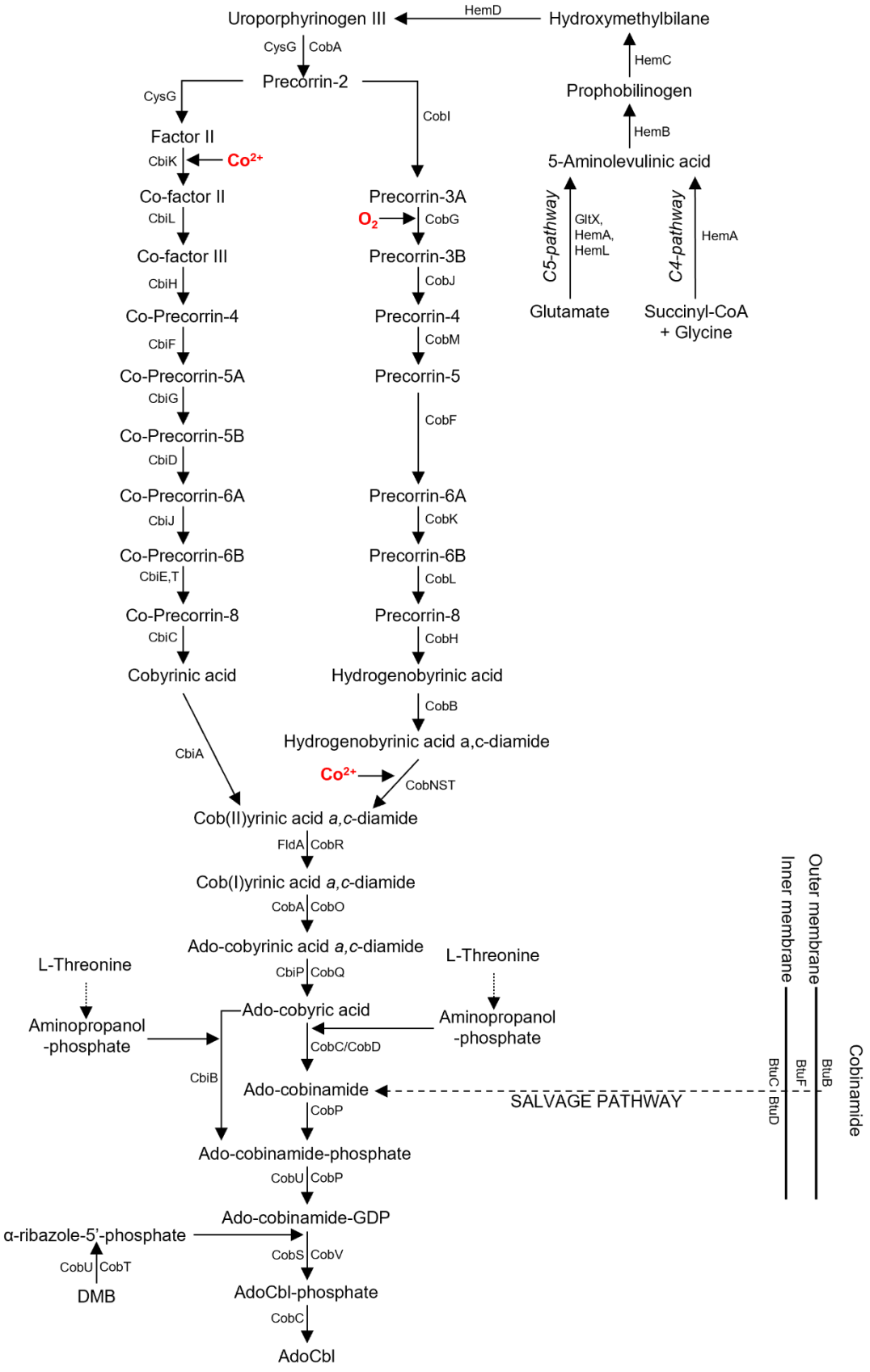


Figure 1.5. Schematic overview of cobalamin biosynthesis.

1.5.2.1.1 From 5-ALA to UroIII

5-ALA, the first general precursor for all known tetrapyrrole derivatives, is synthesized through C4 or C5 pathways (Figure 1.6). The C4 pathway (or shemin pathway) is found in non-photosynthetic eukaryotes and some bacteria such as α-proteobacteria. In this pathway, 5-ALA is formed from a condensation reaction of glycine and succinyl-CoA [129], catalyzed by 5-aminolevulinic acid synthase [130]. The C5 pathway is utilized in higher plants, algae and majority of bacteria, producing 5-ALA from glutamate [131, 132]. Some bacteria, such as *P. freudenreichii*, have been reported to use both pathways [133]. The generated 5-ALA is stepwise transformed to porphobilinogen (PBG), hydroxymethylbilane (HMB) and UroIII via condensation, polymerization and cyclization reactions [127] (Figure 1.6). The UroIII represents the first branch point at which the biosynthetic pathways for other pigments of life including heme and chlorophyll diverge from B12 synthesis [17, 127].

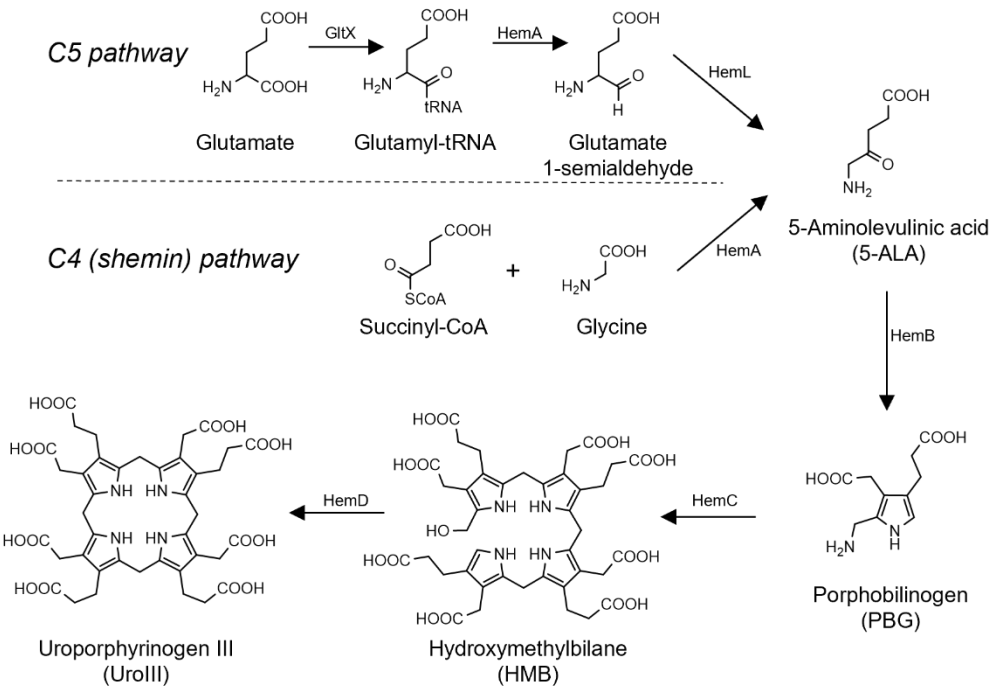


Figure 1.6. Two routes for the biosynthesis of 5-ALA and subsequent conversion into UroIII.

1.5.2.1.2 Corrin ring synthesis

Formation of precorrin-2, the diverging point of two pathways

UroIII is transformed into precorrin-2 via two methylation reactions [126]. Precorrin-2 marks the second branch point where the anaerobic and aerobic pathways diverge and it is also the last common precursor shared by siroheme, coenzyme F430 and heme d1 [127]. The intermediates afterwards are unique to cobalamin synthesis.

From Precorrin-2 to cobyrinic acid a,c-diamide

In the anaerobic pathway (Figure 1.7), precorrin-2 is converted to sirohydrochlorin (also known as factor II), followed by an early chelation of cobalt into the macrocycle to form cobalt cofactor II (also known as cobalt-precorrin-2) [17, 127]. After methylation at C-20 of this cobalt complex to give cobalt factor III (also known as cobalt-precorrin-3), ring contraction is triggered by the methylation at C-17 position and as the result lactonization between ring A acetate and C-20 [17]. The generated product is cobalt-precorrin-4 and it goes through a series of ring modification reactions to afford cobalt-precorrin-8 [134]. A methyl-rearrangement reaction then takes place, which completes the methylation pattern found in the final cobalamin structure, generating cobyrinic acid [134]. Cobyrinic acid is amidated to give cobyrinic acid *a,c*-diamide [127, 135], the point at which the two pathways re-join.

In the aerobic pathway (Figure 1.8), precorrin-2 is first methylated to give precorrin-3A [136, 137]. This intermediate is then hydroxylated at the same position using molecular oxygen, concurrently forming a *Ɣ*-lactone with ring A acetate side chain. The corresponding product precorrin-3B acts as the substrate for a concerted process of ring contraction and methylation at C-17 position, generating precorrin-4 [17, 138]. This compound is stepwise transformed to hydrogenobyrinic acid *a,c*-diamide [17]. In the next step, this molecule chelates cobalt into the macrocycle to generate the cobyrinic acid *a,c*-diamide [17, 139].

From cobyrinic acid a,c-diamide to adenosylcobyric acid

The remaining steps of corrin ring synthesis are shared by both pathways. After the adenosylation of cobalt and further sequential amidation reactions, adenosylcobyric acid (AdoCby) is formed, marking the completion of the corrin ring structure [126, 127].

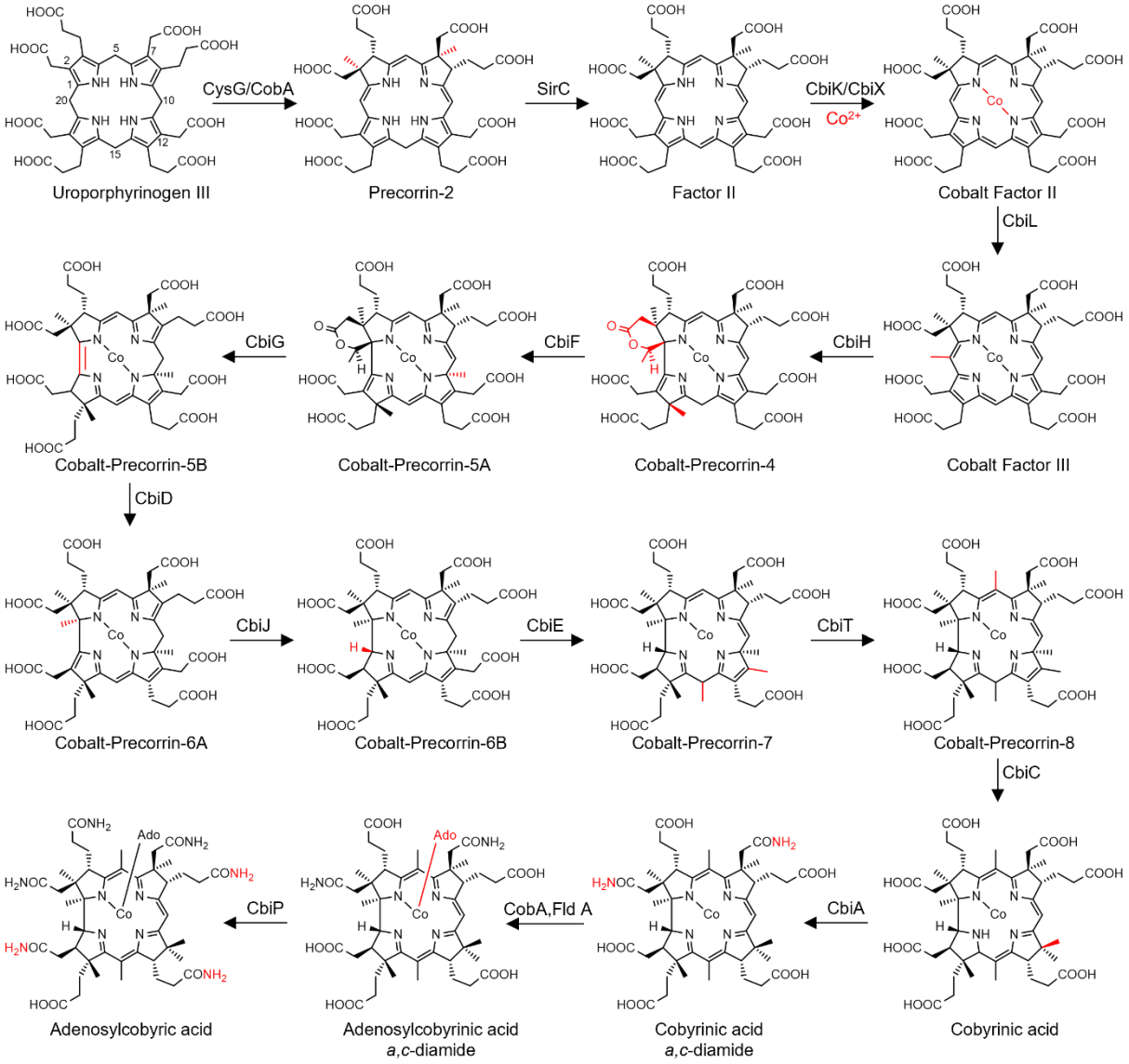


Figure 1.7. The anaerobic pathway for corrin ring biosynthesis.

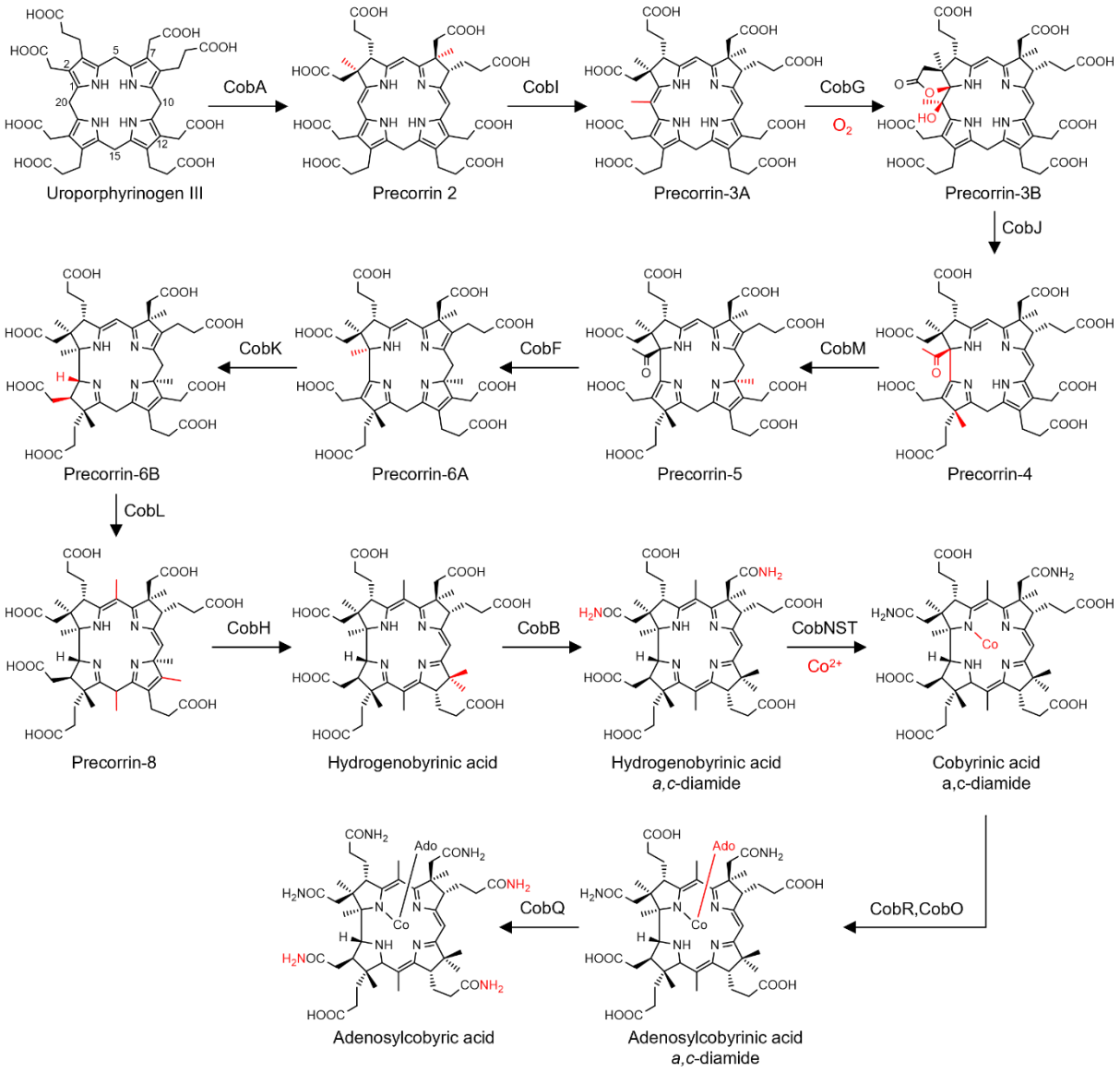


Figure 1.8. The aerobic pathway for corrin ring biosynthesis

1.5.2.1.3 Lower ligand attachment

To form the "complete" corrinoid structure, the last part of the biosynthesis is dedicated to attaching the lower ligand to the corrin ring. An aminopropanol group derived from L-threonine is attached to AdoCby, yielding adenosylcobinamide phosphate (AdoCbi-P). In the next steps, AdoCbl is formed via a so-called nucleotide loop assembly (NLA) pathway consisting of three steps: i) activation of the precursor adenosylcobinamide (AdoCbi), ii) activation of the lower ligand DMB and iii) joining of the activated precursors to generate AdoCbl [17] (Figure 1.9).

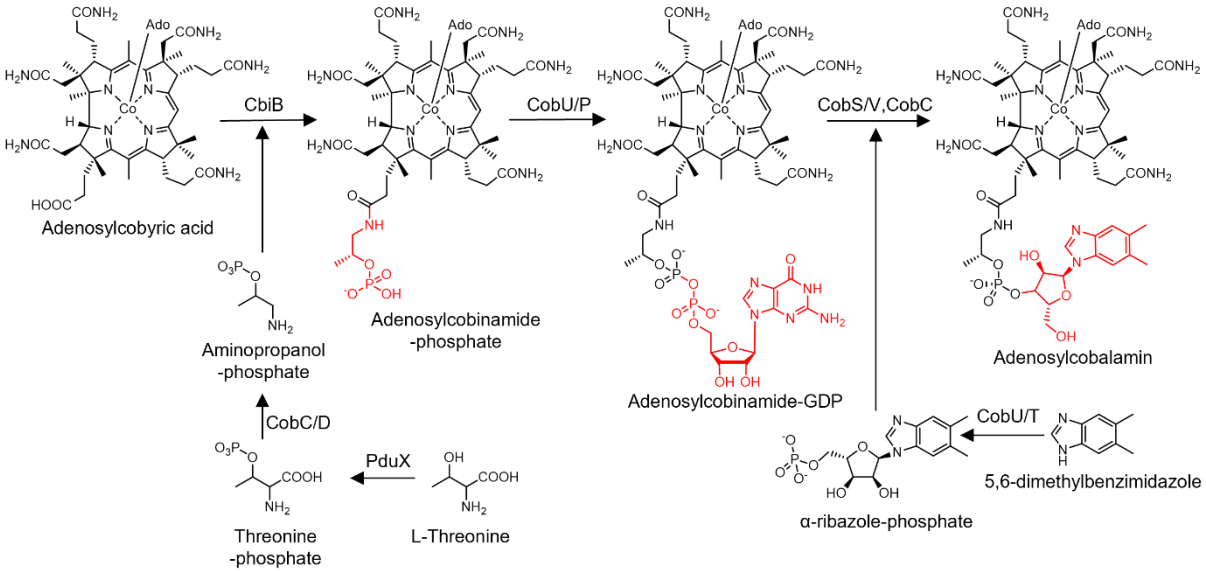


Figure 1.9. The final stage of cobalamin biosynthesis.

1.5.2.1.4 Formation of DMB, the authentic lower ligand of cobalamins

Two distinct pathways (Figure 1.10) are known for producing DMB, the lower ligand of cobalamins. One requires molecular oxygen and the other one does not. In the oxygen-dependent pathway, reduced flavin mononucleotide ($FMNH_2$) is cannibalized to DMB by a single enzyme BluB [140]. The oxygen-independent pathway constructs DMB from glycine [141], glutamine [142] and formate [143]. Methionine provides the methyl groups on the DMB molecules via SAM [101]. A gene cluster *bzaABCDE*, which was first identified in *Eubacterium limosum* [101], directs the formation of DMB branching from thiamin and purine biosynthesis. A recent research using *bza* operons comprising *bzaA-bzaB-cobT-bzaC* from *Moorella thermoacetica* suggested that ribosylation of 5-hydroxybenzimidazole (5-OHBza) might precede methylation steps of the

benzimidazole ring to form DMB, proposing a new synthesis and activation pathway of benzimidazolyl lower ligands in oxygen-independent biosynthesis [144].

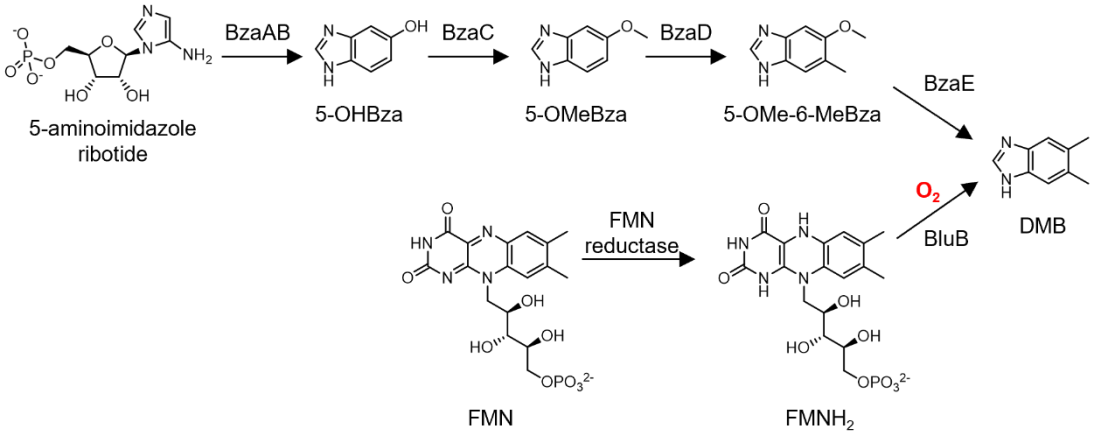


Figure 1.10. Oxygen-independent (upper) and oxygen-dependent (lower) pathways of DMB biosynthesis. 5-OHBza, 5-hydroxybenzimidazole; 5-OMeBza, 5-methoxylbenzimidazole; 5-OMe-6MeBza, 5-methoxy-6-methylbenzimidazole; FMN, flavin mononucleotide; $FMNH_2$, reduced flavin mononucleotide.

1.5.2.1.5 Formation of cobalamin analogues

As demonstrated in Figure 1.1, there is a broad diversity of cobamides possessing a lower ligand other than DMB. The activation of these alternative bases is similar to that of DMB before attachment. The gene product responsible for the reaction (e.g., CobT in *S. enterica*) limits the range of lower ligands that can be activated and incorporated into the cobamide structure, determining cobamide diversity. The specificity of CobT homologue is dictated by both the availability of lower ligand compounds and metabolic requirements of a specific organism [126].

Current understanding of the origins of these alternative lower ligand bases found in different cobamides is still rather limited. In some anaerobes, a combination of *bza* genes is responsible for the formation of certain benzimidazolyl bases including 5-OH-Bza, 5-OMeBza and 5-OMe, 6-MeBza [101]. The sources of purinyl bases for cobamides are still unclear and may be attributed to purine biosynthetic pathways for the basic nucleotides. In terms of phenolyl bases, it was reported that *p*-cresol is synthesized from tyrosine in *Sporomusa ovata* [145] but the pathway to phenol is still not elucidated. It has to be noted that some reported cobamides were obtained from guided biosynthesis with exogenous supplementation of base compounds into growth media [21, 146]. In some

cases, the specific cobamides formed via guided biosynthesis may cause inhibition of certain metabolisms requiring other cobamides as cofactors and thus lead to reduced viability of the bacteria [100, 147].

1.5.2.2 Salvage pathway

Some bacteria and archaea have the capability of salvaging incomplete corrinoids from the environment and further convert them to AdoCbl. Extracellular cobinamide is imported into cells via highly specific transport systems and is then adenosylated to AdoCbi before further transformation following two salvage pathways [148]. In bacteria (cobinamide salvage pathway I), a bifunctional enzyme with kinase and guanylyltransferase activities (e.g., CobU in *S. typhimurium* or Cob P in *P. denitrificans*) phosphorylates the amino-propanol moiety of AdoCbi, yielding AdoCbi-P [148]. In archaea (cobinamide salvage pathway II), AdoCbi is first converted to AdoCby, which is then condensated with aminopropanol-phosphate to form AdoCbi-P [149]. From AdoCbi-P to AdoCbl, the steps are identical to these of the *de novo* biosynthesis. Worth noting, some bacteria and archaea also obtain complete corrinoids from the surroundings and remodel them into desired cobamides [99]. More detailed reviews about the salvage pathway are found elsewhere [148, 150].

1.5.3 Microbial production of B12 by *Propionibacterium freudenreichii*

Due to the complexity of chemical synthesis of vitamin B12, large scale B12 production exclusively relies on biosynthetic fermentation processes. Until now, only a few bacteria and archaea have been identified as authentic B12 producers in nature [151]. Among all, *P. freudenreichii* (anaerobic pathway) and *P. denitrificans* (aerobic pathway) are most often employed for the manufacturing of B12 because of their naturally outstanding B12 productivity, rapid growth, and simple nutritional requirements [46]. Moreover, biosynthetic pathways have been extensively studied in both species, providing solid experimental evidence and genetic accessibility for their applications. Compared with *P. denitrificans*, *P. freudenreichii* has its distinct advantages; it has GRAS (generally recognized as safe) status approved by the FDA (U.S. Food and Drug Administration) and QPS (qualified presumption of safety) status by EFSA and thus is also suitable for food biofortification purposes. Therefore, *P. freudenreichii* is not only extensively used for industrial biotechnological synthesis but also has been employed in a growing number of food bio-fortification applications [49, 152]. The yield of B12 using *P. freudenreichii* could

reach as high as about 300 mg/L, applying various optimization strategies such as strain selection, metabolic engineering, and medium and process optimization [46, 151].

Originally isolated from Swiss-type cheese, *P. freudenreichii* is a gram-positive, rod-shaped bacterium that belongs to the genus *Propionibacterium*, to the family Propionibacteriaceae, and to the phylum Actinobacteria. In the past, *P. freudenreichii* was divided into two subspecies, namely *P. freudenreichii* subsp. *freudenreichii* and *P. freudenreichii* subsp. *Shermanii*. However, the subdivision is no longer justified based on the phylogenetic analyses and phenotypic properties [153, 154]. Thus, the subspecies mentioned in the literature are not specified and generally described under *P. freudenreichii* in this chapter.

1.5.3.1 Wood-Werkman cycle and *de novo* B12 biosynthesis in *P. freudenreichii*

The name "Propionibacterium" originates from the characteristic feature of all Propionibacteria of producing large amounts of propionic acid during growth. In the so-called Wood-Werkman cycle (Figure 1.11), pyruvate obtained via glycolysis of substrates such as glucose is stepwise transformed by a reverse TCA cycle to succinyl-CoA and eventually to the propionic acid [154, 155]. The reaction isomerizing succinyl-CoA to *R*-methylmalonyl-CoA in the cycle is catalysed by methylmalonyl-CoA mutase which requires AdoCbl as coenzyme. The dependency of AdoCbl in the pathway is associated with the natural production of cobalamins in *P. freudenreichii*.

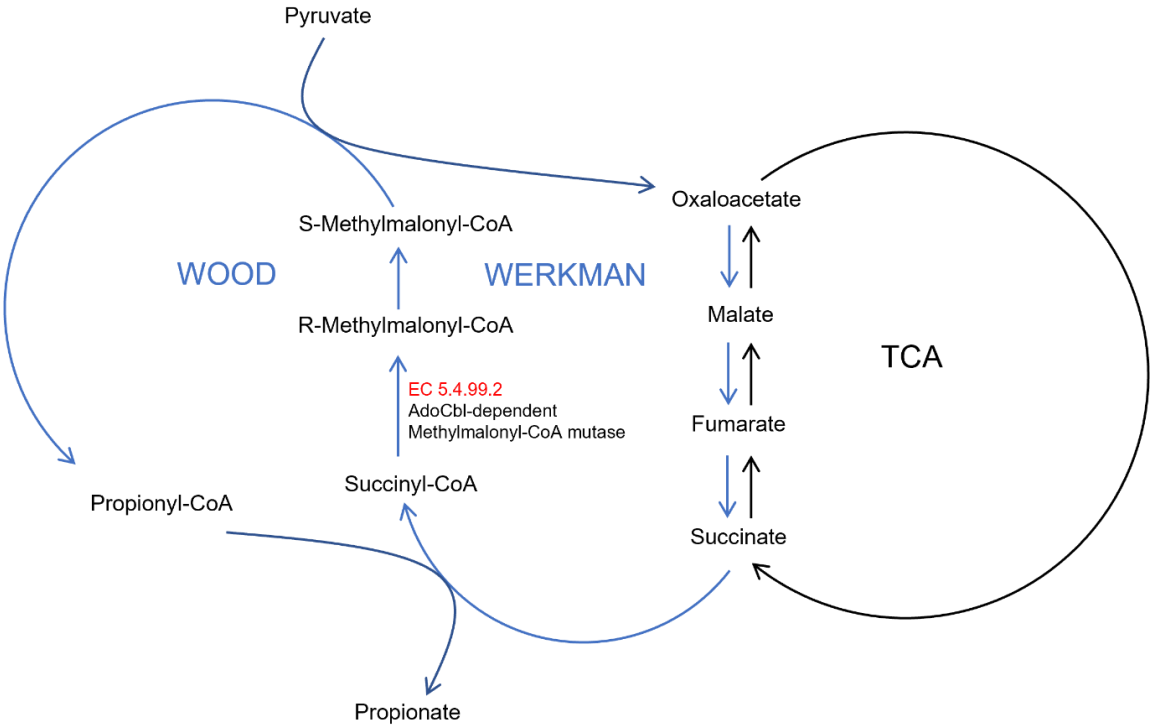


Figure 1.11. Schematic overview of Wood-Werkman cycle (in blue) and tricarboxylic acid (TCA) cycle (in black) in *P. freudenreichii* [155].

The *de novo* biosynthesis of B12 in *P. freudenreichii* is featured by a unique combination of the anaerobic and aerobic pathways. More details about the two pathways are given in section 1.5.2. Irrespective of presence or absence of oxygen, *P. freudenreichii* synthesizes the corrin-containing intermediates exclusively via the anaerobic pathway but relies on the oxygen-dependent cannibalization of $FMNH_2$ to form DMB [46, 156]. In the final NLA pathway, the formation and activation of DMB are catalysed by a fusion enzyme BluB/CobT2 [102]. The fusion of these two activities enables the efficient transformation of the lower ligand DMB produced within the same enzymatic complex to form the authentic cobalamin with the reaction equilibrium being shifted strongly to the product side [102].

1.5.3.2 Genetical background and nutritional requirements of *P. freudenreichii*

For microbial production of any compound, a good knowledge of the biosynthetic pathway and the corresponding genes is highly beneficial for controlling, optimizing and troubleshooting the processes. The genes responsible for the complete biosynthetic pathway of B12 have been characterized for *P. freudenreichii* [155]. In fact, the complete

genomes of *P. freudenreichii* DSM 4902 (CIRM-BIA1^T) and *P. freudenreichii* DSM 20271^T have been successfully sequenced and announced by Falentin et al. [155] and by Koskinen et al. [157], respectively. Moreover, the draft genomes of 21 *P. freudenreichii* strains were described by Loux et al. [158] using *P. freudenreichii* DSM 4902 genome as a reference and later the draft genome of *P. freudenreichii* ITG P20 was published as well [159]. Subsequently, Deptula et al. announced completed genomes of 20 *P. freudenreichii* strains using PacBio sequencing platform [160]. The genomic analysis not only shed light on the complete description of B12 biosynthetic pathway in *P. freudenreichii* but also explained important aspects in metabolism and nutritional requirements of the species.

P. freudenreichii has a very complex metabolism but demonstrates few nutritional requirements. Experimentally, *P. freudenreichii* is prototrophic for all amino acids and nucleotides, and it can grow in minimal media containing a carbon and energy source, ammonium as the sole nitrogen source, minerals and two to four vitamins (pantothenate, biotin, thiamin, and *p*-aminobenzoic acid) [155, 161]. Based on the genome data of strain CIRM-BIA1^T, the complete biosynthetic pathways of all amino acids were reconstituted [155]. Additionally, biosynthetic pathways for all vitamins except biotin and pantothenate are complete in strain CIRM-BIA1^T, which is in accordance with previous observations that all Propionibacteria strains require biotin and pantothenate for growth [155, 161]. The genome annotation also confirmed the ability of the strain to ferment a wide variety of carbon substrates including various sugars, alcohols and acids via different pathways. The characteristic cobalamin-utilizing Wood-Werkman cycle could be completely reconstituted in the strain CIRM-BIA1^T as well. The complete and draft genome data further provided insights into other features of the species such as the strain-dependent carbohydrate utilization patterns, adaptation to the environments and capability of long-term survival.

1.5.3.3 Fermentation processes using *P. freudenreichii* for B12 production

P. freudenreichii usually grows under anaerobic to microaerophilic conditions, but interestingly it possesses all the genes needed to construct a respiratory chain [155]. The species has the capability to use molecular oxygen as electron acceptor [161] and produces pyruvate from propionate via a reverse methylmalonyl-CoA pathway under aerobic conditions [162]. The production of B12, however, was reported to stop under

aerobic conditions and resumed when switched back to anaerobic conditions [162]. It seems that high yields of B12 production in *P. freudenreichii* are only possible under very low oxygen levels [46]. However, molecular oxygen is needed for the formation of DMB if it is not externally supplied in the fermentation broth. Therefore, a two-stage cultivation is commonly employed for the industrial production of B12 using *P. freudenreichii* [46]. The first stage, which usually lasts for 3 days, is carried out under very low oxygen concentrations for the bacteria to grow and to synthesize B12 intermediate lacking the lower ligand. Subsequently in the second stage, aeration of the culture is performed for 1-3 days, allowing for the oxygen-dependent formation of DMB that later is activated and linked to the precursor to form the complete cobalamin structure. Generally, the incubation processes are performed at 30 ºC, close to the optimum growth temperature for Propionibacteria. Due to the high amounts of organic acids produced during growth, neutralization is crucial to maintain the culture pH, commonly in the range 6.5-7, to prevent inhibition caused by low pH and undissociated acetic and propionic acids [151].

1.5.3.4 Optimization on B12 production using *P. freudenreichii*

In order to improve the yields of B12 production by *P. freudenreichii*, various strategies and tools have been investigated, mainly including strain selection, metabolic and genetic engineering, and medium and process optimization. As B12 production is highly strain-dependent, the screening of a large natural biodiversity of strains have been performed to identify authentic and efficient producers [163]. Additionally, a common strategy is to use random mutagenesis (e. g. UV exposure) to generate strains that produce B12 in high yields and that have practical advantages for industrial production [46]. The metabolic and genetic engineering of *P. freudenreichii* to improve B12 production is not easy due to the complexity of the biosynthetic pathway. Several expression systems have been developed and applied for metabolic engineering of Propionibacteria [164]. Piao et al. [165] overexpressed several genes involved in the B12 biosynthesis in *P. freudenreichii* and achieved 1.5- to 1.9-fold increase in yields. Expressing heterologous genes in *P. freudenreichii* has also been performed to increase the yields by 2.2-fold [165]. Genome shuffling has been conducted by Zhang et al. [166] to produce a strain of *P. freudenreichii* that could produce a higher yield of 2.8 mg/L compared to the original yield of 1.8 mg/L by the parent strain. Various other metabolic and genetic engineering technologies such as protein engineering, inactivation or down

regulation of genes and bypass or removal of cobalamin riboswitches also demonstrate great potential to maximize the efficiency of *P. freudenreichii* for B12 production [125]. Alternatively, constructing a heterologous biosynthetic pathway in model organisms that can be easily genetically manipulated is a promising strategy to produce B12 in high yields [167]. A comprehensive review about recent advances on strategies and tools from synthetic biology and metabolic and genetic engineering to increase microbial cobalamin production has been presented previously by Fang et al. [125].

