Quantitative Determination of Vitamin B_{12} in Plants

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von

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"Οὐδὲν τοῖς θαρροῦσιν ἀνάλωτον"
(There is nothing impossible to him who will try)

– Alexander the Great (King of Macedonia, 356-323 B.C)

Michail Nakos

Erklärung zur Dissertation

gemäß §6(1) der Promotionsordnung der Naturwissenschaftlichen Fakultät der Gottfried Wilhelm Leibniz Universität Hannover

für die Promotion zum Dr. rer. nat.

Hierdurch erkläre ich, dass ich meine Dissertation mit dem Titel „Quantitative determination of vitamin B_{12} in plants“ selbstständig verfasst und die benutzten Hilfsmittel und Quellen sowie gegebenfalls die zu Hilfeleistungen herangezogenen Institutionen vollständig angegeben habe.

Die Dissertation wurde nicht schon als Masterarbeit, Diplomarbeit oder andere Prüfungsarbeit verwendet.

Hannover, November 2015
Michail Nakos

Abstract

Vitamin B_{12} is an essential nutrient in human dietary, as it has an important role in promoting carbohydrate and normal fat metabolism, as well as it is essential in the formation of red blood cells, and the normal functioning of the nervous system. Naturally occurring vitamin B_{12} is found mainly in animal-based foods (meat, milk and eggs), but it is well-known for its absence in plants and fruits. A couple of plant-based foods such as particular types of dried lavers (*nori*) and certain species of mushrooms contain certain amounts of vitamin B_{12}. Actinorhizal plants, such as *Hippophae rhamnoides* are symbiotic with actinobacteria *Frankia alni*, they are potential producers of vitamin B_{12}. Due to modern vegetarian and vegan nutrition in the last decades, more and more attention has been paid to natural vitamin B_{12} sources of plant origin. Based on increased demands of strict vegetarians for more plant-sourced vitamin B_{12} and due to the high risk of vitamin B_{12} deficiency in certain risk groups, an investigation of vitamin B_{12} content in plant sources was carried out.

An extraction protocol based on previous non-plant methods for the isolation and purification of vitamin B_{12}, was optimized and specialized for plant matrices. Afterwards the analysis was performed with a developed and validated high performance liquid chromatographic method HPLC–UV for the determination of vitamin B_{12} (cyanocobalamin), and a high performance liquid chromatographic method HPLC–MS/MS for the determination of cyanocobalamin analogues. The method was successfully applied to several plant samples and extracts, *Hippophae rhamnoides* juice and vitamin B_{12} tablets in comparison to animal based systems (veal liver).

The vitamin B_{12} concentration was determined by RP-HPLC with UV detection and the active vitamin B_{12} was separated from pseudo-vitamin B_{12} by an HPLC–MS/MS method, after prior matrix isolation by immunoaffinity chromatography (IAC). Vitamin B_{12} was extracted from plants and berries in the presence of sodium cyanide, to transform all forms of cobalamin into cyanocobalamin. A diode array detector (DAD) was used to monitor vitamin B_{12}, after its chromatographic separation under gradient elution with a mobile phase consisting of acetonitrile and trifluoroacetic acid (TFA) 0.025 % (w/v). The method demonstrated linear response over a large range of concentrations with a regression coefficient $r^2 > 0.999$. LOD and LOQ values were 0.004 µg/ ml and 0.014 µg/ ml, respectively. The system precision of the method was evaluated at different concentration levels and the relative standard deviation for standard solutions of cobalamin ranged from 0.53 – 7.37 %, for plant samples the precision varies between 0.7 and 0.8 % (n=6). The specificity of the method was demonstrated by the retention characteristics, UV spectra and by comparing the peak purity with an external standard solution. Significant amounts of vitamin B_{12} in plants were detected in *Hippophae rhamnoides* (37 µg/ 100 g dry

weight), in *Inula helenium* (11 µg/ 100 g dry weight), in *Agropyron repens* (26 – 23 µg/ 100 g) and trace amounts in black mustard (1.52 µg/ 100 g).

Keywords: vitamin B_{12} (cobalamin), plant-based foods, *Hippophae rhamnoides*, *Frankia alni*, extraction protocol, immunoaffinity chromatography (IAC), HPLC-UV, HPLC-MS/MS

Kurzbeschreibung

Vitamin B_{12} ist ein essentieller Nährstoff in der menschlichen Ernährung, der eine wichtige Rolle in dem Kohlenhydrat- und normalen Fettmetabolismus spielt. Desweiteren ist es auch essentiell bei der Bildung roter Blutkörperchen und der Normalfunktion des Nervensystems beteiligt. Natürlich vorkommendes Vitamin B_{12} wird hauptsächlich in tierischen Lebensmitteln (Fleisch, Milch und Eiern) gefunden, jedoch nicht in Pflanzen und Früchten. Einige pflanzenbasierte Lebensmittel, wie spezielle Typen getrockneten Seetangs (*nori*), sowie bestimmte Arten von Pilzen enthalten eine geringe Menge an Vitamin B_{12}. Aktinorrhiza-Pflanzen, wie *Hippophae rhamnoides,* bilden eine Symbiose mit den Actinobakterien *Frankia alni*, und sind potentielle Erzeuger von Vitamin B_{12}. Auf Grund moderner vegetarischer und veganer Ernährung in den letzten Jahrzehnten, wird mehr und mehr Aufmerksamkeit auf natürliche Vitamin B_{12} Quellen pflanzlichen Ursprungs gerichtet. Basierend auf der steigenden Nachfrage strikter Vegetarier nach mehr pflanzenlichem Vitamin B_{12}, sowie dem hohen Risiko an Vitamin B_{12}-Mangel in bestimmten Risikogruppen, wurde eine Untersuchung des Vitamin B_{12}-Gehalts aus pflanzlichen Quellen durchgeführt.

Ein auf nicht-pflanzlichen Methoden basierendes Extraktionsprotokoll zur Isolierung und Reinigung von Vitamin B_{12} wurde für Pflanzenmatrizen optimiert und spezialisiert. Die anschließende Analyse wurde mit einer entwickelten und validierten Hochleistungsflüssigkeitschromatographiemethode HPLC für die Bestimmung von Vitamin B_{12} (Cyanocobalamin) durchgeführt. Um Cyanocobalaminanaloge zu bestimmen wurde eine Hochleistungsflüssigkeitschromatographiemethode gekoppelt mit einer Massenspektrometrie HPLC-MS/MS verwendet. Die Methode konnte erfolgreich auf verschiedene Pflanzenproben und Extrakten, *Hippophae rhamnoides* Saft und Vitamin B_{12}-Tabletten im Vergleich zu tierisch basierten Systemen (Kalbsleber) angewendet werden.

Anschließend an eine Matrix Isolation durch Immunaffinitätschromatographie (IAC) wurde die Vitamin B_{12}-Konzentration mittels RP-HPLC mit UV-Detektion bestimmt und das aktive Vitamin B_{12} wurde durch eine HPLC-MS/MS Methode von dem Pseudo-Vitamin B_{12} getrennt. Vitamin B_{12} wurde aus Pflanzen und Früchten in der Gegenwart von Natriumcyanid extrahiert, um alle Formen von Cobalamin zu Cyanocobalamin zu transformieren. Nach einer chromatographischen Trennung mit Hilfe einer Gradietenelution mit einer mobile Phase bestehend aus Acetonritil und 0,025 % (w/v) Trifluoressigsäure (TFA), wurde ein Diodenarray-Detektor (DAD) genutzt, um Vitamin B_{12} zu detektieren. Die Methode zeigte Linearität über einen großen Konzentrationsbereich mit einem Regressionskoeffizienten $r^2 > 0{,}999$. Die LOD und LOQ Werte betrugen 0,004 µg/ml und 0,014 µg/ml. Die Systempräzision der Methode wurde bei verschiedenen Konzentrationen evaluiert und die relative Standardabweichung varierte zwischen 0,7 und 0,8 (n=6). Die Spezifität des

Verfahrens wurde durch die Retentionseigenschaften, UV-Spektren und den Vergleich der Peakreinheit mit einem externen Standard nachgewiesen. Signifikante Mengen von Vitamin B_{12} konnten in den Pflanzen *Hippophae rhamnoides* (37 µg/ 100 g Trockengewicht), in *Inula helenium* (11 µg/ 100 g Trockengewicht), in *Agropyron repens* (26 – 23 µg/ 100 g) und Spuren in schwarzem Senf (1.52 µg/ 100 g) detektiert werden.

Stichwörter: vitamin B_{12} (cobalamin), pflanzenbasierte Lebensmittel, *Hippophae rhamnoides*, *Frankia alni*, Extraktionsprotokoll, Immunaffinitätschromatographie (IAC), HPLC-UV, HPLC-MS/MS

List of Abbreviations

AAS	Atomic absorption spectroscopy
Ado	Adenosyl
ANOVA	Analysis of variance
AOAC	Association of analytical communities
Bio	Bioproducts
BIA	Biomolecular interaction analysis
^{o}C	Celsius
Cbl	Cobalamin
CE	Capillary electrophoresis
CNCbl	Cyanocobalamin
cGMP	Current good manufacturing practices
CoA	Coenzyme A
CRF	Code of federal regulations
d	Day
DAD	Diode array detector
DNA	Deoxyribonucleic acid
DMBI	5,6-Dimethylbenzimidazole
DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid
e.g	Exempli gratia
ESI	Electrospray ionization
etc.	Et cetera
et al.	et aliae, “and others”
FDA	Food and drug administration
FIA	Flow injection analysis
Fig.	Figure
FRET	Fluorescence resonance energy transfer
FW	Fresh weight
g	Grams
GC	Gas chromatography
GC	Guanine-Cytosine
GmbH	Gesellschaft mit beschränkter Haftung, “limited liability company“
h	Hours
Hcy	Hyperhomocysteinemia
HPLC	High-performance liquid chromatography
IAC	Immuno affinity chromatography
ICH	International conference on harmonization
ICP	Inductively coupled plasma
i.e.	illud est
IF	Intrinsic factor
Kg	Kilogram
LC	Liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantification
LSD	Least significant difference
MBA	Microbiologal assay

mg	Milligram
µg	Microgram
mm	Millimeter
µm	Micrometer
mM	Millimolar
MMA	Methyl malonic acid
MS	Mass spectrum
m/z	Mass-to-charge ratio
ng	Nanograms
RDA	Recommended daily allowance
rel. AU	Relative arbitrary units
RNA	Ribonucleic acid
RP	Reverse phase
rpm	Rounds per minute
RSD	Relative standard deviation
R.T	Room temperature
RT	Retention time
R.V.C	Rotational vacuum concentrator
SIM	Single ion mode
S/N	Signal to noise ratio
SPE	Solid phase extraction
TC	Transcobalamin
TFA	Trifluoroacetic acid
t_R	Retention time
U.S	United states
USP	United states pharmacopeia
UV	Ultraviolet
V/V	Volume per volume
Vis	Visible
W/V	Weight per volume

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1. Introduction

In the developed world consumers are increasingly health conscious and aware of their nutritional requirements. At the same time, in the developing world malnutrition can result in dietary deficiencies, particularly in vitamins, lack of sufficient amounts any of them can cause severe physiological problems[1].

Malnutrition based diseases such as pernicious anemia, which was first described in 1824 had fatal prognosis, prior to the discovery of its treatment. It took about one hundred years before Whipple, Minot and Murphy discovered that after treatment with liver "…all the patients showed a punctual, rapid, and distinct remission of their anemia…" and that liver must contain an anti-pernicious anemia factor[2]. In this period that can be described as the early medicinal era of vitamin B_{12} Whipple, Minot and Murphy in 1934 shared the Nobel Prize for Medicine and Physiology. Folkers at Merck, Sharp and Dohme and, soon afterwards, Smith at Glaxo, after the Second World War, isolated and crystallized that factor, which was named vitamin B_{12}[2]. Dorothy Hodgkin was awarded the Nobel Prize in Chemistry in 1964 for using X-ray crystallography to determine its structure[3]. In 1972, Woodward and Eschenmoser reported the total synthesis of vitamin B_{12} as a result of a collaborative effort involving more than one hundred scientists over eleven years[4,5]. During the vitamin B_{12} *crystallographical age* (1948 – 1985) the chemistry of vitamin B_{12} upsurged, until the crystal structure determination of several B_{12}-dependent enzymes which play an important role in vitamin B_{12} enzymology[6,7,8]. Finally, the structure of transcobalamin (TC)[9] has provided the structural basis for designing appropriate Cbl-based bioconjugates for imaging of tumours as well as targeted and selected delivery of anti-tumor agents to malignant cells[10].