Apart from altering the B12-producing strains, many studies have been carried out to optimize the medium composition and the cultivation processes to enhance the B12 yields and productivity of *P. freudenreichii*. The influences of supplementing key components such as cobalt and DMB have often been addressed [46, 168, 169]. In addition, selection of carbon sources, addition of minerals, amino acids, vitamins and precursors, blue light, co-fermentation and co-cultivation have been shown to affect the cobalamin production [150]. Moreover, investigations on B12 production by *P. freudenreichii* in food-like growth conditions are emerging due to the great potential of *P. frendenreichii* in food biofortification [170, 171]. Recently, Liu et al. [172] evaluated the effects of medium composition on B12 production by *P. freudenreichii* and they were able to explain the changes on B12 production by corresponding differences in the overall cellular metabolism, applying a metabolomic approach. Attempts on cultivation processes combined with different techniques and bioreactors (suspended and planktonic cells, batch, fed-batch, continuous, semi-continuous) have been made in order to efficiently produce B12 in large-scale fermentation and to reduce production costs [151, 164].

1.5.4 Preparation of labelled cobalamin compounds

Due to the extreme complexity of total chemical synthesis of cobalamin and related compounds, researchers often combine biological and chemical procedures to produce corrinoids of desired structures in laboratory scales. The complex corrin-derivatives, such as cobyric acid and cobinamides, are usually obtained from microbiological sources. The various upper and lower ligands can be tailor-made chemically. The different parts are assembled via chemical [116, 173], enzymatic [174] or biological ways [21, 100, 175] to form a complete structure.

Regarding the preparation of labelled cobalamins, current studies applied similar methodologies mentioned above and introduced labels mainly to the upper or lower ligands. Applying guided biosynthesis, labelled lower ligands were supplied to model microorganisms to prepare the target compounds [21, 94]. Alternatively, labelled upper ligands (e.g., labelled cyanide $^{13}C^{15}N^-$) were incorporated into the cobalamin structures via chemical reaction with co(I)balamin [176, 177].

There are important criteria to consider when preparing stable-isotope labelled cobalamin compounds for the purpose of SIDA. Generally, a sufficient mass shift is required for the labelled standards in order to avoid overlaps between analytes and labelled standards during mass spectrometric analysis. Only if the labelled standard has significantly higher mass increment than the analyte, the interfering influence of natural isotopes can be avoided. Multiple occurrences of natural isotopes can be expected when regarding the high numbers of carbon, nitrogen, and oxygen atoms in cobalamins and have to be taken into account for the synthetic strategy. The approach of labelling the lower ligand is suitable if enough labels are incorporated into the structure. However, the chemical synthesis of the labelled lower ligand is rather complex, time consuming and expensive. Moreover, the later guided biosynthesis does not guarantee a unified labelling in the final product. Labelling the upper ligand is straightforward but not sufficient for introducing enough labels into the cobalamin structure for SIDA.

Using the biosynthetic competence of microorganisms for synthesizing the labelled cobalamin completely is another straightforward alternative. This approach has been shown to be effective for producing labelled metabolites with complex structures such as ^{13}C-labelled *Alternaria* toxins [178]. In the case of cobalamins, it seems possible to prepare ^{15}N-labelled cobalamins by cultivating *P. freudenreichii* in chemically defined medium containing ^{15}N-ammonium as the sole nitrogen source, considering its low nutritional requirements described earlier (section 1.5.3.2).

2 Objectives of the thesis

The general objective of the present thesis was to develop an analytical method that surpasses current limitations on B12 analysis with the application of LC-MS/MS and SIDA. The developed method should be able to accurately quantify naturally occurring cobalamins in foods, a goal which has not yet been realized in the past.

As the development of SIDA for cobalamins faces a great obstacle of lacking commercially available stable-isotope labelled standards, the first aim of the thesis was to utilize the biosynthetic power of *P. freudenreichii* to prepare four ^{15}N-labelled cobalamin compounds, namely [^{15}N]-OHCbl, [^{15}N]-AdoCbl, [^{15}N]-CNCbl and [^{15}N]-MeCbl. More specifically, suitable chemically defined media and incubation schemes should be developed and optimized for the ^{15}N-labelling purpose. Subsequently, further extraction, purification and chemical modification protocols should be developed to isolate the targeted compounds. Eventually, the prepared ^{15}N-labelled cobalamins should be characterized to check their labelling patterns and isotopic purities.

The second aim of the present thesis was to establish the first multiple SIDA method for analyzing the four major cobalamins in foods using the self-made ^{15}N-labelled cobalamins. For this purpose, sample preparation protocol as well as a reliable LC-MS/MS method with the application of SIDA should be developed to quantitively determine trace levels of OHCbl, AdoCbl, CNCbl and MeCbl in meat samples. The newly developed method should be thoroughly validated based on a blank matrix to assess the method performance.

The third aim of the thesis was to compare quantification of B12 in foods based on the native cobalamins or as total CNCbl after cyanidation conversion. A conversion SIDA method that determines total CNCbl after cyanidation conversion should be developed and validated. The conversion SIDA method should be further compared with the multi-SIDA method addressing the native cobalamins.

3 Materials and methods

The materials and methods concerning the preparation of the ^{15}N-labelled cobalamins and the development of the native SIDA method were described in the respective publications [179, 180]. The following sections describe materials and methods that are relevant to the development and application of the conversion SIDA method relying on cyanidation conversion.

3.1 Materials, chemicals, and samples

Soybean flour (type I, cat. no. S9633), *α*-amylase from *Aspergillus oryzae* (EC 3.2.1.1, cat No. 10065), papain from *papaya latex* (EC 3.4.22.2, cat no. P4762) and certified reference material BCR 487 (lyophilized pork liver; European Commission, Geel, Belgium) were obtained from Sigma-Aldrich (Steinheim, Germany).

Acetic acid for LC-MS (≥ 99.8%), glacial acetic acid (HPLC grade) and ammonium acetate for LC-MS (≥ 99.0%) were purchased from VWR International (Ismaning, Germany). Water (LC-MS and HPLC grade) and methanol (HPLC grade) were obtained from TH. Geyer (Renningen, Germany). Methanol (LC-MS grade) was purchased from Honeywell (Seelze, Germany). Sodium cyanide (≥ 97%) and sodium acetate trihydrate (≥ 99.0%) were received from Sigma-Aldrich (Steinheim, Germany).

Analytical standards of CNCbl, OHCbl·HCl, AdoCbl, and MeCbl were purchased from Sigma-Aldrich (Steinheim, Germany). The isotopically labelled compound [$^{15}N_{13}$]-CNCbl was self-prepared via biosynthesis, chemical modification, and chromatographic purification [179].

Fresh pork fillet (300 g) was purchased from a local butcher shop (Freising, Germany) in minced form. After lyophilization (Alpha 1-2 LDplus, Martin Christ Gefriertrocknungsanlagen, Osterode am Harz, Germany), the sample was ground using a kitchen grinder (EGK 200, Rommelsbacher, Dinkelsbühl, Germany), thoroughly homogenized, and stored at -20 °C in the dark until further analysis. The previous meat samples (pork fillet, beef fillet, lamb fillet and chicken breast) that have been analyzed by the native SIDA method for individual vitamers [180] were again analyzed for total B12 using the developed conversion SIDA method.

3.2 Preparation and concentration determination of standard solutions

The preparation of standard solutions and the determination of their concentrations were performed following the procedures developed for the native SIDA method [180] with slight modifications.

Stock solutions of unlabelled CNCbl were prepared by dissolving 1 mg of the reference compound in 10 mL of water. The accurate concentration of the CNCbl stock solution was determined by UV spectroscopy after further dilution with water (1:3, v/v). The isotopically labelled [$^{15}N_{13}$]-CNCbl was redissolved separately in water to obtain a stock solution (~10 µg/mL). For the preparation of samples, the stock solution was further diluted to prepare working solutions in the range of 0.01 and 0.5 µg/mL suitable for spiking purposes. Using the LC-MS/MS, the accurate concentrations of [$^{15}N_{13}$]-CNCbl solutions used for each extraction were routinely determined by measuring a calibrator solution comprising unlabelled CNCbl (~50-100 ng/mL) and labelled [$^{15}N_{13}$]-CNCbl (~50-100 ng/mL). For this purpose, the CNCbl stock solution was diluted and further mixed with [$^{15}N_{13}$]-CNCbl to obtain the calibrator. The concentrations of [$^{15}N_{13}$]-CNCbl can be calculated using the LC-MS/MS response function with the known amounts of CNCbl previously determined by the UV spectroscopy.

For the determination of conversion rate, stock solutions of OHCbl, AdoCbl and MeCbl were prepared by dissolving 1 mg of respective reference compound in 10 mL of water. The accurate concentrations of OHCbl, AdoCbl and MeCbl stock solutions were determined by HPLC-DAD using CNCbl as internal standard. For this purpose, a working solution consisting of the four cobalamins, prepared by mixing different stock solutions, was used for the HPLC-DAD measurements. The detailed conditions of HPLC-DAD are shown in the publication of the native SIDA method [180]. The accurate concentration of CNCbl was obtained from UV spectroscopy measurements. Based on the known concentration of CNCbl, the concentrations of the other three cobalamins could be calculated using the HPLC-DAD response functions listed in Table 3.1 [180]. For the spiking purpose, a working solution composed of OHCbl, AdoCbl and MeCbl (~200 ng/mL, respectively) was prepared by diluting and mixing respective stock solutions. 200 µL of the working solution was used for spiking.

The stock solutions of CNCbl were prepared monthly and the stock solutions of the other cobalamins were prepared every 2 months. For the storage, the stock solutions were

pre-divided into 1 mL aliquots. For each extraction, freshly thawed aliquots were used. All standard solutions were stored at -20 °C in the dark.

Table 3.1. HPLC-DAD response functions for OHCbl, AdoCbl and MeCbl [180].

Analyte	λ (nm)	HPLC-DAD response curve [a]	R^2
OHCbl	351	$y = 1.1518x - 0.0428$	0.9998
AdoCbl	377	$y = 0.8036x - 0.0055$	0.9999
MeCbl	341	$y = 1.0648x - 0.0137$	0.9999

Note: [a], y are peak area ratios [A(A)/A(IS)] and x are mass concentration ratios [ρ(A)/ρ(IS)]; CNCbl as internal standard (IS).

3.3 Sample preparation of the conversion SIDA method

The sample preparation protocol was developed based on the native SIDA method of the present thesis [180] as well as previous conversion-based methods [64, 65, 69, 177]. Various sample preparation methods were tested on the newly purchased pork fillet sample during method development. The detailed procedures for each method are described below. A summary highlighting the differences between the methods are presented in Table 3.2. Method D was the optimized method eventually adopted for the method validation and the preparation of the real samples.

Method A: 1 g of lyophilized and homogenized meat sample, 5 mg of papain, 5 mg of α-amylase were weighed into a 50 mL amber extraction vial (Duran, Mainz, Germany). [$^{15}N_{13}$]-CNCbl was added as internal standard in amounts based on the expected total contents of cobalamins in the samples to fall inside the calibration range. 25 mL of extraction buffer (50 mM sodium acetate buffer, pH = 4) and 500 µL of sodium cyanide solution (1%, w/v) were added to the sample. The sample mixture was vortexed and further stirred on a magnetic stirrer (IKA, RO 15 power, Staufen, Germany) at room temperature (RT) for 20 min and then incubated in a shaking water bath (GFL 1092, Burgwedel, Germany) at 37 °C for 1 h. Afterwards, the sample homogenate was boiled for 10 min, cooled in an ice-water bath, and transferred to a 50 mL amber centrifuge tube. The residue in the extraction vial was rinsed with 5 mL of extraction buffer and was further incorporated into the centrifuge tube. The sample was centrifuged (Eppendorf 5810R, Hamburg, Germany) for 20 min (3220 × g, RT). The supernatant was retained and then paper filtered (Whatman 597$^{1/2}$) before further purification. The

filtrate was purified using immunoaffinity column following the same protocol from the native SIDA method [180]. Briefly, the filtrate was loaded onto the column followed by washing the column with 10 mL of water. Afterward, the cobalamins were eluted twice using 2 mL of methanol each time with black flushing. The eluate was dried at 40 °C under nitrogen using an evaporator system (EC2, VLM, Bielefeld, Germany). The sample was reconstituted in 300 µL of LC-MS water and filtered through a PVDF membrane (Ø13 mm, 0.22 µm, Ahlstrom Munksjö, Helsinki, Finland) before LC-MS/MS analysis. All steps were performed under subdued light conditions.

Method B: The same procedures from method A were adopted for method B but different light conditions were used. Clear extraction vials and centrifuge tubes were used instead of the amber ones. The whole extraction process, including stirring, incubation and boiling, was performed under normal laboratory light conditions. After the extraction, subdued light was again employed for the immunoaffinity purification process.

Method C: The same procedures and light conditions of method B were adopted for method C except for an extended boiling time of 30 min after the incubation.

Method D: On the basis of method C, Method D incorporated an additional cyanidation step. After paper filtration, 100 µL of sodium cyanide solution (1%, w/v) was added to the filtrate for a second cyanidation step. Subsequently, the filtrate was shaken horizontally at 300 rpm (Shaker KL 2, Edmund Bühler, Bodelshausen, Germany) for 10 min under normal laboratory light conditions. Afterwards, the filtrate was kept in dark before the immunoaffinity purification that was further conducted under the subdued light conditions.

Table 3.2. Summary of differences between extraction methods A-D.

	Extraction steps			
Method	**Stirring (20 min, RT)**	**Incubation (1 h, 37 °C)**	**Boiling**	**2nd cyanidation after paper filtration**
A	Subdued light	Subdued light	Subdued light, 10 min	Not applied
B	Normal light	Normal light	Normal light, 10 min	Not applied
C	Normal light	Normal light	Normal light, 30 min	Not applied
D	Normal light	Normal light	Normal light, 30 min	Addition of 100 µL of 1% NaCN (w/v);10 min shaking under normal light

3.4 Calibration and quantification of LC-MS/MS

The established LC-MS/MS response curve for CNCbl from the native SIDA method [180] in the present thesis was used for the quantification. The calibration function for CNCbl was $y = 0.001x^2 + 1.0643x - 0.0276$ ($R^2 = 0.9999$) between molar ratios of analyte and internal standard [n(A)/n(IS)] of 0.01 and 115.61. The *X* axis is the molar ratio [n(A)/n(IS)] and the *Y* axis is the peak area ratio[*A*(A)/*A*(IS)].

After being converted into CNCbl during extraction, cobalamins in the samples were quantified as total CNCbl using [$^{15}N_{13}$]-CNCbl as internal standard.

3.5 Method validation of the conversion SIDA method

3.5.1 LOD and LOQ

The LOD and LOQ of the conversion SIDA method were determined according to Vogelgesang and Hädrich [109] in a revised way using the so-called surrogate analyte approach [181]. The self-prepared [$^{15}N_{13}$]-CNCbl was spiked at four different levels (0.2, 0.7, 1.4, and 2.1 ng/g) to the soy flour as the surrogate analyte for CNCbl. Each spiking level was prepared in quadruplicate. CNCbl was added as internal standard for quantification.

For the quantification of [$^{15}N_{13}$]-CNCbl, a reverse LC-MS/MS response curve was established by plotting peak area ratios [*A*([$^{15}N_{13}$]-CNCbl)/*A*(CNCbl)] against the molar ratios [n([$^{15}N_{13}$]-CNCbl)/n(CNCbl)] from previous LC-MS/MS measurements that were used to establish the LC-MS/MS response curve of CNCbl for the native SIDA method [180]. A linear response curve ($y = 1.0088x - 0.014$, $R^2 = 0.9999$) in molar ratios [n([$^{15}N_{13}$]-CNCbl)/n(CNCbl)] between 0.043 and 8.65 was obtained. In this case, the *Y* axis is the peak area ratio [*A*([$^{15}N_{13}$]-CNCbl)/*A*(CNCbl)] and the *X* axis is the molar ratio [n([$^{15}N_{13}$]-CNCbl)/n(CNCbl)]. The linearity in the given range was confirmed by the Mande's fitting test [182]. Using this reverse LC-MS/MS response curve, the LOD and LOQ were calculated from [$^{15}N_{13}$]-CNCbl instead of CNCbl, circumventing the problem of lacking absolute cobalamin-free blank matrices.

3.5.2 Recovery

The recoveries of CNCbl were determined by analyzing spiked pork fillet samples using the conversion SIDA method. The pork fillet sample was spiked with CNCbl at three

different levels (7.5, 37.6, and 74.7 ng/g) in triplicate. The recoveries were calculated for CNCbl as follows:

$$\text{Recovery (\%)} = \frac{\text{found amount of total CNCbl (ng/g)} - \text{endogenous amount of total CNCbl (ng/g)}}{\text{spiked amount (ng/g)}} \times 100\%$$

3.5.3 Precision

A pork fillet sample containing all four cobalamins was analyzed to determine the precisions of the conversion SIDA method. The inter-injection precision was determined by multiple injection of one sample (n = 6). The intra-day precision was obtained from quadruplicate analysis (n = 4) of the pork fillet sample using triplicate injections. For the inter-day precision, the pork fillet sample was analyzed in quadruplicate over three weeks (n = 3) using triplicate injections.

3.5.4 Analysis of reference material BCR 487

The developed conversion SIDA method was applied to analyze the reference material BCR 487 to further check the accuracy of the method. In order to compare the performances of the native and the conversion SIDA methods, the BCR 487 was as well analyed by the native SIDA method [180]. A sample amount of 0.1 g was used for the extractions applying the conversion method, whereas 0.2 g was adopted for the extractions using the native SIDA method.

3.6 LC-MS/MS

A Nexera X2 UHPLC system (Shimadzu, Kyoto, Japan) coupled to a triple quadrupole mass spectrometer (LCMS 8050, Shimadzu, Kyoto, Japan) was used for the LC-MS/MS analysis. The same LC and MS parameters from the native SIDA method [180] were used for the LC-MS/MS measurements. MRM transitions of OHCbl, AdoCbl and MeCbl were kept in the LC-MS/MS method for monitoring the residues of unconverted cobalamins.

3.7 Determination of conversion rate

A pork fillet sample was analyzed for total CNCbl applying the conversion SIDA method before and after being spiked with OHCbl (35.0 ng/g), AdoCbl (27.3 ng/g) and MeCbl (39.5 ng/g). The spiked amount of each cobalamin was determined by the HPLC-DAD

method routinely used for the concentration determination of the standard solutions [180]. The conversion rate was calculated as follows:

$$\text{Conversion rate (\%)} = \frac{\text{total CNCbl of spiked sample (ng/g)} - \text{total CNCbl of original sample (ng/g)}}{\text{total spiked amount calculated as CNCbl equivalent (ng/g)}} \times 100\%$$

4 Results

4.1 Production of four ^{15}N-labelled cobalamins via biosynthesis using *Propionibacterium freudenreichii*

Published as: Wang, M., Asam, S., Chen, J., Ehrmann, M., and Rychlik, M. (2021). Production of Four ^{15}N-Labelled Cobalamins via Biosynthesis Using *Propionibacterium freudenreichii*. *Frontiers in Microbiology* 12(2256). doi: 10.3389/fmicb.2021.713321.

4.1.1 Summary

In nature, cobamides are exclusively produced by a subset of prokaryotes. One of these prokaryotes is *Propionibacterium freudenreichii*, a species known for producing authentic cobalamins that are biologically active for humans. Utilizing the biosynthetic competence of *P. freudenreichii* in combination with chemical modifications, four specifically ^{15}N-labelled cobalamins were successfully prepared.

First, a CDM containing ammonium sulphate as a sole nitrogen source except three essential vitamins was developed and optimized for long-term stable growth of *P. freudenreichii*. Together with various incubation schemes, the medium was further optimized for cobalamin production in *P. freudenreichii*. With the application of the final optimized ^{15}N-CDM and incubation process, fully ^{15}N-labelled OHCbl and AdoCbl were obtained in *P. freudenreichii* with volumetric yields of 312 ± 29 µg/L and 635 ± 102 µg/L, respectively. In addition, specific partially ^{15}N-labelled cobalamins were produced with the employment of an optimized anaerobic process with DMB-supplemented ^{15}N-CDM and the corresponding volumetric yields obtained were 96 ± 18 µg/L and 990 ± 210 µg/L, respectively for [^{15}N, $^{14}N_2$]-OHCbl and [^{15}N, $^{14}N_2$]-AdoCbl. The labelled cobalamins were then extracted from bacteria and purified by SPE and semi-preparative HPLC. Cyanidation and methylation reactions were conducted to prepare [^{15}N]-CNCbl and [^{15}N]-MeCbl, respectively. The purified substances were characterized by HPLC-UV and LC-MS/MS. The results indicated that the labelled compounds have high chromatographic and isotopic purities and also have sufficient ^{15}N-enrichment for the differentiation from natural isotopologues during mass spectrometric measurements.

The labelled substances can be used for future applications requiring isotopically labelled standards such as SIDA. The alternative biosynthetic approach can be inspiring

for future studies addressing the preparation of other labelled metabolites with complex structures.

4.1.2 Author contribution

Mengle Wang conducted an extensive literature research and conceived the idea of using *P. freudenreichii* to prepare labelled cobalamin compounds. Mengle Wang further designed all experiment plans and performed all microbiological experiments. Moreover, Mengle Wang developed and optimized the bacterial extraction protocol and all analytical methods used in the study. Under the supervision of Mengle Wang, Jianqi Chen performed certain bacterial extraction and purification experiments. The data analysis and interpretation were mainly conducted by Mengle Wang. In addition, Mengle Wang wrote and revised the manuscript. Michael Rychlik, Stefan Asam and Matthias Ehrmann provided scientific support during the whole period of research and contributed to the revision of the manuscript.

4.2 Development of stable isotope dilution assays for the analysis of natural forms of vitamin B12 in meat

Published as: Wang, M., Asam, S., Chen, J., and Rychlik, M. (2021). Development of Stable Isotope Dilution Assays for the Analysis of Natural Forms of Vitamin B12 in Meat. *Journal of Agricultural and Food Chemistry* 69(36), 10722-10730. doi: 10.1021/acs.jafc.1c03803.

4.2.1 Summary

Reliable analytical methods for B12 analysis are lacking, especially ones addressing individual bioactive vitamers in complex matrices. SIDA, the highly advocated technique in food analysis, has not yet been applied to cobalamins, mainly restricted by the critical bottleneck of the unavailability of corresponding stable-isotope labelled internal standards. As this bottleneck has been cleared with the successful preparation of four ^{15}N-labelled cobalamins via biosynthesis, a multiple SIDA method was further developed for the simultaneous determination of OHCbl, AdoCbl, CNCbl and MeCbl in real foods.

First, sample preparation was optimized with enzymatic treatment to extract cobalamins from complex meat matrices. Papain and α-amylase, both from non-animal origins, were used in the extraction after confirmed to be analyte-free. Further purification and concentration were performed using specific immunoaffinity columns. Afterwards, the cobalamins were analyzed by LC-MS/MS and quantified using SIDAs. The developed method was thoroughly validated to assess the sensitivity, recovery, accuracy and reproducibility. For calibration of the SIDAs, response functions from quadratic regressions were used in molar ratios [n(analyte)/n(internal standard)] between 0.01 and 86.72 for OHCbl, between 0.01 and 96.15 for AdoCbl, between 0.01 and 115.61 for CNCbl, and between 0.01 and 121.88 for MeCbl. Utilizing a cobalamin-free soy flour sample as surrogate blank matrix, limits of detections ranging from 0.19 to 0.58 ng/g and limits of quantifications ranging from 0.68 to 1.73 ng/g were obtained. For all analytes, recoveries at three spiking levels in the surrogate matrix were in the range from 82% to 121%. Analyzing a pork sample, intra-day and inter-day precisions for all cobalamins were below 6% and 11% RSD, respectively. The total B12 content of a certified reference material (pork liver) quantified by the developed method resulted in a deviation < 1% from the certified value. The developed method was applied to analyze samples from common categories of meat. The predominant vitamers in all samples were AdoCbl and OHCbl. MeCbl was present in all samples but in lower concentrations.

CNCbl was found only in the pork fillet and chicken breast at trace levels. Therefore, the contribution of native CNCbl to total B12 content was negligible.

Altogether, the developed method was sensitive, accurate, reliable and robust for all investigated cobalamins. The application of the method in meat samples provided valuable quantitative information on the natural vitamer distributions in common types of meats. The developed method has great potential in future studies requiring differentiation of individual B12 vitamers.

4.2.2 Author contribution

Mengle Wang conducted the critical literature review, conceptualized the research, and designed the experiments. Mengle Wang performed the experiments of method development. The method validation was conducted by Mengle Wang and Jianqi Chen. Moreover, Mengle Wang analyzed all meat samples applying the developed method. Besides, data evaluation and interpretation were mainly conducted by Mengle Wang. In addition, Mengle Wang wrote and revised the manuscript. Michael Rychlik and Stefan Asam provided great support for the research and for the revision of the manuscript.

4.3 Development and validation of a conversion SIDA method using cyanidation for the determination of total B12 in meat

4.3.1 Sample preparation during method development

The total CNCbl contents determined by applying different sample preparation methods (details in section 3.3) are summarized in Table 4.1.

Table 4.1. Total CNCbl contents of the tested pork fillet sample applying different extraction protocols (details in section 3.3).

Extraction method	Total CNCbl content (ng/g)
A	13.1
B	24.1
C	27.2
D	29.9

4.3.2 Method validation

4.3.2.1 LOD, LOQ, recoveries and precisions

The validation results concerning sensitivity, reproducibility and accuracy of the conversion SIDA method are summarized in Table 4.2.

Table 4.2. Limit of detection, limit of quantification, recoveries and precision of the conversion SIDA method

Analyte	LOD (ng/g)	LOQ (ng/g)	Recovery (%)			Precision (% RSD)		
			Level 1 (n = 3)	Level 2 (n = 3)	Level 3 (n = 3)	Inter-injection (n = 6)	Intra-day (n = 4)	Inter-day (n = 3)
CNCbl	0.09*	0.29*	111 ± 8	107 ± 5	101 ± 2	2	2	4

Note: *, values determined using surrogate analyte [$^{15}N_{13}$]-CNCbl

4.3.2.2 Analysis of reference material BCR 487

The application of the conversion SIDA method on the certified reference material generated a total CNCbl content of 843 ± 13 ng/g (n = 4). The obtained LC-MS/MS chromatograms (see Figure 4.1) did not show any residual peaks resulting from unconverted cobalamins. The total B12 as CNCbl equivalent obtained by applying the native SIDA method was 907 ± 26 ng/g (n = 3).

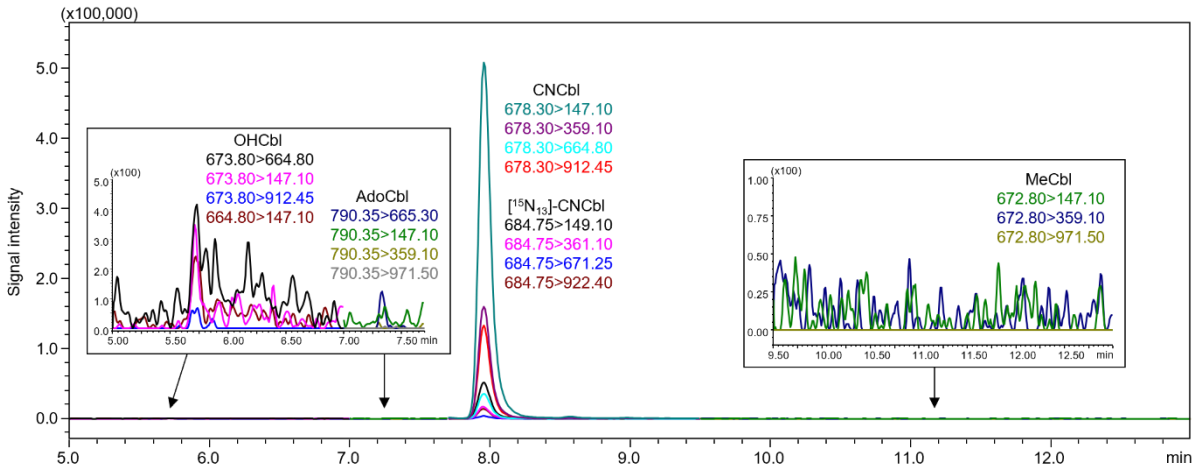


Figure 4.1. LC-MS/MS Chromatogram of the BCR 487 applying the conversion SIDA method.

4.3.3 Analysis of real meat samples

The total CNCbl contents of real meat samples applying the validated conversion SIDA method are shown in Table 4.3. The total B12 contents of the respective meat samples determined previously by the native SIDA method are displayed as well for reference. The LC-MS/MS chromatograms of the beef fillet sample and the lamb fillet samples (see Figure 4.2 as an example) showed peaks of unconverted OHCbl, AdoCbl and MeCbl, indicating incomplete conversion during sample preparation. No residual peaks of OHCbl, AdoCbl and MeCbl were observed on the LC-MS/MS chromatograms of the pork fillet and the chicken breast samples.

Table 4.3. Total CNCbl contents of meat samples determined applying the conversion SIDA method.

Sample	Total CNCbl by conversion SIDA method (ng/g)	Total B12 by native SIDA method* (ng/g)
Pork fillet	16.93 ± 0.25 (n = 4)	22.92 ± 1.33 (n = 12)
Beef fillet	21.91 ± 1.04 (n = 3)	30.45 ± 0.08 (n = 3)
Lamb fillet	52.66 ± 0.97 (n = 3)	71.79 ± 2.93 (n = 4)
Chicken breast	4.72 ± 0.48 (n = 3)	9.44 ± 1.04 (n = 4)

Note: *, total B12 calculated as CNCbl equivalent previously determined by applying the native SIDA method [180].

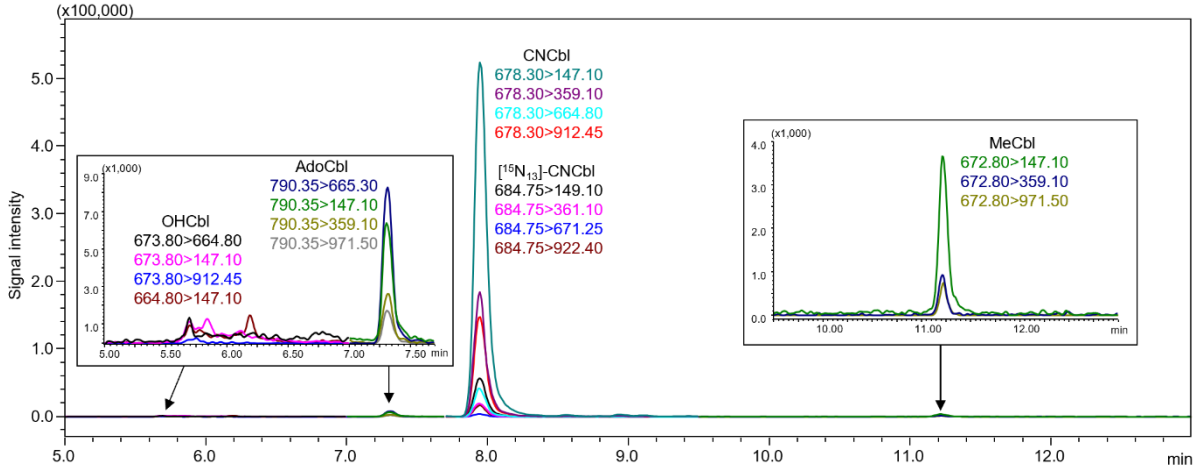


Figure 4.2. LC-MS/MS chromatogram of the lamb fillet sample applying the conversion SIDA method**.**

4.3.4 Conversion rate

Using the equation displayed in section 3.7, a conversion rate of 81 ± 2% (n = 3) was obtained. The LC-MS/MS chromatograms of the spiked pork fillet samples revealed presence of unconverted OHCbl, AdoCbl and MeCbl in the treated sample solutions.

5 Discussion

In the present thesis, a multi-SIDA method was pursued to simultaneously determine four cobalamins in foods. The development of SIDA encountered a great hurdle of poor commercial availability of the stable-isotope labelled internal standards. Overcoming the obstacle, ^{15}N-labelled cobalamin compounds were successfully obtained by smart utilization of biotechnological processes using *P. freudenreichii*. With the application of home-made ^{15}N-labelled cobalamins from biosynthesis, the first multiple SIDA method capable of quantitating individual vitamers was established. The sensitivity, accuracy, and robustness of the newly developed method were proven by the results from method validation. Application of the method to real meat samples further reflected the performance of the novel method and meanwhile provided valuable quantitative information on natural vitamer distribution. The multi-SIDA method analyzing native cobalamins was further compared with a conversion SIDA method that determines total B12 as CNCbl after cyanidation conversion. The application of native SIDA method demonstrated better reliability and suitability for B12 quantification in foods without the uncertainties in the cyanidation process. The obtained results are discussed in the following sections.

5.1 Biosynthesis of ^{15}N-cobalamins by *P. freudenreichii*

5.1.1 Development of chemically defined medium and cultivation schemes

To introduce labelling into the cobalamin structures via *de novo* biosynthesis, developing a suitable growth medium containing the target isotopes is a pre-requisite. Based on the chemical structures of cobalamins, it is feasible to label the compounds with different possibilities, for example with the commonly adopted isotopes of ^{13}C, ^{15}N or ^{2}H. Considering the numbers of each atom in the structures, labelling cobalamins with ^{15}N is a good choice since a full labelling (e.g., M+18 in the case of AdoCbl) will be adequate for avoiding isotopic overlaps during the future MS measurements but will not generate “too heavy” labelled analogues that might cause severe isotope effects. More importantly, according to the published nutritional requirements of *P. freudenreichii* (more details in section 1.5.3.2), it was rather promising to prepare fully ^{15}N-labelled cobalamins by growing *P. freudenreichii* in a CDM containing ^{15}N-ammonium as the sole nitrogen source. Therefore, the present thesis first tried to develop a CDM that meets the following criteria: i) the CDM contains ammonium as the sole nitrogen source except the

essential vitamins, ii) the CDM supports long-term stable growth of *P. freudenreichii* throughout consecutive transfers, iii) reasonable biomass of *P. freudenreichii* should be obtained when grown in the CDM, and iv) cobalamins are present in the bacterial cells grown in the CDM and ideally in high intracellular concentrations. The first two criteria are important for the final isotopic purity of the produced ^{15}N-cobalamins. Serial transfer in CDM contributes to diminishing carry-over of non-labelled nitrogenous compounds from the preculture complex medium. The other two criteria are crucial for the final yields of ^{15}N-labelled cobalamins via biosynthesis.