Vitamin B_{12} belongs to a group of strictly related compounds called corrinoids. All corrinoids (including all cobalamins) are considered B_{12} analogues. Many corrinoids, and possibly even some cobalamins, are not useable by human vitamin B_{12} enzymes. These are considered inactive vitamin B_{12} analogues. Different forms of vitamin B_{12} are similar in the Co central ion, the four parts of the corrin ring and a dimethylbenzimidazole group, but differ in the sixth site which may contain a cyano group (–CN), hydroxyl group (–OH), methyl group ($–CH_3$) and/or a 5' deoxyadenosyl group (–Ado)[11]; It is the only water-soluble vitamin that can be stored in the liver for many years[12]. The biological active forms of vitamin B_{12} are methylcobalamin and adenosylcobalamin. However, the synthetic cyanocobalamin, as the most stable form of vitamin B_{12}, is the form mainly used in pharmaceuticals, supplements and in the fortification of foods. Cyanocobalamin is converted in human metabolism to the biological active form of methylcobalamin by ilea enterocytes[13].

Vitamin B_{12} is acting as a co-enzyme and plays an important role in promoting carbohydrate, protein and normal fat metabolism, it is essential in the formation of red blood cells, the normal functioning of the nervous system and on the translocation of the methyl group in DNA synthesis[14,15,16]. Although vitamin B_{12} deficiency in developed countries is uncommon and unlikely to develop in healthy human beings (except in strict vegetarians), studies have shown that deficiency may lead to megaloblasts (i.e., abnormal cell growth that results in anemia); symptoms include excessive tiredness, listlessness, breathlessness, and poor resistance to infections. Extended deficiency leads to nerve degeneration and irreversible neurological damage. Causes of deficiency may comprise nutritional imbalance (among vegetarians), malabsorption syndromes and other gastrointestinal problems[17]. According to the Institute of Medicine (National Academies, USA), the recommended daily allowance (RDA) for the vitamin B_{12} is 2.4 µg/ d[18]. Vegeterians are at higher risk of vitamin B_{12} deficiency than non-vegetarians[19]. The deficiency frequency among vegetarians were estimated as 62 % in pregnant women, 25 – 86 % in children, 21 – 41 % in adolescents, and 11 – 90 % in elderly subjects, by review of the 18 reports evaluating vitamin B_{12} status of vegetarians[20].

Cobalamin is unique, in a sense of its *de novo* synthesis, production appears to be restricted only to some bacteria and archea. These vitamin B_{12}-producing microorganisms form the biological source of vitamin B_{12}. Cobalamin provides a nutritional requirement for animals and protists although they do not synthesize it, whereas plants, neither require nor synthesize it[1]. There is evidence that nitrogen fixing actinobacteria *Frankia alni* produce the vitamin B_{12} and these bacteria form nodule endophytes in woody trees and shrubs. *Frankia alni* is symbiotic with actinorhizal plants (comprising of eight families and 25 genera, and containing more than 220 species)[21]. Owing to this symbiosis, content of vitamin B_{12} is possible to be found also in plants[22]. Therefore, the vitamin is found in foods fermented by such bacteria, in plants symbiotic with *Frankia alni*, or derived from the tissues of animals which have ingested B_{12}-containing foods. Likewise, ruminant animals can obtain cobalamin from certain bacteria in their microflora which synthesize the vitamin, and consequently the liver of such animals is a rich source of this specific vitamin. Actinorhizal plants such as *Hippophae rhammnoides* and *Myrica* which are symbiotic with actinobacteria *Frankia alni* are potential producers of vitamin B_{12} and its analogues[22,23].

Major sources of vitamim B_{12} are liver, meat, milk, eggs, fish, oysters and clams. Although vitamin B_{12} is well-known for its absence in plant source foods (apart from plants that have been contaminated with soil or have been exposed to foods containing vitamin B_{12}[24,25]), edible species of mushrooms including black trumpet (*Craterellus cornucopioides*) and golden chanterelle (*Cantharellus cibarius*), contain

noticeable amounts of vitamin B_{12} (1.09 – 2.65 µg/ 100 g dry weight), in comparison with other species of wild mushrooms that contain no vitamin B_{12} or trace amounts. The corrinoids of these mushrooms have been identified as vitamin B_{12}[26,27]. On the other hand, certain species of edible cyanobacteria such as *Spirulina*, *Aphanizomenon* and *Nostoc* contain significant amounts of vitamin B_{12} analogues (pseudo-B_{12}) which are known to be biologically inactive in human, e.g. commercially available tablets of *Spirulina* contain 127 – 244 µg/ 100 g vitamin B_{12} analogues[28]. Moreover, widely consumed edible algae such as dried green (*Enteromorpha sp*.) contain (63.6 – 69.2 µg/ 100 g of dry weight) and purple lavers (*Porphyra sp*.) contain considerable amounts of vitamin B_{12} analogues (133 µg/ 100 g dry weight), however the biological activity of those algae derived corrinoids in human still remains unclear[29,30,31]. Fermented foods have been also tested, such as Tempeh (type of soybean) which contains 0.7 – 8.0 µg/ 100 g vitamin B_{12}[32], Sauerkraut (7.2 µg/ 100 g) and fenugreek juice fermented with lactic acid bacteria (12.5 µg/ 100 ml)[33].

Some of the vitamins B_{12} analogues, apart from the fact being biological inactive, can also block the vitamin B_{12} metabolism in mammalian cells[34]. Due to the limited availability of natural sources of vitamin B_{12}, and because in most cases the biological activity of the cobalamins is uncertain, further research in the field of plant-based sources is on demand. Finding new plant-based consumable products might help all kinds of vegetarians to have an extra alternative to consume vitamin B_{12}. Besides, fortified products or vitamin B_{12} supplements contain artificial vitamin B_{12} (cyanocobalamin), which has been generated by a derivatization of the highly toxic potassium cyanide. Additionally, artificially vitamin B_{12}-enriched products may not be compatible with the philosophy of vegetarians.

2. Aim and Scope

Despite the general agreement that plants can not synthesize vitamin B_{12}, there are reports that some plants contain detectable amounts of vitamin B_{12}[35]. Robbins et al., (1950)[36] suggested that vitamin B_{12} found in plants may in fact be of microbial (soil) origin. The medium-sized company Teutopharma GmbH, in the 90s launched a plant-based product from *Hippophae rhamnoides* berries, claiming that is contained amounts vitamin B_{12} similar to that in pig liver, one of the richest sources of vitamin B_{12}. Their hypothesis was that the symbiosis of *Hippophae rhamnoides* with the nodule of *Frankia alni* could cumulate vitamin B_{12} into the berries of the plant. Intensive research in collaboration with the TCI confirmed that *Hippophae rhamnoides* contains vitamin B_{12}[22]. Through regular quality controls the company found that the quantity of vitamin B_{12} had decreased over the years. They assumed either that the exhaustive usage of soil or climate change were the cause for this decrease.

The introduction of high-performance liquid chromatography (HPLC) has facilitated the analysis of vitamin B_{12} in several matrices such as meat products, infant powder, nutritional supplements, but the determination of vitamin B_{12} and its analogues in plants and berries is still a great challenge. Owing to very low concentrations of vitamin B_{12} in plants, the sensitivity of the analytical method and the sample preparation are essential steps. Thus, before this thesis began there was a clear need for carefully documented methods for analyzing vitamin B_{12} (CN-cobalamin) as well as for new data on their occurrence in plants and berries.

The aim of the thesis was on the one hand to establish an analytical method for the investigation and determination of vitamin B_{12} in a variety of natural plant matrices, on the other hand to identify and separate the active forms of cobalamins from inactive analogues of vitamin B_{12}. HPLC-UV alone is not sensitive enough to detect vitamin B_{12} in a natural matrix that contains several interfering compounds. Due to the need for accurate determination of vitamin B_{12}, a combined purification and concentration step with an immunoaffinity column should be developed in this work. The optimized analytical protocol for plant matrices should be based on the established “non-plant” methods[37,38,13,26]. Heudi et al. have shown that the method is a good alternative to the standard microbiological assay (MBA) for cobalamin determination in food products such as milk-based infant formula powder[38]. Other research groups like Marley et al. have applied the immunoaffinity method for the analysis of vitamin B_{12} enriched products[13]; or for determining the vitamin B_{12} content of different meat products (Guggisberg et al.)[37]. Watanabe et al. have implemented immunoaffinity columns after a solid phase extraction and a concentrating step in their determination of the vitamin B_{12} content of common edible mushrooms[26].

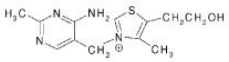
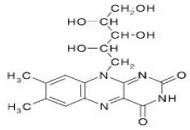
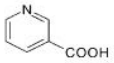
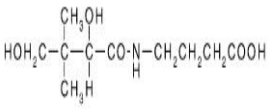
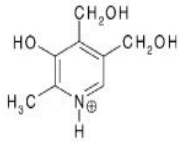
This thesis consists of three parts: The first part of the thesis reviews the vitamin B_{12} literature mainly focuses on vitamin B_{12}, the different vitamin B_{12} forms and functions, their role and the edible sources that can be found. The second part is the experimental section, in which an HPLC-UV method for analyzing cobalamin in various food items was developed, coupled with an HPLC-MS/MS method for separating the different forms of vitamin B_{12} and identifying the real vitamin B_{12}. In the third part the optimized assay was applied to defense the vitamin B_{12} concentration in various plant sources. The last section consists of the conclusion and outlook of this thesis.

3. Theoretical Background

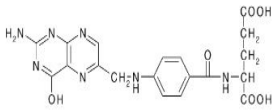
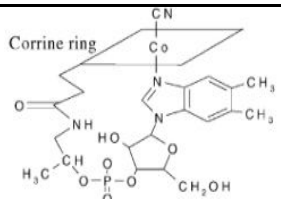
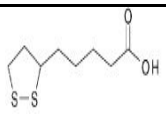
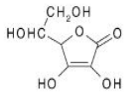
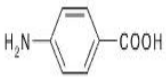
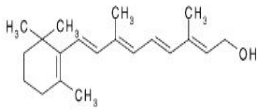
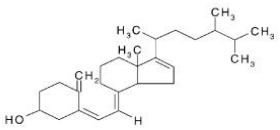
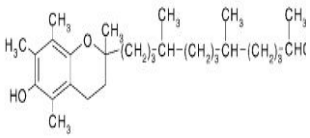
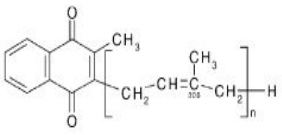
3.1. Vitamins

Vitamins are a wide group of organic compounds that are needed only in minor quantities. However they are essential constituents of food required for the normal growth, self-maintenance, and functioning of human and animal bodies[39]. Humans are not able to synthesize most vitamins and they consequently have to be obtained exogenously[40]. Vitamins are usually divided into two general classes: the fat-soluble vitamins, such as A, D, E and K_1, and the water-soluble vitamins, B_1, B_2, B_6, nicotinamide, pantothenic acid, biotin, folic acid, B_{12} and C (Table 3.1)[41]. Usually, a balanced diet can adequately supply the vitamin requirement of the body. A deficiency can result in hypovitaminosis and, if more severe, in avitaminosis[42]. A consequence of insufficient supply of vitamins by food intake, is not the only reason for hypovitaminosis or avitaminosis, but can be caused also by disturbances in resorption, by stress and by disease. The extent of vitamin supply can be assessed by determining the vitamin content in blood plasma, or by measuring a biological activity which is dependent on the presence of a vitamin[42].

Table 3.1: Chemical structures, basic biological activities and recommended daily doses of water- and fat-soluble vitamins[41]

Vitamin	Structure	Coenzyme or active form Function promoted	Recommended daily dose
Water Soluble			
Thiamine (Vitamin B_1)		Thiamine Pyrophoshate (TPP) Aldehyde group transfer	1.0 – 1.5 mg per day
Riboflavin (Vitamin B_2)		Flavin mononucleotide (FAD), Flavin adenine dinucleotide (FAD) Electron transfer	1.3 – 1.8 mg per day
Nicotinic acid (Vitamin B_3)		Nicotinamide adenine Dinucleotide (NAD, NADP)	15 – 20 mg per day
Pantothenic acid (Vitamin B_5)		Coenzyme A (CoA) A cyl group transfer	10 – 100 mg per day
Pyridoxine (Vitamin B_6)		Pyridoxal phosphate Amino group transfer	2 mg per day

Tab. 3.1 continued

Vitamin	Structure	Coenzyme or active form Function promoted	Recommended daily dose
Folic acid		Tetrahydrofolic acid One-carbon group transfer	0.4 mg per day
Cyanocobalamin (Vitamin B_{12})		Coenzyme B_{12} 1,2 shift of hydrogen atoms	2.4 mg per day
Thioctic acid		Lypoullysine Hydrogen atom and acyl group transfer	150 – 600 mg per day
L – ascorbic acid (Vitamin C)		- Cofactor in hydroxylation	60 mg per day
p-aminobenzoic acid (RABA)		- Antioxidant	50 mg per day
Fat Soluble			
Trans-retinol (Vitamin A)		11- *cis* - retinal Visual cycle	0.8 – 1.0 mg per day
Vitamin D_3		1,25-Dihydroxycholecalciferol Calcium and phosphate metabolism	0.08 mg per day
α-tocopherol		- Antioxidant	8 – 10 mg per day
Vitamin K_1		- Prothormbin biosynthesis	0.07 – 0.08 per day

3.2. Vitamin B_{12}

3.2.1. Structure and Chemistry of Vitamin B_{12}

Vitamin B_{12} (Fig. 3.1) belongs to a group of compound (corrinoids), which all contain a complex ring system with cobalt as a central atom[37]. The central cobalt atom is complexed to four pyrrole rings, a lower a-ligand donated by the DMBI (5,6-dimethylbenzimidazole) and an upper b-ligand. The upper b-ligand of the cobalt, which projects above the plane of the molecule, is covalently bound to one of several groups, designated, L[43] (Figure 3.2)[44]. This vitamin includes three naturally occurring forms: desoxyadenosyl group (adenosylcobalamin or coenzyme B_{12}), CH_3-(methylcobalamin) and OH-(hydroxycobalamin) and one chemically transformed form CN-(cyanocobalamin), differing only in the upper ligand[45].