As the purpose of the CDM in the present thesis is very specific, relevant literature is scarce, and thus the development of the CDM had to go through a "try-and-error" process. First, a minimal defined medium (Medium M) was designed on the basis of defined media previously reported for the growth of *P. freudenreichii* with some modifications [183, 184]. After omission of amino acids and nucleotides, the resulted medium M contained sodium lactate as the carbon source, ammonium sulphate as the sole nitrogen source, mineral salts and eight vitamins, which met the first criterion mentioned earlier as well as the reported minimum nutritional requirements of *P. freudenreichii*. However, the bacteria failed to grow in the medium M after two generations and thus such a composition could not support a stable and long-term growth. Addition of some organic acids from TCA cycle, whose stimulating effects for the growth of Propionibacteria have been mentioned previously [185], solved this problem. The organic acids are non-nitrogenous and thus did not introduce undesirable non-labelled nitrogen into the medium. Subsequently after further omission of non-essential vitamins, CDMs satisfying the first two criteria were obtained and were further tested for cobalamin biosynthesis.

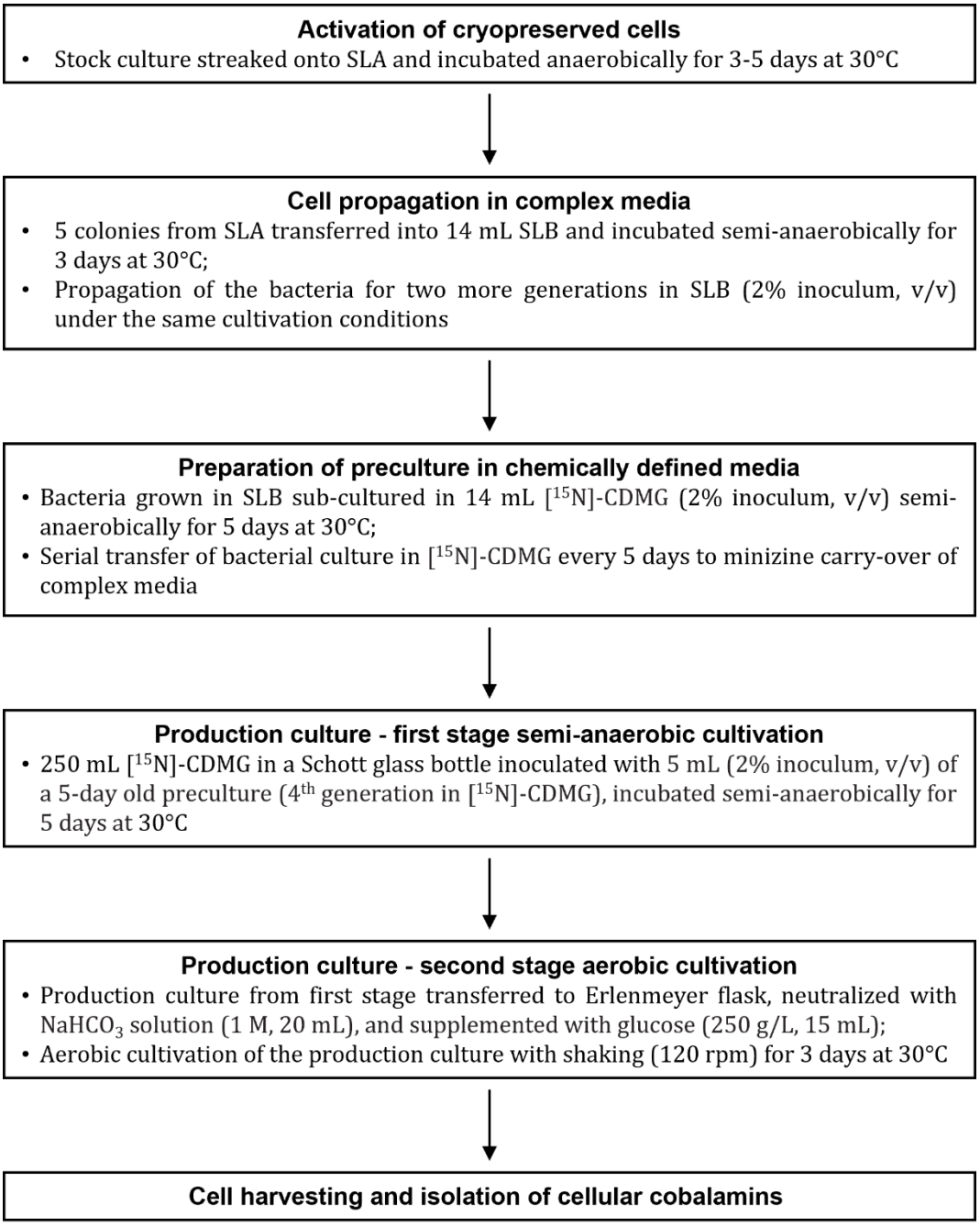
The biosynthesis of cobalamins in bacteria is not only influenced by the medium composition but also by the cultivation processes. Previous knowledge on medium and process optimization was mostly derived from studies using complex media and thus could not be easily transferred to the present thesis targeting at using a nutrient-limited CDM. Therefore, *P. freudenreichii* was grown under different cultivation schemes in the CDM to evaluate if the developed CDM could further support *in vivo* cobalamin biosynthesis. The cultivation processes were designed based on the published biosynthetic pathway of *P. freudenreichii* (details in section 1.5.3) and comprise of two

phases: i) anaerobic/semi-anaerobic phase for the accumulation of cobalamin intermediates lacking the lower ligand and ii) aerobic phase for the DMB formation and attachment. The bacterial cells from each cultivation were extracted and analyzed for the metabolic profiles with the focus on cobalamins and relevant intermediates.

HPLC-DAD analysis of the cell extracts revealed that under semi-anaerobic conditions, cobalamin precursors were found in bacterial cells with AdoCbi being the most abundant form. With an additional aerobic phase, the peak of AdoCbi diminished while the peak of AdoCbi-GDP increased. However, the cobalamin precursors were depleted instead of being further transformed into AdoCbl even with an extended period of 7-day aerobic cultivation. The presence of FMN and flavin adenine dinucleotide (FAD), which are the precursors for DMB in the oxygen-dependent way (section 1.5.2.1.4), excluded the possibility of a lack of available precursors for the lower ligand in the cells grown in the CDM. Subsequently, *P. freudenreichii* was cultured semi-anaerobically in the CDM with supplementation of DMB at the beginning or first supplied after 7-day of semi-anaerobic incubation. The corresponding cell extracts contained OHCbl and AdoCbl, revealing that *P. freudenreichii* was able to incorporate exogenously supplied DMB to form AdoCbl when cultured in CDM. Therefore, the reason for the incomplete cobalamin biosynthesis could be an insufficient supply of DMB needed for the last step and the latter probably as a result of an ineffective DMB transformation *in vivo*.

Despite that stable and continuous growth of *P. freudenreichii* was achieved in the CDM, low cell biomasses were obtained for all batch cultivations regardless of DMB supplementation. In addition, the pH values of the spent broth did not drop below 6.5, indicating that the limited cell growth was not caused by inhibition of produced acids and could be due to exhaustion of carbon sources. Moreover, the fact that cell biomasses and pH values of DMB-supplemented and non-supplemented cultures were similar, suggesting that the formation of intact cobalamins did not contribute to enhancing the growth of *P. freudenreichii* in CDM. By providing additional carbon source (10 g/L of glucose) to CDM (CDMG), the growth of *P. freudenreichii* was significantly enhanced, which confirmed the previous assumption. However, the precursors AdoCbi and AdoCbi-GDP were still present in high levels after a 5-day anaerobic and 3-day aerobic incubation.

Previous studies reported that the optimum pH for *P. freudenreichii* is between 6-7 [161, 186, 187] and growth decreases significantly below pH 5.5 and ceases below pH 5 [188]. Due to the high amounts of acids produced during fermentation, periodical or continuous adjustment of pH by neutralization is often employed for industrial production of B12 using *P. freudenreichii* [46]. In the present thesis, the culture pH dropped to 5.5 after a 5-day semi-anaerobic incubation in the CDMG. The low pH could affect the transformation of precursors to AdoCbl during the aerobic phase. Therefore, a neutralization step was introduced after the 5-day semi-anaerobic phase and additional glucose was supplied to the culture for providing more energy for cellular metabolic activities. With this optimized incubation scheme, *P. freudenreichii* was able to grow in the CDMG to a biomass of 13.7 g/L after a 5-day anaerobic and 3-day aerobic cultivation. Moreover, the majority of precursors were successfully converted into cobalamins. Therefore, the developed CDMG together with the optimized cultivation scheme (scheme I) could fulfill the four criteria listed earlier for the ^{15}N-labelling purpose.



Scheme I. Cultivation process for preparing fully ^{15}N-labelled cobalamins using *P. freudenreichii*. SLA, sodium lactate agar; SLB, sodium lactate broth; CDMG, chemically defined medium supplemented with glucose.

5.1.2 Strategy for specific partial labelling of cobalamins

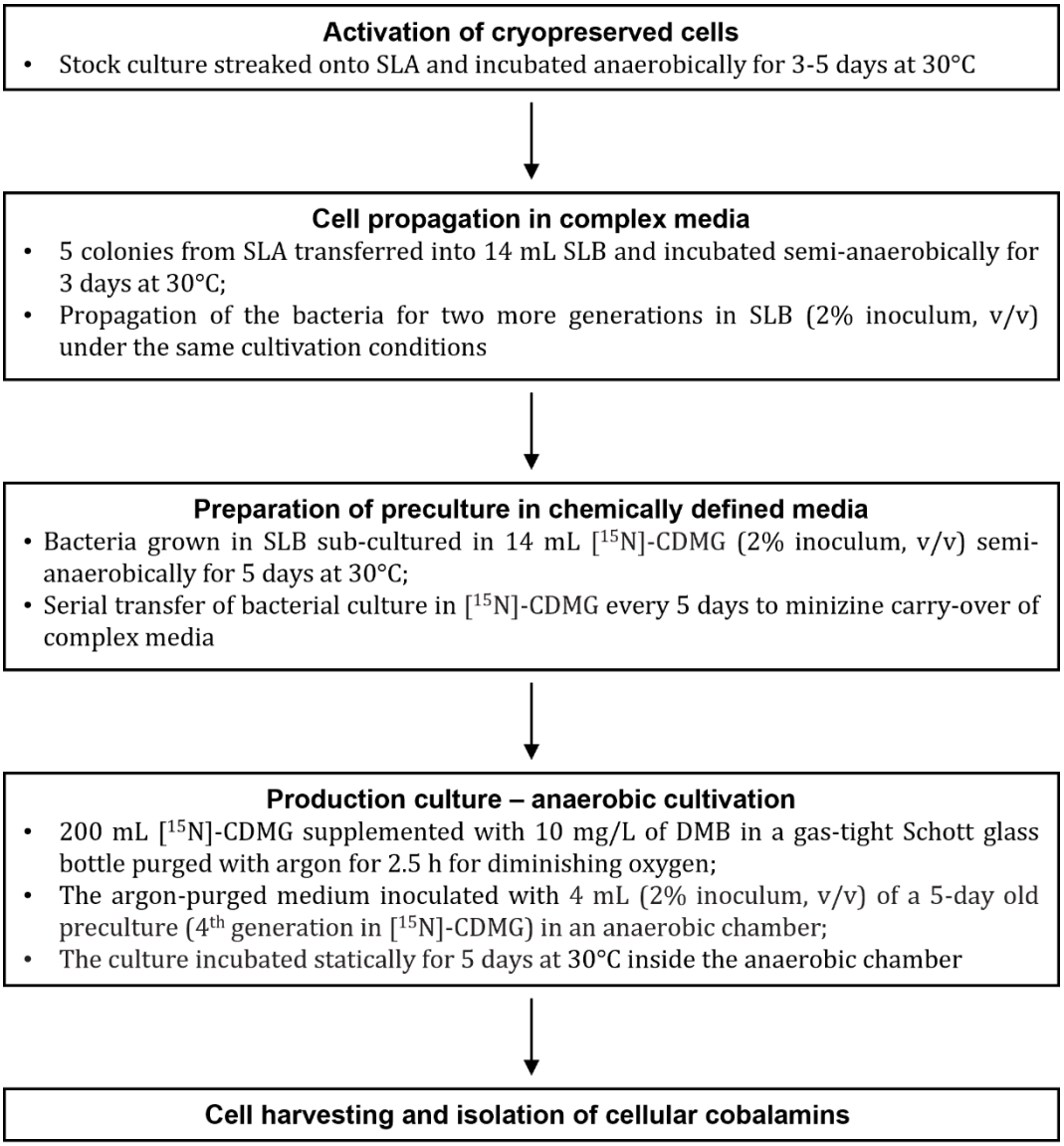
The application of CDMG with the optimized two-phase cultivation process (scheme I) was intended to prepare fully ^{15}N-labelled cobalamins. However, ideally the labelling pattern for ^{15}N-OHCbl should be different from the other labelled cobalamins. Apart from being a naturally occurring vitamer, OHCbl also can be the degradation product from other cobalamin forms. When handling cobalamins, such a degradation is

unavoidable. Therefore, the use of fully ^{15}N-labelled OHCbl as internal standard could be problematic due to the inevitable degradation of other fully ^{15}N-labelled internal standards. To address this issue, a dual isotope design should be adopted for OHCbl quantification by using a labelled internal standard that can be clearly distinguished by LC-MS/MS from the fully labelled degradation product. Therefore, the cultivation scheme was further revised to produce specifically labelled [^{15}N, $^{14}N_2$-DMB]-OHCbl that possesses an unlabelled lower ligand.

To prepare [^{15}N, $^{14}N_2$-DMB]-OHCbl in high isotope purity, the incubation scheme should be modified so that *P. freudenreichii* uses unlabelled DMB from the media rather than synthesizing the labelled one *in vivo*. For this purpose, incubating *P. freudenreichii* in strictly anaerobic conditions would be an effective approach to block the *in vivo* DMB formation. In the present thesis, it was observed that growth of *P. freudenreichii* in CDMG under strictly anaerobic conditions was rather limited and eventually ceased after three generations when no exogenous DMB was supplied. This phenomenon is expected as the synthesis of intact cobalamins is obstructed in the absence of trace oxygen under the strictly anaerobic conditions. The obstructed cobalamin synthesis might be responsible for the diminished bacterial growth and *P. freudenreichii* ceases growth as soon as the cobalamin carry-over from previous generations is depleted. Therefore, supplementation of DMB is necessary for continuous cell growth and effective cobalamin biosynthesis under the strictly anaerobic conditions. However, supplementing DMB into the inoculum media is not preferable for the labelling purpose as the unlabelled nitrogen from DMB might be incorporated into the corrin ring structure in the later batch cultivations via unknown metabolic pathways.

Eventually, a cultivation scheme was developed as the result of a compromise between isotopic purity and cobalamin productivity. The preparation of preculture was performed under semi-anaerobic conditions using CDMG to ensure a good growth of *P. freudenreichii*. The following anaerobic batch cultivation was conducted using DMB-supplemented CDMG. The batch media was previously purged with argon and stored in the anaerobic chamber to eliminate dissolved oxygen that can be used for in *vivo* DMB biosynthesis and the whole inoculation and incubation process was performed in the anaerobic chamber to minimize oxygen exposure. The batch incubation time was shortened to 5 days in order to lower the risk on utilization of DMB by other metabolic pathways than cobalamin biosynthesis. With this optimized cultivation scheme (scheme

II), an acceptable cell biomass of 11.70 g/L was eventually obtained with pH of the culture dropping to 5.6 after incubation. More importantly, the majority of cobalamin intermediates were transformed into cobalamins in the harvested cells.



Scheme II. Cultivation process for preparing [^{15}N, $^{14}N_2$-DMB]-cobalamins using *P. freudenreichii*. SLA, sodium lactate agar; SLB, sodium lactate broth; CDMG, chemically defined medium supplemented with glucose; DMB, 5,6-dimethylbenzimidazole.

5.1.3 Analysis of cobalamin related metabolites in bacterial cells

In the present thesis, suitable analytical approaches were required to analyze cobalamin related metabolites in the bacterial cells. The information of cobalamin intermediates was essential for assessing the status of B12 biosynthesis in cells grown in different media or under different cultivation conditions. Due to the complex biosynthetic

pathway of B12, a wide range of cobalamin relevant intermediates could be potentially present in the harvested cells. Many of these compounds have similar structures and must be distinguished from one another by selective and specific methods. In the present thesis, these compounds were analyzed employing a combination of different analytical techniques. To obtain the comprehensive profiles of cobalamin relevant compounds, the cell extracts were analyzed by LC coupled with DAD and quadrupole time-of-fight mass spectrometry (QTOF-MS). The LC conditions were optimized to separate metabolites of interest to a large extent. The peaks corresponding to the cobalamin intermediates could be easily noticed due to their unique UV-Vis absorption patterns derived from corrin-containing structures. The characteristic peaks on MS and MS/MS spectra further provided insightful structural information. In terms of sample preparation, bacterial cells were boiled in an extraction buffer and further cleaned up by SPE using C18 columns. For analytes with commercial primary standards being available, which are FAD, FMN, riboflavin and cobalamins in the present thesis, peaks of these analytes from bacterial cells were compared to the peaks of respective reference compounds for further confirmation. The cobalamins in the cell extracts were additionally measured by LC-ESI-MS/MS monitoring specific mass transitions for checking the authenticity. In order to gain knowledge on cellular contents of crucial intermediates such as AdoCbi and AdoCbi-GDP, a HPLC-DAD method was further developed. As the detection of the method relies on the inherent chromophores of the analytes, changes on peak intensities of the targeted analytes could effectively reflect the changes on cellular concentrations of the analytes. The developed HPLC-DAD method achieved separation between different cobalamin forms and was also used for quantification of cellular yields of the produced cobalamins with external calibration.

5.1.4 Production, purification, and characterization of ^{15}N-labelled cobalamins

Ammonium sulphate in the CDMG was replaced by $^{15}N_2$-ammonium sulphate to form [^{15}N]-CDMG for the labelling experiments. To prepare fully ^{15}N-labelled cobalamins, *P. freudenreichii* was cultivated in [^{15}N]-CDMG following the incubation scheme I. Afterwards, [^{15}N]-OHCbl was obtained with volumetric and intracellular yields of 312 ± 29 µg/L and 24.0 ± 2.0 µg/g, respectively. The volumetric and intracellular yields for [^{15}N]-AdoCbl were 635 ± 102 µg/L and 48.7 ± 5.9 µg/g, respectively. To produce partially ^{15}N-labelled cobalamins ([^{15}N, $^{14}N_2$-DMB]-cobalamins), *P. freudenreichii* was grown in the DMB-supplemented [^{15}N]-CDMG following the optimized scheme II. In the

harvested cells, 7.4 ± 1.5 µg/g of [^{15}N, $^{14}N_2$-DMB]-OHCbl and 77 ± 15 µg/g of [^{15}N, $^{14}N_2$-DMB]-AdoCbl were obtained. These values corresponded to volumetric yields of 96 ± 18 µg/L and 990 ± 210 µg/L for [^{15}N, $^{14}N_2$]-OHCbl and [^{15}N, $^{14}N_2$]-AdoCbl, respectively.

Despite that *P. freudenreichii* was cultured in the nutrient-limited CDMG in the present thesis, the finally obtained yields of cobalamins were in the same order of magnitude as values from *P. freudenreichii* grown in complex media under laboratory conditions [163, 169, 170]. As a proof of concept, the present thesis only tested the most straightforward cultivation schemes to produce ^{15}N-labelled cobalamins in a simple way. Thus, there is great potential in further improving yields of [^{15}N]-cobalamins by applying various biotechnological strategies. One simple and probably highly effective approach would be employing *P. freudenreichii* strains that naturally produce higher amounts of cobalamins [163]. Further optimization on media composition could be another straightforward alternative. In addition, a more sophisticated process control strategy during the whole cultivation period, such as continuous pH adjustment and oxygen level control, could be another direction as their effectiveness has been previously demonstrated [187, 189]. Furthermore, various metabolic and genetic engineering technologies previously adopted for the general B12 production (details in section 1.5.3.4) could as well be applied for the preparation of ^{15}N-labelled cobalamins in the future.

Considering the low chemical stability of cobalamins, the biosynthesized ^{15}N-labelled cobalamins were kept in the harvested cells at -20 °C for long-term storage. For further preparation of labelled compounds, ^{15}N-labelled cobalamins were extracted from a portion of the cells. The [^{15}N]-AdoCbl was isolated directly by semi-preparative HPLC from cell extracts after SPE clean-up. The preparation of [^{15}N]-CNCbl involved additional steps for cyanidation after the SPE elution. The [^{15}N]-MeCbl was prepared from isolated [^{15}N]-AdoCbl via chemical modifications. Both [^{15}N]-CNCbl and [^{15}N]-MeCbl were further isolated using semi-preparative HPLC. Regarding the [^{15}N,$^{14}N_2$]-OHCbl, a portion of cells from DMB-supplemented [^{15}N]-CDMG was extracted and cleaned up by SPE. The eluant was exposed to light to induce conversion of [^{15}N]-cobalamins into the hydroxyl-form via photodegradation. The final product was as well isolated from the light-treated sample solution using semi-preparative HPLC.

The characterisation of the prepared labelled compounds was performed using HPLC-DAD and LC-ESI-MS/MS. In general, the results showed that the labelled cobalamins were obtained in high chromatographic and isotopic purity with anticipated labelling patterns and thus are perfectly suitable for the future SIDA applications. Taking the isolated [^{15}N]-AdoCbl as an example, the chromatogram from HPLC-DAD analysis revealed a sole major peak which was eluted at the same retention time as the unlabelled AdoCbl. The UV-Vis spectrum of the isolated [^{15}N]-AdoCbl was identical to that of the unlabelled standard. The mass spectrometric analysis of the isolated [^{15}N]-AdoCbl revealed a base peak of *m/z* 799.30 in double charged form. In comparison to the base peak of *m/z* 790.30 ([M+2H]$^{2+}$) for the unlabelled AdoCbl, a mass increment of M+18 (Δ *m/z* = + 9 in double charged forms) was obtained for the labelled compound, confirming that the majority of [^{15}N]-AdoCbl was fully labelled [$^{15}N_{18}$]-AdoCbl. In addition, no spectral overlap between ^{15}N-labelled and unlabelled compounds was observed and no trace of unlabelled AdoCbl was detected in the isolated [^{15}N]-AdoCbl. These results revealed that the serial transfer process in [^{15}N]-CDMG was effective for diminishing carry-over from the complex media. Furthermore, the prepared [^{15}N]-AdoCbl was analysed by LC-MS/MS monitoring characteristic mass transitions involving the ^{15}N-labels for [$^{15}N_{18}$]-AdoCbl. Apart from that a peak corresponding to [$^{15}N_{18}$]-AdoCbl was observed at the expected retention time on the chromatogram, intensity ratios between different mass transitions involving the ^{15}N-labels from [$^{15}N_{18}$]-AdoCbl were comparable to those of unlabelled transitions from AdoCbl. The intensity ratios between different transitions are considered as an important quality control criterion for unequivocal identification in MS/MS. Similar results concerning chromatographic and isotopic purities were obtained for [^{15}N]-CNCbl and [^{15}N]-MeCbl.

The [^{15}N, $^{14}N_2$-DMB]-OHCbl prepared from the cultivation scheme II was as well characterized using HPLC-DAD and LC-MS/MS. The HPLC-DAD analysis confirmed the chromatographic purity of the labelled compound. The MS analysis demonstrated a base peak shift from *m/z* 673.80 ([M+2H]$^{2+}$) to *m/z* 679.30 (Δ *m/z* = +5.5 in double charged forms), indicating a mass increment of M+11 in the labelled compound. The mass increment was in agreement with the anticipated labelling pattern that two nitrogen atoms in the lower ligand DMB were not labelled. The labelling pattern was further confirmed by the peak corresponding to [$^{15}N_{11}$, $^{14}N_2$-DMB]-OHCbl in the obtained LC-MS/MS chromatogram. Due to the sufficient mass increment, the labelled compound

could be separated from the natural isotopologues of OHCbl. In addition, no residual unlabelled OHCbl was detected in the labelled compound. Taken together, the modified biosynthetic strategy successfully yielded specifically labelled [$^{15}N_{11}$, $^{14}N_2$]-OHCbl of high purity. The prepared [$^{15}N_{11}$, $^{14}N_2$]-OHCbl could be differentiated from fully ^{15}N-labelled OHCbl degraded from the other labelled cobalamins when used as internal standard in further SIDA applications. Since the degradation product, i.e. the fully ^{15}N-labelled [$^{15}N_{13}$]-OHCbl, has mass increments starting from M+13, there are no mass interferences from the isotopologues of the degradation product on the monoisotopologue of the [$^{15}N_{11}$, $^{14}N_2$]-OHCbl, which has a mass increment of M+11.

5.2 Development of stable isotope dilution assays for cobalamins in meats

The success on the preparation of individual ^{15}N-labelled cobalamins enabled further development of the first multi-SIDA method for analyzing natural cobalamins. The multi-SIDA method was first established and validated based on meat matrices as meat samples are known for containing all four cobalamins in trace amounts.

5.2.1 Method development

Previously reported LC-MS(/MS) methods for B12 analysis in foods were rather limited and rarely targeted at simultaneous analysis of the native vitamers [105-108, 177, 190]. In the present thesis, several C18-based columns were first evaluated using common mobile phases with or without MS compatible additives including formic acid, acetic acid, ammonium formate and ammonium acetate. It was clearly observed that ion suppression occurred to different degrees when any of the modifiers was present in the mobile phases and the worst cases were seen in the presence of ammonium. However, despite that ion suppression is undesirable and might cause adverse effects on the final sensitivity of the method, proper buffering was found to be crucial to maintain reproducible chromatograms with stable retention times and peak shapes. The present thesis eventually applied a YMC hydrosphere column with mobile phases comprising of water acidified with 0.1% acetic acid and pure methanol. Upon gradient optimization, baseline separation of the four cobalamins was achieved. The targeted peaks demonstrated satisfactory intensities and reproducibility. Moreover, the analytes could be resolved from interfering compounds with the optimized chromatographic conditions when analyzing real samples.

Together with the chromatographic conditions, the MS parameters were optimized for the analysis of the four cobalamins. The soft ionization of the cobalamins in the positive ESI mode generated a wide range of ion species including $[M+H]^+$, $[M+Na]^+$, $[M+2H]^{2+}$, $[M+H+Na]^{2+}$, $[M+3H]^{3+}$ and some other solvent and metal adducts. This phenomenon could be expected considering the multiple nitrogen atoms in the cobalamin structures that can be ionized. The most dominant ion species, $[M+2H]^{2+}$ in the cases of AdoCbl, CNCbl, MeCbl and $[M-OH+H]^{2+}$ in the case of OHCbl, were selected as precursors for the MS/MS fragmentation. The $[M-OH+H]^{2+}$-ion of OHCbl was formed after the loss of the characteristic upper ligand as the result of ion-source fragmentation. To ensure specificity and unequivocal identification, the $[M+2H]^{2+}$-ion of OHCbl was additionally

selected as precursor. In the MS/MS experiments, the fragment ion of $[DMB+H]^+$ derived from the lower ligand was observed for all selected precursors. In addition, a range of characteristic fragments of cobalamins that either derived from the nucleotide part or the corrin-containing core were obtained, similar to the results of previous mass spectrometric studies [94, 105, 177, 190-192].

The multiple reaction monitoring (MRM) mode was used for further identification and quantification purposes. For each analyte, at least three MRM transitions were monitored to ensure unambiguous determination. In the preliminary experiments, the matrix interferences from the real samples were monitored for all the unlabelled and labelled transitions. The quantitative MRM transitions were then chosen taking the specificity and intensity of the transitions as well as the potential matrix interferences from real samples into consideration. For CNCbl and MeCbl, the most characteristic transitions of $[M+2H]^{2+}$ to $[DMB+H]^+$ were adopted as they were the most specific and intense transitions and were not affected by matrix interference. For AdoCbl, the paired transition of $[M+2H]^{2+}$ to $[M-Ado+H]^{2+}$ served the purpose. When it comes to OHCbl, a compromised choice of transition of $[M+2H]^{2+}$ to $[M-OH+H]^{2+}$ was used for quantitation as all the transitions involving the characteristic fragment $[DMB+H]^+$ witnessed severe matrix interferences when analyzing real samples. The source parameters were accordingly optimized to decrease in-source fragmentation so that higher intensity of $[M+2H]^{2+}$ to $[M-OH+H]^{2+}$ for OHCbl could be achieved.

The ^{15}N-labelled internal standards prepared and used in the present thesis have mass shifts of M+11 in the case of $[^{15}N_{11},^{14}N_2$-DMB]-OHCbl, M+18 in the case of $[^{15}N_{18}]$-AdoCbl, and M+13 in the cases of $[^{15}N_{13}]$-CNCbl and $[^{15}N_{13}]$-MeCbl. As the relative abundances of natural isotopologues with mass increments higher than M+5 were negligible for all cobalamins (< 0.2%), there were no obvious spectral overlaps between unlabelled analytes and labelled internal standards. The baseline separation of the analytes avoided spectral overlaps of natural isotopologues from different cobalamins. By applying the dual isotope design for OHCbl, the internal standard ($[^{15}N_{11},^{14}N_2$-DMB]-OHCbl) could be successfully distinguished by the MS from the degradation product ($[^{15}N_{13}]$-OHCbl) of other labelled standards, ensuring the correctness of the quantitation. The degradation product $[^{15}N_{13}]$-OHCbl from other fully labelled cobalamins was observed during the method development by monitoring the corresponding MRM transitions, revealing the necessity of applying the dual isotope design.

5.2.2 Sample preparation

In the present thesis, enzymatic treatment was applied to release bound cobalamins during sample preparation. In the literature, pepsin was commonly used for this purpose [63, 64, 69, 105, 107, 113]. However, the highly sensitive LC-MS/MS system employed in the present thesis detected trace levels of cobalamins in the pepsin, mainly in the form of CNCbl. Therefore, quantification of low levels of cobalamins in food samples was not possible with the application of pepsin. Since animal-derived enzymes always have the potential problem of containing traces of endogenous cobalamins, plant-based alternatives were considered. Papain, after tested to be free of cobalamins, was eventually used for the extraction. The application of α-amylase was important for treating carbohydrate-rich food matrices [69]. Although meat samples were the main target of the present investigation, the sample preparation here also included α-amylase in order to establish a universal method that can be further applied to a wide range of food matrices in the future. The amounts of enzymes as well as the incubation temperature and duration were further optimized to ensure a complete and time-efficient extraction. The application of enzymatic treatment and further paper filtration also contributed to a good speed of sample loading on the immunoaffinity columns.

The necessity and superiority of IAC for CNCbl purification from complex food matrices have been previously reported for the HPLC-UV based methods [63, 64]. In the present thesis, immunoaffinity purification was also found necessary to enrich low levels of native cobalamins in meat samples while effectively removing interfering compounds, despite the use of the highly sensitive LC-MS/MS. A selection of conventional SPE columns were as well tested for the clean-up purpose. However, none of them could deliver comparable performance to the immunoaffinity columns. The application of IAC achieved highly selective enrichment of all investigated cobalamins and resulted in lower baselines and cleaner backgrounds in the LC-MS/MS chromatograms. In spite of the high cost, the immunoaffinity columns remained to be indispensable for effective purification and enrichment of cobalamins from complex food matrices.

As the novel method aimed at determining naturally occurring vitamers in their original forms, the undesirable use of cyanide to convert all cobalamins into CNCbl was avoided during the sample extraction. However, the immunoaffinity column was tailor-made for CNCbl and demonstrated different affinities towards other cobalamin forms [69]. In the

literature [69], when a mixture of OHCbl and CNCbl was loaded to the immunoaffinity column, the recovery of OHCbl dropped from 75% to 45% while the recovery of CNCbl remained at a high level of 90%. A similar phenomenon was also observed in the present thesis. The binding of CNCbl was more stable and reproducible, revealed by a better reproducibility of absolute peak areas of CNCbl between replicate extractions than those of other cobalamins. Moreover, the binding of cobalamins in lower concentrations was affected by the co-existence of other forms present at higher levels of a different order of magnitude, even though the total loading amounts never exceeded the capacity of the immunoaffinity column (1 µg according to the manufacturer). However, as an exception, the binding of CNCbl in lower concentrations was not influenced due to the better interaction with the antibody. These results demonstrated the discrimination effects of the IAC between cobalamin forms. In the present thesis, different losses of analytes caused by the discrimination during the immunoaffinity purification could be compensated by the application of ^{15}N-labelled internal standards. However, when severe discrimination occurs and neither the analyte nor the labelled internal standards are captured by the antibody, false results will be obtained despite of the application of SIDA. Therefore, multiplicate analysis (n = 4) was performed for the real samples and quantitative results were only calculated from the measurements showing peaks of all four internal standards.

5.2.3 Concentration determination of cobalamin standards

The quantification of SIDA relies on correct concentrations of the added internal standards to calculate the concentrations of the analytes. Due to the chemical instability of the cobalamins, the concentrations of ^{15}N-labelled internal standards used for spiking need to be determined routinely for each analysis. For this purpose, a three-step protocol was developed in the present thesis for routine quantification of the standard solutions. First, CNCbl, the most stable form, was chosen as reference standard and quantified using UV spectroscopy at its λ_{max} of 361 nm. The molar extinction coefficient of CNCbl needed for calculation was obtained experimentally using a freshly prepared CNCbl solution, whose concentration was aforehand quantified by quantitative nuclear magnetic resonance (q-NMR). Then, a mixed standard solution of the four cobalamins was measured by HPLC-DAD and the concentrations of OHCbl, AdoCbl and MeCbl could be determined using CNCbl as internal standard. The concentration of CNCbl was known from the previous UV measurements. To guarantee the accuracy of the HPLC-DAD

method, fresh stock solutions of all four vitamers were as well quantified by q-NMR before used for establishing the HPLC-DAD response curves. In the third step, a calibrator solution composed of the four cobalamins and their respective ^{15}N-labelled standards was measured on LC-MS/MS. With the known concentrations of unlabelled standards quantified in the first two steps, the accurate concentrations of ^{15}N-cobalamins could be calculated based on the LC-MS/MS response curves.

The developed protocol for determining concentrations of the standards is comprehensive and necessary. Commercial primary standards of cobalamins suffer from stability issue and thus should be checked for their purity every time rather than relying on the gravimetric measurements and the purity declarations. Moreover, inter-conversion between cobalamin forms could generate errors on quantification if standard solutions of individual cobalamins are measured separately. The novel approach particularly measured mixed standard solutions of four cobalamins to compensate for the potential conversion of AdoCbl and MeCbl into OHCbl. The developed UV spectroscopic method and HPLC-DAD method were also used for the quantification of each ^{15}N-labelled cobalamin standard before they were used for the establishment of the LC-MS/MS response curves. The low concentrations of ^{15}N-labelled cobalamin solutions were not suitable for quantifications by q-NMR.

5.2.4 Calibration and quantification of SIDA

For preparing the response curves of SIDA, varying amounts of analytes (A) were mixed with constant amounts of respective ^{15}N-labelled internal standards (IS) in the molar ratio [n(A)/n(IS)] range of 0.01 to 100. The response curves were obtained by plotting the peak area ratios [A(A)/A(IS)] against the molar ratios [n(A)/n(IS)] from LC-MS/MS measurements. The Mandel's fitting test [182] was used to check non-linearity of linear regression models. In the prepared molar ratio ranges for all analytes, the Mandel's fitting test identified non-linearity and indicated that quadratic regression models were better suited than the linear ones. When a broad calibration range is used, non-linearity is common for response curves based on SIDA as the perfect scenario featured by absolute absence of spectral overlaps between unlabeled analytes and labelled standards is hardly achievable [110]. In the present thesis, it was possible to narrow down the calibration ranges to get the restricted linear regions for all cobalamins. However, considering the varying contents of cobalamins in food samples, it would be preferable

to keep the method as universal as possible for future measurements. Therefore, response functions were eventually obtained using quadratic regression to keep the large calibration ranges. Similar approaches have been demonstrated previously [110, 193, 194]. For the quantification of samples, the functions were mathematically resolved for n(A). With the application of SIDA, the loss and discrimination of analytes during preparation and analysis were compensated for.