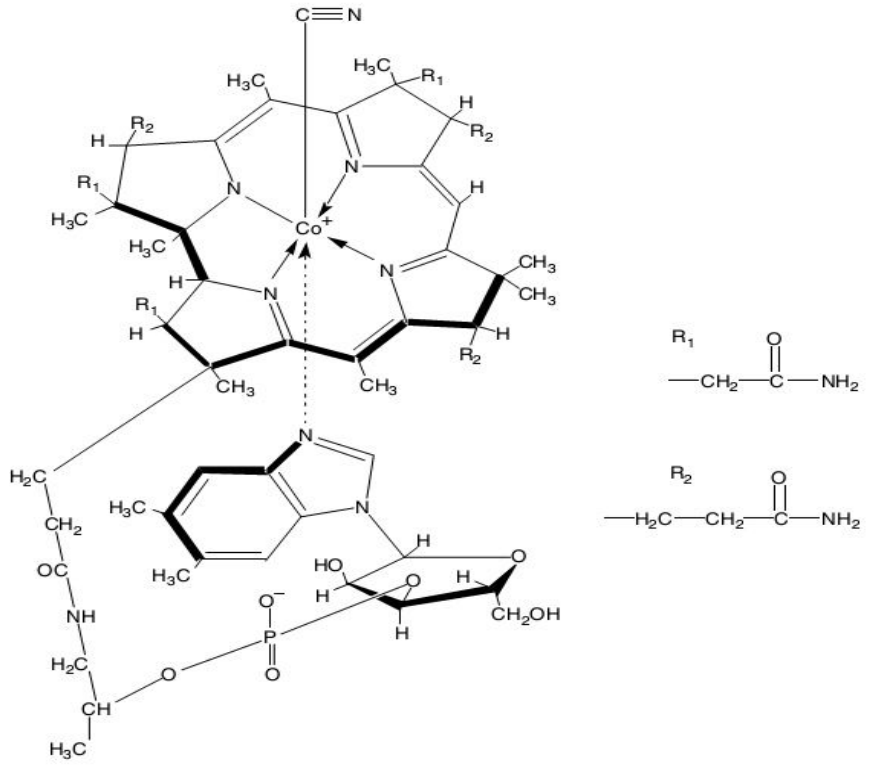


Figure 3.1: The structure of vitamin B_{12} (cyanocobalamin)[43]

From those forms, methylcobalamin and adenosylcobalamin are biologically active. Other forms such as hydroxycobalamin (or aquacobalamin) and cyanocobalamin must be metabolized to either of the two active forms in order to be used in human cells.

The predominant form of vitamin B_{12} in nature is 5'-desoxyadenosylcobalamin and has 5'-desoxyadenosyl as the L-group. In eukaryotes this form is located primarily in the mitochondria. It serves as the cofactor for the enzyme methylmalonyl CoA mutase. The other major form of vitamin B_{12} is methylcobalamin, which is the

predominant form in human plasma and within the cytosol. It serves also as the cofactor for the enzyme methionine synthase. Adenosylcobalamin and methylcobalamin are rapidly converted to hydroxycobalamin (exists in minor amounts in nature) when the carbon-cobalt bond is disrupted by exposure to light. The cobalt atom in hydroxycobalamin is fully oxidized in the Co(III) state, whereas in the 5'-desoxyadenosylcobalamin and methylcobalamin forms the cobalt exists as reduced Co(I) or Co(II)[43]. Cyanocobalamin is the most stable form, is obtained by reacting natural cobalamins with cyanide and it is the major vitamin B_{12} used in fortified foods, nutritional formula and pharmaceutical preparations[46].

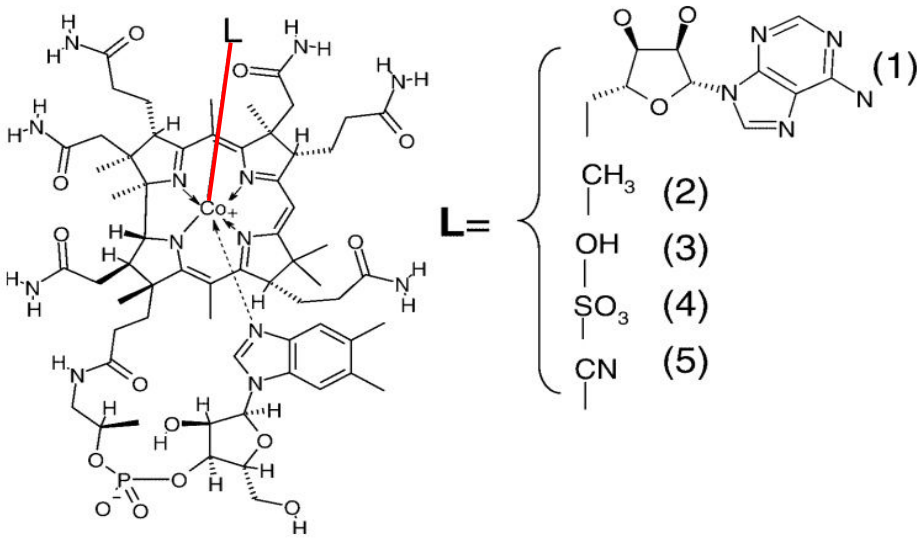


Figure 3.2: Structural formula of vitamin B_{12} and partial structures of vitamin B_{12} compounds. The partial structures of vitamin B_{12} compounds show only those parts of the molecule that differ from vitamin B_{12}. 1) 5'-desoxyadenosylcobalamin; 2) methylcobalamin; 3) hydroxycobalamin; 4) sulfitocobalamin; 5) cyanocobalamin [44]

3.2.2. Analogues of Vitamin B_{12}

Many analogues of vitamin B_{12} exist in nature, and are known with the name corrinoids[47]. Corrinoids carrying a base other than 5,6-dimethylbenzimidazole (DMBI) as the lower ligand (cobalt-coordinated nucleotide) are also found in nature. However, DMBI is essential for the binding of the vitamin to the intrinsic factor for its absorption. As a consequence corrinoids with lower ligand other than DMBI are biologically inactive in humans but have vitamin activity in microorganisms (Figure 3.3)[27]. The corrinoids can be grouped into two major types: (1) cobamides, which contain substitutions in the place of ribose, for example, adenoside; and (2) cobinamides, which lack a nucleotide[43]. It is unclear whether vitamin B_{12} analogues are inert or inhibit vitamin B_{12}-dependent reactions. Organisms such as *Euglena gracilis* and *Lactobacillus leichmannii* can distinguish microbiologically the analogues of vitamin B_{12} (whose growth is sustained by the cobalamins), but not the cobamides

or cobinamides. The sources of vitamin B_{12} analogues are still unknown, whether they come from diet, gut bacteria, or endogenous break-down of vitamin B_{12}. Vitamin B_{12} analogues have been found in fetal blood and tissues[43].

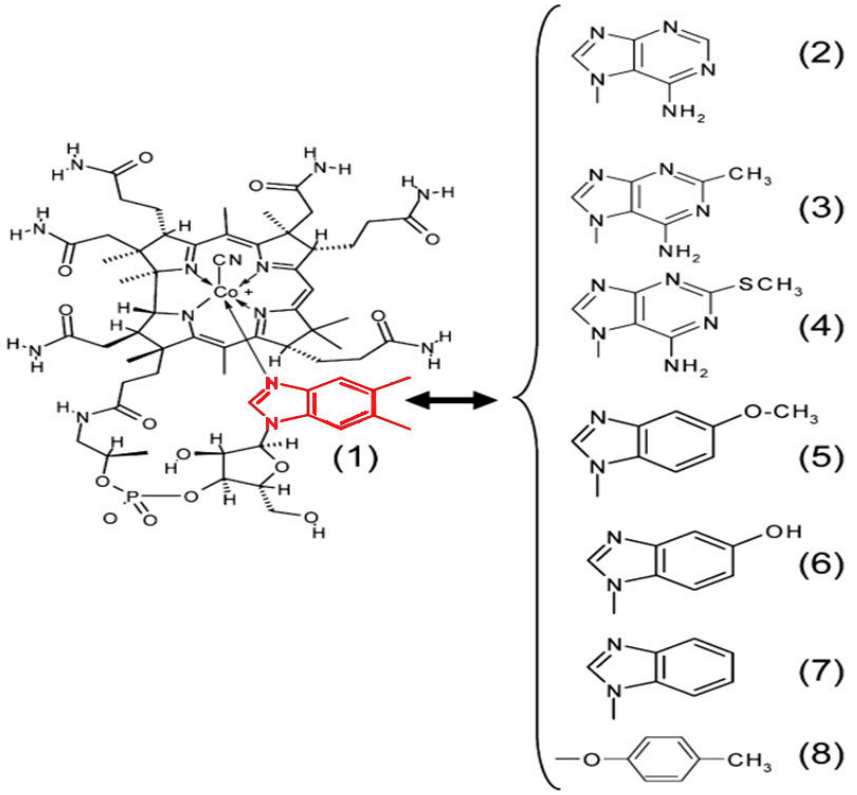


Figure 3.3: Structural formula of vitamin B_{12} and partial structures of B_{12} related compounds showing the portions that differ from vitamin B_{12}: (1) vitamin B_{12} or cyanocobalamin; (2) pseudovitamin B_{12} or factor IV; (3) factor A (2-methyladenylcobamide); (4) factor S (2-methylmer-captoadenylcobamide); (5) factor IIIm (5-methoxybenzimidazolylcobamide); (6) factor III (5-hydroxybenzimidazolylcobamide); (7) BIA (benzimidazolylcobamide); (8) pCC (p-cresolylcobamide)[27]

3.2.3. Stability and Degradation of Vitamin B_{12}

The stability of vitamin B_{12} (cyanocobalamin) depends on different conditions. It is fairly stable in crystalline form and aqueous solution at room temperature when protected from light. On exposure to light, the cyano group is cleaved to produce OH-Cbl, which occurs as aquacobalamin in neutral or acidic solution[48,49]. Because these two forms are equally biologically active, this photolytic reaction does not result in a loss of vitamin B_{12} activity. Cyanocobalamin exhibits optimal stability at pH 4 – 6, even at high temperatures[42]. Hydrolysis under heating in acidic and alkaline conditions results in biologically inactive degraded products. In the presence of strong oxidizing or reducing agents, such as vitamin C or SO_2, iron (II) ions and specific minerals such as copper, vitamin B_{12} is also susceptible to deactivation[50].

3.3. Vitamin B_{12} in Human Metabolism

3.3.1. Digestion and Absorption

In food, vitamin B_{12} is bound to proteins known as R-proteins or R-binders. In order vitamin B_{12} to be absorbed, the peptide bond that binds vitamin B_{12} to the protein carrier must be broken. The process of absorption and digestion is relatively complex, occurs in a few stages and requires sufficient synthesis of hydrochloric acid, proteases and an intrinsic factor (IF), a glycoprotein secreted by the parietal cells of the stomach[17]. The activity of pepsin, an enzyme secreted in the stomach, helps vitamin B_{12} to be released from the R-binders found in foods. Once released, vitamin B_{12} binds with R-binders (also known as haptocorrins) secreted with the saliva. Then, as it bounds to these R-binders, vitamin B_{12} is transported to the small intestine where pancreatic proteases digest the R-binders. After the digestion vitamin B_{12} is ready to be taken up by IF, and to form a IF-B_{12} (or IF-Cbl) complex (Fig 3.4)[17].