5.2.5 Method performance

Prior to real sample analysis, the reliability and the accuracy of the novel method need to be proven by method validation. In the present thesis, a soy flour sample served as surrogate blank matrix for the method validation as animal-derived products always contain traces of cobalamins. The LODs and LOQs of the cobalamins were determined according to Vogelgesang and Hädrich [109]. The LODs of 0.19 to 0.58 ng/g and the LOQs of 0.68 to 1.73 ng/g were obtained for the four cobalamins in the soy flour. Previous validation data on OHCbl, AdoCbl and MeCbl is scarce. Compared to estimated values in the literature [108, 114], the LODs and LOQs of OHCbl, AdoCbl and MeCbl obtained in the present thesis were the lowest so far reported. Regarding the determination of CNCbl, the sensitivity of the novel method was about 11 times higher than an earlier reported LC-MS method for meat and was at least 26 times higher than those previously reported using HPLC with UV or fluorescence detection [63-65, 69, 113]. Besides LODs and LOQs, the recoveries were determined for the cobalamins at three spiking levels in the soy flour. Good and reproducible recoveries of 82-121% were obtained for all analytes. Compared to the literature in which recoveries of 52-68% were obtained for OHCbl, AdoCbl and MeCbl, the application of SIDA in the present thesis clearly improved the accuracy of quantification to a superior level. A pork sample containing the four cobalamins was used for determining various precisions. For all analytes, the inter-injection and intra-day precisions were below 4% and 6%, respectively. The inter-day precisions were in a slightly higher range of 6-11%. The highest RSD of 11% was obtained for CNCbl, whose concentration was the lowest among all analytes in the pork sample. Overall, the various precisions were satisfactory and demonstrated a good reproducibility of the novel method.

The accuracy of the method was further specified by analyzing a certified reference material of pig liver (BCR 487). The total B12 content (1116 ± 14 ng/g calculated as

CNCbl equivalent) determined by the novel method was in agreement with the certified reference value (1120 ± 90 ng/g of CNCbl), demonstrating the outstanding accuracy of the current method. Previously, Chamlagain et al. [65] analyzed the BCR 487 using cyanidation conversion for sample preparation and using HPLC-UV for analysis. They obtained a lower total B12 content of 876 ± 49 ng/g and suggested that the discrepancy between the analyzed and certified values might be attributed to the presence of cobalamin analogues in the reference material. In the present thesis, the application of SIDA ensured the specificity of quantification, which excluded potential influences from analogues or co-eluting compounds. With the confirmation of presence of different cobalamin forms in the reference material by the current method, the lower content reported previously could result from an insufficient conversion of other vitamers into CNCbl during the sample preparation. Since the cyanidation method has been the state of the art for B12 analysis in the recent years, the fact that the completeness of cyanidation conversion is not monitored in routine analysis is rather concerning. Clearly, a complete conversion is a pre-requisite for accurate determination of total B12 content if cyanidation is applied. The developed multi-method measures native cobalamins without cyanidation and thus avoids inaccuracy that might stem from incomplete conversion.

In general, the novel multi-SIDA method demonstrated excellent performance for all four cobalamins. It was the first time that the major cobalamin vitamers in non-fortified foods could be simultaneously quantified in an accurate manner. Applying the novel method, the lowest LOD and LOQ and the best recoveries were obtained for CNCbl. The better performance of the developed method for CNCbl might be attributed to the discrepancies on the bindings of the immunoaffinity columns towards different forms. Further method optimization appeared to be largely restricted by the binding affinity of the current immunoaffinity columns. If the discrimination effects of the binding during immunoaffinity purification could be further eliminated, for example by combining different antibodies tailor-made for different cobalamin forms, the performance of the current method can be further improved.

5.2.6 Analysis of meat samples

The developed SIDA method was applied to analyze cobalamins in common categories of meats including chicken, pork, beef and lamb. The total B12 contents were further

calculated as CNCbl equivalent from contents of investigated cobalamin forms. Compared to values reported in the literature [195], the total B12 contents in meat samples determined using the novel method were in the same order of magnitude. The variances between analyzed contents and literature values within the same category of meat could be caused by various factors including differences on analytical methods, the cuts of meat, and conditions of farming practices. Among all analyzed meat samples, the highest amount of total B12 was found in the lamb fillet, followed by the contents in beef fillet and pork fillet, whereas the chicken breast contained the lowest amount. In the literature [195, 196], similar observations were described that meats from ruminant animals generally contain higher amounts of B12 than meats of monogastric animals due to the higher bacterial activities synthesizing B12 in the rumens. Regarding contents of native cobalamin vitamers in meats, literature values were scarce. Szterk et al. [108] analyzed the four cobalamins in beef liver and beef sirloin using a LC-MS method and reported that OHCbl and AdoCbl were the dominant forms in both samples. However, the recoveries of their method for OHCbl, AdoCbl and MeCbl (52-68%) were too low for accurate quantitation and the method was not sensitive enough to detect the lower levels of MeCbl and CNCbl in beef sirloin. With the novel multi-SIDA method, native vitamer distributions in common meat types were determined quantitatively for the first time in the present thesis. The major forms found in all meats as well as in the reference liver sample (BCR 487) were AdoCbl and OHCbl. MeCbl was detected in lower concentrations in all investigated samples and CNCbl was only present in trace levels in the pork fillet and chicken breast. In this case, the contribution of native CNCbl to the total B12 contents in meats was negligible. The successful application of the novel method in meats provided a comprehensive proof of concept for future application of the SIDA method on different food matrices to verify unconventional sources of dietary B12.

5.3 Method Comparison between conversion and native SIDA method

The fact that cyanidation conversion has been widely applied for total B12 quantification in foods without properly monitoring the completeness of conversion is rather concerning. In the last part of the thesis, a conversion SIDA method (hereafter referred as conversion method) determining total B12 as CNCbl after cyanidation was developed and validated. The conversion method was further compared with the previously developed multi-SIDA method (hereafter referred as native method) for their reliability and suitability for B12 analysis in foods. The following sections discuss the obtained results (see section 4.3) that have not yet been published.

5.3.1 Method development of the conversion method

Aiming at achieving complete conversions of all cobalamins into CNCbl, the sample preparation of the conversion method was developed based on the procedures of the native method. The instrumental parameters of the native method were transferred to the conversion method for the LC-MS/MS measurements. The concentration determination of standard solutions for the conversion method was carried out following the same way as that of the native method. A pork fillet sample was used for the optimization of the extraction. The conversion of cobalamins from different extractions were monitored by determining the contents of total B12 in the pork fillet sample applying SIDA as well as observing the peaks of unconverted OHCbl, AdoCbl and MeCbl on the LC-MS/MS chromatograms. The conversion was considered complete if the following two criteria were both met: i) there were no residual peaks of OHCbl, AdoCbl and MeCbl on the LC-MS/MS chromatograms and ii) there was no further increase on the total B12 contents of the analyzed samples.

In the beginning, cyanide (250 µL of 1% sodium cyanide solution) was added to the extraction buffer in amounts equivalent to the literature value [65] and the whole extraction was performed in amber vials under light-protected conditions. The same procedures from the native method were carried out for the extraction. To minimize photodegradation of the cobalamins, subdued light conditions were often required for the extraction of cobalamins with cyanidation conversion in the literature [64, 65, 69, 177] and were also used for the native method of the present thesis. The extremely light sensitive cobalt-carbon bond in MeCbl and AdoCbl, however, was found to be rather stable in the dark under neutral, diluted acidic and alkaline conditions [197]. In a previous

study [112], MeCbl remained unchanged in the dark in the presence of excess potassium cyanide (molar ratio of 50:1). Therefore, light exposure might be necessary to facilitate the ligand substitution via OHCbl that readily reacts with CN^- to form CNCbl. The obtained results from the first light-protected extraction clearly showed incomplete conversion of AdoCbl and MeCbl into CNCbl. A further increase of the amount of cyanide (500 µL of 1% sodium cyanide solution) did not lead to complete conversion and a total CNCbl content of 13.1 ng/g was obtained (Table 4.1). By repeating the whole extraction under normal laboratory light conditions (method B in section 3.3), the determined total CNCbl increased to 24.1 ng/g. These results suggested that light exposure might be needed for a complete and timely conversion. It is also possible that the amount of cyanide used was not enough for the conversion of all cobalamins in the sample. Moreover, the duration of boiling adopted from the native method was 10 min, which was shorter than the duration of 30 min commonly reported in the literature [64, 65]. Therefore, the extraction of the conversion method was further optimized based on the following parameters: i) duration of light exposure, ii) duration of boiling and iii) the amount of cyanide added. Apart from the degree of conversion, various other factors were also taken into consideration when optimizing the extraction method. First, excess cyanide might lead to the formation of dicyanocobalamin (Di-CNCbl) instead of CNCbl [112]. The increase of the amount of toxic cyanide used also increases the safety concerns of the experiments. In addition, the prolonged light exposure and boiling time might be beneficial for the conversion but also increased the risk of degradation of all cobalamins.

After testing various combinations of the three parameters, an optimized extraction protocol (method D in section 3.3) was eventually obtained which met the two criteria mentioned earlier. First, 500 µL of 1% sodium cyanide solution was added to the mixture of 1 g of sample, 5 mg of papain and 5 mg of α-amylase in 25 mL of extraction buffer (50 mM sodium acetate, pH = 4). The following extraction steps, including stirring (RT, 20 min), incubation (37 °C, 1 h) and boiling (30 min), were performed under normal laboratory light conditions. After centrifugation (3220 x *g*, RT), the supernatant was paper filtered and then an additional 100 µL of 1% sodium cyanide solution was added to the filtrate. Next, the filtrate was shaken continuously in a clear falcon tube for 10 min under light exposure. Afterwards, the sample solution was purified by IAC. The steps for immunoaffinity purification and subsequent handling were kept the same as

for the native method under subdued light conditions. The optimized conversion method was further tested on various meat samples including pork fillet, beef fillet, lamb fillet, chicken breast and a liver sample. None of the analyzed samples showed residual peaks of unconverted cobalamins on the LC-MS/MS chromatograms. The performance of the optimized conversion method was later checked by method validation.

5.3.2 Method validation of the conversion SIDA method

The optimized conversion method was further validated before being compared with the native method. With the cyanidation conversion, the soy flour previously used as the surrogate blank matrix for the validation of the native method showed the peak of CNCbl due to the pre-concentration of all cobalamins into the cyano-form. For the validation according to Vogelgesang and Hädrich [109], a blank matrix was required for the spiking purposes. However, a further investigation of different plant-based matrices, including soy protein isolate, pea protein isolate, pumpkin seed protein isolate, rice protein isolate, almond protein isolate and a self-prepared gluten sample, suggested the ubiquitous presence of traces of cobalamins in foods and the levels could become detectable after being converted and concentrated by cyanidation for the highly sensitive LC-MS/MS of the present thesis. For the determination of LOD and LOQ, the traces of cobalamins in the matrices would cause severe errors in quantifications of low levels of CNCbl when applying the conventional approach.

Under these circumstances, a revised way using the "surrogate analyte approach" [181] was adopted for determining the LOD and LOQ of the conversion method. The [$^{15}N_{13}$]-CNCbl was spiked in varying amounts to the matrices as the surrogate analyte while the unlabelled CNCbl was added as internal standard for the quantification. In this case, the LOD and LOQ were calculated from [$^{15}N_{13}$]-CNCbl but not from CNCbl. The soy flour was chosen as the surrogate blank matrix as it showed the lowest signal of CNCbl among all investigated matrices. By adding proper amounts of internal standard (i.e., CNCbl), the influence of CNCbl stem from endogenous cobalamins in the matrix became negligible for further quantifications. For the quantification of [$^{15}N_{13}$]-CNCbl, a linear reverse response curve was obtained by plotting peak area ratios [A([$^{15}N_{13}$]-CNCbl)/A(CNCbl)] against the molar ratios [n([$^{15}N_{13}$]-CNCbl)/n(CNCbl)] from the previous LC-MS/MS measurements. The linearity of the reverse response curve was confirmed by Mandel's fitting test [182]. It has to be noted that the reverse response function was only used for

the calculations of LOD and LOQ using the surrogate analyte approach. The quantifications of CNCbl in samples were carried out by adopting the original SIDA response curve of CNCbl previously established in the native method (details in section 3.4).

For assessing the recoveries of CNCbl, a pork fillet sample was spiked with CNCbl at three different levels (7.5 ng/g, 37.6 ng/g, and 74.7 ng/g) and the concentrations of CNCbl were then determined by the conversion method. The endogenous amount of CNCbl in the pork sample was as well determined by the conversion method. Similar to the validation of the native method, inter-injection, intra-day and inter-day precisions of CNCbl were obtained by analyzing a pork fillet sample using the conversion method. The native method and the conversion method were both applied to analyze the certified reference material BCR 487 in order to compare accuracies of the two methods. The validation results of the native method and the conversion method are summarized in Table 5.1.

Table 5.1. Method validation results of the native method and the conversion method.

Method	LOD (ng/g)	LOQ (ng/g)	Recovery (%)	Precisions (% RSD)	CNCbl in BCR 487 (ng/g)
Native	0.19	0.68	98-101	4-11	907 ± 26
Conversion	0.09	0.29	101-111	2-4	843 ± 13

The performances of the conversion method and the native method were generally comparable when only comparing the respective validation results for the target analyte CNCbl (Table 5.1). The conversion method demonstrated a slightly higher sensitivity for CNCbl as lower LOD and LOQ were obtained. This was expected as cyanidation conversion acts as a pre-concentrating step to enrich lower levels of cobalamins in foods. The surrogate analyte approach adopted for determining the LOD and LOQ might also contribute to the lower LOD and LOQ of the conversion method. The reproducibility of the conversion method for CNCbl was slightly better than that of the native method, reflected by the lower RSDs of the various precisions. This was also expected as the native method determined CNCbl natively present in the pork sample in concentrations much lower than the contents of total CNCbl pre-concentrated from different forms. The spiking amounts and matrices used for determining the recoveries of the two methods varied between each other. Therefore, a direct comparison between the obtained values

is not feasible. In general, the recoveries of the two methods were both rather satisfactory at their respective spiking levels.

With the application of the conversion method, a total B12 content of 843 ± 13 ng/g was obtained for the reference material BCR 487. This value was lower than the certified value of 1120 ± 90 ng/g and was also lower than the previously determined content of 1116 ± 14 ng/g by the native method. In order to check if the variance on the B12 contents was caused by the analytical method or due to the changes of cobalamins during the storage, the BCR 487 was again analyzed by the native method. The total B12 content obtained was 907 ± 26 ng/g, which was lower than the previous content determined by the same method and was in agreement with the value from the conversion method. Therefore, the lower content of 843 ± 13 ng/g from the conversion method was not caused by the analytical method but by the degradation of cobalamins in the reference material.

In general, both native and conversion methods achieved satisfactory performances in terms of CNCbl determination in foods. However, whether the slightly better sensitivity and robustness brought by the cyanidation conversion is worthy of sacrificing information on vitamer distribution remains questionable.

5.3.3 Analysis of real meat samples by the conversion method

For comparison, the validated conversion method was further used to analyze the real meat samples that have been previously determined by the native method. The obtained total B12 contents from both methods are displayed in Figure 5.1. In general, the total B12 contents of all samples determined by the conversion method were lower than their corresponding values obtained using the native method. The LC-MS/MS chromatograms of the beef fillet and lamb fillet samples applying the conversion method showed residual peaks of OHCbl, AdoCbl and MeCbl, revealing incomplete conversions of other cobalamins into CNCbl during the preparation of these samples. The encountered incomplete conversions were not expected as all meat samples showed complete conversion previously when qualitatively analyzed by the LC-MS/MS during the method development. These results indicate that the conversion of cobalamins might not be reproducible and was probably influenced by ambient conditions such as the intensity of natural light. Alternatively, it could also be possible that the monitoring of conversion by LC-MS/MS was not reliable when unconverted cobalamins were only

present in trace levels due to the variance on day-to-day performance of the instrument and the discrimination of immunoaffinity columns towards different cobalamins during the sample preparation.

During the validation of the conversion method, the issue of incomplete conversion was not noticed as the experiments for LOD, LOQ and recoveries were based on spiking CNCbl that requires no further conversion. Therefore, despite of the good results from the method validation for CNCbl, the conversion method could not deliver accurate quantifications of total B12 contents as the use of [$^{15}N_{13}$]-CNCbl could only compensate for the losses of CNCbl but not for the incomplete conversion of cobalamins during the sample preparation. Similarly in the literature [63-65, 69, 108], HPLC-UV and LC-MS(/MS) methods using cyanidation conversion were only validated based on CNCbl without taking the conversion into consideration. Therefore, the accuracies of previous results regarding total B12 determination are questionable.

A complete conversion of other cobalamins into CNCbl is the prerequisite for accurate determination of total B12 by the conversion method. In order to gain more insights into the issue of conversion, the pork fillet sample was spiked with OHCbl (35.0 ng/g), AdoCbl (27.3 ng/g) and MeCbl (39.5 ng/g) and then measured by the conversion method for the determination of conversion rate. Using the conversion method, a conversion rate of 81% was eventually obtained and the spiked sample showed traces of OHCbl, AdoCbl and MeCbl on the LC-MS/MS chromatogram. Previously, no unconverted cobalamins were observed in the measurements of the original pork sample. These results indicate that the rate of conversion in the pork sample was either concentration dependent or the unconverted cobalamins in original pork sample were present in levels that could not be detected by the LC-MS/MS in a reproducible way. Interestingly, the BCR 487 contained much higher contents of OHCbl, AdoCbl and MeCbl but demonstrated a complete conversion during the analysis (details in section 4.3.2.2). Thus, the conversion appeared to be matrix dependent.

Further investigation is required to elucidate the reasons responsible for the phenomenon of incomplete and unstable conversion. However, the suitability and reliability of analytical methods based on the principle of cyanidation conversion for total B12 determination is generally in question. Due to the influence of light, concentrations of cobalamins and sample matrices on the conversion, further

optimization to achieve a sample preparation protocol that can be universally applied for analyzing various food samples with cobalamins present in a wide range of concentrations is rather difficult. In addition, the monitoring of conversion poses another challenge as the LC-MS/MS adopted here already represents the state of the art for identification of low levels of cobalamins but still suffers from the reliability issue. Moreover, cobalamins are known for their chemical instability. Instead of being converted into CNCbl, cobalamins might also degrade in the presence of cyanide during the extraction. Thus, the potential analyte loss of unconverted cobalamins should also be investigated. Without all the uncertainties caused by the cyanidation conversion, the native method developed in the present thesis clearly provides a more straightforward, reproducible, and reliable way of determining B12 in foods when compared to the conversion method.

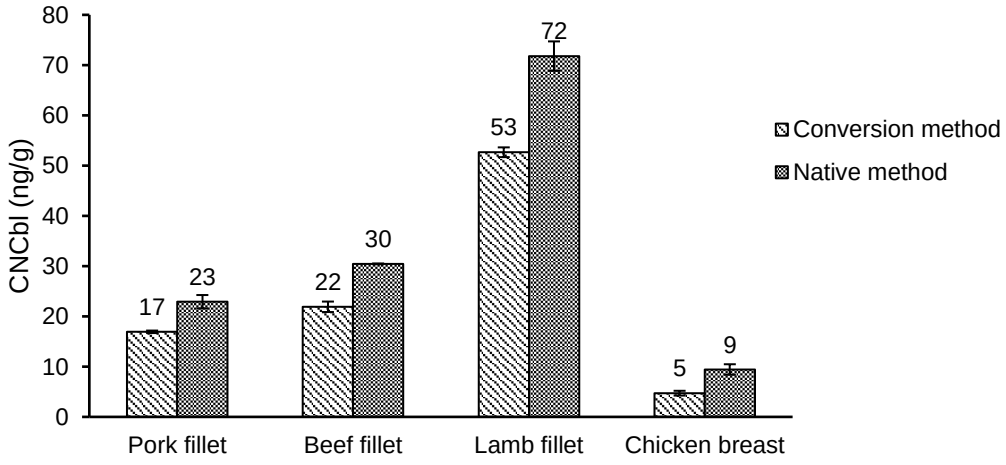


Figure 5.1. Total B12 contents of meats determined by the conversion and the native method.

6 Summary and outlook

Stable isotope dilution assay (SIDA) has been long-awaited for the analysis of naturally occurring cobalamins in foods for its undoubtful superior specificity, sensitivity and ability to compensate for analyte loss and matrix interferences. However, the development of SIDA for cobalamins was restricted by the poor commercial availability of isotopically labelled internal standards. Therefore, the first part of the present thesis focused on the preparation of ^{15}N-labelled cobalamins via biosynthesis using the bacterium *Propionibacterium freudenreichii*. A chemically defined medium (CDM) containing ammonium sulphate as the sole nitrogen source except three essential vitamins was developed, which could support growth of *P. freudenreichii* throughout continuous transfers. The CDM was further optimized together with the cultivation schemes for the *in vivo* production of cobalamins. The profiles of cellular metabolites were monitored by HPLC-DAD and LC-QTOF-MS to assess the stage of cobalamin biosynthesis. With the optimized CDM (CDMG) in which ammonium sulphate was substituted by ^{15}N-labelled ammonium sulphate and the application of an optimized 2-stage cultivation scheme consisting of a semi-anaerobic and an aerobic phase, fully ^{15}N-labelled AdoCbl with a volumetric yield of 635 ± 102 µg/L and fully ^{15}N-labelled OHCbl with a volumetric yield of 312 ± 29 µg/L were obtained. In order to improve the purity of the labelled substances, serial transfers in ^{15}N-lablled CDMG were conducted for the preparation of the precultures. In addition, a second cultivation scheme under anaerobic conditions was developed for the preparation of specifically labelled [^{15}N, $^{14}N_2$]-cobalamins using [^{15}N]-CDMG supplemented with the lower ligand DMB. The obtained volumetric yields were 990 ± 210 µg/L and 96 ± 18 µg/L, respectively for [^{15}N, $^{14}N_2$-DMB]-AdoCbl and [^{15}N, $^{14}N_2$-DMB]-OHCbl. Eventually, fully ^{15}N-labelled AdoCbl was directly isolated from cell extracts by SPE and semi-preparative HPLC. [^{15}N]-CNCbl and [^{15}N]-MeCbl were prepared from isolated [^{15}N]-AdoCbl or [^{15}N]-OHCbl via chemical modifications. [^{15}N, $^{14}N_2$-DMB]-OHCbl was isolated by semi-preparative HPLC from light-treated cell extracts after SPE purification. The final ^{15}N-labelled cobalamins demonstrated high chromatographic and isotopic purities as well as anticipated labelling patterns.

In the present thesis, preliminary optimizations were carried out for the biosynthetic production of ^{15}N-labelled cobalamins as a pilot test. In future studies, there is great potential to improve the yields of ^{15}N-labelled cobalamins by further optimizing the

media composition, applying more sophisticated process control strategies or adopting various metabolic and genetic engineering methodologies. The biosynthetic approach might be further extended to produce other cobamides, cobalamin intermediates and their corresponding isotopically labelled substances. Most of these compounds are not commercially available and are highly requested for B12 research not only in the scope of human nutrition but also in the field of microbiology. In addition, the biosynthetic approach might be inspiring for future studies addressing production of other labelled metabolites with complex structures.

With the self-prepared ^{15}N-labelled cobalamins ([$^{15}N_{18}$]-AdoCbl, [$^{15}N_{13}$]-CNCbl, [$^{15}N_{13}$]-MeCbl, [$^{15}N_{11}$,$^{14}N_2$-DMB]-OHCbl) from biosynthesis, a multi-SIDA method was further developed for the quantification of native OHCbl, AdoCbl, MeCbl and CNCbl in foods. The sample preparation protocol was optimized based on meats as the target matrices. The extraction uses α-amylase and papain for enzymatic digestion and the purification takes advantage of the highly selective immunoaffinity columns for simultaneous enrichment of the four cobalamins while removing the interfering compounds from the matrices. The analysis by LC-MS/MS allows specific and sensitive detection of cobalamins and the application of SIDA enables accurate quantification by compensating for analyte loss, variances on ionization efficiency and matrix interferences. The use of [$^{15}N_{11}$,$^{14}N_2$-DMB]-OHCbl as internal standard for OHCbl quantification avoided the errors caused by the degradation of the other labelled standards. Using soy flour as surrogate blank matrix, the developed method was thoroughly validated for all analytes by determining LODs, LOQs, recoveries at three different spiking levels, as well as inter-injection, intra-day and inter-day precisions. The results from method validation revealed low LODs (0.19 to 0.58 ng/g) and LOQs (0.68 to 1.73 ng/g), satisfactory recoveries (82-121%) as well as good precisions (< 11%), demonstrating excellent sensitivity, accuracy and reproducibility of the developed method. The total B12 content of a certified reference material determined by the novel method was in accordance with the certified value, further confirming the accuracy of the developed method. The developed method was applied to analyze various meat samples, providing valuable information on vitamer distribution in common categories of meats.

Due to the fact that reliable methods were lacking, data on individual cobalamin vitamers in foods are scarce. In future studies, the developed SIDA method should be further applied on different categories of foods to update dietary B12 data with contents

of individual vitamers. The novel method might also be further used to identify and verify cobalamins in unconventional foods such as plant-based fermented foods and algal supplements. With modifications, the developed method might also see its potential applications in human studies investigating bioavailabilities of individual vitamers in foods. Due to the high cost of the immunoaffinity columns, further improvements on the sample preparation should be conducted to search for new alternatives to reduce the cost of the analysis.

In the last part of the present thesis, a conversion SIDA method that measures total B12 as CNCbl after cyanidation conversion was developed. The sample preparation protocol of the conversion method was optimized from the procedures of the developed multi-SIDA method (native method) using pork fillet as the model matrix. The major modifications include the use of cyanide during extraction, an extended boiling time of 30 min and the use of normal laboratory light conditions for the whole extraction period. The purification was also performed using IAC and the steps were kept the same as the native method under subdued light conditions. The results from preliminary tests of the conversion method on various meat samples demonstrated a lack of unconverted cobalamins on the obtained LC-MS/MS chromatograms. The conversion method was further subjected to method validation and the obtained results revealed low LOD (0.09 ng/g), low LOQ (0.29 ng/g), good recoveries (101-111%) and satisfactory precisions (2-4% RSD) for the analysis of CNCbl. The reference material BCR 487 was measured by both the native method and the conversion method with consistent results obtained.

The validated conversion method was further used to quantify cobalamins in real meat samples that have been previously determined by the native method. The total B12 contents determined by the conversion method were lower when compared to the corresponding values obtained applying the native method. Contradictory to the preliminary results revealing complete conversions, residual peaks of unconverted cobalamins were observed on the LC-MS/MS chromatograms of beef fillet and lamb fillet. The discrepancy revealed various issues of the conversion method that were not reflected in the method validation. First, the cyanidation conversion might not be stable under normal laboratory conditions susceptible to changes of natural light. Second, the monitoring of conversion by LC-MS/MS, albeit being the state of the art, might not be adequate in trace levels due to the unavoidable variances on sensitivity of the

instrument. Moreover, further investigations on conversation rates revealed that the conversion appeared to be both concentration and matrix dependent, which makes further optimization to obtain a universal sample preparation protocol accommodating a wide range of foods a very challenging task. Altogether, the native method of the present thesis offers better reliability on B12 quantification in foods as uncertainties caused by the cyanidation conversion are avoided by measuring individual cobalamins naturally occurring in foods. Future studies adopting cyanidation conversion should elucidate reasons for the incomplete conversions and further seek for solutions to achieve complete and stable conversions for B12 quantification.

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I. List of abbreviations

5-ALA	5-aminolevulonic acid
5-CH_3-H_4folate	5-methyl-tetrahydrofolate
5,10-CH_2-H_4folate	5,10-methylene-tetrahydrofolate
5-OHBza	5-hydroxybenzimidazole
5-Ome-6MeBza	5-methoxy-6-methylbenzimidazole
5-OMeBza	5-methoxybenzimidazole
A	Analyte
A	Peak area
AdoCbi	adenosylcobinamide
AdoCbi-GDP	adenosylcobinamide-GDP
AdoCbi-P	adenosylcobinamide phosphate
AdoCbl	adenosylcobalamin
AdoCby	adenosylcobyric acid
AI	adequate intake
AOAC	Association of Official Agricultural Chemists
B12	vitamin B12
Cba	cobamide
Cbi	cobinamide
Cby	cobyric acid
CDM	chemically defined medium
CDMG	chemically defined medium supplemented with glucose
CNCbl	cyanocobalamin
DAD	diode array detection
Di-CNCbl	dicyanocobalamin
DMB	5,6-dimethylbenzimidazole
EFSA	European Food Safety Authority

ESI	electrospray ionization
FAD	flavin adenine dinucleotide
FDA	U.S. Food and Drug Administration
FMN	flavin mononucleotide
$FMNH_2$	reduced flavin mononucleotide
GRAS	generally recognized as safe
H_2folate	dihydrofolate
H_4folate	tetrahydrofolate
HC	haptocorrin
HMB	hydroxymethylbilane
holo-TC	holo-Transcobalamin
HPLC	high performance liquid chromatography
HPLC-DAD	high performance liquid chromatography coupled with diode array detection
IAC	immunoaffinity chromatography
ICP	inductively coupled plasma
ICP-MS	inductively coupled plasma mass spectrometry
IF	intrinsic factor
IOM	Institute of Medicine
IS	Internal standard
LC	liquid chromatography
LC-ESI-MS(/MS)	liquid chromatography coupled with electrospray ionization (tandem) mass spectrometry
LC-ICP-MS	liquid chromatography coupled with inductively coupled plasma mass spectrometry
LC-MS(/MS)	liquid chromatography coupled with (tandem) mass spectrometry
LOD	limit of detection
LOQ	limit of quantification
m/z	mass-to-charge ratio

MBA	microbiological assay
MeCbl	methylcobalamin
MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
n	amount of substance [mol]
NLA	nucleotide loop assembly
OHCbl	hydroxocobalamin
P.	*Propionibacterium*
PBG	porphobilinogen
q-NMR	quantitative nuclear magnetic resonance
QPS	qualified presumption of safety
QTOF-MS	quadrupole time-of-fight mass spectrometry
RDA	recommended dietary allowance
RSD	relative standard deviation
RT	room temperature
SAH	*S*-adenosyl-homocysteine
SAM	*S*-adenosyl-methionine
SIDA	stable isotope dilution assay
SLA	sodium lactate agar
SLB	sodium lactate broth
SPE	solid phase extraction
TC	transcobalamin
TCA	tricarboxylic acid
TOF	time-of-flight
(U)HPLC	(ultra-) high performance liquid chromatography
(U)HPLC-UV	(ultra-) high performance liquid chromatography coupled with UV detection

UroIII	uroporphyrinogen III
USDA	U.S. Food and Drug Administration
UV	ultraviolet
UV-Vis	ultraviolet-visible

II. List of figures and schemes

III. List of tables

IV. List of publications and presentations

Publications

Wang, M., Asam, S., Chen, J., Ehrmann, M., and Rychlik, M. (2021). Production of Four ^{15}N-Labelled Cobalamins via Biosynthesis Using *Propionibacterium freudenreichii*. *Frontiers in Microbiology* 12(2256). doi: 10.3389/fmicb.2021.713321.

Wang, M., Asam, S., Chen, J., and Rychlik, M. (2021). Development of Stable Isotope Dilution Assays for the Analysis of Natural Forms of Vitamin B12 in Meat. *Journal of Agricultural and Food Chemistry* 69(36)**,** 10722-10730. doi: 10.1021/acs.jafc.1c03803.

Oral presentations

Analysis of natural forms of vitamin B12 in foods by LC-MS/MS. 29th Doktorandenseminar Hohenroda, Hohenroda, Germany, 01/2019.

Development of stable isotope dilution assays for the analysis of four naturally occurring cobalamins in foods. The 6th International Vitamin Conference, Copenhagen, Denmark, 09/2021.

Stable isotope dilution analysis of natural vitamin B12 forms. Forschungsseminar der Lebensmittelchemie, Freising, Germany, 12/2021.

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VI. Appendix

Appendix A: Production of four ^{15}N-labelled cobalamins via biosynthesis using *Propionibacterium freudenreichii*

frontiers in Microbiology

ORIGINAL RESEARCH
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Production of Four ^{15}N-Labelled Cobalamins via Biosynthesis Using *Propionibacterium freudenreichii*

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Cobalamins (vitamin B12) are required by humans for their essential roles as enzyme cofactors in diverse metabolic processes. The four most common cobalamin vitamers are hydroxocobalamin (OHCbl), adenosylcobalamin (AdoCbl), methylcobalamin (MeCbl), and cyanocobalamin (CNCbl). Humans are not able to synthesise cobalamins *de novo* and thus must acquire them from external sources. Therefore, a reliable and robust analytical method to determine the cobalamins in dietary sources is highly required. For such a purpose, stable isotope dilution assays (SIDAs) with LC-MS/MS are most suited due to their superior sensitivity, specificity, and ability to compensate for matrix effects and analyte loss during sample work-up. However, a critical bottleneck for developing a SIDA method for cobalamins is the availability of stable isotope-labelled internal standards. In the present study, we harnessed the potential of *Propionibacterium (P.) freudenreichii* for the biosynthesis of ^{15}N-labelled cobalamins. First, we developed a chemically defined medium (CDM) containing ammonium sulphate as a single nitrogen source except three essential vitamins that supported long-term stable growth of *P. freudenreichii* throughout continuous transfers. The CDM was further optimised for cobalamin production under different incubation schemes. With the optimised CDM and incubation scheme, fully ^{15}N-labelled cobalamins were obtained in *P. freudenreichii* with a final yield of 312 ± 29 μg/L and 635 ± 102 μg/L, respectively, for [^{15}N]-OHCbl and [^{15}N]-AdoCbl. Additionally, an optimised incubation process under anaerobic conditions was successfully employed to produce specifically labelled [^{15}N, $^{14}N_2$]-cobalamins, with a yield of 96 ± 18 μg/L and 990 ± 210 μg/L, respectively, for [^{15}N, $^{14}N_2$]-OHCbl and [^{15}N, $^{14}N_2$]-AdoCbl. The labelled substances were isolated and purified by solid phase extraction and semi-preparative HPLC. Chemical modifications were carried out to produce [^{15}N]-CNCbl and [^{15}N]-MeCbl. Eventually, ^{15}N-labelled compounds were obtained for the four cobalamin vitamers in high chromatographic and isotopic purity with desired ^{15}N-enrichment and labelling patterns, which are perfectly suited for future use in SIDAs or other applications that require isotopologues.

Keywords: biosynthesis, *Propionibacterium freudenreichii*, chemically defined medium, vitamin B12, cobalamins, ^{15}N-labelling

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INTRODUCTION

Cobalamin, commonly known as vitamin B12 (B12), has one of the most complex structure of all vitamins, which is composed of a cobalt central atom coordinated with a tetracyclic corrin system, an upper β-ligand (cyano, methyl, hydroxyl, or adenosyl group) and 5,6-dimethylbenzimidazole (DMB) as the lower α-ligand (**Figure 1**). In human physiology, methylcobalamin (MeCbl) and adenosylcobalamin (AdoCbl) are required as cofactors for the metabolic functions of two enzymes: methionine synthase (E.C. 2.1.1.13) and methylmalonyl-CoA mutase (E.C. 5.4.99.2) (Kolhouse and Allen, 1977). Hydroxocobalamin (OHCbl) and cyanocobalamin (CNCbl) can be converted into these two forms by human metabolism after absorption. The two mentioned enzymes play vital roles in various metabolic reactions involved in DNA synthesis, branched-chain amino acid and odd-chain fatty acid metabolisms (Stabler, 1999). In nature, cobalamins are exclusively produced by certain bacteria and archaea (Roth et al., 1996). In human nutrition, the major dietary sources for cobalamins are animal-derived foods due to the natural food-chain enrichment. Plant-derived foods are considered to be devoid of cobalamins unless fermented with certain bacteria. Inadequate intake or malabsorption results in cobalamin deficiency, affecting the normal functions of the blood and nervous system (Green et al., 2017). To prevent cobalamin deficiency, one of the most straightforward approach is monitoring the dietary intake to meet the recommended dietary allowance (RDA) of 2.4 μg/day for adults and 0.9 to 1.8 μg/day for children (Green and Miller, 2013). For such a purpose, robust, reliable, and sensitive analytical methods for the determination of cobalamins in known and potential cobalamin-containing food sources are highly required.