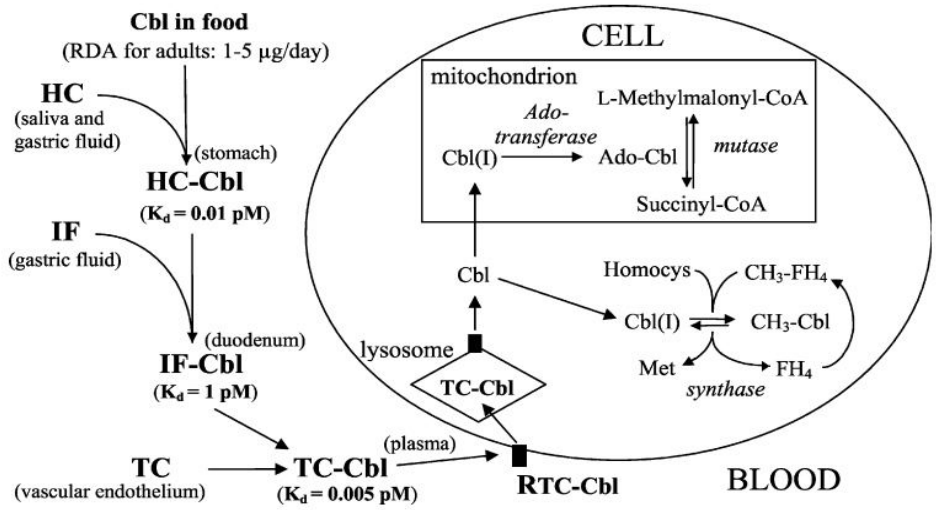


Figure 3.4: Absorption, cellular uptake and enzymatic processes involving cobalamins (Cbl) in mammals. The intracellular trafficking to the two mammalian Cbl-dependent enzymes have been reviewed recently[6]

For the absorption of vitamin B_{12} into the blood stream two separate mechanisms have been found:

I. The first mechanism is a receptor-mediated endocytosis that takes place in the distal ileum. The IF-Cbl receptor can be expressed only in the distal ileum of the intestines. Once inside the enterocytes vitamin B_{12} is released from the IF and then binds to holo-transcobalamin II protein (TCII), a protein carrier synthesized in the microvascular endothelium of the ileal villi[17].

II. The second absorption mechanism of vitamin B_{12} is the mass-action pharmacologic mechanism[51]. When vitamin B_{12} exists in large amounts, such as in the case of ingesting B_{12} supplements, about 1 % or less of free vitamin

B_{12} is absorbed by diffusion across the epithelial ileum. IF and bypasses IF-Cbl receptors are not required in this process[17].

3.3.2. Metabolic Function

Several vitamin B_{12} dependent enzymes can be found in bacteria and algae. However, in mammalian cells there are only two. The first one involves the transfer of a methyl group from methylcobalamin to methionine synthase, a reaction that converts homocysteine to methionine. In this reaction, vitamin B_{12} activates folate, whereas methionine is required for DNA methylation. The other enzyme, methylmalonyl CoA mutase, uses adenosylcobalamin for converting L-methylmalonyl-coenzyme A (CoA) to succinyl-CoA. This conversion plays an important role in the extraction of energy from proteins and fats[17,52].

3.3.3. Deficiency

Although vitamin B_{12} deficiency is uncommon and unlikely to develop in healthy human beings in developed countries, groups of people with poor diets such as: elderly, vegans or ovo-lacto vegetarians, can develop deficiencies of vitamin B_{12}. The most common causes are related to inadequate IF production, atrophic gastritis, interference with the ileal uptake of vitamin B_{12} due to disease, resection or interference by bacterial overgrowth, drug-nutrient interactions as well as some less common genetic defects[17].

Table 3.2: Health Conditions / Symptoms Associated with Vitamin B_{12} Deficiency[17]

Types	Results	Clinical Manifestations
Hematological	Pernicious anemia Megaloblastic anemia Leukopenia Thrombocytopenia	Large, immature red blood cells, lower than the normal amount of white blood cells, abnormally low number of platelets in the bloodstream
Neurological	Demyelination Neuropathy	Nervousness Striking behavioral changes Paranoia Tingling Fatigue Paralysis Memory loss Numbness
Psychiatric		Irritability Mild memory impairment Depression Psychosis / Personality change

Tab. 3.2 continued

Health Conditions		Multiple sclerosis Parkinson disease Alzheimer's disease Dementia Cancer Occlusive vascular diseases Congenital heart defects Osteoporosis Failure to thrive Premenstrual syndrome Neural tube defects

Symptoms of vitamin B_{12} deficiency can be grouped into several categories, including neurological, hematological, and psychiatric effects. Extended deficiency leads to nerve degeneration and irreversible neurological damage. Characteristic symptoms of the above categories of vitamin B_{12} deficiency are listed in Table 3.2. Very often symptoms of vitamin B_{12} deficiency are misdiagnosed, and that is because vitamin B_{12} deficiency mimic symptoms of other health conditions such as Alzheimer's disease, amyotrophic lateral sclerosis, diabetic peripheral neuropathy, spinal cord compression, alcoholic peripheral neuropathy, and congestive heart failure[53,54]. Classical symptoms of vitamin B_{12} deficiency include synthesis of large, immature, oblong-shaped erythrocytes (megaloblasts) and myelin deterioration in both central and peripheral nervous systems[54].

3.3.4. Recommendation

According to the Institute of Medicine (National Academies, USA), the recommended daily allowance (RDA) for the vitamin B_{12} is 2.4 µg/ d[17,55]. The actual daily amount that is needed to maintain a sufficient serum level to promote erythropoiesis and other blood functions is considerably smaller, however this recommendation assumes a 50 % absorption rate of vitamin B_{12} from the amount ingested with foods[17]. Also they state that so far there is no sufficient scientific evidence to set an upper intake level for vitamin B_{12} consumption[56].

3.4. Main Types of Vegetarian Diets

As it is stated from vegetarians "all animal flesh must be excluded". There are several types of vegetarianism, the most common are listed in Table 3.3. Fruitarianism allows only fruit, nuts, seeds and plant, which can be gathered without harming the plant. Raw veganism includes fresh fruit, nuts, seed and vegetables, excluding every cooking process. Su vegetarianism apart from animal products excludes also vegetables from the *Allium* family (onion, spring onions, garlic, scallions and leeks).

In addition, strict vegans and Su vegetarians avoid products that may use animal ingredients (not included in product label) or products which use animal products in their manufacturing (cheeses that use animal enzymes, gelatin (from animal skin, bones and connective tissue, etc.)[57].

Table 3.3: Dietary Composition of Main Types of Vegetarianism[57]

	meat	fish	egg	dairy	honey	*Allium* family
vegan[a]	x	x	x	x	x	✓
ovo-vegetarian	x	x	✓	x	✓	✓
lacto-vegetarian	x	x	x	✓	✓	✓
ovo-lactovegetarian	x	x	✓	✓	✓	✓
raw vegan[b]	x	x	x	x	x	x
Su vegetarian[c]	x	x	x	x	x	x
fruitarian[d]	x	x	x	x	x	x

[a]Excludes all animal flesh and animal products, milk, honey, eggs. May also exclude any products tested on animals, also any clothing from animals. [b]Includes only fresh and uncooked fruit, nuts, seeds, and vegetables, excludes vegetables in the *Allium* family. Vegetables can be cooked only up to a certain temperature. [c]Includes only fruit, nuts, seeds, and other plant matter that can be gathered without harming the plant. [d]Excludes all animal products as well as vegetables in the *Allium* family.

3.5. Bioavailability of Vitamin B_{12} in Vegetarians and Elderly People

From the perspective of nutrient intake, vegetarian diets are usually rich in carbohydrates, *ω*-6 polyunsaturated fatty acids, dietary fibers, carotenoids, folic acid, vitamin C, vitamin E, and magnesium (Mg). On the other hand, these diets are relatively low in proteins, saturated fatty acids, *ω*-3 polyunsaturated fatty acids (particularly eicosapentaenoic and docosahexaenoic acids), vitamin A (retinol), vitamin B_{12}, vitamin D_3 (chlolecalciferol), zinc, iron, and calcium (Table 3.4)[20,57,58].

Table 3.4: Nutrient imbalance in vegetarian diets

Rich	Low
Fiber	Vitamin A
Vitamin C	Vitamin D_3
Vitamin E	Vitamin B_{12}
Folate	Iron
Magnesium	Cholesterol
ω-6 Polyunsaturated fatty acids	*ω*-3 Polyunsaturated fatty acids
Carbohydrates	Saturated fatty acids

Vegetarians have due to an increased consumption of fruits, vegetables, whole-grains, legumes, nuts, and various soy products typically a lower body mass index,

lower serum cholesterol levels, and low blood pressure[59]. Compared with non-vegetarians, vegetarians also have: reduced rates of mortality due to ischemic heart disease (lower blood cholesterol), have lower incidences of hypertension, stroke, type 2 diabetes, and certain cancers[59]. Pawlak et al.[24] showed that vegetarians are able to develop vitamin B_{12} depletion or deficiency despite their demographic characteristics, place of residency, age, or type of vegetarian diets. The reason is that non-animal derived products are generally poorly absorbed. Characteristic examples are: the bioavailability of vitamin B_{12} from cooked eggs is 3.7 – 9.2 %, the vitamin B_{12} content of various types of milk is very low (approximately 0.3 – 0.4 µg /100 g)[60], and significant losses of vitamin B_{12} occur during the processing of milk[61]. These observations explain why vitamin B_{12} deficiency is relatively common in lacto-ovo-vegetarians.

A considerable proportion of elderly people with low serum B_{12} levels without pernicious anemia have been recently reported to have malabsorption of protein-bound B_{12} (food-bound B_{12} malabsorption). Vitamin B_{12} malabsorption occurs with certain gastric dysfunctions, particularly atrophic gastritis with low stomach acid secretion[62]. However, vitamin B_{12} deficiency can be undetected in vegetarians because their diets are rich in folic acid, which may covers vitamin B_{12} deficiency until severe health problems occur[63]. Thus, strict vegetarians and elderly people are at higher risk for developing cobalamin deficiency, and in order to prevent that, consuming vitamin B_{12} fortified products or vitamin B_{12} containing supplements, can be a good measure of prevention.

3.6. Vitamin B_{12} in Foods

Vitamin B_{12} in foods can be found in several forms, including adenosylcobalamin, hydroxycobalamin, methylcobalamin, cyanocobalamin, and sulfitocobalamin[64,44]. Hydroxycobalamin and methylcobalamin are better absorbed than the other forms, however adenosylcobalamin and hydroxocobalamin are the predominant sources in foods[65]. Major contributors of vitamin B_{12} in the human diet are animal-based foods such as meat, fish, eggs and dairy products. Vitamin B_{12} is accumulated in animal tissues or milk, primarily due to gut microbial activities and feed supplements[45]. Apart from animal-based sources, vitamin B_{12} can be found in very small amounts exclusively in plant foods such as cereal, breads, pies, and even cookies, due to either contamination during processing, adding of small amounts of ingredients derived from products of animal origin (milk solids) or fortification. However, the amount of cobalamin in the majority of these foods, except foods fortified with vitamin B_{12}, is negligible[17].

3.6.1. Animal-Derived Food Sources Containing Vitamin B_{12}

Meat organs such as liver and clams contain the highest amounts of vitamin B_{12}. The content of vitamin B_{12} in the liver varies from about 3.3 µg in whole chicken liver to

more than 82 µg in 100 g of beef liver[17]. Most other meats contain between 1.2 – 3.5 µg of vitamin B_{12} per 100 g. Bioavailability of vitamin B_{12} from meats ranges from 10 % in liver to as much as 90 % in ground-cooked mutton patties[17]. The vitamin B_{12} content of fish ranges from 3.0 – 8.9 µg/ 100 g. The dark muscle of skipjack is considered as one of the richest sources of vitamin B_{12} (159 µg/ 100 g) of fishes[44]. Milk contains between 0.3 – 0.4 µg vitamin B_{12} per 100 g. The absorption rate of vitamin B_{12} from milk is around 65 %. Heat has a destructive effect on vitamin B_{12}. The boiling process of milk can destroy 30 – 50 % of vitamin B_{12} depending on the duration of cooking, while pasteurization destroys 5 – 10 % of vitamin B_{12}. The vitamin B_{12} content of dairy products such as cheese or cottage cheese recovers between 20 – 60 % in relation to that originally presented in milk. The whole egg contains between 0.9 – 1.4 µg /100 g vitamin B_{12}. Most of the vitamin B_{12} in an egg is found in the yolk. The bioavailability of vitamin B_{12} from eggs depends on the preparation method (egg, scrambled egg, boiled egg) and ranges from about 4 % to a little more than 9 %[17,44].

3.6.2. Plant-Derived Food Sources Containing Vitamin B_{12}

In the United States many foods are fortified with vitamin B_{12} and comprise a high proportion of the dietary vitamin B_{12} intake[56]. This includes cereals such as Kellogg's Special K, Wheat Bran Flakes, Total, Total Raisin Bran, All Bran Original, soymilk, and soy meat analogues such as MorningstarFarms Burger Crumbles. These foods contain from less than the RDA to more than 200 % of the RDA of vitamin B_{12}. Fortified breakfast cereal with folic acid, vitamins B_{12} and B_6 has been recommended by several research groups as it increases the blood concentrations of these vitamins and decreases the total homocysteine concentrations in the plasma of elderly subjects[66]. Hence, vitamin B_{12}-fortified breakfast cereals may be a particularly valuable source of vitamin B_{12} for vegetarians[20]. However, vegetarians avoid strongly also processed foods in addition to animal products. Thus, the identification of plant-derived food sources that naturally contain a large amount of vitamin B_{12} became necessary in order to prevent vitamin B_{12} deficiency in vegetarians[20].

3.6.2.1. Vitamin B_{12}-Enriched Beans and Vegetables Using Organic Fertilizers or Hydroponics

The addition of an organic fertilizer such as cow manure significantly increased the vitamin B_{12} content of spinach leaves, i.e., approximately 0.14 µg/ 100 g fresh weight[20]. Nevertheless, the consumption of several hundred grams of fresh spinach would be insufficient to meet the RDA of 2.4 µg/ day for adult humans[56,67].