Common analytical methods for cobalamins include microbiological assays (MBA) (Kelleher and Broin, 1991), high performance liquid chromatography with UV detection (HPLC-UV) (Heudi et al., 2006; Marley, 2009; Guggisberg et al., 2012; Chamlagain et al., 2015) and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) (Szterk et al., 2012; Zironi et al., 2013). The reported methods suffer from different drawbacks: (i) lack of sensitivity requiring high sample amounts for food analysis, (ii) lack of specificity to distinguish cobalamins from structural analogues with vitamin activity (vitamers) and other inactive corrinoids, and (iii) lack of accuracy due to the low recoveries caused by matrix interference, especially in MS measurements. Therefore, it has become common practice to determine cobalamins not in their natural forms but as CNCbl after treating the sample with cyanide. Some of the mentioned analytical challenges can be improved by this technique, but only by accepting the loss of information about the natural compositions of cobalamins in the food sample. However, optimising the LC-MS/MS technique is another option, as this technique already is sufficiently sensitive

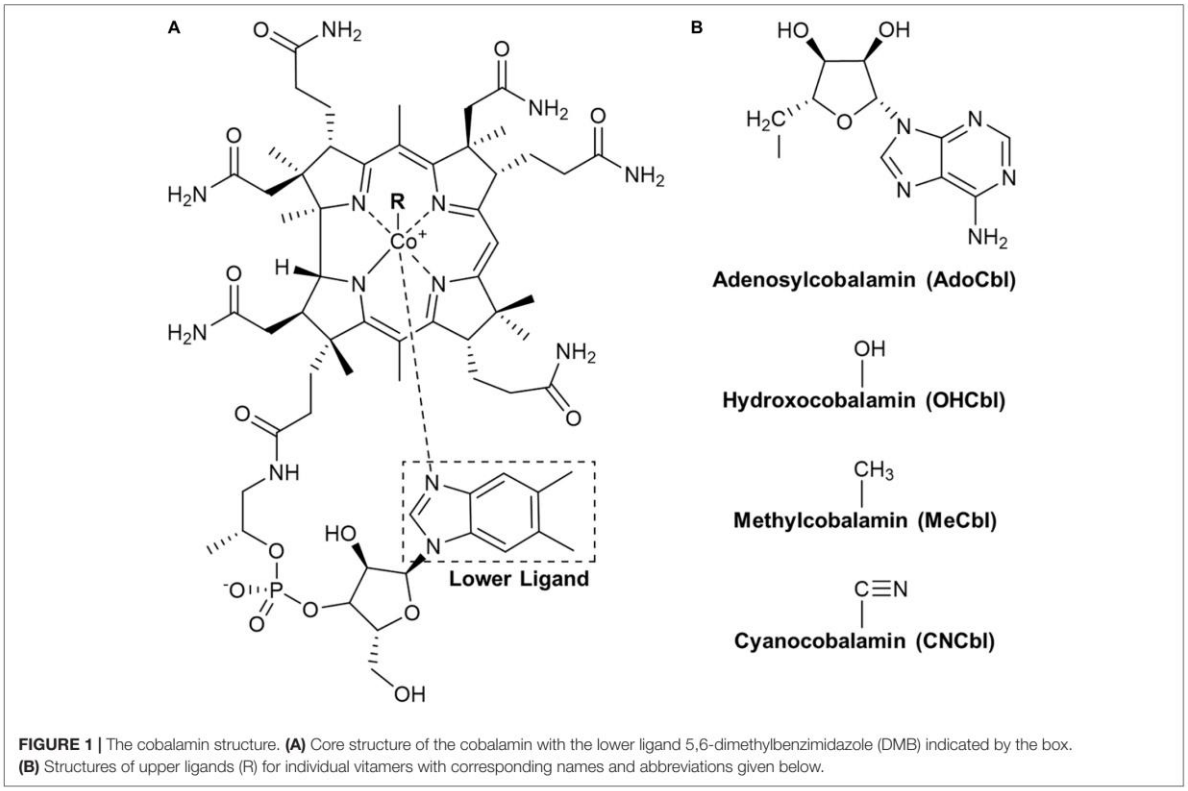

FIGURE 1 | The cobalamin structure. **(A)** Core structure of the cobalamin with the lower ligand 5,6-dimethylbenzimidazole (DMB) indicated by the box. **(B)** Structures of upper ligands (R) for individual vitamers with corresponding names and abbreviations given below.

and specific. Deficiencies in accuracy can be solved by using stable isotope dilution assays (SIDAs). With the application of stable isotope-labelled internal standards, losses during sample work-up and matrix effects during MS measurements are completely compensated due to the identical physical and chemical properties of the analyte and their corresponding isotopologues (Asam and Rychlik, 2015). To date, SIDA has not been applied to native cobalamins in food samples.

The critical bottleneck for developing a SIDA method for cobalamins is the availability of stable isotope-labelled internal standards. The chemical synthesis of cobalamin, with more than 70 synthesis steps, is extremely complicated and expensive (Woodward, 1973; Eschenmoser and Wintner, 1977). Therefore, the industrial production of cobalamins exclusively relies on microbial production (Martens et al., 2002). Researchers in this field often combine biological and chemical approaches to prepare cobalamin-related compounds, with the complex corrin ring structure obtained from biological sources (Friedrich et al., 1960; Stupperich et al., 1988; Fonseca and Escalante-Semerena, 2001; Allen and Stabler, 2008; Crofts et al., 2013; Widner et al., 2017). Regarding the preparation of labelled cobalamins, current publications were mainly based on the following two approaches: (i) isotopes were introduced via chemical synthesis in the lower ligand structure before supplementation to model bacteria for the guided biosynthesis of the labelled target compound (Carkeet et al., 2006; Allen and Stabler, 2008) or (ii) labelled upper ligands (e.g., labelled cyanide $^{13}C^{15}N^-$) were used for reaction with co(I)balamin, a reduced form, to produce the final labelled compound (Kolhouse et al., 1991; D'Ulivo et al., 2017). However, the chemical synthesis of isotopically labelled lower ligands can be complex, time consuming, and expensive. Moreover, guided biosynthesis does not always guarantee a unified labelling in the final product. Labelling the upper ligand is practical when a limited number of labels are needed, but not sufficient for labelling cobalamins for the purpose of SIDA. In order to avoid mass spectrometric overlaps between analytes and labelled standards, a sufficient mass shift is required for the labelled standard.

Among all efficient cobalamin producers, *Propionibacterium (P.) freudenreichii* is one of the most common species used to produce cobalamins in industrial scale (Martens et al., 2002). It is also preferred due to its generally recognised as safe (GRAS) and qualified presumption of safety (QPS) status. Moreover, *P. freudenreichii* is a hardy bacterium that has low nutritional requirements and can survive and adapt to various environments (Thierry et al., 2011). *P. freudenreichii* is prototrophic for all amino acids and nucleotides and is able to grow, under anaerobic conditions, in a minimal media containing ammonium as the sole nitrogen source, if a carbon source, minerals and two to four vitamins (pantothenate, biotin, thiamin, and p-aminobenzoic acid) are supplied (Falentin et al., 2010).

Thus, the preparation of labelled cobalamins seems possible by fermentation of *P. freudenreichii* in a chemically defined medium (CDM) and replacing the sole nitrogen source with ^{15}N. Based on the reported biosynthetic pathway of cobalamins (Fang et al., 2017), ^{15}N may be introduced stepwise starting from the first precursor L-glutamate via C5 pathway into the final product via *de novo* synthesis, as *P. freudenreichii* is self-sufficient for this amino acid.

Therefore, the aim of our study was: (i) to develop a chemical defined medium that allows biosynthetic preparation of ^{15}N-labelled cobalamins by *P. freudenreichii* in high isotopic purity, (ii) isolation of the biosynthesised compounds as pure substances, and (iii) structural characterisation of the labelled compounds to check their suitability as potential internal standards in SIDAs.

MATERIALS AND METHODS

Strains, Chemicals, and Solvents

Propionibacterium freudenreichii subsp. *freudenreichii* DSM 20271^T (DSM 20271) was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany) and was kept as cryopreserved stock culture at −80°C in glycerol.

All chemicals were obtained from Sigma-Aldrich (Steinheim, Germany) unless otherwise specified. Tween 80 was purchased from GERBU Biotechnik GmbH (Heidelberg, Germany). [$^{15}N_2$]-ammonium sulphate [$(^{15}NH_4)_2SO_4$, 99%] was purchased from Eurisotop (Saarbrücken, Germany). Tryptone, yeast extract and agar were purchased from Roth (Karlsruhe, Germany). HPLC-UV grade solvents were obtained from VWR (Ismaning, Germany). LC-MS grade solvents were from Honeywell (Seelze, Germany).

Growth Media and General Incubation Conditions

Complex Media

Sodium lactate broth (SLB) contained 10 g of tryptone, 5 g of yeast extract and 16.7 g of sodium DL-lactate syrup (60%, w/w) per litre. The pH of the medium was adjusted to 7.0 using 4 M NaOH before autoclaving at 118°C for 10 min. For the preparation of sodium lactate agar (SLA), 15 g of agar per litre was additionally added to form the solid medium.

Chemically Defined Media (CDM)

The composition of the CDM is given in **Table 1**. The pH was adjusted to 7.0 by 4 M NaOH and the medium was sterilised via filtration (CytoOne Bottle Top Filtration Unit, 0.2 μm, PES, Starlab GmbH, Hamburg, Germany). For CDM fortified with glucose (CDMG), 10 g/L of glucose was further added while preparing the medium. For the preparation of ^{15}N-labelled CDMG ([^{15}N]-CDMG), $(^{15}NH_4)_2SO_4$ was substituted for unlabelled $(NH_4)_2SO_4$.

Incubation Conditions

All cultures were grown at 30°C. Semi-anaerobic incubation was performed statically in tightly closed screw-cap Schott bottles or centrifuge tubes under normal atmosphere. Aerobic incubation was carried out in Erlenmeyer flasks under shaking conditions (120 rpm). Anaerobic incubation was performed inside a Bactron anaerobic chamber (Sheldon Manufacturing Inc., Cornelius, OR, United States) statically.

TABLE 1 | Composition of chemically defined medium developed in the current study.

Components	Concentration (g/L)
Sodium DL-lactate*	12
$(NH_4)_2SO_4$	3
K_2HPO_4	8.7
KH_2PO_4	6.8
$MgSO_4.7H_2O$	0.2
$FeSO_4.7H_2O$	0.01
$MnSO_4.H_2O$	0.02
$ZnSO_4.H_2O$	0.01
NaCl	0.2
$CaCl_2.2H_2O$	0.132
$CoCl_2.6H_2O$	0.002
Calcium pantothenate	0.001
Biotin	0.001
Thiamin.HCl	0.001
Sodium acetate trihydrate	6
Sodium pyruvate	1
α-ketoglutaric acid	1
Succinic acid	1
Myo-inositol	0.1
Tween 80	0.5

**20 g of sodium DL-lactate syrup (60%, w/w) used.*

TABLE 2 | Conditions of different cultivation schemes.

Scheme	Medium	Culture conditions
a	CDM	7-day semi-anaerobic
b	CDM	14-day semi-anaerobic
c	CDM	7-day semi-anaerobic 1-day aerobic
d	CDM	7-day semi-anaerobic 2-day aerobic
e	CDM	7-day semi-anaerobic 3-day aerobic
f	CDM	7-day semi-anaerobic 7-day aerobic
g	CDM + 10 μM DMB	14-day semi-anaerobic
h	CDM	7-d semi-anaerobic 10 μM DMB added* 3-day aerobic
i	CDMG	5-day semi-anaerobic
j	CDMG	5-day semi-anaerobic 3-day aerobic
k	CDMG	5-day semi-anaerobic $NaHCO_3$ (1 M, 20 mL) added* Glucose (250 g/L, 15 mL) added* 3-day aerobic

**Added after the semi-anaerobic phase.*

Cell Activation and Preculture Preparation

The frozen stock culture was thawed and streaked onto SLA for activation. The inoculated agar plates were incubated in a sealed jar with a generated anaerobic atmosphere (AnaeroGen, Thermo Fisher Scientific, Madison, WI, United States) for 3–5 days. Subsequently, five single colonies from the plates were transferred into 14 mL SLB and incubated semi-anaerobically for 3 days. The bacteria were further propagated in 14 mL SLB (2% inoculum, v/v) for two more generations under the same incubation conditions before transferring to the CDM or CDMG.

Growth Observation in CDM

To assess the growth of *P. freudenreichii* in CDM, 14 mL CDM inoculated with 2% (v/v) of the preculture grown in SLB described above was incubated semi-anaerobically for 5 days. If apparent sedimentation of cells were observed in the bottom of the centrifuge tube, next propagation was carried out by transferring 2% (v/v) of the culture after mixing to a fresh 14 mL CDM for next generation. Purity of the cultures were checked periodically by streaking out onto the SLA.

In vivo Cobalamin Biosynthesis Under Different Incubation Schemes

Propionibacterium freudenreichii was grown in CDM or CDMG under different cultivation schemes (**Table 2**) for the optimisation of *in vivo* cobalamin biosynthesis. The cultivations were performed in 250 mL Schott bottles for the semi-anaerobic phase and were transferred to 500 mL Erlenmeyer flasks with cotton plugs for the aerobic phase when applied. For each cultivation, 250 mL of medium was used. The inocula (2%, v/v) were 4th generation culture grown in CDM. For each scheme, two biological replicate cultures were used.

Production of Fully and Partially ^{15}N-Labelled Cobalamins

Two-Phase Incubation for Production of ^{15}N-Cobalamins in [^{15}N]-CDMG (Full Labelling)

For biosynthesis of fully ^{15}N-labelled cobalamins, *P. freudenreichii* was grown in [^{15}N]-CDMG. After activation in SLB, the strain was sub-cultured in 14 mL [^{15}N]-CDMG (2% inoculum, v/v) by serial transfer every 5 days to minimise carry-over of complex media. The cultures were maintained under semi-anaerobic conditions. Afterward, 250 mL media was inoculated with 5 mL (2% inoculum, v/v) of a 5-day old preculture (4th generation in [^{15}N]-CDMG) for a larger-scale batch fermentation. The inoculated batch culture was first incubated semi-anaerobically for 5 days and then incubated aerobically with shaking (120 rpm) for another 3 days. Before the aerobic incubation period, the culture was neutralised with 20 mL of 1 M $NaHCO_3$ solution and further supplemented with 15 mL of 250 g/L glucose stock solution.

Production of [^{15}N, $^{14}N_2$-DMB]-Cobalamin in DMB-Supplemented [^{15}N]-CDMG Under Anaerobic Conditions (Specific Partial Labelling)

To produce partially labelled cobalamins with unlabelled lower ligand, [^{15}N]-CDMG supplemented with 10 mg/L DMB was used as the fermentation broth. 200 mL of DMB-supplemented [^{15}N]-CDMG was employed in a 250 mL high pressure glass bottle (Schott, Mainz, Germany) for each cultivation. The bottles were sealed gas tight by butyl rubber lids.

Before inoculation, the media were purged with a stream of argon for 2.5 h using two sterile cannulas. The flow of argon was introduced via a long cannula reaching below the liquid level to thoroughly displace air inside the media while stirring. The other cannula was placed above the liquid level as a gas outlet. The gas composition of the headspace was monitored by a gas analyser (PA 7.0, Witt-Gasetechnik, Witten, Germany). After 2.5 h of constant gassing, the oxygen was no longer detected in the headspace. Then, the gas flow was stopped, and the bottles were simultaneously locked gas tight.

Inoculation was performed inside the anaerobic chamber to minimise oxygen exposure. Media were inoculated with 4 mL of a 5-day old preculture semi-anaerobically propagated in 14 mL of [^{15}N]-CDMG (4th generation). Afterward, a static incubation was carried out inside the anaerobic chamber for 5 days.

Cell Harvesting

The cells were harvested by centrifugation (10,000 × *g*) at 4°C for 20 min and washed once with phosphate buffer saline (PBS, pH = 7.4). Afterward, cell pellets were stored at −20°C until further use.

Extraction and Purification of Bacterial Cells

Wet bacterial cells (200 mg for analytical purposes and 400 mg for preparative purposes) were weighed into a 25 mL extraction vial and was mixed with 10 mL of extraction buffer (50 mM sodium acetate buffer, pH = 4.0). The mixture was thoroughly vortexed and further stirred with magnetic stir bar for 20 min at room temperature. Afterward, the homogenate was heated in a boiling water bath for 30 min and cooled immediately in an ice-water bath. The sample was further incubated at 37°C for 1 h in a light-protected shaking water bath. After cooling down in the ice-water bath, the sample mixture was transferred to a 50 mL centrifuge tube. The residue in the extraction vial was washed twice each with 3 mL of extraction buffer and then transferred into the centrifuge tube. The sample was centrifuged for 30 min (4°C and 3,220 × *g*). The supernatant was collected for solid phase extraction (SPE) using C18 columns (500 mg, 6 mL, Discovery^{R} DSC-18, Supelco, Bellefonte, PA, United States). The columns were first activated with 6 mL of methanol and equilibrated with 6 mL of extraction buffer before a complete loading of the sample solutions. After washing the columns again with 2 mL of extraction buffer, an elution step was carried out using 3 mL of MeOH/H_2O (70/30; v/v). The eluant was evaporated to dryness under a stream of nitrogen at 40°C and stored at −20°C until further use.

Further Preparation of ^{15}N-Labelled Cobalamin Standards

Preparation of [^{15}N]-AdoCbl

The cells from [^{15}N]-CDMG were extracted as described in section "Extraction and Purification of Bacterial Cells." [^{15}N]-AdoCbl was isolated by semi-preparative HPLC as described below.

Preparation of [^{15}N]-CNCbl

After extraction of cells and clean-up of the extracts from [^{15}N]-CDMG incubation as described in section "Extraction and Purification of Bacterial Cells," the eluent was exposed to ambient light for 40 min before adding 30 μL of NaCN solution (1% in water, w/v). The mixture was left to react at room temperature protected from light for 2 h. The solution was manually shaken every 30 min to ensure complete mixing. Afterward, the mixture was dried at 40°C under a stream of nitrogen and stored at −20°C before further purification. After reconstitution in water, the produced [^{15}N]-CNCbl was isolated from the reaction solution by collecting corresponding peaks from semi-preparative HPLC as described below.

Preparation of [^{15}N]-MeCbl

The reduction and methylation procedures were adopted from Brown et al. (1984) with some changes. A portion of isolated pure [^{15}N]-AdoCbl was reconstituted in 1 mL of distilled water and photolysed to [^{15}N]-OHCbl by exposure to ambient light for 40 min. The solution was then degassed with argon for 1 h in a sealed glass vial. Subsequently, a freshly prepared $NaBH_4$ solution (25 mg/mL, 200 μL) in NaOH (0.1 M, pre-purged with argon) was added dropwise to the solution through a septum and the reaction was left to proceed at room temperature under argon for 30 min. Afterward, methyl iodide (50 μL) was added to the reaction vial, and the mixture was left to react at room temperature under argon for 30 min. Acetone (200 μL) was added to quench residue $NaBH_4$ in the solution. The mixture was then dried at 40°C under a stream of nitrogen and stored at −20°C before further purification. After reconstitution in water, the produced [^{15}N]-MeCbl was isolated from the reaction solution by collecting corresponding peaks from semi-preparative HPLC as described below.

Preparation of [^{15}N, $^{14}N_2$-DMB]-OHCbl

The cells obtained from DMB-supplemented [^{15}N]-CDMG were extracted and purified as previously described in section "Extraction and Purification of Bacterial Cells." After the elution step of SPE, the sample solution was exposed to ambient light for 40 min with thorough vortexing every 10 min. The light treated eluant was then dried at 40°C under nitrogen and reconstituted in 300 μL of water for further purification. The partially labelled [^{15}N, $^{14}N_2$]-OHCbl was isolated by collecting corresponding peaks from semi-preparative HPLC as described below.

In addition, we also isolated fully ^{15}N-labelled [^{15}N]-OHCbl from cells obtained from [^{15}N]-CDMG incubation. The cells were extracted as described in section "Extraction and Purification of Bacterial Cells" and [^{15}N]-OHCbl was isolated by collecting corresponding peaks from semi-preparative HPLC as described below.

Analytical and Semi-Preparative HPLC-DAD

HPLC-DAD was performed on a Shimadzu HPLC system (Shimadzu, Kyoto, Japan) consisting of an auto-sampler (SIL-20A), a liquid chromatograph (LC-20AD) and a diode array detector (SPD-M20A). A YMC Triart C18 column

(150 × 3.0 mm, 3 μm, Dinslaken, Germany) was used for chromatographic separation at 30°C. Two different linear gradients based on different combinations of mobile phases were used for the analytical and preparative purposes, respectively.

For the analysis of cobalamins and cobalamin precursors in bacterial cultures during method development and optimisation, 50 mM ammonium acetate buffer (pH = 4) and methanol were used as mobile phases A and B, respectively. The gradient started at 10% B and linearly increased to 40% B in the first 22 min. The gradient was held at 40% B for 2 min before it was raised to 95% within 2 min. After it maintained at 95% B for 2 min, the gradient returned to 10% B within 2 min and the column was equilibrated for 10 min before next injection. The flow rate was 0.3 mL/min and the injection volume was 20 μL. The cellular concentrations of OHCbl and AdoCbl were determined by external calibration based on the calibration graphs in the **Supplementary Figures 8, 9**).

For the preparation of ^{15}N-labelled cobalamins, chromatographic separation was performed using 25 mM sodium acetate buffer (pH = 4) and methanol as mobile phases A and B, respectively. The gradient also started at 10% B and increased to 40% B in the first 30 min. Afterward, the gradient followed the same path as for the analytical method. However, a different flow rate of 0.45 mL/min was used for preparative purposes.

LC-MS/MS

Liquid chromatography coupled to tandem mass spectrometry analyses were performed with a Shimadzu Nexera X2 UHPLC-LCMS 8050 triple quadrupole mass spectrometer under positive electrospray ionisation (ESI) mode. Briefly, samples were injected into a Hydrosphere C18 column (150 × 30 mm, 3 μm, YMC, Dinslaken, Germany) at 30°C. The mobile phases A and B were 0.1% acetic acid in water and pure methanol, respectively. The detailed LC and MS parameters are summarised in the **Supplementary Material**.

UHPLC-Q-TOF-MS

UHPLC-Q-TOF-MS analyses were performed on a Waters Acquity UHPLC System (Waters, Eschborn, Germany) coupled to a Bruker maXis UHR-ToF-MS with an Apollo II ESI source (Bruker Daltonics, Bremen, Germany). A YMC Triart C18 column (150 × 3.0 mm, 3 μm, YMC, Dinslaken, Germany) was used for separation at 30°C. 10 mM ammonium formate buffer (pH = 4) and methanol were used as mobile phases A and B, respectively. The detailed operating parameters are shown in the **Supplementary Material**.

RESULTS AND DISCUSSION

Development of Chemically Defined Medium for Stable Growth of *P. freudenreichii*

For the preliminary experiments, a minimal defined medium (medium M, **Supplementary Table 2**) was employed on the basis of defined media previously reported for the growth of *P. freudenreichii*, with some modifications (Glatz and Anderson, 1988; Dherbécourt et al., 2008). This medium met the reported minimum nutritional requirements of *P. freudenreichii* (Vorobjeva, 1999; Falentin et al., 2010), as it contained sodium lactate as the carbon source, ammonium sulphate as the sole nitrogen source, mineral salts and eight vitamins. Amino acids and nucleotides were not included as they were proven to be non-essential for *P. freudenreichii* to grow.

When inoculated from SLB (2% inoculum, v/v), the *P. freudenreichii* was able to grow in medium M for two

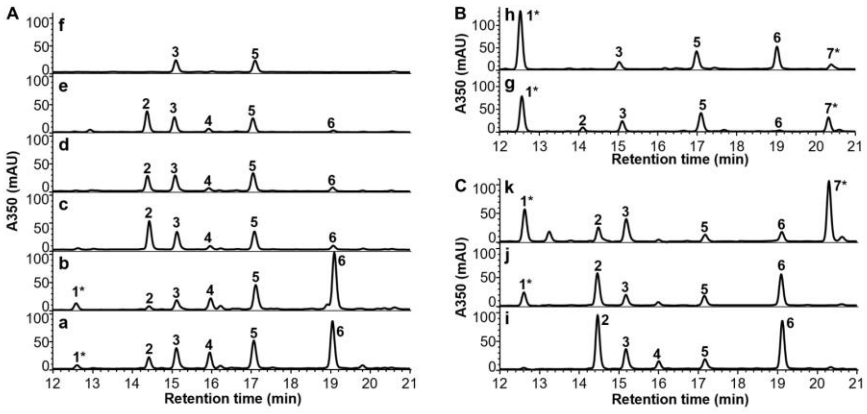

FIGURE 2 | HPLC-DAD chromatograms of cell extracts of *P. freudenreichii* grown in CDM or CDMG under different cultivation schemes indicated on the upper left corner (details in **Table 2**). The identities of the numbered peaks were determined by comparing to the standards of OHCbl (1) and AdoCbl (7) or by UHPLC-QTOF-MS in terms of AdoCbi-GDP (2), FAD (3), AdoCbi-P (4), FMN (5) and AdoCbi (6) (see **Supplementary Figures 1–6**). **(A)** *P. freudenreichii* grown in CDM following cultivation schemes a–f. **(B)** *P. freudenreichii* grown in CDM with the addition of DMB before and after semi-anaerobic incubation following cultivation schemes g and h, respectively. **(C)** *P. freudenreichii* grown in CDMG following cultivation schemes i–k. Asterisk* indicates intact cobalamin structures.

generations but failed to continue after the 3rd transfer. However, we aimed to develop a CDM that supports long-term stable growth of *P. freudenreichii* with ammonium as sole nitrogen source throughout consistent transfers, as the CDM was intended for later ^{15}N-labelling experiments. Serial transfers contribute to decreasing carry-over of non-labelled compounds from the preculture complex medium, i.e., SLB, leading to a better isotopic purity of the final products. The medium M, therefore, was not adequate for this purpose. To improve the growth, organic acids from TCA cycle were added to the medium as their stimulatory effects have been previously reported (Ferguson et al., 1978). These organic acids were non-nitrogenous and therefore did not introduce additional nitrogen into the medium. However, non-essential vitamins were omitted to further remove nitrogen-containing nutrients other than ammonium sulphate. Eventually, a stable and continuous growth of *P. freudenreichii* over 10 generations was observed in the final CDM listed in **Table 1**. The developed CDM was further tested for cobalamin production.

In vivo Cobalamin Biosynthesis Under Different Incubation Schemes

Cobalamin Precursors Formed in *P. freudenreichii* Grown in the CDM

In order to evaluate if the developed CDM can further support *in vivo* cobalamin biosynthesis, *P. freudenreichii* was grown under different cultivation schemes (**Table 2**) in the CDM, and cells were extracted and analysed by HPLC-DAD. The cultivation schemes were designed based on the reported biosynthetic pathway of cobalamins in *P. freudenreichii*, which is divided into two phases: (i) anaerobic/semi-anaerobic phase for the synthesis of cobalamin precursors from δ-aminolevulinic acid (ALA) until formation of 5′-deoxyadenosylcobinamide-GDP (AdoCbi-GDP) and (ii) aerobic phase that requires molecular oxygen for the conversion of flavin mononucleotide (FMN) into the lower ligand DMB before attachment to the corrin ring via a nucleotide loop. The metabolite profiles of cells from different batch cultures are summarised in **Figure 2**. The identities of relevant metabolite peaks were determined based on UHPLC-QTOF-MS measurements and UV-Vis spectra (**Supplementary Figures 1–5**).

HPLC-DAD analysis demonstrated that mainly cobalamin precursors were present in cell extracts when *P. freudenreichii* was grown in CDM under semi-anaerobic conditions up to 14 days. The most abundant precursor detected was 5′-deoxyadenosylcobinamide (AdoCbi). Corresponding peaks from 5′-deoxyadenosylcobinamide-phosphate (AdoCbi-P) and AdoCbi-GDP were also observed, but in lower intensities. Detectable levels of OHCbl were found in cells from semi-anaerobic incubation phase, which is to be expected as the fermentation was not conducted under strictly anaerobic conditions. When a second aerobic phase was employed during cultivation, the intensity of AdoCbi-GDP increased while the peak of AdoCbi diminished. Unexpectedly, the synthesised AdoCbi-GDP was accumulated in the cells without being further converted into AdoCbl when incubated aerobically up to 3 days. Moreover, the cobalamin precursors were completely depleted in the cells after an extended aerobic incubation period of 7 days. Interestingly, FMN and flavin adenine dinucleotide (FAD), the direct and indirect precursors of DMB biosynthesis (Taga et al., 2007), were found in all cell extracts from different cultivation schemes. These results suggest that the incomplete cobalamin biosynthesis was not caused by a lack of available precursors in the cells grown in the CDM.

Complete Cobalamins Synthesised in *P. freudenreichii* Grown in the CDM With DMB Supplementation

To investigate if DMB was the limiting precursor for the further conversion of AdoCbi-GDP to AdoCbl, *P. freudenreichii* was cultured in the CDM supplemented with DMB under semi-anaerobic conditions for 14 days in two ways. 10 μM of DMB was either added to CDM at the beginning of the incubation or first supplied to the culture after 7 days of semi-anaerobic incubation. HPLC-DAD analysis showed that AdoCbl and OHCbl were present in cell extracts from both cultivations (**Figure 2B**), indicating that *P. freudenreichii* was able to incorporate provided DMB to form AdoCbl when grown in the CDM. Therefore, it appeared to us that the *in vivo* DMB synthesis was not effective in *P. freudenreichii* incubated in the CDM, resulting in an insufficient supply of lower ligand needed for the final step of cobalamin synthesis. The reasons for the ineffective DMB synthesis, however, remain to be elucidated. Surprisingly, cell biomasses and pH of the DMB-supplemented cultures were similar to the values of the non-supplemented ones after 14-day semi-anaerobic incubation (**Figure 3**). These results suggest that

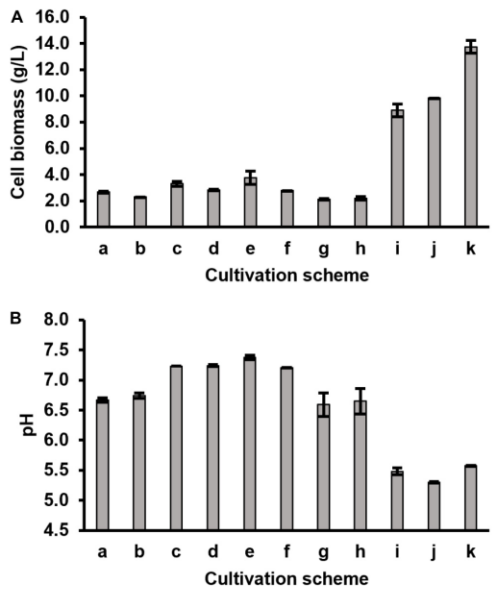

FIGURE 3 | Harvested cell biomass **(A)** and pH **(B)** of the spent broth from different cultivation schemes. Two biological replicates for each scheme ($n = 2$).

the growth of *P. freudenreichii* in CDM was not enhanced when intact cobalamins were synthesised inside the cells.

Enhanced Cell Growth and Completed Cobalamin Synthesis in CDMG

Although CDM could support stable growth of *P. freudenreichii* for generations, low cell biomasses were obtained during all batch cultivations using CDM with or without DMB supplementation (**Figure 3**). The pH values of the cultured media did not drop below 6.5 (originally pH 7.0), indicating that the limited cell growth could be due to exhaustion of carbon sources rather than to inhibition by produced acids. Therefore, glucose (10 g/L in final media) was added as extra carbon source to CDM (CDMG) to check if the growth of *P. freudenreichii* can be improved. After 5-day of semi-anaerobic incubation in CDMG (scheme i in **Table 2**), cell biomass increased from 2.7 g/L (7-day semi-anaerobic incubation, scheme a in **Table 2**) to 8.9 g/L, with pH value of the culture dropping to 5.5 (**Figure 3**). The addition of glucose, therefore, significantly enhanced the cell growth. HPLC-DAD analysis showed that the CDMG cell extracts from semi-anaerobic phase contained both AdoCbi-GDP and AdoCbi in abundant amounts (**Figure 2C**). With an additional 3-day aerobic incubation, OHCbl was observed in the cells while AdoCbi-GDP and AdoCbi were still present in high levels (**Figure 2C**).

As previously reported, the optimum pH for *P. freudenreichii* to grow is between pH 6 and 7 (Hsu and Yang, 1991; Ye et al., 1996; Vorobjeva, 1999) and growth decreases distinctly at pH values below 5.5 and ceases below pH 5 (Hettinga and Reinbold, 1972). To maintain the culture pH around 7, periodical neutralisation of fermented acids by alkaline agents is a common practice during industrial B12 production using *P. freudenreichii* (Martens et al., 2002). Therefore, we introduced a neutralisation step using 20 mL of 1 M $NaHCO_3$ after 5-day semi-anaerobic incubation. Afterward, additional glucose (3.75 g) was added to the culture for providing more energy for cell growth and cobalamin synthesis before the 3-day aerobic phase. By doing so, the cell biomass further increased to 13.7 g/L (**Figure 3A**) and the majority of precursors were converted into intact cobalamins in harvested cells (**Figure 2C**). The culture pH afterward dropped to 5.6.

Anaerobic Incubation for Specific Partial Labelling

The Necessity of Using Partially ^{15}N-Labelled OHCbl for SIDA

OHCbl exists not only as a natural cobalamin but also can be the degradation product from other naturally occurring forms.