Researchers attempted to prepare vitamin B_{12}-enriched vegetables by treating them with a solution that contains high levels of vitamin B_{12}. Thus, the plant vitamin B_{12} contents increased significantly, suggesting that vitamin B_{12}-enriched vegetables may

be particularly beneficial to vegetarians[68,69]. However, artificially vitamin B_{12}-enriched vegetables may not be compatible with the philosophy of vegetarians.

3.6.2.2. Fermented Beans and Vegetables

The content of vitamin B_{12} in soybeans is very low, with the exception of a fermented soybean-based food called tempeh that contains a considerable amount of vitamin B_{12} (0.7–8.0 µg/ 100 g)[32]. The increased vitamin B_{12} content of tempeh during its production may occur from bacterial contamination[70]. Other fermented soybean products such as Natto[71], Korean soybean products (doenjang, chungkookjang, and kochujang) contain minute amounts of vitamin B_{12} (0.1 − 1.9 µg/ 100 g)[72]. Also trace amounts of vitamin B_{12} were found in broccoli, asparagus, Japanese butterbur, mung bean sprouts, tassa jute, and water shield[73]. Fermented Korean vegetables such as kimuchi contain traces (<0.1 µg/ 100 g) of vitamin B_{12}[72,74]. Various fermented tea leaves contain trace amounts of vitamin B_{12} (0.1 − 0.5 µg/ 100 g dry weight)[75,76]. The juice of fenugreek (*Trigonella foenum graecum*) leaves can be enriched with vitamin B_{12} (12.5 µg/ 100 ml) by certain lactic fermentations[77]. The addition of *Propionibacteria* to cabbage during sauerkraut production results in higher concentrations of vitamin B_{12} (7.2 µg/ 100 g)[33].

3.6.2.3. Edible Algae

Several types of edible algae are consumed worldwide as food sources. However, the most widely consumed edible algae are dried green (*Enteromorpha sp*.) and purple lavers (*Porphyra sp*.) and contain substantial amounts of vitamin B_{12} (approximately 133 µg/ 100 g dry weight)[29,30]. Apart from these two, other species of edible algae contain no vitamin B_{12} or only trace amounts[78]. The algal corrinoid compounds of dried purple and green lavers were purified and confirmed as vitamin B_{12}[79,80]. A significant amount (133.8 µg/ 100 g dry weight) of vitamin B_{12} was found in dried Korean purple laver (*Porphyrasp*.). However, seasoned and toasted laver products contain lower amounts of vitamin B_{12} (approximately 51.7 µg/ 100 g dry weight)[30]. The biological activity of lyophilized purple laver (*Porphyra yezoensis*) was investigated in vitamin B_{12}-deficient rats, and the results indicated that vitamin B_{12} is bioavailable in rats[81]. A nutritional study of six vegan children who had consumed vegan diets including brown rice and dried purple laver (nori) for 4 – 10 years suggested that the consumption of nori may prevent vitamin B_{12} deficiency in vegans[82]. Also biologically active vitamin B_{12} in *Chlorella* tablets (eukaryotic microalgae *Chlorella sp*.) have been used in human food supplements[31]. Edible cyanobacteria such as *Spirulina*, *Aphanizomenon*, and *Nostoc* are described in the nutritional labels of dietary supplements that contain high levels of vitamin B_{12}. However, the substantial amounts of vitamin B_{12}, measured in these commercially available supplements, were determined using microbiological methods, which cannot separate vitamin B_{12} from vitamin B_{12} inactive analogues[83]. These supplements often contain large amounts of pseudovitamin B_{12}, which is biologically

inactive in humans. Therefore, edible cyanobacteria and their products should not be recommended as sources of vitamin B_{12} for vegetarians.

3.6.2.4. Edible Mushrooms

Many species of wild mushrooms are widely consumed due to dietary importance of their nutritional and medicinal characteristics[84,85]. In European countries six wild edible mushroom species are well known among vegetarians. Porcini mushrooms (*Boletus sp*.), parasol mushrooms (*Macrolepiota procera*), oyster mushrooms (*Pleurotus ostreatus*), and black morels (*Morchella conica*) contain zero or trace levels (0.01 − 0.09 μg/ 100 g dry weight) of vitamin B_{12}. On the other hand, black trumpet (*Craterellus cornucopioides*) and golden chanterelle (*Cantharellus cibarius*) mushrooms contain considerable levels of vitamin B_{12} (1.09 − 2.65 μg/ 100 g dry weight)[26]. Using the combination of immunoaffinity columns and LC/ESI-MS/MS, purified corrinoid compounds from *C. cornucopioides* or *C. cibarius* were identified as vitamin B_{12}. Consumption of approximately 100 g dried black trumpet (approximately 1 kg of fresh mushroom with 90 % moisture content), is recommended in order to reach the RDA levels for adults (2.4 μg/ day), although ingestion of such large amounts would not be feasible[27]. Nevertheless, moderate mushroom intake may slightly contribute to the prevention of severe vitamin B_{12} deficiency in vegetarians[27].

3.7. Industrial Production of Vitamin B_{12} from Bacteria

Over the last years, vitamin B_{12} has received much attention because of the increasing demand on the world market. Due to highly complicated and costly chemical synthesis of vitamin B_{12}, the commercial vitamin B_{12} produced exclusively via biosynthetic fermentation processes. For microbial production of vitamin B_{12}, there are two types of processes, the aerobic and the anaerobic fermentation[86]. Cultures of the anaerobic bacteria *Propionibacterium shermanii* or *Propionibacterium freudenreichii* etc. as well as cultures of the aerobic bacteria *Pseudomonas denitrificans* have been used successfully for the production of vitamin B_{12}. The above bacteria have been used from industrial companies due to their rapid growth and high productivity[87]. The implementation of random mutagenesis is a common strategy for generating strains that produce high yields of vitamin B_{12}[87]. It has also been reported that cobalt-resistant strains of propionibacteria have enhanced vitamin B_{12} production[88]. Different metabolic engineering strategies have been applied to increase vitamin B_{12} production in P. *freudenreichii*[89]. A recombinant *P. freudenreichii* strain harbouring a plasmid containing *hemA*, from *Rhodobacter sphaeroides*, and homologues of *hemB* and *cobA*, showed 2.2-fold overproduction of vitamin B_{12}[90].

During the microbial production process of vitamin B_{12} the resulting photosensitive compound of methylcobalamin and adenosylcobalamin are derivatized by subsequent cyanidation in the stable form of cyanocobalamin, and further purified with sequential extraction methods using organic solvents[91]. Nowadays, in the developed world contemporary consumers as well as vegans or vegetarians, demand increasingly Bio products and products with the least possible industrial processing. As a consequence, the research as well as the market has turned to find sources of high vitamin B_{12} content from plant products. Hence, nutritional supplements (plant-derived) with high amounts of vitamin B_{12} will be on demand for people that follow diets such as veganism or vegetarianism, which suffer from vitamin B_{12} absence. Developing markets such as Indian and Chinese promote many plant-based products labeled with vitamin B_{12} and products enriched with vitamin B_{12}. In most cases those claims are wrong, because they do not measure only the biologically active form of vitamin B_{12} but also inactive analogues of cobalamin. An important factor for the misleading labels is the analytical method used for the determination of vitamin B_{12}, more specifically, the microbiological assay (MBA) does not differentiate between active forms of vitamin B_{12} and other corrinoids[45]. Before vegetarianism and veganism became way of life for the modern people of developed countries, a medium-sized pharmaceutical company in Lower Saxony (Germany) Teutopharma GmbH, came in the early 90's with a pioneer idea that the symbiosis of nitrogen-fixing actinomycete *Frankia* with actinorhizal plants may give the opportunity for a plant to be a potential producer of vitamin B_{12}. As a matter of fact, based on this concept they discovered that the sea buckthorn (*Hippophae rhamnoides*), as a species of the family of actinorhizal plants, contains vitamin B_{12}[92]. The company as pioneer in natural health-promoting products led the market and promoted sea buckthorn as a unique natural source of vitamin B_{12} from plants matrices, without the addition of artificial cyanocobalamin. Due to insufficiency of the developed analytical methods and high deviations of the measured concentrations, which led to uncertainty (concerning the real vitamin B_{12} content of the plant), the production and distribution of the sea buckthorn was intermitted by the company in 2010[93,94].

3.8. *Frankia*

Frankia is a genus of soil gram-positive actinomycetes, famous for its ability to form N_2-fixing root nodule symbioses with actinorhizal plants. It is a filamentous free-living bacterium found in root nodules or in soil[95]. The classification of the genus *Frankia* has been made in the order of Actinomycetales, on the basis of morphology, cell chemistry, and 16S rRNA sequences[96]. *Frankia* is the only species of *Frankeniaceae* with the capacity to fix nitrogen. This microaerophilic bacterium is characterized by a high guanine-cytosine (GC) % content and a slow growth rate[97].

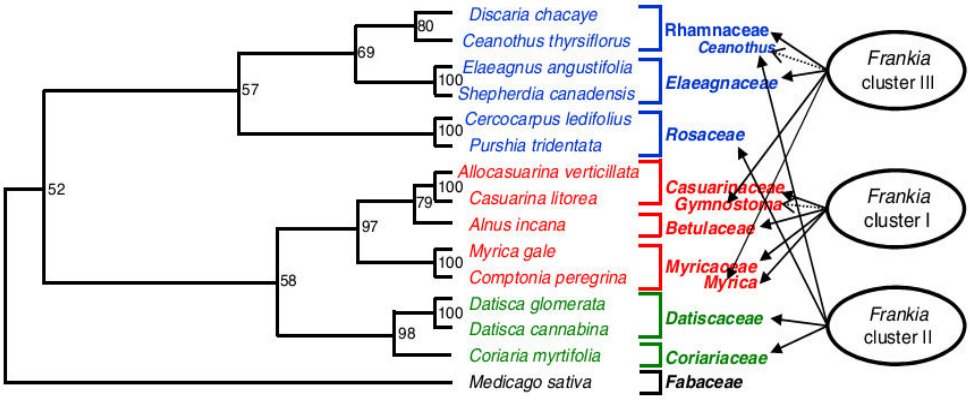


Figure 3.5: The host specificity in actinorhizal symbioses is shown in a simplified scheme adapted from Benson and Clawson[98]; the numbering of *Frankia* clusters is adapted from Normand et al[94]. Legumes (Fabales) are indicated in black, Fagales in red, Rosales in blue and Cucurbitales in green. Thick arrows connecting a *Frankia* cluster with a group of plants indicate that members of this cluster are commonly associated with the plants. Dashed arrows indicate that members of this cluster have been isolated from or detected in an effective or ineffective nodule of a member of the plant group at least once. It should be pointed out that not all strains isolated from, or identified in, an actinorhizal nodule can reinfect the corresponding plant species[99].

The *Frankia* genus includes four phylogenetic clusters[94]. These clusters reflect *Frankia*–host range and *Frankia*–host–plant biogeography. *Frankia* strains in Cluster I are associated with *Alnus*, *Myrica*, *Comptonia* and *Casuarina* hosts. In Cluster II *Frankia* strains occur in nodules of *Coriaria*, *Datisca*, *Purshia*, *Ceanothus*, and *Dryas*. *Colletia* with the other actinorhizal *Rhamnaceae* of South America, *Morella*, *Gymnostoma* and the *Elaeagnaceae* are nodulated by cluster III *Frankia* strains (Fig 3.5). An additional cluster IV group of strains forms a broad group of a typical *Frankia* (non-infective and/or non-nitrogen-fixing) obtained from actinorhizal plants[100].

Frankia as a generic name was proposed by Brunchorst (1886 – 1888) for the microorganism in nodules of *Alnus* and *Elaeagnaceae*[95]. Due to difficulties in isolating *Frankia* into pure cultures, knowledge about *Frankia* was previously restricted to its symbiotic stage. Pommer (1959)[101] most likely made the first successful and well described isolation from *Alnus glutinosa* nodules, but his cultures were unfortunately lost[95]. After twenty years was reported from Callaham et al. (1978)[102] isolation of *Frankia* CpI1 from *Comptonia peregrina*. After that, many isolates of *Frankia* occurred but not from all actinorhizal plant species. In case *Frankia* isolates are not available, crushed root nodules or soil must be used as inoculum. When *Frankia* is investigated in soil via a trap plant assay, the *Frankia* strain(s) are able to nodulate the plants[95].

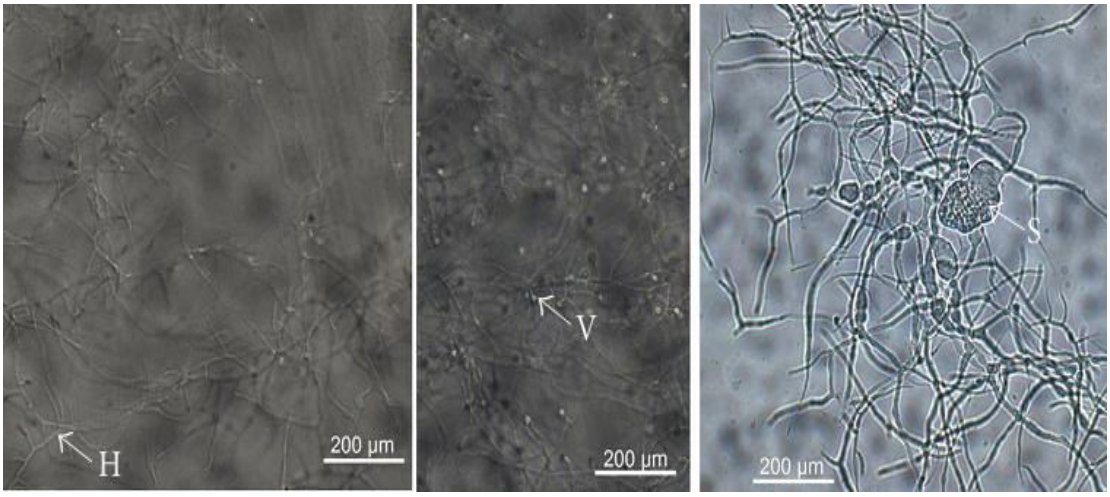


Figure 3.6: Structures of *Frankia* bacteria H: hyphae, V: vesicle, and S: sporangia[97]

Depending on the culture conditions, in liquid culture *Frankia* forms hyphae and multilocular sporangia which are located on hyphae either terminal or intercalary[97]. Ultrastructure showed that sporangia are multilocular and contain the spores, the effective propagules of the bacteria, and the hyphae, free living structures are hollow. The site of nitrogenous activity are the vesicles, which are generally formed when nitrogen is very limited in the medium (Figure 3.6). *Frankia inoculum* is easier to conserve than *Rhizobium inoculums* due to the presence of resistant structure in culture[103]. However, the morphology of *Frankia* in the nodule varies according to host plant. The bacterial partner is responcible for the size of hyphae and the presence or the absence of vesicles.[104].

A perennial root organ called nodule, in which bacteria are hosted and nitrogen is fixed, is formed due to the symbiosis between actinorhizal plants and *Frankia*. In nature, actinorhizal nodule can have various forms and colors[105]. Two types of nodule formation occur in actinorhizal symbiosis: the intercellular and the extracellular infection[106].

3.9. Actinorhizal Plants

Plants nodulated by *Frankia* strains are known as actinorhizal plants; most are trees or woody shrubs. They are found in all continents apart from Antarctica. More than 194 different plant species have been identified as hosts and are classified among 8 different families and 4 subclasses of flowering plants (Table 3.5)[107].

Table 3.5: Actinorhizal Plant Genera[107]

Subclass	Family	Genus	Numbers of nodulated species
Hamamelidae	Betulaceae	*Alnus*	47
Hamamelidae	Casuarinaceae	*Allocasuarina*	54
		Casuarina	16
		Ceuthostoma	2
		Gymnostoma	18
Hamamelidae	Myricaceae	*Comptonia*	1
		Myrica	28
Rosidae	Elaeagnaceae	*Elaeagnus*	38
		Hippophae	2
		Shepherdia	2
Rosidae	Rhamnaceae	*Ceanothus*	31
		Colletia	4
		Discaria	5
		Kentrothamnus	1
		Retanilla	2
		Talguenea	1
		Trevoa	2
Rosidae	Rosaceae	*Cercocarpus*	4
		Chamaebatia	1
		Cowania	1
		Dryas	1
		Purshia	2
Magnoliidae	Coriariaceae	*Coriaria*	5
Dilleniidae	Datiscaceae	*Datisca*	2

The actinorhizal plants have the tendency to grow in marginal soils and play an important role as pioneer species in early successional habitats. These plants inhabit in a variety of climates and ecosystems, including arctic tundra (*Dryas*), forests (*Alnus, Comptonia, Casuarina, Criaria*, and *Sheperdia*), coastal dunes (*Casuarina, Hippophae, Myrica,* and *Elaeagnus*), riparian habitats (*Alnus, Myrica*, and *Datisca*), and chapparal (*Ceanothus*, *Comptonia, Cercocarpus, Cowania*, and *Purshia*). Nitrogen fixation amounts, by actinorhizal plants, are estimated in similar levels to legumes (40-350 kg N-ha^{-1} yr^{-1})[108] and some species can return up to 70 % of this nitrogen to the ecosystem[109]. Additionally to their importance as pioneering species, they play a significant role in land reclamation and stabilization, as sources of timber, biomass, fuelwood, and pulpwood, and as nurse crops for other economically important species[107].

3.10. *Hippophae rhamnoides*

Hippophae rhamnoides (sea buckthorn) belongs in the family *Elaeagnaceae*, is a deciduous nitrogen-fixing spiny shrub or small tree between 2 – 4 m high. It is widely distributed on the Eurasian continent, on river banks and coastal dunes (such as Baltic coast of Finland or on the western coast and along the gulf of Bothnia in Sweden) and in Asia throughout the Himalaya regions including India, Nepal and Bhutan and in the northern parts of Pakistan and Afganistan[110]. The botanical genus name of sea buckthorn is *Hippophae*. The name *Hippophae* in greek, literally means "*shiny horse*" because the ancient Greeks used to feed it to their prize racehorses to keep them sleek and healthy[111]. This unique and valuable plant has recently gained worldwide attention, mainly for its medicinal and nutritional potential. *Hippophae rhamnoides* has yellow or orange berries (Fig 3.7), brown or black rough bark and a thick grayish-green crown. Leaves are alternate, narrow, and lanceolate with a silver-grey color on the underside. This plant is ideal for soil erosion control, land reclamation, wildlife habitat enhancement, and farm shelterbelt protection, with nodule rooting capability of fixing nitrogen from the atmosphere[112]. It is a unisexual plant, in which male and female plants are different. *Hippophae rhamoides* needs about 4 – 5 years from the appearance of the first shoots from the seeds to the beginning of fruit and peaks at the 7-8th year of plant life. With intermittent pruning it remains productive for 30 years. It flowers in April and the fruiting season is from August to October[113]. The plant can tolerate temperatures from – 43 to 40°C, grows in areas with mean annual temperatures ranging from 4.7 to 15.6°C and with annual precipitation ranging from 250 to 800 mm[114]. Like all *Elaeagnaceae* species *Hippophae rhamoides* has the capability of holding soil in fragile slopes, has no need for good arable land for its cultivation and has symbiotic relationships with nitrogen fixing actinomycetes known as *Frankia* in its root nodules[115]. The long networking roots of *Hippophae rhamoides* can also transform insoluble organic matters in the soil in its more soluble state. In many cases, five year old plants have a taproot of up to 3 m. However in forest areas with abudance of water can grow up to 15 m. Deep and horizontal roots extending between 6 and 10 m. The root turion starts to grow seedlings from the horizontal roots creating many new plants, after a period of 2 to 3 years[110]. The symbiotic relationship between sea buckthorn and nitrogen fixing actinomycetes enables sea buckthorn to be planted in marginal soil[115]. In addition it is also a salt-tolerant plant as has been demonstrated in many places e.g., Central Asia, Siberia, Azerbaijan and China where populations of *Hippophae* grow well on the soils of wastelands, deserts and dunes of the seashore that have highly concentrated salt contents[116].

Figure 3.7: Sea buckthorn (*Hippophae rhamnoides L.)* in India early September, branch bearing orange-red berries, thorn and leaves[117]

A large number of bioactive substances can be found in all parts of *Hippophae rhamnoides* such as vitamins (A, C, E, K, riboflavin, folic acid, B_{12}[92]), carotenoids (α, β, δ-carotene lycopene), phytosterols (ergosterol, stigmasterol, lansterol, amyrins), organic acids (malic acid, oxalic acid), polyunsaturated fatty acids and some essential amino acids[117]. Sea buckthorn contains vitamin C 3 to 16 times higher than that of the Kiwi fruit (*Actinidia sinensis*) which is very famous for its high vitamin C content[110].

Table 3.6: Elemental Composition of *Hippophae rhamnoides* Berries/Juice[118]

element	range (µg/ml)	average	references
potassium	100-806	497	Tong et al. (1989)
	0.147-0.209	0.168	Zhang, W., et al. (1989)
calcium	64-256	143	Tong et al. (1989)
	93.9-173	113	Zhang, W., et al. (1989)
phosphorus	82.1-206	131	Zhang, W., et al. (1989)
magnesium	39.8-103	70.4	Zhang, W., et al. (1989)
	53.3-165	88.9	Tong et al. (1989)
sodium	17.7-125	76.9	Zhang, W., et al. (1989)
	18.0-89.8	48.5	Tong et al. (1989)
cobalt	≤0.1		Zhang, W., et al. (1989)
	0.01-0.09	0.034	Tong et al. (1989)
chromium	0.108-0.287	0.178	Zhang, W., et al. (1989)
	0.47-1.00	0.699	Tong et al. (1989)
copper	0.158-0.653	0.384	Zhang, W., et al. (1989)
	<10		Liu and Liu (1989)
manganese	1.17-2.60	1.67	Zhang, W., et al. (1989)
	0.81-3.86	1.27	Tong et al. (1989)
nickel	0.115-0.357	0.237	Zhang, W., et al. (1989)
	0.39-0.09	0.189	Tong et al. (1989)

Tab. 3.6 continued

element	range (μg/ml)	average	references
strontium	0.19-0.616	0.429	Zhang, W., et al. (1989)
	0.08-0.45	0.195	Tong et al. (1989)
vanadium	0.002-0.009	0.0069	Zhang, W., et al. (1989)
iron	4.13-10.9	7.58	Zhang, W., et al. (1989)
	5.93-161	28.2	Tong et al. (1989)
molybdenum	0.03-0.058	0.044	Zhang, W., et al. (1989)
	1.18	1.18	Tong et al. (1989)
zinc	0.431-1.25	0.763	Zhang, W., et al. (1989)
	2.09-6.31	3.29	Tong et al. (1989)
tin	0.045-0.259	0.170	Zhang, W., et al. (1989)
selenium	7.96-11.3	9.21	Zhang, W., et al. (1989)
	0.94-1.11	1.02	Zhao et al. (1989)
boron	0.43-1.38	1.06	Zhang, W., et al. (1989)
barium	0.168-0.362	0.244	Zhang, W., et al. (1989)
aluminum	2.2-16.7	7.88	Zhang, W., et al. (1989)
titanium	0.103-0.814	0.407	Zhang, W., et al. (1989)
lithium	0.132-0.303	0.203	Zhang, W., et al. (1989)
	0.06-0.15	0.09	Tong et al. (1989)
cadmium	<0.05	<0.05	Zhang, W., et al. (1989)
	0.002-0.015	0.0048	Tong et al. (1989)
arsenic	<0.5	<0.5	Liu and Liu (1989)
lead	0.431-0.761	0.551	Zhang, W., et al. (1989)
	<1	<1	Liu and Liu (1989)
	0.06-0.27	0.010	Tong et al. (1989)

There are many elements and trace elements in *Hippophae rhamnoides.* As listed in Table 3.6 which is a synopsis of individual element levels contained either in plant berries or from the plant juice. Bounous and Zaninni (1988) found that fruit maturity affects N, Ca, K, Na, Mg, Cu, Fe, Zn, and Mn contents[119]. An average weight for each berry is 0.016 g, and contains 11.0 % (w/w) moisture and 14.2 % (w/w) oil. Because of its various rich nutrients the berries of sea buckthorn are usually used to make beverages, which are beneficial for many group of people such as sportsmen, manual workers, children, the aged and pregnant women by building up their strength[116].

4. Experimental Part

The determination of vitamin B_{12} in food and dietary supplement products is routinely carried out by various analytical methods. However, for the analysis of vitamin B_{12} two aspects have to be considered, the quantity of vitamin B_{12} and activity of vitamin B_{12}. The quantity of vitamin B_{12} is not necessarily proportional to the activity of vitamin B_{12}; thus, the distinguishing between them is important. Number of ways are available to measure the quantity of vitamin B_{12}, but only a few bioassays are available to assess vitamin B_{12} activity such as macrocytic anemia improvements, Hyperhomocysteinemia (Hcy) reduction, and Methylmalonic acid (MMA) reduction[120].

For the quantitative determination of vitamin B_{12} the "official" methods are the Microbiological assays (MBA), which are the oldest and the most commonly used. In Methods of Analysis for Nutrition Labeling, three official methods are identified (AOAC Method 960.46, 952.20, and 986.23) which are suitable for the determination of vitamin B_{12} in a wide range of food matrices and vitamin preparations. The Microbiological assays count on the proliferation of bacterial cultures with the help of vitamin B_{12} (bacterial growth rate depends on vitamin concentration in the test medium)[121,122]. However, these methods have several disadvantages such as the low precision. The growth takes several days and they are not capable of distinguishing between true cobalamins and cobalamin analogues.