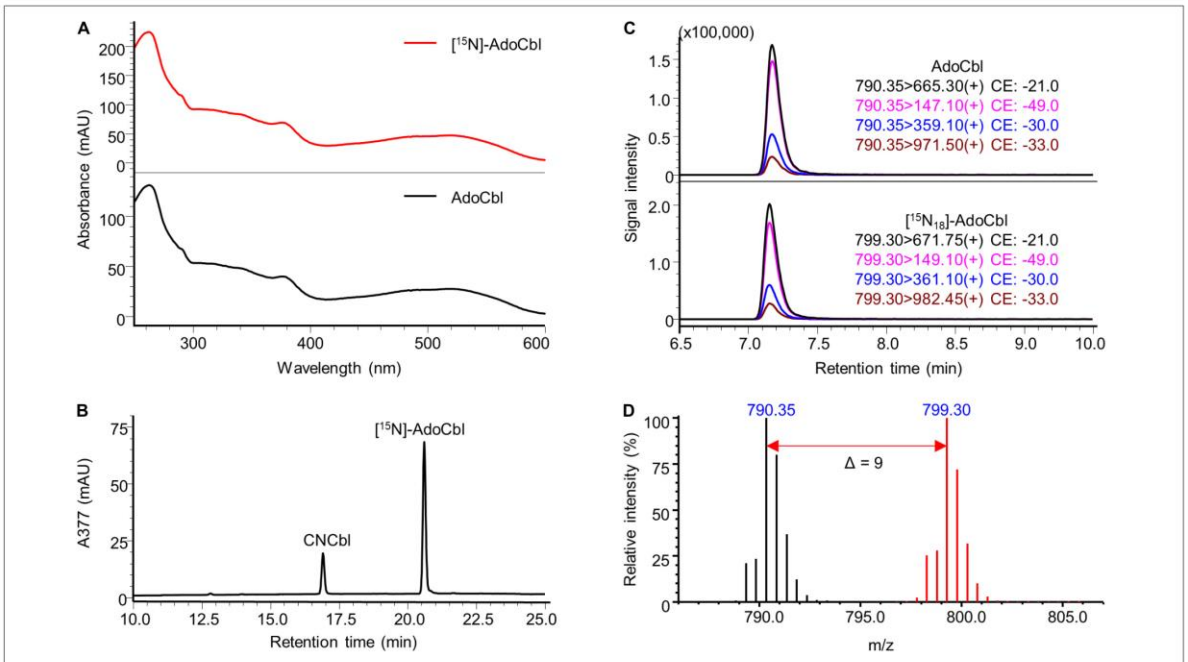

FIGURE 4 | Characterisation of prepared [^{15}N]-AdoCbl. **(A)** UV-Vis spectrum of the prepared [^{15}N]-AdoCbl in comparison to that of the AdoCbl standard. **(B)** HPLC-DAD analysis at a wavelength of 377 nm of the prepared substance showed a major peak at the same retention time of unlabelled AdoCbl standard (see **Supplementary Figure 6**). CNCbl shown on the chromatogram was added as internal standard to determine the absolute concentration of [^{15}N]-AdoCbl. **(C)** LC-MS/MS analysis of [^{15}N]-AdoCbl monitoring mass transitions corresponding to [$^{15}N_{18}$]-AdoCbl (lower trace). The LC-MS/MS chromatogram of unlabelled AdoCbl is shown as a reference (upper trace). **(D)** Overlaid mass spectra of [^{15}N]-labelled AdoCbl (red) and unlabelled AdoCbl (black). The mass shift in $[M + 2H]^{2+}$ values indicated by the red arrow confirmed the labelling of 18 nitrogen atoms. MS spectra were acquired in selected ion monitoring (SIM) mode with a m/z interval of 0.5.

It has to be noted that this degradation is inevitable when handling cobalamins. Therefore, if fully ^{15}N-labelled OHCbl is used as internal standard for the analysis of OHCbl in future LC-MS/MS applications, the unavoidable generation of fully ^{15}N-labelled OHCbl from other fully labelled internal standards will cause severe errors on the accurate quantification of OHCbl. Therefore, it is necessary to use a labelled internal standard that can be clearly distinguished by LC-MS/MS in a so-called dual isotope design from the fully labelled degradation product. One of the easiest approaches to solve this issue is to use specifically ^{15}N-labelled OHCbl with an unlabelled lower ligand as internal standard. For such a reason, we further optimised the cultivation scheme to produce specifically labelled [^{15}N, $^{14}N_2$-DMB]-OHCbl, even though we were able to prepare fully ^{15}N-labelled OHCbl from cells grown in the [^{15}N]-CDMG cultivation.

P. freudenreichii Ceased Growth in Strict Anaerobic Conditions Without DMB Supplementation

To obtain partially labelled cobalamins in high purity, the procedures for biosynthesis must be modified for *P. freudenreichii* to use unlabelled DMB from the media rather than synthesising the labelled one *in vivo*. As the conversion of FMN to DMB requires molecular oxygen, incubation of *P. freudenreichii* in strictly anaerobic condition would be an effective way to block the *in vivo* DMB synthesis. Therefore, we tried to grow *P. freudenreichii* in CDMG under strictly anaerobic conditions without DMB supplementation. However, we observed that the growth was rather limited and even ceased after three generations. Under semi-anaerobic conditions, the presence of trace oxygen supported the growth of *P. freudenreichii* in CDM and CDMG, but only low amounts of cobalamins were synthesised and cobalamin precursors were the major forms in the cultures (**Figure 2**). Under strictly anaerobic condition without provided DMB, the synthesis of intact cobalamins is obstructed. It may be assumed that this is the reason for the decreased growth. When the cobalamin carry-over from previous generations is depleted, the growth ceases. Supplementing DMB is therefore necessary for reasonable production of cobalamins. However, supplementing DMB into the inoculum media for enhancing cell growth under anaerobic incubation is not ideal. The unlabelled nitrogen from DMB might be introduced into the corrin ring via unknown metabolic pathways, resulting in non-unified labelling of final compounds in the later batch cultivations. In a comprised way, *P. freudenreichii* was

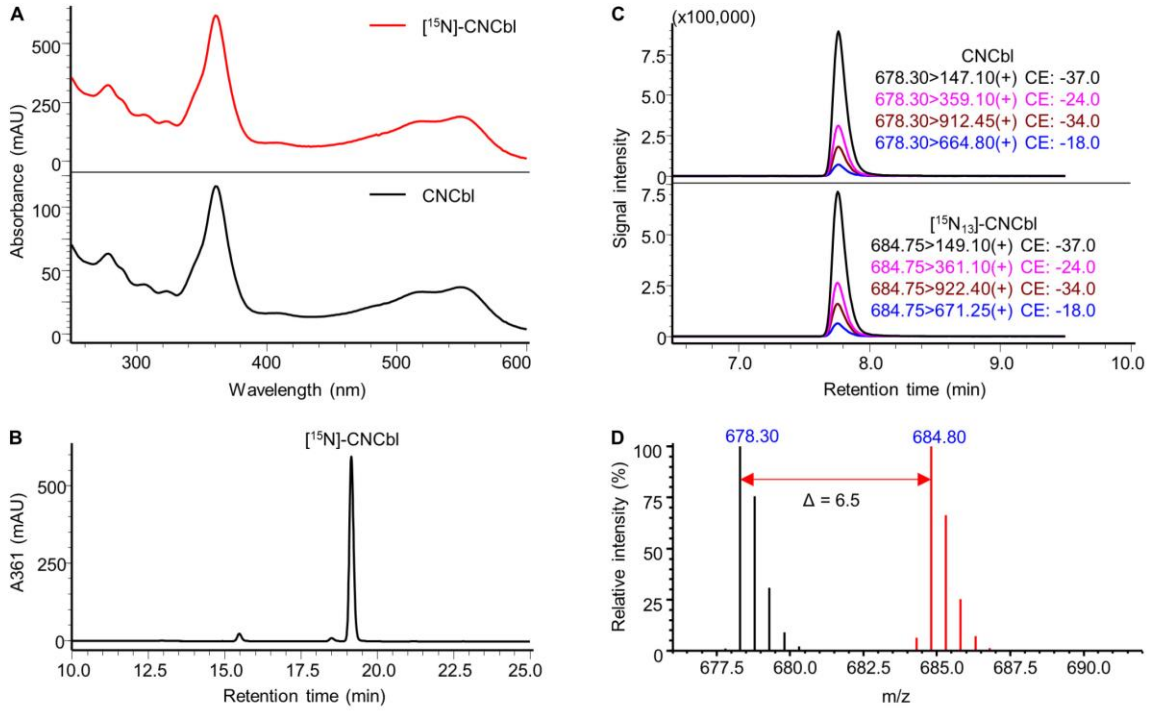

FIGURE 5 | Characterisation of prepared [^{15}N]-CNCbl. **(A)** UV-Vis spectrum of the prepared [^{15}N]-CNCbl in comparison to that of the CNCbl standard. **(B)** Preparative HPLC-DAD at a wavelength of 361 nm of the product from cyanidation reaction showed a major peak of [^{15}N]-CNCbl, which has an identical retention time to that of the CNCbl standard under the same elution conditions (see **Supplementary Figure 7**). **(C)** LC-MS/MS analysis of [^{15}N]-CNCbl monitoring mass transitions corresponding to [$^{15}N_{13}$]-CNCbl (lower trace). The LC-MS/MS chromatogram of unlabelled CNCbl is shown as a reference (upper trace). **(D)** Overlaid mass spectra of [^{15}N]-labelled CNCbl (red) and unlabelled CNCbl (black). The mass shift in $[M + 2H]^{2+}$ values indicated by the red arrow confirmed the labelling of 13 nitrogen atoms. MS spectra were acquired in selected ion monitoring (SIM) mode with a m/z interval of 0.5.

sub-cultured in CDMG under semi-anaerobic conditions first to ensure a good growth in the inoculum before inoculating into batch media of CDMG supplemented with DMB for anaerobic cultivation. Special care was taken to guarantee strict anaerobic conditions. The batch media was previously purged with argon and stored in the anaerobic chamber to reduce dissolved oxygen. Inoculation and incubation were performed in the anaerobic chamber to minimise oxygen exposure. Furthermore, the incubation time was kept as short as 5 days to minimise the possibility of DMB involved in other pathways than being incorporated into the cobalamins as lower ligand.

The cell biomass from the batch fermentation was 11.7 g/L with pH of medium dropping to 5.6. This revised cultivation scheme was eventually adopted to produce the partially labelled cobalamins using DMB-supplemented [^{15}N]-CDMG.

Yields of ^{15}N-Labelled Cobalamins

P. freudenreichii was grown in [^{15}N]-CDMG to produce fully ^{15}N-labelled cobalamins following the previously developed procedures. The intracellular yields of [^{15}N]-OHCbl and [^{15}N]-AdoCbl in the harvested cells were 24.0 ± 2.0 μg/g (n = 3) and 48.7 ± 5.9 μg/g (n = 3), respectively. The corresponding volumetric yields of [^{15}N]-OHCbl and [^{15}N]-AdoCbl were 312 ± 29 μg/L and 635 ± 102 μg/L, respectively. Regarding the production of partially ^{15}N-labelled cobalamins ([^{15}N, $^{14}N_2$-DMB]-cobalamins), the cells grown in the DMB-supplemented [^{15}N]-CDMG contained 7.4 ± 1.5 μg/g (n = 2) of [^{15}N, $^{14}N_2$]-OHCbl and 77 ± 15 μg/g (n = 2) of [^{15}N, $^{14}N_2$]-AdoCbl. The corresponding volumetric yields were 96 ± 18 μg/L and 990 ± 210 μg/L, respectively, for [^{15}N, $^{14}N_2$]-OHCbl and [^{15}N, $^{14}N_2$]-AdoCbl.

The obtained yields are comparable to the results of Hugenschmidt et al. (2010), who reported a B12 yield of 900 μg/L for the same strain (DSM 20271) grown in a complex medium based on whey permeate with cobalt and DMB supplementation. According to Hugenschmidt et al. (2010), the DSM 20271 was not among the best B12 producing strains as a maximum B12 production of 2.5 mg/L was obtained among 37 natural *P. freudenreichii* strains investigated. Therefore, the production of ^{15}N-labelled cobalamins can be further improved by using more capable *P. freudenreichii* strains in the future. Continuously controlling culture pH over the fermentation period might be another effective approach with respect to improving B12 yields as Quesada-Chanto et al. (1998) obtained significantly higher

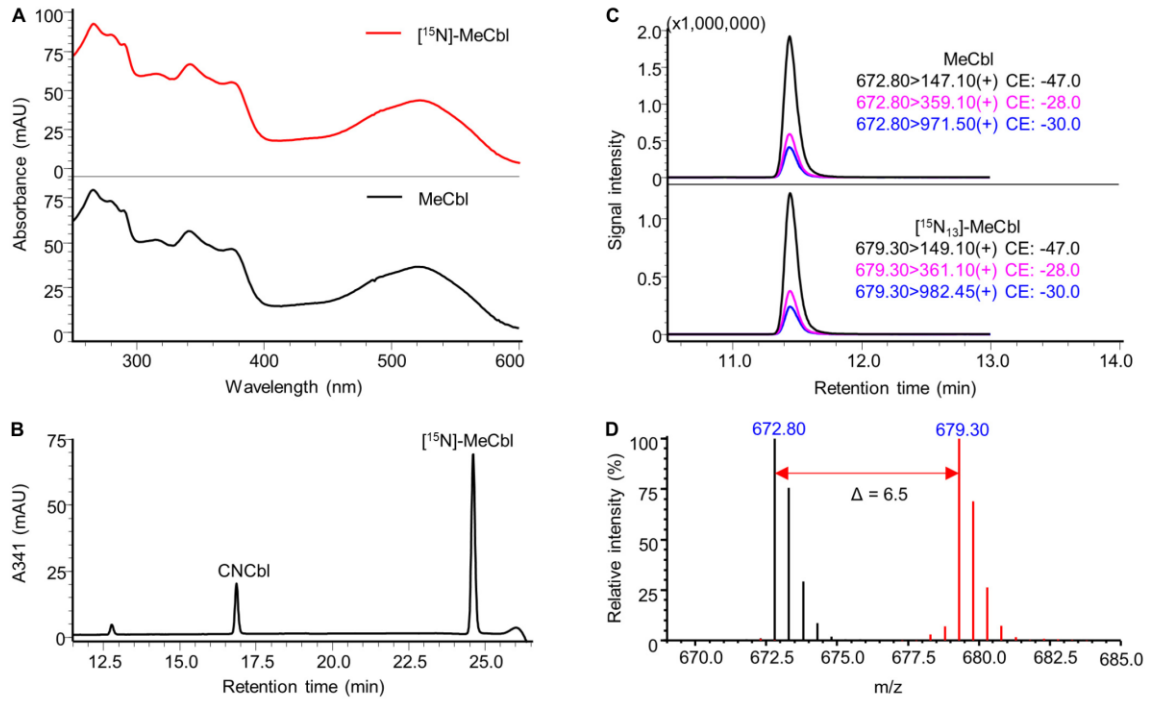

FIGURE 6 | Characterisation of prepared [^{15}N]-MeCbl. **(A)** UV-Vis spectrum of the prepared [^{15}N]-MeCbl in comparison to that of the MeCbl standard. **(B)** HPLC-DAD analysis at a wavelength of 341 nm of the product from methylation reaction showed a major peak of [^{15}N]-MeCbl at the same retention time of unlabelled MeCbl standard (see **Supplementary Figure 6**). **(C)** LC-MS/MS analysis of [^{15}N]-MeCbl monitoring mass transitions corresponding to [$^{15}N_{13}$]-MeCbl (lower trace). The LC-MS/MS chromatogram of unlabelled MeCbl is shown as a reference (upper trace). **(D)** Overlaid mass spectra of [^{15}N]-labelled MeCbl (red) and unlabelled MeCbl (black). The mass shift in $[M + 2H]^{2+}$ values indicated by the red arrow confirmed the labelling of 13 nitrogen atoms. MS spectra were acquired in selected ion monitoring (SIM) mode with a m/z interval of 0.5.

B12 yields of 2.7 mg/L for the DSM 20271 with automatic pH adjustment to 6.5 during the whole fermentation period.

Isolation and Characterisation of Labelled Compounds

The biosynthesised ^{15}N-labelled cobalamins were kept in the harvested cells at −20°C for long-term storage due to the low chemical stability of cobalamins. A portion of the cells was extracted for further preparation of the labelled compounds. Isolation of the labelled cobalamins from bacterial cells after SPE clean-up and further chemical modifications were performed using semi-preparative HPLC.

The identity of the prepared labelled compounds was confirmed by HPLC-DAD and LC-MS/MS in comparison to respective unlabelled standards. The characterisation of [^{15}N]-AdoCbl is summarised in **Figure 4**. The UV-Vis spectrum of the isolated [^{15}N]-AdoCbl was identical to that of the unlabelled AdoCbl (**Figure 4A**). The HPLC-DAD chromatogram of [^{15}N]-AdoCbl showed a single major peak eluting at the same time as the unlabelled AdoCbl (**Figure 4B** and **Supplementary Figure 6**), indicating a high chromatographic purity. Mass spectrometric results confirmed that the majority of [^{15}N]-AdoCbl was fully labelled [$^{15}N_{18}$]-AdoCbl, as the base peak shifted from m/z 790.30 ([M + 2H]$^{2+}$) to m/z 799.30 (Δ m/z = + 9 in double charged forms). As expected, there was no spectral overlap between labelled and unlabelled compounds. Moreover, unlabelled AdoCbl was not at all detected in the [^{15}N]-labelled product, proving that no carry-over from the inoculum took place, which might also have been an issue for mass spectrometric interferences (**Figure 4D**). A peak correspondingx to [$^{15}N_{18}$]-AdoCbl, which eluted at the same retention time of unlabelled AdoCbl, was observed when analysed by LC-MS/MS in the multiple reaction monitoring (MRM) mode (**Figure 4C**). The intensity ratios between different labelled mass transitions from [$^{15}N_{18}$]-AdoCbl were comparable to those of unlabelled transitions from AdoCbl, thus meeting an important quality control criterion in tandem mass spectrometry for unequivocal identification. Taken together, the labelled compound was obtained in high purity and is perfectly suited for future use in SIDA or other applications that require isotopologues. Similar results were obtained for [^{15}N]-CNCbl and [^{15}N]-MeCbl (**Figures 5**, **6**).

In terms of [^{15}N, $^{14}N_2$-DMB]-OHCbl prepared from DMB-supplemented [^{15}N]-CDMG under anaerobic conditions, the base peak shifted from m/z

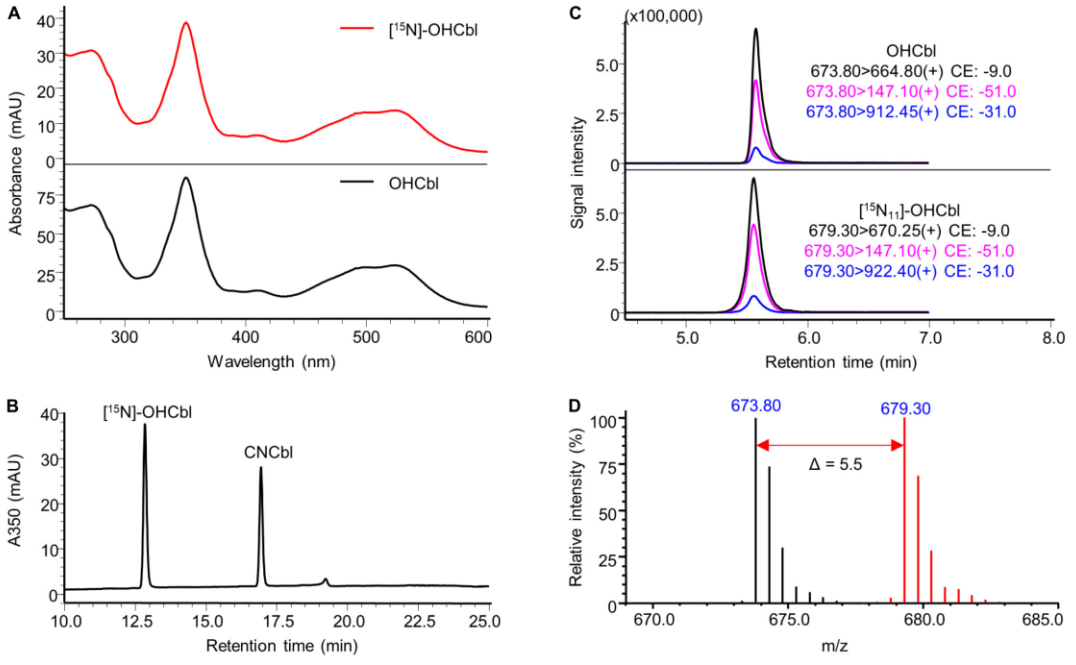

FIGURE 7 | Characterisation of prepared [^{15}N, $^{14}N_2$-DMB]-OHCbl. **(A)** UV-Vis spectrum of the prepared [^{15}N, $^{14}N_2$-DMB]-OHCbl in comparison to that of the OHCbl standard. **(B)** HPLC-DAD analysis at a wavelength of 350 nm of the prepared substance showed a major peak at the same retention time of unlabelled OHCbl standard (see **Supplementary Figure 6**). CNCbl shown on the chromatogram was added as internal standard to determine the absolute concentration of [^{15}N, $^{14}N_2$-DMB]-OHCbl. **(C)** LC-MS/MS analysis of [^{15}N, $^{14}N_2$-DMB]-OHCbl monitoring mass transitions corresponding to [$^{15}N_{11}$, $^{14}N_2$-DMB]-OHCbl (lower trace). The LC-MS/MS chromatogram of unlabelled OHCbl is shown as a reference (upper trace). **(D)** Overlaid mass spectra of [^{15}N, $^{14}N_2$-DMB]-OHCbl (red) and unlabelled OHCbl (black). The mass shift in [M + 2H]$^{2+}$ values indicated by the red arrow confirmed the labelling of 11 nitrogen atoms. MS spectra were acquired in selected ion monitoring (SIM) mode with a m/z interval of 0.5.

673.80 ($[M + 2H]^{2+}$) to m/z 679.30 (Δ m/z = + 5.5 in double charged forms) in the LC-MS spectra (**Figure 7**), indicating a fully ^{15}N-labelled corrin ring and the existence of two unlabelled nitrogen atoms in the lower ligand DMB. Residues of unlabelled OHCbl were not detected in the labelled compound. The corresponding peak of [$^{15}N_{11}$, $^{14}N_2$-DMB]-OHCbl in the LC-MS/MS chromatogram further confirmed the anticipated labelling. The synthesised [^{15}N, $^{14}N_2$-DMB]-OHCbl had sufficient mass increment that does not overlap with natural isotopologues of OHCbl (**Figure 7D**). Taken together, the modification of the biosynthetic strategy yielded specifically labelled [^{15}N, $^{14}N_2$]-OHCbl with the desired labelling pattern that allows the differentiation from fully labelled [^{15}N]-OHCbl as degradation product from other labelled cobalamins.

CONCLUSION

We successfully prepared four ^{15}N-labelled cobalamin vitamers via a biosynthetic approach utilising *P. freudenreichii* in combination with chemical modifications. First, we developed a CDM containing ammonium sulphate as the sole nitrogen source that supports stable growth of *P. freudenreichii*. The CDM was further optimised together with the incubation process for a successful *in vivo* cobalamin biosynthesis. The optimised CDM (CDMG) and incubation process were applied to produce ^{15}N-labelled cobalamins by substituting $(NH_4)_2SO_4$ with $(^{15}NH_4)_2SO_4$ in the medium. Fully ^{15}N-labelled [^{15}N]-AdoCbl was isolated directly from cell extracts by semi-preparative HPLC. [^{15}N]-CNCbl and [^{15}N]-MeCbl were prepared with further cyanidation and methylation reactions, respectively, using isolated [^{15}N]-AdoCbl or [^{15}N]-OHCbl from cell extracts. Specifically labelled [^{15}N, $^{14}N_2$-DMB]-OHCbl was successfully obtained from *P. freudenreichii* cells grown in DMB-supplemented [^{15}N]-CDMG with an optimised incubation process under anaerobic conditions. After semi-preparative HPLC purification, all four labelled compounds demonstrated high HPLC and MS purity with expected ^{15}N-enrichment. The developed biosynthetic production of ^{15}N-labelled cobalamins is simple, effective, and economical. Future improvement on B12 yield is possible by further optimisation on process control, such as static control of culture pH, and by selecting *P. freudenreichii* strains that have better B12 producing capability.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

MW, ME, and MR conceptualised the research and designed the experiments. MW developed the CDM and analytical methods, performed all microbiological experiments, characterised labelled compounds, and wrote the manuscript. MW and JC performed extractions and purifications of bacterial cells, and were responsible for all preparative HPLC experiments. SA, ME, and MR revised the manuscript. All authors contributed to data analysis and interpretation.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.713321/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Appendix B: Supplementary material: Production of four ^{15}N-labelled cobalamins via biosynthesis using *Propionibacterium freudenreichii*

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Supplementary Material

1 LC-MS/MS

A linear gradient was used for elution as follows: 0-2 min, 10% B; 2-3 min, increase to 30% B; 3-11 min, increase to 50% B; 11-12.5 min, 50% B; 12.5-14 min, increase to 99% B; 14-15 min, 99% B; 15-17 min, return to 10% B; 17-27 min, 10% B. The flow rate was 0.3 mL/min. The MS source parameters were set as follows: ESI (+) mode, interface voltage of 4 kV, interface temperature of 340 °C, heat block temperature of 400 °C, desolvation line temperature of 90 °C, nebulizing gas flow of 3 L/min, heating gas flow of 10 L/min, drying gas flow of 10 L/min. Collision-induced dissociation (CID) gas pressure was set at 310 kPa from 0 to 9 min and changed to 270 kPa from 9 min until the end of the run. The detailed MRM parameters are summarized in Table S1.

2 UHPLC-Q-TOF-MS

A linear gradient was used for separation as follows: 10 min pre-run time, 10% B; 0-20 min, increase to 40% B; 20-22 min, 40% B; 22-26 min, increase to 95% B; 26-28 min, 95% B; 28-30 min, return to 10%. The flow rate was 0.3 mL/min. The MS source parameters were set as follows: ESI (+) mode, capillary voltage of 4.5 kV, end plate offset of 500 V, dry heater of 200 ºC, nitrogen flow rate of 10 L/min, and nebulizer pressure of 2.0 bar. Data acquisition was performed in data dependent acquisition (DDA) mode with fragmentation of the three most abundant peaks per scan at a mass range from m/z 50 to m/z 1500. The injection volume was 5 µL in partial loop mode. The mass spectrometer was calibrated by injecting ESI-L Low Concentration Tuning Mix (Agilent Technologies, Santa Clara, CA, USA). Internal calibration of the spectra was performed by injecting ESI-L Low Concentration Tuning Mix (1:4 diluted in acetonitrile), introduced by a switching valve.

Supplementary Material

List of supplementary tables and figures:

Table S1. MRM parameters of LC-MS/MS for unlabelled and labelled cobalamins.

Analyte	Precursor ion m/z	Product ion m/z	Q1 Pre-bias (V)	CE (V)	Q3 Pre-bias (V)	Dwell time (ms)
OHCbl	673.80	664.80	36.0	9.0	20.0	70.0
		147.10	20.0	51.0	28.0	70.0
		912.45	20.0	31.0	28.0	70.0
[$^{15}N_{11}$, $^{14}N_2$-DMB]-OHCbl	679.30	670.25	36.0	9.0	20.0	70.0
		147.10	20.0	51.0	28.0	70.0
		922.40	20.0	31.0	28.0	70.0
AdoCbl	790.35	665.30	22.0	21.0	26.0	70.0
		147.10	22.0	49.0	26.0	70.0
		359.10	22.0	30.0	26.0	70.0
		971.50	24.0	33.0	38.0	70.0
[$^{15}N_{18}$]-AdoCbl	799.30	671.75	22.0	21.0	26.0	70.0
		149.10	22.0	49.0	26.0	70.0
		361.10	22.0	30.0	26.0	70.0
		982.45	24.0	33.0	38.0	70.0
CNCbl	678.30	147.10	34.0	37.0	28.0	70.0
		359.10	34.0	24.0	14.0	70.0
		664.80	20.0	18.0	20.0	70.0
		912.45	20.0	34.0	28.0	70.0
[$^{15}N_{13}$]-CNCbl	684.75	149.10	34.0	37.0	28.0	70.0
		361.10	34.0	24.0	14.0	70.0
		671.25	20.0	18.0	20.0	70.0
		922.40	20.0	34.0	28.0	70.0
MeCbl	672.80	147.10	20.0	47.0	28.0	100.0
		359.10	36.0	28.0	26.0	100.0
		971.50	20.0	30.0	38.0	100.0
[$^{15}N_{13}$]-MeCbl	679.30	149.10	20.0	47.0	28.0	100.0
		361.10	36.0	28.0	26.0	100.0
		982.45	20.0	30.0	38.0	100.0

3

Table S2. Composition of the minimum defined medium (medium M).

	Components	**Concentration (g/L)**
Carbon source	Sodium DL-lactate*	12
Nitrogen source	$(NH_4)_2SO_4$	3
Minerals	K_2HPO_4	8.7
	KH_2PO_4	6.8
	$MgSO_4.7H_2O$	0.2
	$FeSO_4.7H_2O$	0.01
	$MnSO_4.H_2O$	0.02
	$ZnSO_4.H_2O$	0.01
	NaCl	0.2
	$CaCl_2.2H_2O$	0.132
	$CoCl_2.6H_2O$	0.002
Vitamins	Calcium pantothenate	0.001
	Biotin	0.001
	Thiamin.HCl	0.001
	Nicotinic acid	0.001
	Riboflavin	0.001
	Pyridoxal phosphate	0.001
	p-aminobenzoic acid	0.0002
	Folic acid	0.00002
Stimulating agents	Myo-inositol	0.1
	Tween 80	0.5

**20 g of sodium DL-lactate syrup (60%, w/w) was used.*

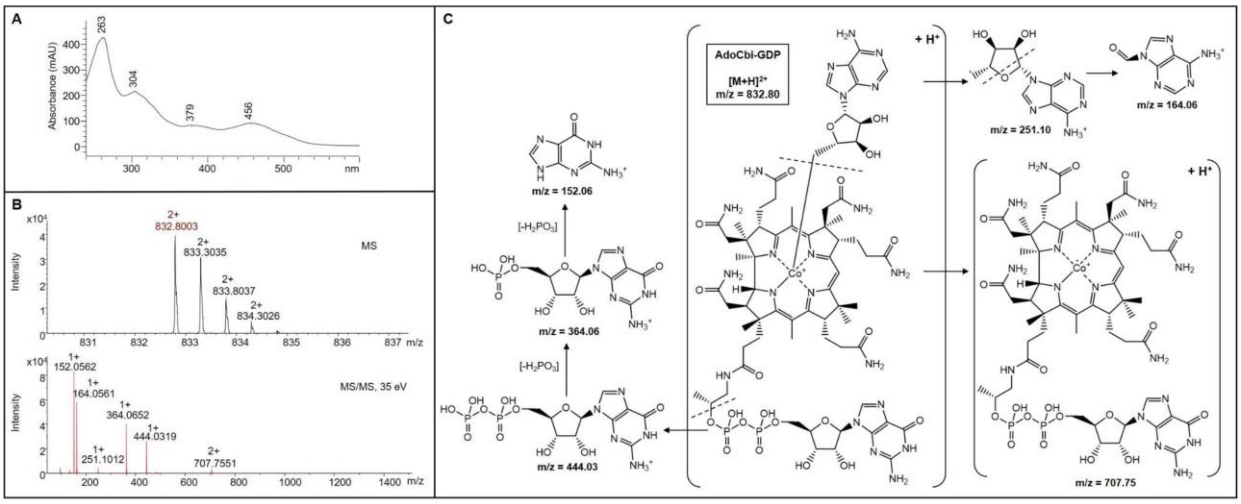

Figure S1. UV-Vis spectrum (A) and UHPLC-QTOF-MS analysis (B) of the peak #2 (AdoCbi-GDP) on Figure 2 with the proposed fragmentation pattern (C). "1+" and "2+" on the MS spectra (B) indicate singly and doubly charged ions, respectively.

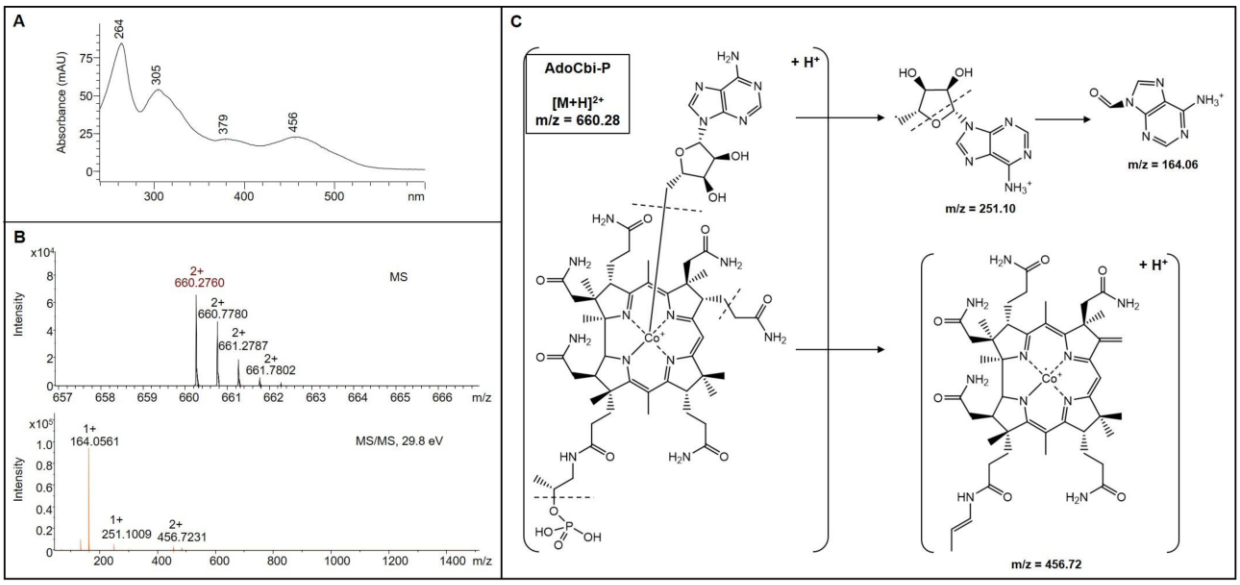

Figure S2. UV-Vis spectrum (A) and UHPLC-QTOF-MS analysis (B) of the peak #4 (AdoCbi-P) on Figure 2 with the proposed fragmentation pattern (C). "1+" and "2+" on the MS spectra (B) indicate singly and doubly charged ions, respectively.

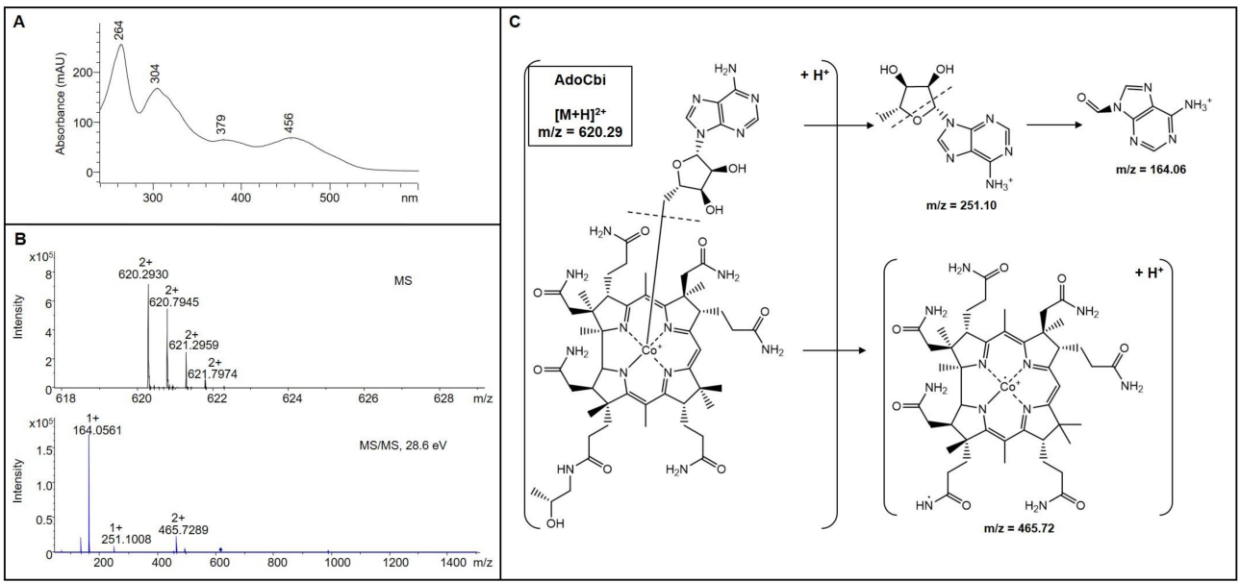

Figure S3. UV-Vis spectrum (A) and UHPLC-QTOF-MS analysis (B) of the peak #6 (AdoCbi) on Figure 2 with the proposed fragmentation pattern (C). "1+" and "2+" on the MS spectra (B) indicate singly and doubly charged ions, respectively.