Several studies on the quantification of vitamin B_{12} in different matrices have been reported in the literature. Most of them include radioisotopic assays[123] or methods based on chromatorgraphic techniques. Other methods include atomic absorption spectrometry (AAS)[124], inductively coupled plasma mass spectrometry (ICP-MS)[14], capillary electrophoresis (CE)[125], chemiluminescence techniques[126], biomolecular interaction analysis (BIA)[127] and a combination of fluorescence resonance energy transfer (FRET) and flow-injection analysis (FIA)[128].

Liquid chromatography (LC) serves as a very efficient technique for the determination of vitamin B_{12}, with UV or other detectors, such as fluorescence and electrochemical detection, but are less sensitive than the microbiological method. Due to their poor sensitivity, these methods are suitable for fortified food analyses but not for natural matrix determinations. However, with clean up and pre-concentration steps the ability to measure low concentrations of vitamin B_{12} can be significantly increased[11]. ICP-MS is one of the most sensitive detection methods for the analysis of trace level metal concentrations. Thus, the combination of LC-ICP-MS provides extremely selective detection, and matrix interference was minimized as the determination of vitamin B_{12} is accomplished through the measurement of the Co content[11]. Moreover, a combination of HPLC – electrospray ionization (ESI) – MS

can determine vitamin B_{12} in food products and in multivitamin tablets at the nanogram level[25].

In the following chapters two analytical methods, an HPLC-UV and an HPLC-MS/MS, were developed for the determination of cobalamins and the active forms of vitamin B_{12} in natural matrices. HPLC-UV alone is not sensitive enough to detect vitamin B_{12} in a natural matrix that contains several interfering compounds. Due to the need for accurate determination of vitamin B_{12}, a combined purification and concentration step with an immunoaffinity column were applied.

4.1. Analytical Determination of Vitamin B_{12} in Plants

Sofar, Heudi et al. have shown that an HPLC-UV method combined with immunoaffinity column (concentration step) is a good alternative to the standard microbiological assay (MBA) for cobalamin determination in food products such as milk-based infant formula powder[38]. Other research groups like Marley et al. have applied this immunoaffinity method for the analysis of vitamin B_{12} enriched products[13]; or for determining the vitamin B_{12} content of different meat products and salami (Guggisberg et al.)[37] Watanabe et al. have implemented immunoaffinity columns (IAC) after a solid phase extraction and a concentrating step in their determination of the vitamin B_{12} content of common edible mushrooms[78].

Based on established non-plant analytical methods[37,38,13,26] and an immunoaffinity column (IAC) protocol for the determination of cobalamin in plants, developed in a dissertation by O. Kysil. (Institut of Technical Chemistry at Leibniz University of Hanover)[22], an analytical assay was optimized and specialized to isolate and analyse quantitatively the vitamin B_{12} content in plants. The analysis of vitamin B_{12} in plants is complex: the concentration of vitamin B_{12} is generally negligible or very low (in the range of 0 − 13 µg/ 100 g) and vitamin B_{12} exists in nature in several cobalamin forms with different degrees of stability. Therefore, the isolation, extraction, purification and analysis of vitamin B_{12} from a complex matrix such as plants and berries, with reproducible results and validation studies was not easy to reach. In terms of better understanding and for the sake of simplicity, the justification of the analytical method development, is divided into four main parts. Each part of the method will be studied separately in the following chapters. The Figure 4.1 below demonstrates the protocol of vitamin B_{12} assay as a flow chart.

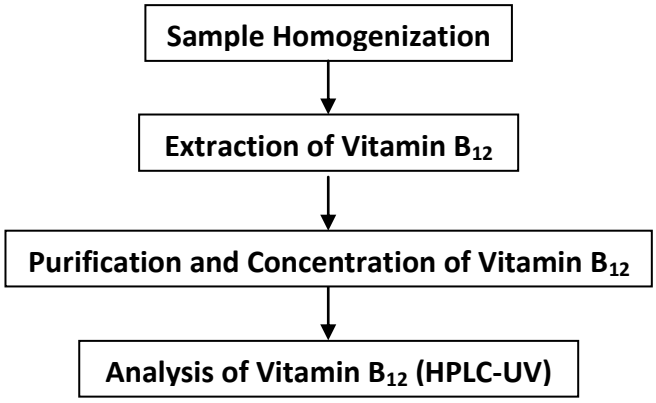


Figure 4.1: General flow chart for vitamin B_{12} determination

4.2. Development and Optimization of Vitamin B_{12} Assay (stepwise process)

4.2.1. Sample Homogenization

Homogenization of samples is an early step in the process of isolation and quantification of analytes. Chemical, mechanical or physical methods are available for homogenization, but generally chemical methods are preferred for many sample types (e.g., *E. coli* and cultured cells). However, solid specimens, such as seeds, and heavily encased samples are not efficiently disrupted chemically. With such resilient samples mechanical and physical methods that rely on grinding, shearing, beating and shocking are used[129]. Mechanical homogenizers, manual homogenizers, mortar and pestles, sonicators, mixer mills, and vortexers are several of the more common tools used for mechanical and physical disruption. Homogenization, has a significant impact on the final results of a process.

It is usually desirable to render solid samples into a fine powder state for the following reasons:

i) A sample in a fine powder state is more homogeneous, allowing more representative sub-sampling with greater precision and accuracy if carefully mixed.
ii) A fine powdered sample dissolves faster and is easier to extract because of its greater surface area.

In our protocol, the choice of the appropriate homogenization method was crucial. In terms of differentiation, each sample that has been tested had different degrees of hardness. Thus, the developed method for reducing the particle size or grinding of solid samples had to be effective enough in a variety of samples such as plants, berries, juices, meat and tablets, etc. All the samples were treated as described in the sample preparation procedures (Appendices 6.1.2), as well as blank spikes of cobalamin. The effectiveness of each method was investigated by measuring the liberation of vitamin B_{12} from the analysed matrix applying the protocol of the

method. For the homogenization of the samples and the liberation of the encased vitamin B_{12}, four different methods were tested in different combinations. General characteristics of each grinding instrument are described below:

i) **Mortar & Pestle (Cryogenic Grinding)**[129]**:** Mortar & Pestle are used for grinding samples frozen in liquid nitrogen. This cryogenic grinding makes the sample brittle and fracture easily, but it also preserves analytes that are heat labile or which may rapidly degrade upon liberation, such as RNA. With dry grinding, it is possible to generate very small particles. However, they are indispensable for grinding solids at room temperature.

ii) **Mixer Mill**[130]**:** This grinding technique enhances the reproducibility and efficiency of grinding as well as the size reduction of the sample, which can reach levels of ~5 µm. A mixer mill can mix and homogenize powders and suspensions in only a few seconds. It is also perfectly suitable for the disruption of biological cells as well as for DNA/RNA or analytes extraction.

iii) **Disperser**[131]**:** Dispersing is a technique for mixing at least two substances that do not dissolve or hardly dissolve in each other and do not chemically react with one another. The goal while producing a dispersion is to create a "satisfactorily" fine distribution in the continuous phase, and to disperse evenly the processed analyte so as the percentage of it to remain constant for any measured quantity.

iv) **Ultrasonic bath**[132]**:** Ultrasonic homogenizing is a mechanical process to reduce small particles in a liquid so that they become evenly small and distributed. The objective is to reduce small particles in a liquid to improve uniformity and stability. These particles (disperse phase) can be either solids or liquids.

The initial protocol of the method (Appendices 6.1.1) used as a grinding method a mortar & pestle with liquid nitrogen (cryogenic grinding). Due to insufficient homogenization of the sample *Hippophae rhamnoides* and to unreproducible analytical results, other methods were decided to be used, to see if they have better impact on the homogenization of the sample (optical inspection) and on the liberation of the encased vitamin B_{12} (analytically measured). An insufficient homogenization of the sample may have an impact on the recovery studies as well as on the precision of the method. Experiments were performed with samples of *Hippophae rhamnoides* with a defined amount (9 g) and the use of various combinations of different grinding methods (Fig 4.2). Apart from the initial grinding method (mortar & pestle), three new grinding methods were studied: mill homogenizer, disperser and ultrasonic bath. The chosen grinding method

combinations had as a basis the mill homogenizer instrument, and had been tested in all possible combinations.

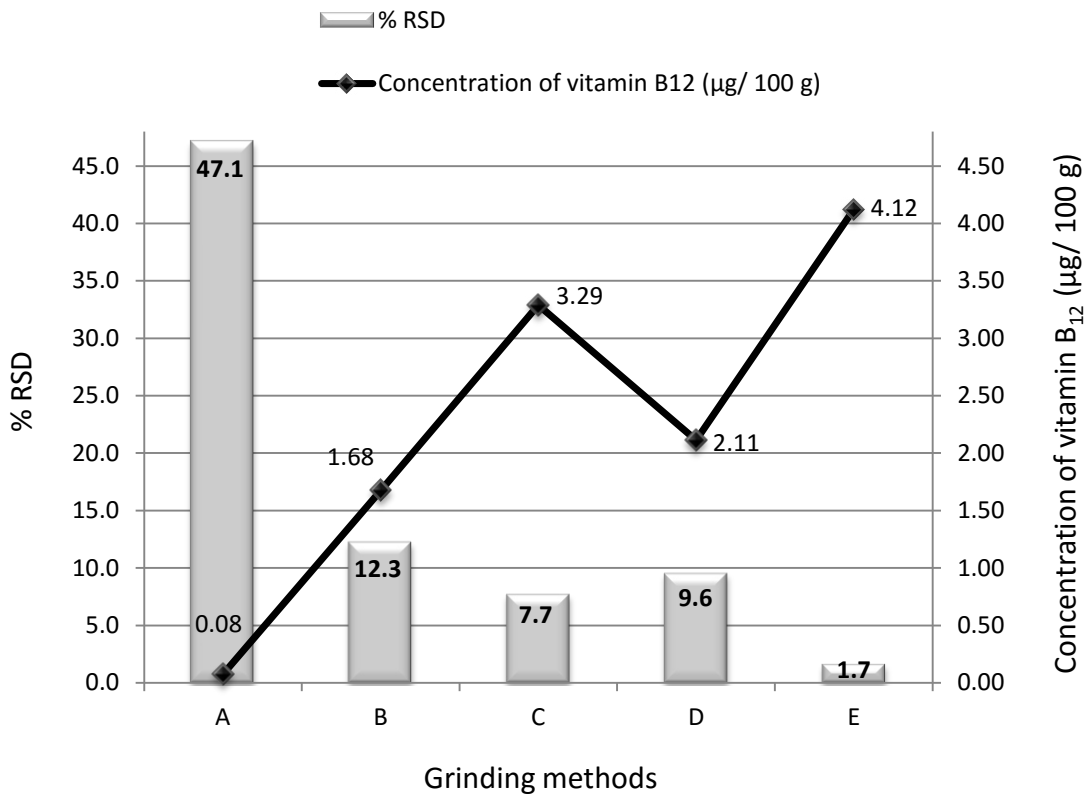


Figure 4.2: The influence of the grinding methods on the concentration (µg/ 100 g) of the extracted vitamin B_{12} from 9 g *Hippophae rhamnoides* berries and on the % RSD between replicate measurements. Grinding methods: A) mortar & pestle - cryogenic grinding B) mill homogenizer, C) mill homogenizer + disperser, D) mill homogenizer + ultrasonic bath, E) mill homogenizer + disperser + ultrasonic bath

The use of the initial grinding protocol (mortar & pestle - cryogenic grinding) in *Hippophae rhamnoides*, yielded concentrations of vitamin B_{12} that ranged between 0.05 – 0.10 µg/ 100 g dry weight, whereas the relative standard deviations between the replicate measurements was very high (~ 47 % RSD). After the application of different grinding methods the % RSD (Fig. 4.2) were reduced in grinding method B to 12.3 % RSD, in grinding method C to 7.7 % RSD, in grinding method D to 9.6 % RSD and the combination of all (method E) disclosed among a set of replicate measurements the lowest value of 1.7 % RSD.

Moreover, the investigation of other grinding methods (see Fig. 4.2) indicated that each of them contributed positively to the increase of the determinable concentration of the vitamin B_{12}, with the combination of all (method E) to extract the highest amounts of vitamin B_{12}. More specifically, in comparison with the initial

grinding method (method A), the use of mill homogenizer increased the extracted vitamin B_{12} twenty one fold (1.68 μg/ 100 g), the combination of mill homogenizer and disperser resulted in forty one fold higher concentrations (3.29 μg/ 100 g), the combination of mill homogenizer with ultrasonic bath increased the concentration of vitamin B_{12} twenty six fold (2.11 μg/ 100 g). The combination of all grinding methods (method E) disclosed the highest determinable vitamin B_{12} value 4.12 μg/ 100 g.

Based on the above results (Fig. 4.2), every grinding method revealed some weaknesses. The use of mortar & pestle alone, showed that is not capable to get an even grind and to reduce well enough the sample size. The use of mill homogenizer alone, decreased the % RSD among replicate measurements and contributed to the increase of the extracted vitamin B_{12}, but the deviation among measurements was still high. The uniformity of the sample might be better than the use of mortar & pestle alone, but not good enough to reduce the size of the sample in order to extract all the encased vitamin B_{12}. The combination of mill homogenizer with the disperser (griding method C) as well as the combination of mill homogenizer with the ultrasonic bath (grinding method D), improved significantly the % RSD among replicate measurements and the extracted amount of vitamin B_{12}. However, only the combination of all available methods (grinding method E) achieved to overcome the weaknesses of individual methods and to increase the extracted vitamin B_{12} amount with a parallel decrease of the % RSD among replicate measurements.