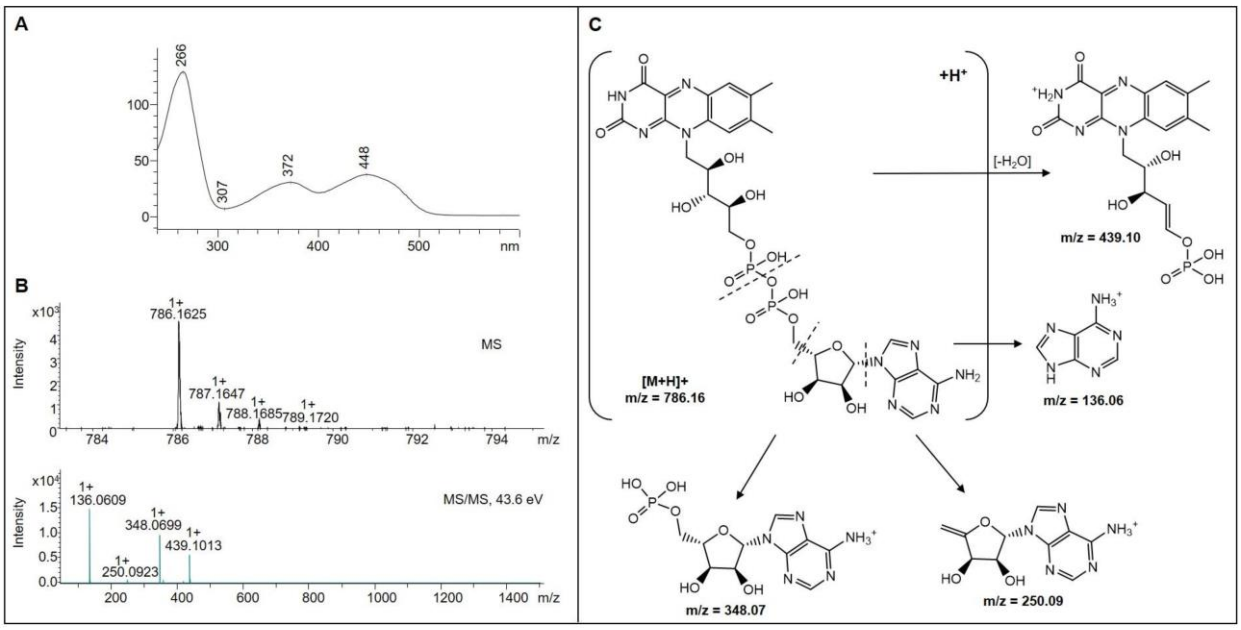

Figure S4. UV-Vis spectrum (A) and UHPLC-QTOF-MS analysis (B) of the peak #3 (FAD) on Figure 2 with the proposed fragmentation pattern (C). "1+" on the MS spectra (B) indicates singly charged ions.

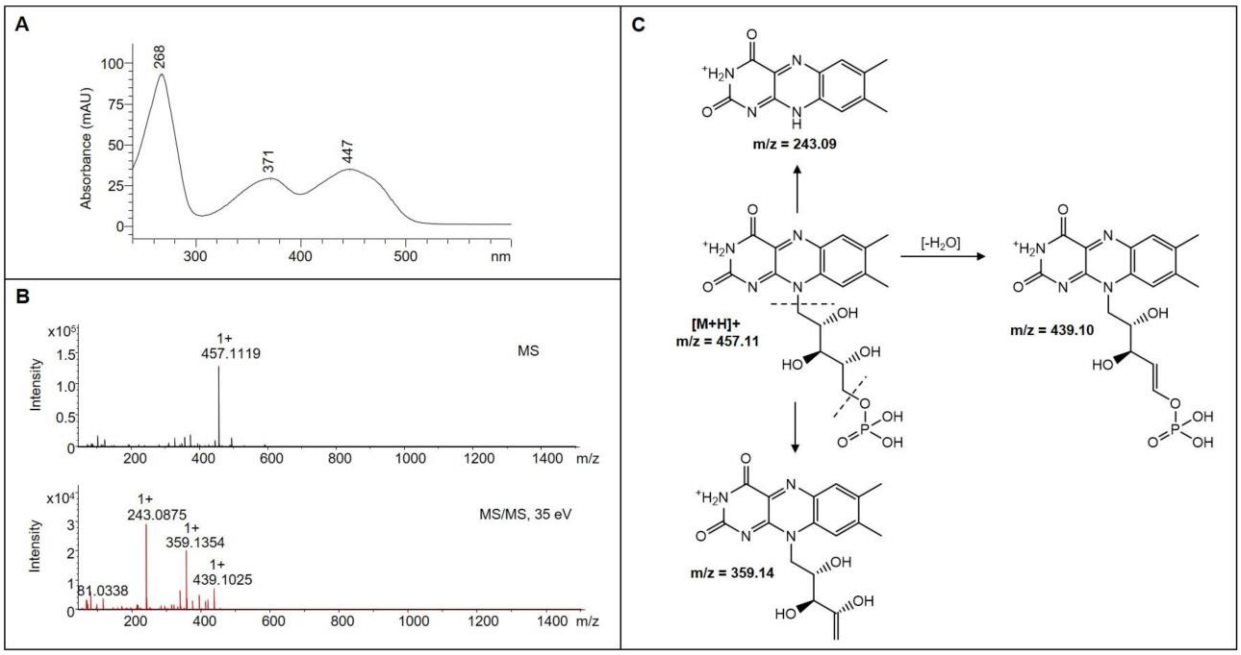

Figure S5. UV-Vis spectrum (A) and UHPLC-QTOF-MS analysis (B) of the peak #5 (FMN) on Figure 2 with the proposed fragmentation pattern (C). "1+" on the MS spectra (B) indicates singly charged ions.

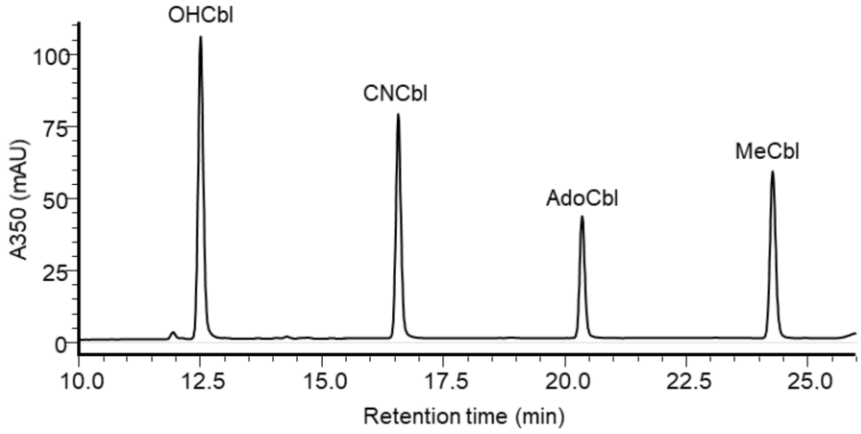

Figure S6. HPLC-DAD chromatogram of a standard solution composed of four cobalamin standards under analytical conditions.

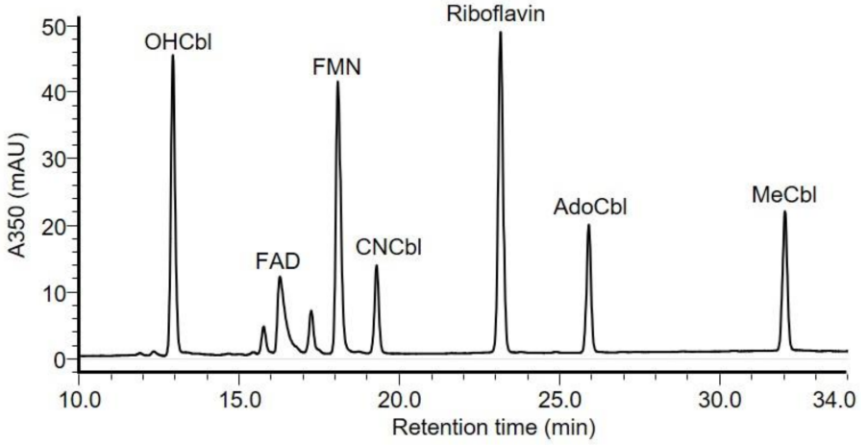

Figure S7. HPLC-DAD chromatogram of a standard solution composed of seven standards under semi-preparative conditions.

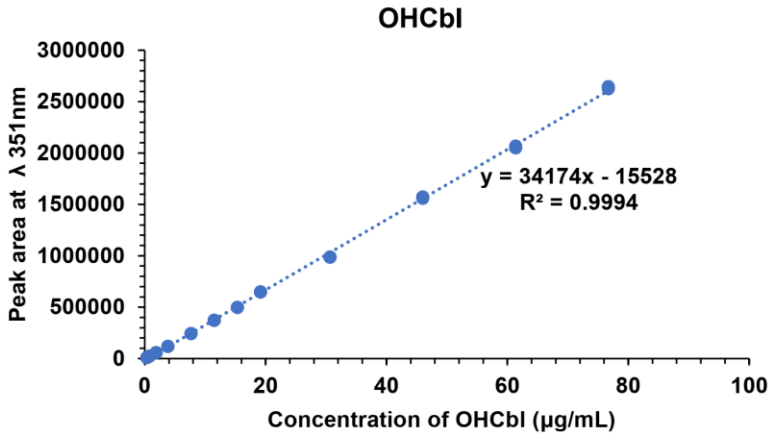

Figure S8. Calibration curve of OHCbl for HPLC-DAD.

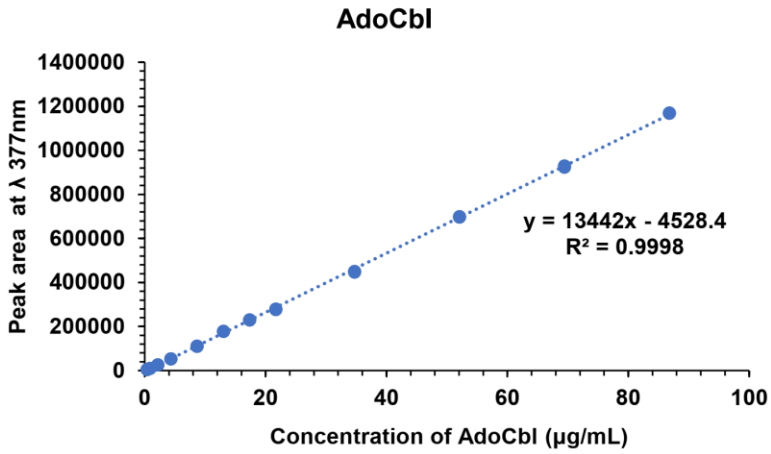

Figure S9. Calibration curve of AdoCbl for HPLC-DAD.

Appendix C: Development Development of stable isotope dilution assays for the analysis of natural forms of vitamin B12 in meat

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Development of Stable Isotope Dilution Assays for the Analysis of Natural Forms of Vitamin B12 in Meat

Mengle Wang,* Stefan Asam, Jianqi Chen, and Michael Rychlik

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ABSTRACT: The first multiple stable isotope dilution assay method was developed for the simultaneous determination of four cobalamins, namely, hydroxocobalamin (OHCbl), adenosylcobalamin (AdoCbl), methylcobalamin (MeCbl), and cyanocobalamin (CNCbl), in their native forms. The sample preparation was optimized with enzyme treatment and immunoaffinity purification. The analysis was performed by LC-MS/MS using respective ^{15}N-labeled cobalamins as internal standards. Method validation resulted in limits of detection ranging from 0.19 to 0.58 ng/g and limits of quantification ranging from 0.68 to 1.73 ng/g. Recoveries at three levels were between 82 and 121%. Intra-day and inter-day precisions were below 6% and 11% RSD, respectively. The analysis of a reference material resulted in a variance of <1% from the certified value. The newly developed method demonstrated excellent sensitivity, recovery, accuracy, and reproducibility and was further applied to quantitate the four cobalamins in various meats.

KEYWORDS: *native vitamin B12, cobalamin, ^{15}N-label, LC-MS/MS, quantitation*

1. INTRODUCTION

Cobalamins, commonly known as the generic term of vitamin B12 (B12), are a family of structurally similar compounds. The fundamental core structure of cobalamins is a cobalt-centered tetracyclic corrin ring system to which an upper β-ligand and a lower α-ligand are coordinated (Figure 1). The lower ligand (Figure 1, boxed) for all cobalamins is 5,6-dimethylbenzimidazole (DMB), a structure crucial for the binding of the molecules to an intrinsic factor for intestinal absorption.[1] The upper ligand differs to form individual vitamers including hydroxocobalamin (OHCbl), adenosylcobalamin (AdoCbl), methylcobalamin (MeCbl), and cyanocobalamin (CNCbl). It has to be noted that CNCbl is not a natural vitamer, but it is commonly used for supplementation due to its superior stability and also as an analytical target after all natural vitamers have been converted into this form. In mammalian cells, AdoCbl and MeCbl are the forms functioning as cofactors for methionine synthase (EC 2.1.1.13) involved in DNA synthesis and methylmalonyl-CoA mutase (EC 5.4.99.2) involved in branched-chain amino acid and odd-chain fatty acid metabolism,[2,3] respectively. OHCbl and CNCbl can be used after conversion to these two cofactor forms in the human body. Some structurally similar compounds found in nature that have the same core structure as cobalamins but have a lower ligand of different chemical variants (e.g., benzimidazole, purine, and phenol derivatives) other than DMB are considered biologically inactive in human organisms[4−7] and are categorized as cobalamin analogues. Cobalamins and their analogues are collectively called cobamides.

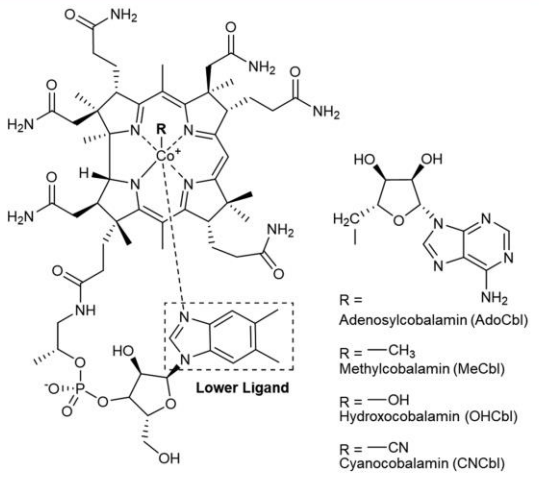

Figure 1. Structure of cobalamin. For all cobalamins, the lower ligand indicated by the box is 5,6-dimethylbenzimidazole (DMB). The upper ligand (R) differs to form individual vitamers including hydroxocobalamin (OHCbl), adenosylcobalamin (AdoCbl), methylcobalamin (MeCbl) and cyanocobalamin (CNCbl).

From a nutritional point of view, B12 is one of the most critical vitamins, as its natural production is restricted to certain bacteria and archaea.[8] Due to natural food-chain enrichment, animal-derived foods (i.e., meat, milk, egg, fish) are considered to be the predominant dietary sources of cobalamins. Plant-based foods are lacking cobalamins unless contaminated or processed with certain bacteria. Therefore,

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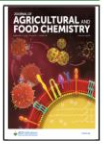

vegetarians, vegans, and elderly population with gastrointestinal malfunctions are potentially endangered by cobalamin deficiency, with symptoms ranging from mild anemia to severe neurological manifestations.[9] With the currently growing populations of vegans, vegetarians, and the elderly, this issue can be expected to become even more relevant in the future. Although cobalamin deficiency can be treated with oral supplements or injection therapies, neurological damages cannot be reversed. Thus, cobalamin deficiency should be prevented by monitoring the dietary intake to meet the recommended values of 2.4 μg/day for adults and 0.9 to 1.8 μg/day for children.[10] For vegetarians and vegans with dietary restrictions on animal-based foods, the search for novel B12 dietary sources that are not of animal origin is highly requested. Alternatively, B12 can be introduced into plant-based foods via biofortification using specific bacterial strains.[11] For both cases, it is important to confirm the presence of authentic cobalamins rather than non-bioactive analogues using reliable and specific analytical methods.

For the analysis of B12 in foods, three major challenges have to be considered: (i) the low concentrations of cobalamins in non-fortified foods, (ii) the occurrence of different vitamers with even lower individual concentration, and (iii) the chemical instability of cobalamins leading to degradation and inter-conversion between forms. Current analysis of cobalamins in foods are carried out using various techniques,[12] among which the microbiological assay (MBA)[13] and high-performance liquid chromatography (HPLC)-UV[14−17] being the most common ones. Recently, a new method using liquid chromatography (LC) and inductively coupled plasma (ICP)-MS has been developed for analyzing B12 in human milk.[18] These approaches, however, suffer from different drawbacks. The MBA is based on the growth of bacteria, such as *L. leichmannii*, which reveals a reasonable sensitivity but lacks the specificity and selectivity. Therefore, false or overestimated results may be obtained in the presence of analogues (e.g., pseudovitamin B12), other inactive corrinoids, or even some nucleic acids.[19] Furthermore, the MBA requires sterile working conditions and can be challenging for analytical chemists, who are not experienced in performing microbiological experiments. The HPLC-UV methods often lack the necessary sensitivity, which requires high sample amounts for analysis even after sample preparation using immunoaffinity purification that enriches the analytes. Moreover, the UV detection also lacks specificity as interferences, such as cobalamin analogues, can be confused with the target analytes due to the similar absorption patterns. In addition, nearly all currently applied HPLC-UV methods make use of conversion of natural B12 vitamers into CNCbl during sample treatment, which results in a loss of information on the natural distribution of different vitamers, in particular, the important native contents of the two relevant co-enzyme forms, i.e., AdoCbl and MeCbl. The novel method using LC coupled with ICP-MS has improved sensitivity and specificity. However, the improvement on specificity is limited as the measurements are based on the cobalt atom instead of the intact structure of cobalamins.

The most selective detection in the field of liquid chromatographic (LC) methods is offered by mass spectrometry (MS). Therefore, some publications tried to establish LC-MS-based methods for cobalamin analysis.[20−24] However, the developed methods in most cases were only applied to simple matrices like vitamin supplements and often suffer from matrix effects when analyzing real food samples. Most often, low recoveries and reduced sensitivity can be observed, as the signal intensity in MS is highly dependent on ionization efficiency and the latter is often compromised by interferences from matrix components. In this regard, stable isotope dilution assays (SIDAs) reveal particular advantages in the method portfolio and have been shown to be superior to other methods due to the use of stable isotope-labeled analogues of the analytes to compensate for losses, discriminations, and mass spectrometric interferences.[25] So far, SIDA has not been applied to cobalamin analysis in real foods.

Therefore, the aim of the present study was to develop and validate the first SIDA method for the simultaneous and reliable determination of four cobalamins in their native forms and to apply the method for the analysis of meat samples.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents. Papain from *papaya latex* (EC 3.4.22.2, cat no. P4762), α-amylase from *A. oryzae* (EC 3.2.1.1, cat No. 10065), soybean flour (type I, cat. no. S9633), and certified reference material BCR 487 (lyophilized pork liver; European Commission, Geel, Belgium) were obtained from Sigma-Aldrich (Steinheim, Germany).

Analytical standards of OHCbl·HCl, CNCbl, AdoCbl, and MeCbl were received from Sigma-Aldrich (Steinheim, Germany). The isotopically labeled standards ([$^{15}N_{11}$, $^{14}N_2$-DMB]-OHCbl, [$^{15}N_{18}$]-AdoCbl, [$^{15}N_{13}$]-CNCbl, and [$^{15}N_{13}$]-MeCbl) were prepared in our laboratory as recently reported.[26]

2.2. Samples. Fresh pork fillet, beef fillet, lamb fillet, and chicken breast were bought from a local butcher shop (Freising, Germany) in minced form. More than 200 g was purchased for each sample. The samples were lyophilized (Alpha 1-2 LDplus, Martin Christ Gefriertrocknungsanlagen, Osterode am Harz, Germany), thoroughly ground in a kitchen grinder (EGK 200, Rommelsbacher, Dinkelsbühl, Germany), intensively homogenized, and then stored at −20 °C in the dark until further treatments.

2.3. Preparation of Standard Solutions. Stock solutions of unlabeled cobalamins were prepared by dissolving 1 mg of the respective reference material in 10 mL of water. The stock solutions were divided into 1 mL aliquots and stored at −20 °C in the dark. For each extraction, one aliquot from each solution was freshly thawed and the concentrations were further determined using UV spectroscopy and HPLC-DAD. First, the stock solution of CNCbl was diluted with water (1:3, v/v) and measured by UV spectroscopy for the accurate concentration. Then, the concentrations of OHCbl, AdoCbl, and MeCbl were determined by HPLC-DAD using CNCbl as internal standard. A working solution of four cobalamins, prepared by mixing water (150 μL), CNCbl (100 μL), OHCbl (250 μL), AdoCbl (250 μL), and MeCbl (250 μL) stock solutions, was used for the HPLC-DAD measurements. The concentrations of OHCbl, MeCbl, and AdoCbl were calculated using the response functions listed in Table 1 based on the concentration of CNCbl determined by UV spectroscopy. Details about UV spectroscopy and the preparation of the HPLC-DAD response curves are described in the Supporting Information.

Table 1. HPLC-DAD Response Curves of OHCbl, AdoCbl, and MeCbl[a]

analyte	HPLC-DAD response curve	λ (nm)	R^2
OHCbl	$y = 1.1518x - 0.0428$	351	0.9998
AdoCbl	$y = 0.8036x - 0.0055$	377	0.9999
MeCbl	$y = 1.0648x - 0.0137$	341	0.9999

[a] y are peak area ratios [A(A)/A(IS)] and x are mass concentration ratios [ρ(A)/ρ(IS)]; CNCbl as IS.

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Table 2. MRM Parameters for Cobalamins and Respective Internal Standards[a]

analyte	precursor (m/z)	product (m/z)	dwell time (ms)	Q1 pre-bias (V)	CE (V)	Q3 pre-bias (V)	acquisition time (min)
OHCbl	673.80	**664.80**^{2}	70.0	36.0	9.0	20.0	5.0−7.0
		147.10^{1}	70.0	20.0	51.0	28.0	
		912.45^{1}	70.0	20.0	31.0	28.0	
	664.80*	147.10^{1}	70.0	34.0	50.0	30.0	
[$^{15}N_{11}$, $^{14}N_{2}$-DMB]-OHCbl	679.30	**670.25**^{2}	70.0	36.0	9.0	20.0	5.0−7.0
		147.10^{1}	70.0	20.0	51.0	28.0	
		922.40^{1}	70.0	20.0	31.0	28.0	
	670.30*	147.10^{1}	70.0	34.0	50.0	30.0	
AdoCbl	790.35	**665.30**^{2}	70.0	22.0	21.0	26.0	7.0−7.7
		147.10^{1}	70.0	22.0	49.0	26.0	
		359.10^{1}	70.0	22.0	30.0	26.0	
		971.50^{1}	70.0	24.0	33.0	38.0	
[$^{15}N_{18}$]-AdoCbl	799.30	**671.75**^{2}	70.0	22.0	21.0	26.0	7.0−7.7
		149.10^{1}	70.0	22.0	49.0	26.0	
		361.10^{1}	70.0	22.0	30.0	26.0	
		982.45^{1}	70.0	24.0	33.0	38.0	
CNCbl	678.30	**147.10**^{1}	70.0	34.0	37.0	28.0	7.7−9.5
		359.10^{1}	70.0	34.0	24.0	14.0	
		912.45^{1}	70.0	20.0	34.0	28.0	
[$^{15}N_{13}$]-CNCbl	684.75	**149.10**^{1}	70.0	34.0	37.0	28.0	7.7−9.5
		361.10^{1}	70.0	34.0	24.0	14.0	
		922.40^{1}	70.0	20.0	34.0	28.0	
MeCbl	672.80	**147.10**^{1}	100.0	20.0	47.0	28.0	9.5−13.0
		359.10^{1}	100.0	36.0	28.0	26.0	
		971.50^{1}	100.0	20.0	30.0	38.0	
[$^{15}N_{13}$]-MeCbl	679.30	**149.10**^{1}	100.0	20.0	47.0	28.0	9.5−13.0
		361.10^{1}	100.0	36.0	28.0	26.0	
		982.45^{1}	100.0	20.0	30.0	38.0	

[a]All precursor ions are double charged, product ions indicated with superscript "1" are single charged and double charged when indicated with superscript "2". The first mass transition of each compound, highlighted in bold, was used for quantitation. The other mass transitions were monitored as qualifiers. *, Precursor ions formed with the cleavage of upper ligand (i.e., −OH group) during ionization.

The isotopically labeled standards were redissolved separately in LC-MS water as stock solutions (~10 μg/mL, respectively). Working solutions were obtained by further dilutions in the range of 0.5 and 0.01 μg/mL for spiking during sample extraction. To establish the LC-MS/MS response curves, the absolute concentration of the [$^{15}N_{13}$]-CNCbl stock solution was determined by UV spectroscopy and the absolute concentrations of the other labeled stock solutions were determined by HPLC-DAD using CNCbl as internal standard. The response functions listed in Table 1 were used for calculation. For routine analysis, the concentrations of labeled standards were determined for each extraction by measuring a calibrator solution composed of unlabeled and isotopically labeled standards. For that, the unlabeled stock solutions were diluted to 0.5 μg/mL and further mixed with the labeled internal standards. The concentration of internal standards can be calculated with known amounts of unlabeled standards.

All standard solutions were stored at −20 °C in the dark. CNCbl stock solutions were prepared monthly, whereas OHCbl, AdoCbl, and MeCbl stock solutions were used within 2 months. The stock solutions were separated into aliquots for storage, and freshly thawed aliquots were used for each extraction. The residue from thawed aliquots was discarded and not used for the next extraction. The stability of the CNCbl stock solution was verified over 1 month by UV spectroscopy.

2.4. LC-MS/MS. The LC-MS/MS measurements were performed on a Nexera X2 UHPLC system (Shimadzu, Kyoto, Japan) coupled to a triple quadrupole mass spectrometer (LCMS 8050, Shimadzu, Kyoto, Japan) using electrospray ionization (ESI) in positive mode. A Hydrosphere C18 column (150 × 3.0 mm, 3 μm, YMC, Dinslaken, Germany) with a C18 guard column (4 × 2.0 mm, Phenomenex, Aschaffenburg, Germany) was used for chromatographic separations at 30 °C. Mobile phases were composed of 0.1% acetic acid in water (solvent A) and 100% methanol (solvent B). A linear gradient was programmed for separation as follows: 0−2 min, 10% B; 2−3 min, 10−30% B; 3−11 min, 30−50% B; 11−12.5 min, 50% B; 12.5−14 min, 50−99% B; 14−15 min, 99% B; 15−17 min, 99−10% B; 17−27 min, 10% B. The flow rate was 0.3 mL/min, and the injection volume was 5 μL.

MS interface parameters were set as follows: interface voltage, 4 kV; interface temperature, 340 °C; desolvation line temperature, 90 °C; heat block temperature, 400 °C; nebulizing gas flow, 3 L/min; heating gas flow, 10 L/min; and drying gas flow, 10 L/min. The collision-induced dissociation (CID) gas pressure was 310 kPa for OHCbl, AdoCbl, and CNCbl and was set to 270 kPa for MeCbl. The acquisition was performed in scheduled multiple reaction monitoring (MRM) mode with details summarized in Table 2. Both quadrupoles Q1 and Q3 were set to unit resolution. The interface and MRM parameters were optimized by direct injection of each unlabeled standard at a concentration of 1 μg/mL. The optimized interface parameters, voltages, and collision energies of mass transitions were adopted for respective isotopically labeled internal standards. LabSolutions software (Shimadzu, Kyoto, Japan) was used for system control, data acquisition, and processing.

2.5. Sample Preparation. The extraction and purification were developed based on previous methods[14−16,27] with modifications.

A total of 1 g of lyophilized, finely ground, and homogenized meat sample (or 0.2 g of BCR 487 pork liver), 5 mg of papain, and 5 mg of α-amylase were weighted into a 50 mL amber extraction vial (Duran, Mainz, Germany). The isotopically labeled internal standards were added in amounts based on the expected contents of respective analytes in the sample to fall inside the calibration range. Extraction buffer (25 mL, 50 mM sodium acetate buffer, pH = 4.0) was then

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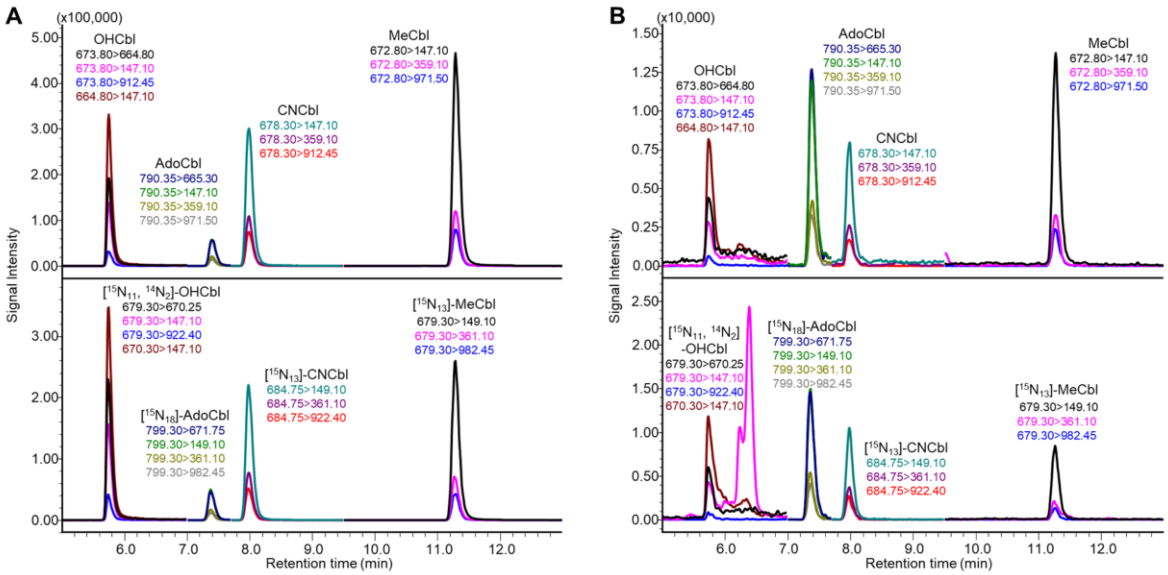

Figure 2. LC-MS/MS chromatograms of a mixed standard solution composed of cobalamins and respective internal standards (A) and a pork sample (B). MRMs of analytes displayed on the upper traces and MRMs of labeled standards displayed on the lower traces.

added to the sample, and the mixture was vortexed and further stirred with a magnetic stir bar for 20 min at room temperature (RT) for equilibrating. Afterward, the homogenate was incubated in a shaking water bath (GFL 1092, Burgwedel, Germany) at 37 °C for 1 h. Then, the sample was heated for 10 min in a boiling water bath, cooled in an ice-water bath, and transferred into a 50 mL amber centrifuge tube. The extraction vial was washed with 5 mL of extraction buffer, which was also transferred to the centrifuge tube. Subsequently, the sample was centrifuged (Eppendorf 5810R, Hamburg, Germany) for 20 min (3220 × *g*, RT). The supernatant was collected and then paper filtered (Whatman $597^{1/2}$) before further clean-up.

Immunoaffinity columns (IAC, EASI-Extract Vitamin B12, R-Biopharm, Glasgow, UK) were used for purification. The whole filtrate was passed through the column, and the column was washed with 10 mL of water. After drying the column under vacuum, the cobalamins were eluted twice using 2 mL of methanol each time with back flushing into an amber sample vial. For back flushing, a syringe was used to purge the solvent upward for three times during elution, in order to extend the contact time between methanol and IAC. The eluate was dried under a stream of nitrogen at 40 °C in an evaporator system (EC2, VLM, Bielefeld, Germany). The residue was reconstituted in 300 μL of LC-MS water and membrane filtered (PVDF, 0.22 μm) prior to LC-MS/MS analysis.

Papain and α-amylase were checked to confirm the absence of endogenous cobalamins before using them for extraction. All extraction and purification processes were performed under subdued light and protected from direct sunlight.

2.6. Calibration and Quantitation of LC-MS/MS. All cobalamins were quantified using corresponding ^{15}N-labeled compounds as internal standards (IS).

The calibration points were prepared by mixing varying amounts of analytes (A) with constant amounts of respective internal standards (IS) in 13 different molar ratios (n(A)/n(IS)) between 0.01 and 100 (1:100, 1:50, 1:20, 1:10, 1:5, 1:2, 1:1, 2:1, 5:1, 10:1, 20:1, 50:1, and 100:1). The response curves were obtained by plotting the peak area ratios [A(A)/A(IS)] against the molar ratios [n(A)/n(IS)] from LC-MS/MS measurements. Linear and quadratic regressions were performed, and non-linearity was checked by Mandel's fitting test.[28] Mandel's fitting test is an F test that compares the residual variances of the linear regression model with that of the quadratic model.

The contents of cobalamins in all samples were calculated based on the LC-MS/MS response curves. All contents reported in the present study are based on dry matter.

2.7. Method Validation. Soybean flour was analyzed to confirm the absence of target cobalamins and was further used as a surrogate blank matrix for method validation due to its similarity to the composition of meat.

2.7.1. LOD and LOQ. LODs and LOQs were determined according to Vogelgesang and Hadrich.[29] The blank matrix was spiked with unlabeled analytes at four different levels (1.91, 4.78, 11.95, and 19.12 ng/g for OHCbl; 0.48, 1.92, 3.30, and 4.80 ng/g for AdoCbl; 0.23, 0.91, 1.60, and 2.28 ng/g for CNCbl; 0.50, 1.99, 3.62, and 4.97 ng/g for MeCbl), covering the range from slightly above the estimated LODs to concentrations 10-fold higher. Each spiking level was prepared in triplicate. The samples were analyzed as described above.

2.7.2. Recovery. The recoveries of each cobalamin were determined in soybean flour at three different levels, respectively. Soybean flour was spiked with OHCbl (4.98, 11.95, and 19.12 ng/g), AdoCbl (3.30, 11.78, and 18.86 ng/g), CNCbl (0.92, 5.71, and 9.13 ng/g), and MeCbl (3.61, 12.92, and 20.67 ng/g). Each spiking level was prepared in triplicate. Analysis was performed as previously described. The recoveries were calculated as the ratio between found and spiked amounts.

2.7.3. Precision. A pork fillet sample containing all four cobalamins was used for inter-injection (n = 6), intra-day (n = 3), and inter-day (n = 3, quadruplicate analysis each week for 3 weeks) precision measurements.

3. RESULTS AND DISCUSSION

3.1. Method Development. *3.1.1. Concentration Determination of Cobalamin Standards.* Cobalamins are known for their chemical instability that includes degradation as well as inter-conversion into other vitamers. This affects not only endogenous cobalamins in food but also standards used for quantitation in analytical chemistry. Therefore, commercial primary standards should be checked for their purity every time before use rather than relying on the simple gravimetric measurements and the purity declaration of the manufacturer. To do so, we adopted an approach that has been successfully

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applied by our group previously to the analysis of folate vitamers that encounter the same stability problem.[30] For such a purpose, we established an HPLC-DAD method for the routine quantification of the unlabeled standard solutions of CNCbl, OHCbl, AdoCbl, and MeCbl. CNCbl, the most stable form, was chosen as reference standard and quantified using UV spectroscopy after gravimetric preparation of the stock solution. The CNCbl standard solutions were also checked regularly by HPLC-DAD to ensure the absence of interfering compounds that might cause errors on the UV read-outs. This CNCbl standard solution was further used as internal standard for the quantification of the other vitamers using the HPLC-DAD. For method development, the stock solutions of all four vitamers used for establishing the HPLC-DAD response curves were quantified using q-NMR to guarantee the utmost accuracy of the response functions (details in the Supporting Information). Thus, the absolute concentration of the stock solutions could be determined daily based on the UV measurement of CNCbl and HPLC-DAD measurements of the other vitamers using the response curves. To address the inter-conversion problem, the cobalamins were not measured separately, but a mixed standard solution of all four cobalamins was particularly used for the quantification to compensate for the potential conversion of AdoCbl and MeCbl into OHCbl. Similar to the fact that the concentration of the stock solutions of the unlabeled cobalamins has to be checked daily using the method described above, the concentration of the labeled standards also has to be supervised by measuring individual responses using LC-MS/MS.

3.1.2. Chromatography. We evaluated several reverse-phase C18 columns using common mobile phases with or without the addition of common MS compatible modifiers including formic acid, acetic acid, ammonium acetate, and ammonium formate. Ion suppression was observed for all additives tested in the mobile phases. However, proper buffering of the mobile phases was found necessary to keep the retention times of the cobalamins stable. Eventually, the YMC hydrosphere column was chosen as good and reproducible chromatograms were obtained by using 0.1% acetic acid in the aqueous phase. With this method, all four cobalamin peaks were baseline separated with satisfactory intensities and were further resolved from interfering compounds in the real samples (Figure 2).

3.1.3. Mass Spectrometry. In the positive ESI mode, all cobalamins under study gave various ion species with different charge states and the double charged $[M + 2H]^{2+}$-ions were the most abundant ones for AdoCbl, CNCbl, and MeCbl (m/z 790.30, 678.30, and 672.80, respectively). However, the most intense peak for OHCbl was the signal of the $[M-OH + H]^{2+}$-ion (m/z 664.80) as the result of in-source fragmentation, i.e., the cleavage of the β-ligand (−OH) bound to cobalt.

For the MS/MS method, the double charged species of the highest intensity were chosen as precursors for each analyte. For OHCbl, the less-intense doubly protonated molecule $[M + 2H]^{2+}$ without the loss of the hydroxyl group (m/z 673.80) was also selected as a precursor for fragmentation to ensure specificity and unequivocal identification. The proposed fragmentation patterns are shown in Figures S1−S4 (Supporting Information).

In the preliminary experiments, mass transitions of OHCbl from both m/z 673.80 ($[M + 2H]^{2+}$) and m/z 664.80 ($[M-OH + H]^{2+}$) were monitored when analyzing the surrogate matrix and meat samples. Matrix interference was observed near the retention time of OHCbl and $[^{15}N_{11},^{14}N_2$-DMB]-OHCbl, leading to elevated baselines of transitions m/z 673.80 → 147.10 ($[M + 2H]^{2+}$ to $[DMB + H]^+$) for the analyte and m/z 679.30 → 147.10 ($[M + 2H]^{2+}$ to $[DMB + H]^+$) and m/z 670.30 → 147.10 ($[M-OH + H]^{2+}$ to $[DMB + H]^+$) for the internal standard (Figure 2B). Therefore, transitions involving $[DMB + H]^+$ as the product ion, despite of the high signal intensity and the formation of a characteristic fragment for cobalamins, was not used for quantitative purposes. Instead, the paired transitions from $[M + 2H]^{2+}$ to $[M-OH + H]^{2+}$, m/z 673.80 → 664.80 for OHCbl and m/z 679.30 → 670.25 for $[^{15}N_{11},^{14}N_2$-DMB]-OHCbl, were used for quantification, as clean spectra and reproducible results were obtained. By lowering the desolvation line temperature of the MS, the intensities of mass transitions based on the $[M + 2H]^{2+}$-ion (m/z 673.80) of OHCbl increased substantially as the in-source fragmentation diminished (Figure S5), ensuring high sensitivity for OHCbl analysis. For the other cobalamins, the mass transition showing the highest intensity was chosen for quantification and two to three additional transitions for qualification. The optimized MRM parameters are summarized in Table 2.

For all four cobalamins, the relative abundances of natural isotopologues with mass increments higher than M + 5 were found to be negligible (<0.2%). There were no m/z overlaps between unlabeled and labeled compounds, as the labeled ones have mass shifts of M + 11 in the case of $[^{15}N_{11},^{14}N_2$-DMB]-OHCbl, M + 18 in the case of $[^{15}N_{18}]$-AdoCbl, and M + 13 in the case of $[^{15}N_{13}]$-CNCbl and $[^{15}N_{13}]$-MeCbl.

However, spectral overlap could occur between different cobalamins if they are not chromatographically separated. For instance, the natural isotopologue of M + 2 (m/z 673.80; $[M+2H]^{2+}$; relative abundance of 30.3%) from MeCbl could interfere with the MRM transitions of OHCbl (e.g., m/z 673.80 → 147.10). In the same way, the natural isotopologue of M + 2 (m/z 679.30; $[M+2H]^{2+}$; relative abundance of 30.5%) from CNCbl cannot be distinguished by low-resolution quadrupole MS from $[^{15}N_{11},^{14}N_2$-DMB]-OHCbl (m/z 679.30; $[M + 2H]^{2+}$) and $[^{15}N_{13}]$-MeCbl (m/z 679.30; $[M + 2H]^{2+}$) due to the same m/z of precursor ions. Since a complete chromatographic separation of cobalamins was achieved in this study, this type of interference was successfully circumvented during MS measurements.

Another point, which has to be taken into consideration when using stable isotope-labeled cobalamins, is that the internal standard of OHCbl has to have a different labeling pattern than the other labeled standards. The reason for this is the unavoidable degradation of all cobalamins to OHCbl. For example, $[^{15}N_{13}]$-CNCbl will degrade to $[^{15}N_{13}]$-OHCbl during sample preparation, which will lead to inaccuracy for the determination of OHCbl. Therefore, we used an internal standard with a different labeling pattern ($[^{15}N_{11},^{14}N_2$-DMB]-OHCbl) for the determination of natural OHCbl in this study.

3.2. Sample Treatment. Previous instrumental methods for the analysis for cobalamins[14−17,20,22] were mainly based on the quantitation of CNCbl as a whole, and thus cyanide must be used during the extraction to convert all cobalamins into the cyano-form. In the present study, the cobalamins were extracted and analyzed as their native forms. Therefore, the undesirable handling with cyanide was avoided.

Enzyme treatment was applied to release bound forms of cobalamins during extraction. Pepsin, commonly used in previous extraction methods[14−16,20,22,27] was found to contain

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Table 3. Limits of Detection, Limits of Quantification, Recoveries, and Precisions of the SIDA Method

			recovery (%)			precision (RSD %)		
analyte	LOD (ng/g)	LOQ (ng/g)	level 1 (n = 3)	level 2 (n = 3)	level 3 (n = 3)	inter-injection (n = 6)	intra-day (n = 4)	inter-day (n = 3)
OHCbl	0.58	1.73	104 ± 3	118 ± 2	121 ± 1	3	6	8
AdoCbl	0.33	1.00	96 ± 5	82 ± 6	92 ± 11	4	1	6
CNCbl	0.19	0.68	98 ± 3	102 ± 2	101 ± 1	4	4	11
MeCbl	0.30	1.15	114 ± 4	105 ± 1	100 ± 3	2	4	9

cobalamins (mainly CNCbl) at trace levels. In this case, accurate quantitation of cobalamins in samples with low levels was not possible. Alternatively, proteases from non-animal origins were considered and plant-based papain was eventually chosen for the current method as it was tested to be cobalamin-free. Previously reported chromatographic interferences from papain in HPLC-UV[27] did not cause problems due to the application of the highly specific LC-MS/MS method. To develop an economical and time-efficient extraction method, different enzyme amounts (1, 2, 5, and 10 mg), incubation temperatures (37 and 60 °C), and incubation durations (0.5, 1, and 2 h) were investigated taking the low stability of cobalamins into consideration (Table S1 in Supporting Information). Finally, 5 mg of papain and 5 mg of α-amylase were used for each sample hydrolysis at 37 °C for 1 h as the further increase in the enzyme amount or incubation time did not show a difference on the determined contents of cobalamins in real samples. Further decreases in the enzyme amount or incubation time caused difficulties in later filtration. Both the enzyme treatment and further paper filtration were indispensable for a good loading speed of sample extracts on the immunoaffinity columns.

For the sample purification, a highly selective method is necessary for concentrating low levels of cobalamins in meat samples while removing components that cause matrix interferences in LC-MS/MS. The use of immunoaffinity columns demonstrated sufficient selectivity, whereas working with other conventional SPE cartridges, including C18 (DSC-18, Supelco, Bellefonte, PA, USA), HLB, WCX, and MCX (Oasis, Waters, Milford, MA, USA), showed a lack of efficacy. Similar results were reported in previous studies.[14,16]

3.3. Calibration of SIDA. Mandel's fitting test detected non-linearity of the linear regression and indicated that quadratic functions were more suitable than linear functions in the prepared molar ratio ranges for all analytes. The non-linearity is common for response curves based on SIDA when a large calibration range is used.[31] It has to be emphasized that the coefficient of determination (R^2) is not adequate for testing the linearity of calibration functions and instead statistical tests like Mandel's fitting test should be systematically applied during the method validation.[32,33]

In the present study, we decided to rather use quadratic regression than to limit the calibration range to achieve linearity because it was our purpose to keep the method as universal as possible for the analysis of samples with varying contents of cobalamins. The calibration functions were therefore obtained using quadratic regression in molar ratios $[(n(\mathrm{A})/n(\mathrm{IS})]$ between 0.01 and 86.72 for OHCbl ($y = -0.0004x^2 + 1.0194x - 0.1481$, $R^2 = 0.9998$), between 0.01 and 96.15 for AdoCbl ($y = 0.0008x^2 + 0.9691x - 0.0918$, $R^2 = 0.9996$), between 0.01 and 115.61 for CNCbl ($y = 0.001x^2 + 1.0643x - 0.0276$, $R^2 = 0.9999$), and between 0.01 and 121.88 for MeCbl ($y = 0.0025x^2 + 1.1117x - 0.4379$, $R^2 = 0.9997$). For all the LC-MS/MS response curves, the Y axis is the peak area ratio $[A(\mathrm{A})/A(\mathrm{IS})]$ and the X axis is the molar ratio $[n(\mathrm{A})/n(\mathrm{IS})]$. For the quantitation of samples, the curves were mathematically resolved for $n(\mathrm{A})$.

3.4. Method Validation. LODs, LOQs, recoveries, and precisions for all analytes of the developed method are summarized in Table 3. For the determination of LODs and LOQs according to Vogelgesang and Hadrich,[29] a blank matrix is required for spiking purposes. As animal-derived products always contain traces of cobalamins, a soybean flour sample was chosen as the surrogate matrix after being confirmed as blank by LC-MS/MS analysis. Using this blank matrix, the LODs ranged from 0.19 to 0.58 ng/g and the LOQs ranged from 0.68 to 1.73 ng/g. The sensitivity of the SIDA method for CNCbl was at least 26 times better than those previously reported using HPLC-UV[14−17] or HPLC with fluorescence detection.[27] Moreover, it was about 11 times more sensitive than an earlier reported LC-MS method for meat.[21] Validation data for OHCbl, AdoCbl, and MeCbl in the literature are rather limited. To the best of our knowledge, the LODs and LOQs obtained for these analytes in our study were the lowest so far reported, when compared to previous estimated values.[21,24]

Recoveries were determined at three different spiking levels in the surrogate matrix. For all analytes, good and reproducible recoveries were obtained in the range from 82 to 121% (Table 3). Without the application of SIDA, the recoveries for OHCbl, AdoCbl, and MeCbl in meat have been reported to be 68.3, 60.7, and 52.3%, respectively.[21] Therefore, the use of isotopically labeled internal standards clearly improved the accuracy of analysis to a superior level.

Precisions (RSD) were determined by analyzing a pork fillet sample containing OHCbl (11.19 ± 0.90 ng/g), AdoCbl (9.21 ± 0.52 ng/g), CNCbl (1.33 ± 0.15 ng/g), and MeCbl (2.40 ± 0.22 ng/g). As shown in Table 3, inter-injection and intra-day precisions were below 4 and 6%, respectively. Inter-day precisions were slightly higher varying from 6 to 11%. CNCbl had the highest RSD of 11%, which was attributed to its low concentration present in the sample.

The developed method demonstrated the best performance for CNCbl in terms of LOD, LOQ, and recoveries. The LOD and LOQ of CNCbl were about 1.5−5 times lower than values of other analytes, and the recoveries of CNCbl were around 100% at all spiking levels. The discrepancy on the bindings of immunoaffinity columns toward different cobalamin forms might be one of the major reasons for the better values of CNCbl. According to Marley et al.,[15] the antibody demonstrated greater affinity to CNCbl than to OHCbl. Similarly, in our experiments, the binding of CNCbl was found to be more reproducible, mainly revealed by a better reproducibility of absolute peak areas of CNCbl between replicates, than those of other analytes. In addition, the binding of cobalamins in lower concentrations were influenced when other co-existing forms were present at higher levels of a different order of magnitude during immunoaffinity clean-up.

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Table 4. Contents of Cobalamins in Meat and BCR Reference Liver Sample[a]

sample	OHCbl (ng/g)	AdoCbl (ng/g)	CNCbl (ng/g)	MeCbl (ng/g)	Total B12* (ng/g)
pork fillet (n = 12)	11.19 ± 0.90	9.21 ± 0.52	1.33 ± 0.15	2.44 ± 0.22	22.92 ± 1.33
beef fillet (n = 3)	14.54 ± 0.61	12.03 ± 0.66	<LOD (ca. 0.16)	5.28 ± 0.01	30.45 ± 0.08
lamb fillet (n = 4)	35.43 ± 2.59	28.39 ± 0.61	(0.25 ± 0.01)[b]	11.43 ± 0.23	71.79 ± 2.93
chicken breast (n = 4)	4.06 ± 0.35	3.38 ± 0.82	1.69 ± 0.05	(0.76 ± 0.01)[b]	9.44 ± 1.04
BCR pig liver (n = 4)	520.4 ± 9.4	535.5 ± 14.1	12.84 ± 0.34	119.22 ± 0.95	1116 ± 14

[a]All contents are based on dry matter. *, Total B12 content calculated as CNCbl. Values in superscript "b" are values between LOD and LOQ.

This discrimination effect between different cobalamins was observed despite the fact that the total amount of cobalamins loaded was always within the capacity of the immunoaffinity column (i.e., 1 μg according to the manufacturer). However, the binding of CNCbl was not affected due to the better interaction with the antibody. It was reported previously that the recovery of OHCbl dropped from 75 to 45% when loaded in a mixture with CNCbl to the column, whereas the recovery of CNCbl remained higher than 90%.[15] In our case, the application of isotopically labeled internal standards compensated for the discrimination of analytes during immunoaffinity purification. However, false results will be obtained if discrimination becomes very pronounced and neither the analytes nor the internal standards are captured by the antibody. In this case, no signal at all was detected. To solve this problem, multiplicate analysis (n = 4) was carried out for real samples and only measurements with the presence of all four internal standard peaks in the chromatograms were further processed for quantitative results. The binding of cobalamins appears to be a critical factor for further method optimization. If, in the future, the immunoaffinity columns can be produced by integrating different antibodies tailor-made for different forms, the performance of the method will be further improved as more sensitive and reproducible bindings of all forms are anticipated.

3.5. Analysis of Cobalamins in Meat and Liver Samples. The developed SIDA method was applied to analyze a certified reference material BCR 487 (lyophilized pig liver) and different meat samples (Table 4). The total B12 content was also calculated as CNCbl equivalent from the investigated cobalamins.

For the BCR 487, the total B12 content (1116 ± 14 ng/g; calculated as CNCbl) was in accordance with the certified reference value (1120 ± 90 ng/g), confirming the accuracy of the developed method. Previously, Chamlagain et al.[17] reported a lower value of 876 ± 49 ng/g for the BCR 487 using HPLC-UV and suggested that the discrepancy might be due to the presence of cobalamin analogues in the sample. In our case, as the specificity of the analysis was ensured by the application of SIDA, potential influence from analogues or co-eluting compounds was excluded. We confirmed the presence of other cobalamins in the reference material; the lower content in the previous publication might result from an insufficient conversion of other forms to CNCbl. This aspect is somehow surprising as the cyanidation method has been state-of-the-art in the recent years. The cyanidation method will yield correct values only when the conversion is complete. However, the completeness of conversion or its dependence from the matrix is not monitored in routine measurements because it would require the determination of all relevant cobalamins by LC-MS/MS. As our method is capable of measuring the native cobalamins in the samples without cyanidation, this source of inaccuracy is also avoided.

The contents of total B12 in meat samples that we obtained using the SIDA method were in the same level of magnitude as previously reported.[34] In addition to variances due to the analytical method, the differences between analyzed and literature values within the same type of animal can be attributed to various factors including the analytical methods, cut of meat, and conditions of farming practices. Among all investigated meat samples, the lamb fillet contained the highest amount of B12 followed by the beef and pork fillet, while the chicken breast showed the lowest value. These results generally agreed with previous observations of higher B12 contents in ruminant meat than in meat of monogastric animals due to the higher bacterial activities synthesizing B12 in the rumens.[34,35]

Regarding the vitamer distribution, AdoCbl and OHCbl were the predominant forms in all meat and liver samples. MeCbl was present in all samples but in lower concentrations. CNCbl, the synthetic form of B12, was found only in trace levels in the pork fillet and chicken breast and was below LOQ in the beef fillet and lamb fillet samples. In general, the contribution of CNCbl to the total B12 content was negligible. Previously, Szterk et al.[21] reported that OHCbl and AdoCbl were the main forms in beef liver and beef sirloin. However, their method was not sensitive enough to detect the low levels of MeCbl and CNCbl in beef sirloin and their recoveries of OHCbl, AdoCbl, and MeCbl (68.3, 60.7, and 52.3%, respectively) were too low for accurate quantitation. With our SIDA method, vitamer distributions in common categories of meat were determined with reliable quantitative results for individual forms. In future studies, the developed SIDA method can be further applied on different types of food matrices to verify other known and potential sources of dietary B12. Differentiation of B12 vitamers will also open further avenues for investigating their respective bioavailabilities to better assess dietary requirements and nutritive value of different foods and supplements.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.1c03803.

Additional experimental details regarding q-NMR, UV spectroscopy, and HPLC-DAD; results from optimization of incubation conditions and enzyme amounts for sample preparation; proposed fragmentation schemes for all analytes; and influence of desolvation line temperature on the signal intensity of MRM transitions of OHCbl (PDF)

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Notes

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■ ABBREVIATIONS USED

AdoCbl, adenosylcobalamin; B12, vitamin B12; CID, collision-induced dissociation; CNCbl, cyanocobalamin; DAD, diode-array detection; DMB, 5,6-dimethylbenzimidazole; ESI, electrospray ionization; HPLC, high-performance liquid chromatography; ICP, inductively coupled plasma; IS, internal standard; LC, liquid chromatography; LOD, limit of detection; LOQ, limit of quantification; MBA, microbiological assay; MeCbl, methylcobalamin; MS, mass spectrometry; OHCbl, hydroxocobalamin; RT, room temperature; SIDA, stable isotope dilution assay

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Appendix D: Supporting information: Development of stable isotope dilution assays for the analysis of natural forms of vitamin B12 in meat

Supporting Information

Development of stable isotope dilution assays for the analysis of natural forms of vitamin B12 in meat

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Supplementary methods

1 q-NMR

The q-NMR experiments were performed according to Frank et al. (Frank et al., 2014). Commercial cobalamin standards were dissolved in D_2O (700 µl) and filled into NMR tubes (5 × 178 mm, USC-tubes, Bruker, Faellanden, Switzerland). The ^{1}H-NMR measurements were conducted on a Bruker AVIII system (500 MHz, Bruker, Rheinstetten, Germany). The signal assignment was performed according to Brasch et al. (Brasch and Finke, 1999) with focus on the distinct chemical shifts of cobalamins in the aromatic region for further quantitation. L-tyrosine (purity ≥ 99 %, Sigma-Aldrich, Steinheim, Germany) was used as external standard and ERETIC 2 function of TopSpin 3.6.0 (Bruker BioSpin, Faellanden, Switzerland) was used to calculate the concentrations. The accuracy of the q-NMR measurement was within an error limit of ± 2%.

2 UV spectroscopy

2.1 Determination of molar extinction coefficient of CNCbl

A freshly prepared CNCbl solution (~ 1 mg of CNCbl dissolved in 700 µL of D_2O) was measured by q-NMR for the accurate concentration. The solution was further checked with HPLC-DAD for purity and was then diluted to three different concentrations (triplicate dilutions for each concentration) for absorption measurements on a Genesys 10S UV-Vis spectrophotometer (Thermo Fischer Scientific, Madison, Wisconsin, USA) at λ_{max} = 361 nm. Disposable cuvettes (PMMA, VWR, Ismaning, Germany; optical path length (d) = 1 cm) were used and LC-MS Water was measured as blank. The molar extinction coefficient ε was calculated as the mean of the different concentrations using the following equation: ε = absorption/(d [cm]*concentration [mol/L]).

2.2 Determination of CNCbl concentration using UV spectroscopy

CNCbl stock solution (~ 0.1 mg/mL) was 4-fold diluted with water and measured on the Genesys 10S UV-VIS spectrophotometer against LC-MS water at 361 nm. The concentration was calculated using our experimental molar extinction coefficient.

3 Preparation of HPLC-DAD response curves

The HPLC-DAD response curves (Table 1) were established for OHCbl, AdoCbl and MeCbl using CNCbl as the internal standard (IS). First, fresh stock solutions were prepared for each cobalamin and their absolute concentrations were determined by q-NMR. Then, varying amounts of each analyte were mixed with constant amounts of the internal standard (i.e. CNCbl) and further diluted with water to corresponding concentration ranges of 0.38 -76.70 µg/mL for OHCbl, 0.43 - 86.81 µg/mL for AdoCbl and 0.40 - 63.37 µg/mL for MeCbl, with a constant final concentration of 8.26 µg/mL for CNCbl. Response curves were obtained by plotting the peak area ratios [A(A)/A(IS)] against the mass concentration ratios [ρ(A)/ρ(IS)] at respective wavelengths from HPLC-DAD measurements. The response curves were calculated by linear regression and linearity was confirmed by Mandel's fitting test.

4 HPLC-DAD

The concentrations of working standards were determined on a Shimadzu HPLC-PDA system (Shimadzu, Kyoto, Japan) using a Triart C18 column (150 x 30 mm, 3 µm, YMC, Dinslaken, Germany) for separation at 30 °C. 50 mM ammonium acetate buffer (pH = 4) and 100% methanol were used as mobile phases A

and B, respectively. The elution gradient was as follows: 0-22 min, 10-40% B; 22-24 min, 40% B; 24-26 min, 40-95% B; 26-28 min, 95% B; 28-30 min, 95-10% B; 30-40 min, 10% B. The flow rate was 0.3 mL/min and the injection volume was 20 μL**.** The detection covered range from 250-600 nm.

Table S1 Optimization of incubation conditions and enzyme amounts for sample preparation

Extraction batch*	Incubation condition		Enzyme amount (mg)		Cobalamin content in pork (ng/g)**			
	Temperature (°C)	Duration (h)	Amylase	Papain	OHCbl	AdoCbl	CNCbl	MeCbl
#1	**37**	1	10	10	10.97 ± 0.36	9.24 ± 0.38	1.11 ± 0.08	2.22 ± 0.02
	60	1	10	10	10.22 ± 0.44	9.05 ± 0.22	1.18 ± 0.00	2.30 ± 0.12
#2	37	1	**10**	**10**	10.88 ± 0.05	9.50 ± 0.37	3.54 ± 0.18	7.05 ± 0.11
	37	1	**5**	**5**	11.00 ± 0.74	9.82 ± 0.02	3.62 ± 0.06	7.01 ± 0.17
	37	1	**2**	**2**	11.97 ± 0.65	9.41 ± 0.07	4.60 ± 0.34	7.36 ± 0.11
	37	1	**1**	**1**	11.50 ± 0.32	9.75 ± 0.58	4.15 ± 0.12	7.30 ± 0.11
#3	37	**1**	5	5	6.46 ± 0.45	7.48 ± 0.43	1.19 ± 0.06	2.11 ± 0.02
	37	**2**	5	5	5.68 ± 0.63	7.43 ± 0.32	1.23 ± 0.06	2.21 ± 0.04
#4	37	**1**	5	5	11.90 ± 0.68	11.53 ± 0.46	1.47 ± 0.12	1.85 ± 0.01
	37	**0.5**	5	5	11.09 ± 0.25	11.41 ± 0.03	1.42 ± 0.00	1.81 ± 0.05

*Parameters highlighted in bold are tested conditions for each extraction batch. * Different extraction batches were performed on different days using different pork samples. ** Standard deviations derived from duplicate or triplicate injections.*

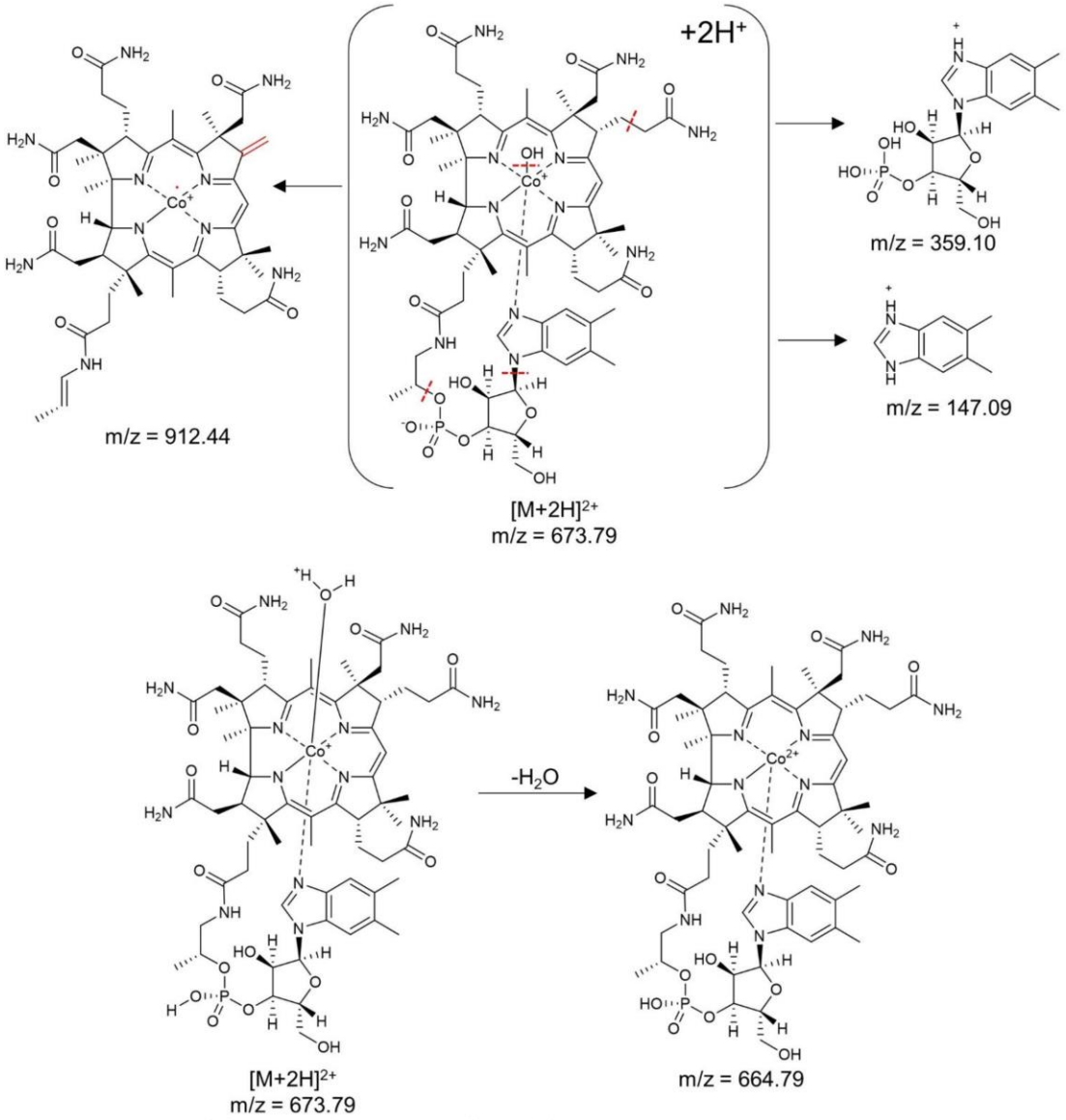

Figure S1 Proposed fragmentation pattern of OHCbl.

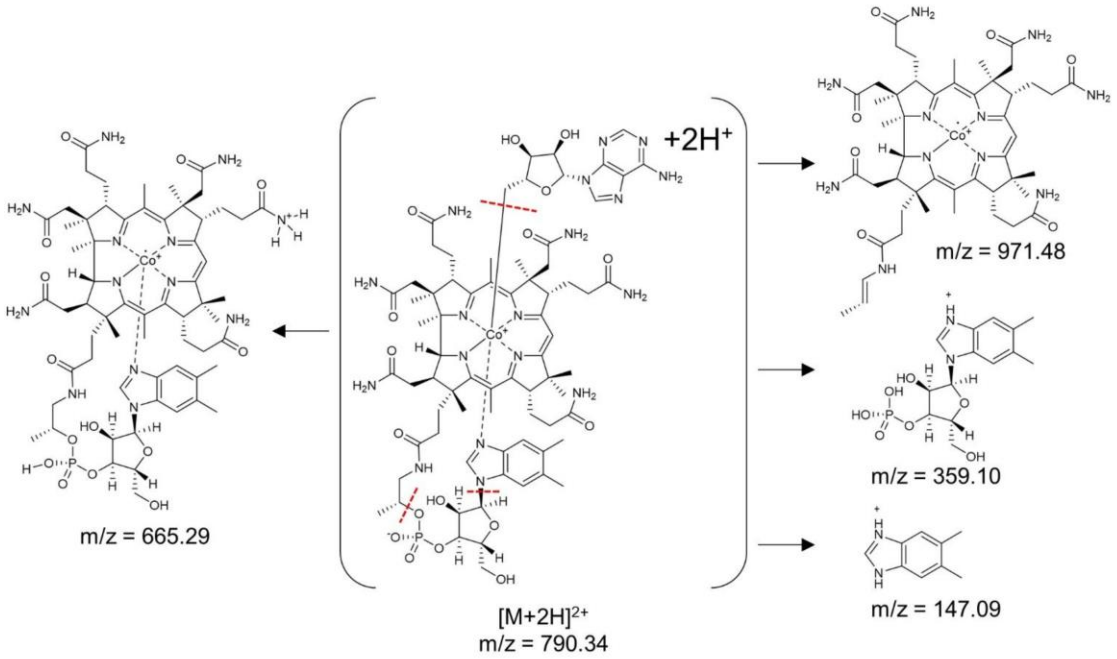

Figure S2 Proposed fragmentation pattern of AdoCbl.

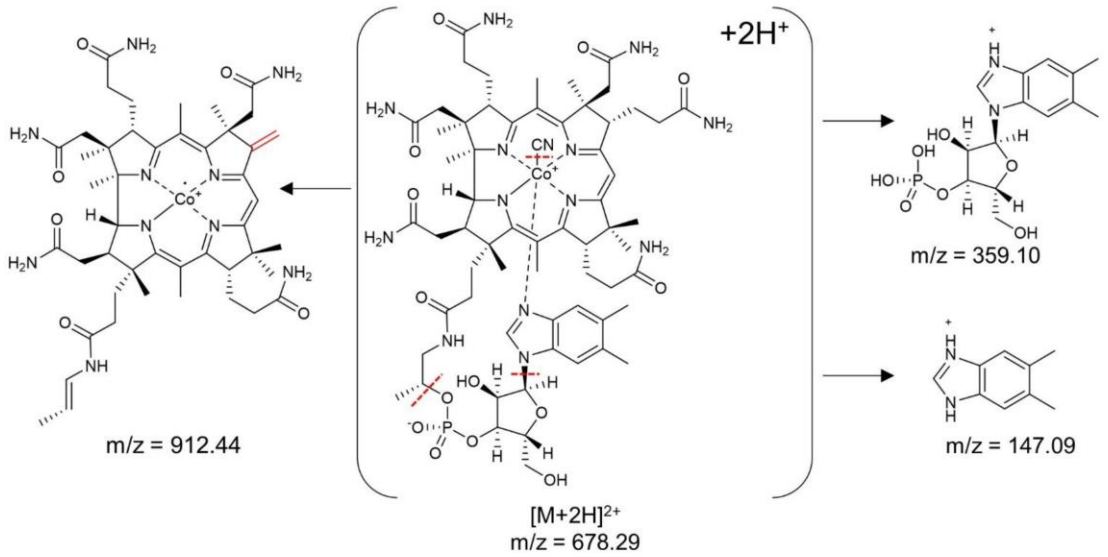

Figure S3 Proposed fragmentation pattern of CNCbl.

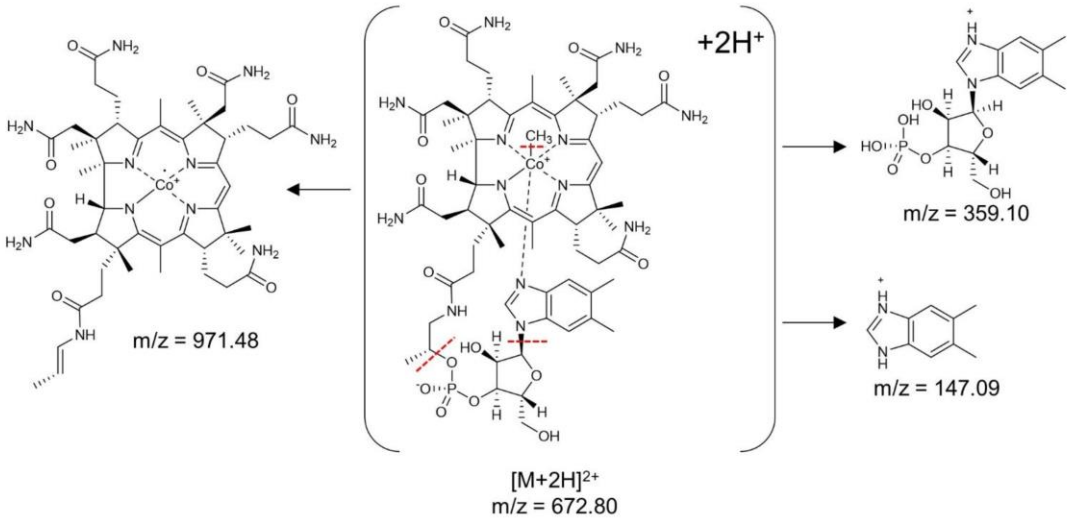

Figure S4 Proposed fragmentation pattern of MeCbl.

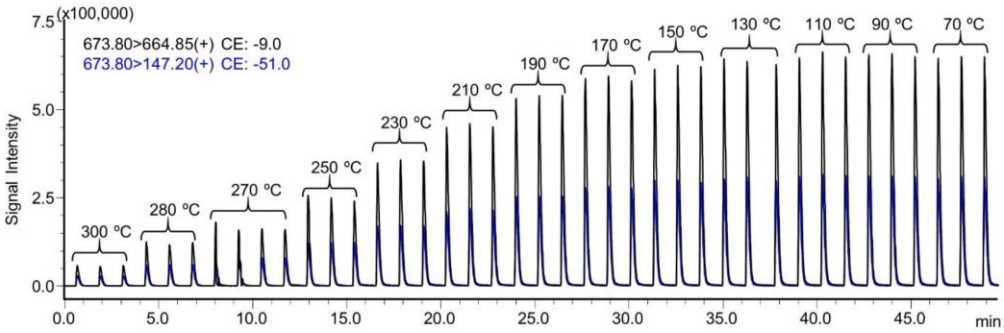

Figure S5 Intensity increase of selected MRM transitions of OHCbl with the temperature decrease of desolvation line (DL) in LC-MS/MS. The DL temperature was indicated above each peak.

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