The application of different grinding methods in conjunction with the increase of vitamin B_{12} concentration in the analysed samples, disclosed that the real content of vitamin B_{12} in *Hippophae rhamnoides* had not been yet revealed. As a consequence of that, it came up the assumption that the real content of vitamin B_{12} might be higher than the expected one. Moreover, If we take under consideration that the immunoaffinty column has a capacity limit of 1.0 μg vitamin B_{12} per column, with optimal amount 0.5 μg/ per column, there was a significant possibility that the processed amount (9 g) contained higher amounts of cobalamins that overloaded the immunoaffinity column.

Thus, a second step of the investigation was to check the influence of the sample amount parameter, in comparison with the previous experiment which was constant (9 g) (Fig. 4.2). Further experiments were performed. The sample amount was adjusted to the column capacity. The following combinations of grinding methods were applied: A) mill homogenizer, B) mill homogenizer + mortar and pestle, C) mill homogenizer + mortar and pestle + disperser, D) mill homogenizer + mortar and pestle + disperser + ultrasound bath, parallel with a reduction of the sample amount from 10 g to 0.5 g following the direction A to D (Fig. 4.3). Only with the use of mill homogenizer as grinding method (grinding method A) and 10.0 g of sample the %

RSD was 14. The combination of the mill homogenizer with a mortar & pestle and sample amount 7.0 g, decreased the % RSD to 11 and increased the extracted vitamin B_{12} (4.9 μg/ 100 g). The grinding method C (mill homogenizer + mortar and pestle + disperser) with 2.0 g sample amount showed a lower value of % RSD (4.63 % RSD) and higher amount of the extracted vitamin B_{12} (12.3 μg/ 100 g). The lowest sample amount (0.5 g) in combination with all the applied grinding methods (D) disclosed the highest extracted amount of vitamin B_{12} (37.0 μg/ 100 g) (Fig. 4.4), with the lowest relative standard deviation (0.7 % RSD) among replicate measurements (Fig. 4.3).

In Figure 4.3 it becomes obvious that as the sample amount (g) reduces and extra grinding methods are added to the grinding process, the % RSD among replicate measurements decreases with parallel increase of the extracted vitamin B_{12} from the plant. The grinding method E of the first experiment (Fig. 4.2) and grinding method D of the second experiment (Fig. 4.3), differ only in the use of mortar and pestle, which was added extra in grinding method D. Apart from that, the basic parameter that differentiates these grinding methods is the sample amount. Even though the % RSD between the methods ranges in similar levels (1.7 – 0.7 % RSD), the vitamin B_{12} extracted from 0.5 g sample yielded nine fold higher concentration (37.0 μg/ 100 g) than the extracted vitamin B_{12} from 9 g (4.1 μg/ 100 g). In this experiment, it was proved the assumption that the constant parameter of sample amount had a significant impact on the extraction of vitamin B_{12} from *Hippophae rhamnoides.* The initial choice of the sample amount (10 – 9 g) was proved unexpectedly large for processing, with content of vitamin B_{12} greater than the capacity limit of the immunoaffinity column. By extension, the large quantity of the sample could also create processing capacity problems with each of the grinding methods, and probably with other steps of the protocol.

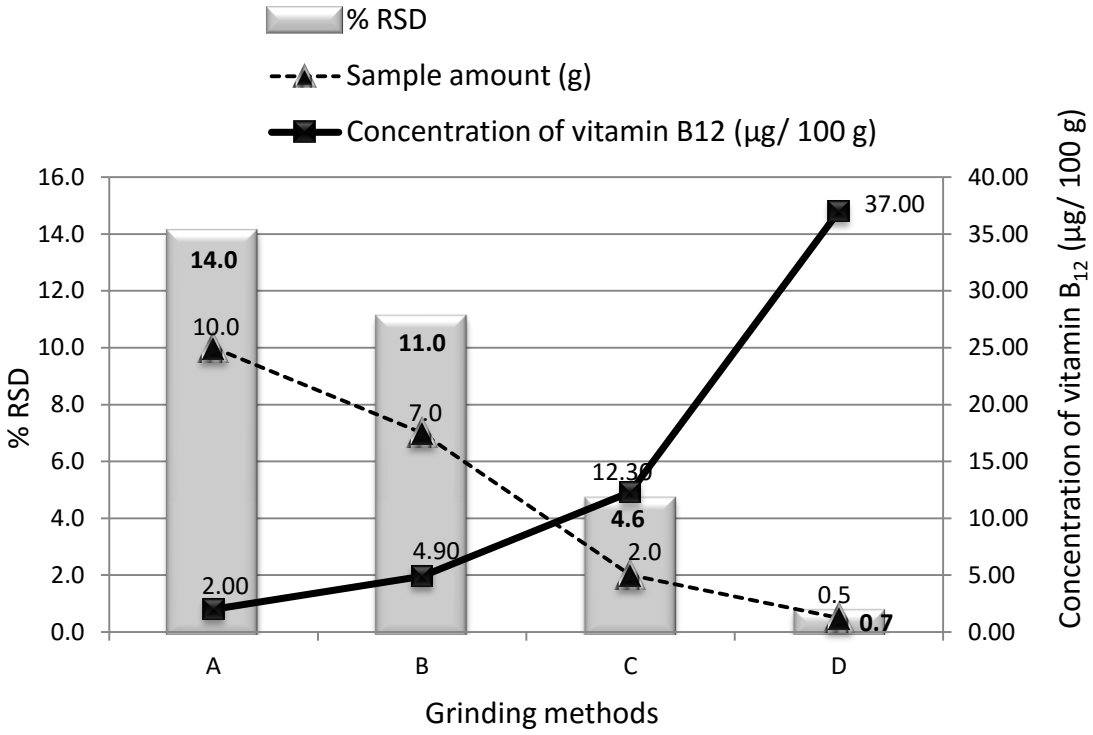


Figure 4.3: Influence of grinding method and sample amount (g) on the determinable concentration (µg/ 100 g) of vitamin B_{12} of *Hippophae rhamnoides* and on the % RSD among replicate measurements. Grinding methods: A) mill homogenizer, B) mill homogenizer + mortar and pestle, C) mill homogenizer + mortar and pestle + disperser, D) mill homogenizer + mortar and pestle + disperser + ultrasonic bath

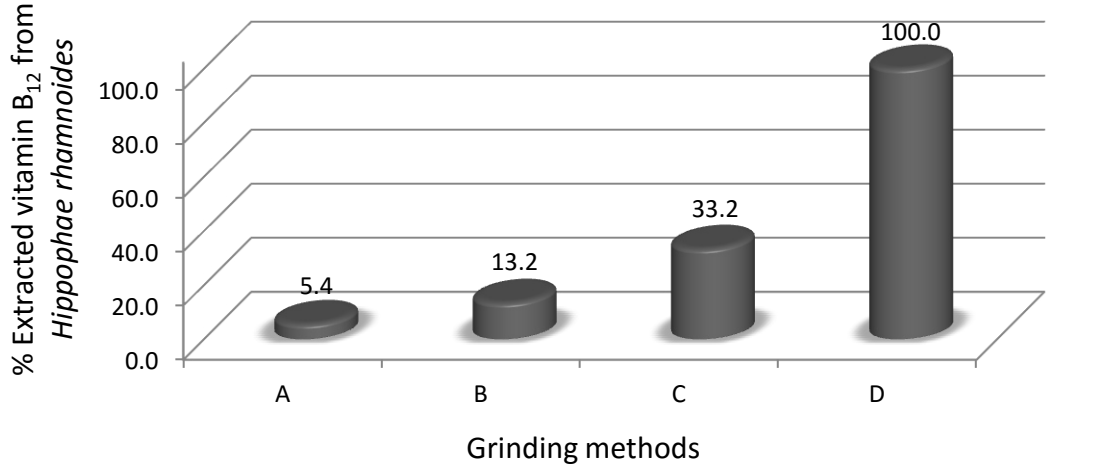


Figure 4.4: % Extracted amount of vitamin B_{12} from *Hippophae rhamnoides* (558) after the application of different grinding methods in different sample amounts: A) mill homogenizer (10 g sample), B) mill homogenizer + mortar and pestle (7 g sample), C) mill homogenizer + mortar and pestle + disperser (2 g sample), D) mill homogenizer + mortar and pestle + disperser + ultrasonic bath (0.5 g sample)

Two of the grinding methods (disperser and ultrasonic bath) were applied also in a liquid sample, to see the influence of the homogenization and the extraction ability in the liquid phase. Liquid extracts of black mustard were tested and followed the optimized protocol (Appendices 6.1.2) in order to measure the concentration of vitamin B_{12}, parallel with testing the influence of the grinding method concerning the extraction ability. Replicate samples were divided in three groups. The first group passed the "Sample Homogenization" step without using any of the existing grinding methods, the second group of replicate samples was processed with a disperser, and the third group of samples was processed with ultrasonic bath. The results as illustrated in Figure 4.5, prove that the application of the disperser and the ultrasonic bath, even in a liquid sample can extract higher amounts of vitamin B_{12}, due to better homogenization and parallel reduction of the particle size of the liquid sample. In addition, a significant finding is that the % RSD (% relative standard deviation) between the replicate samples of the groups was different. The first group "no grinding method" had 7.4 % RSD, the second group "disperser" had ~ 0.0 % RSD and the third one "ultrasonic bath" 8.5 % RSD. Even though, the third group "ultrasonic bath" performed better in terms of the extraction of the vitamin B_{12} (1.65 µg/ 100 ml) from the liquid matrix, the % RSD was much higher (8.5 % RSD) in comparison with the second group, which had approximately 0.0 % RSD. In conclusion, the application of the grinding methods (disperser and ultrasonic bath) supported the extraction of vitamin B_{12} content, and the disperser method contributed significantly to the improvement of % RSD among replicate measurements.

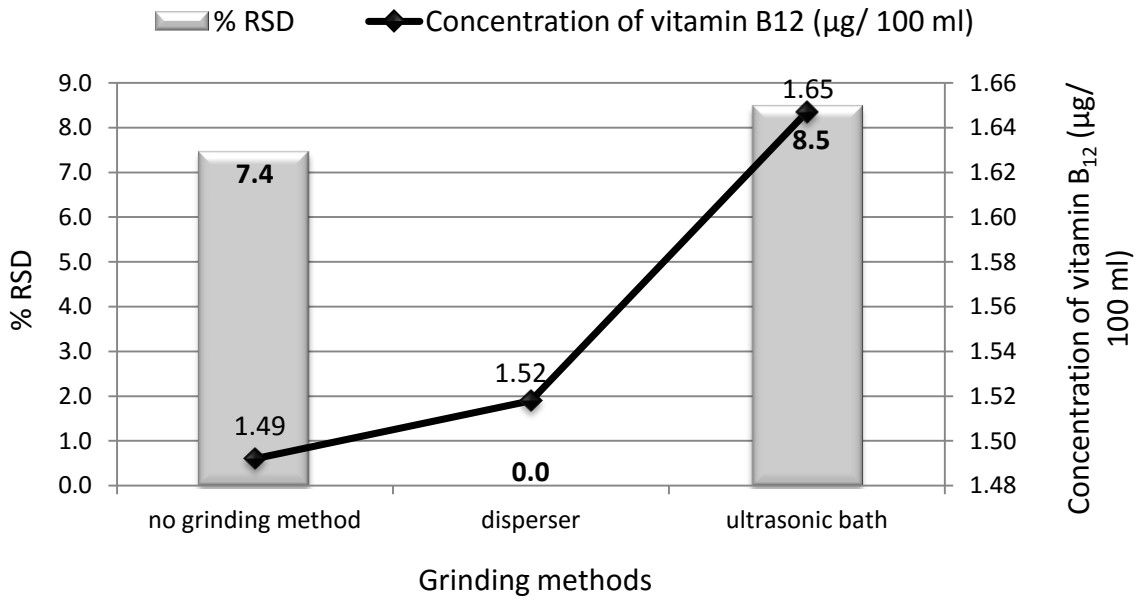


Figure 4.5: Influence of grinding methods on the determinable concentration (µg/ 100 ml) of vitamin B_{12} of black mustard (liquid extract) and on the % RSD among replicate measurements. Grinding methods: no grinding method, disperser and ultrasonic bath

Summary – Discussion

Several samples (solids and liquids) were tested, in order to specify the optimal parameters for an efficient grinding procedure, that can release the encased vitamin B_{12} quantitatively from the measured matrices and can determine the concentration of the vitamin B_{12} in the tested sample. The right method or the right combination of the methods had to comply with the following conditions: to grind the matrix into small particles, release the vitamin B_{12} content from the matrix, protect the measured analyte from heat, mix perfect the powder (homogenize) and dispersed evenly the vitamin B_{12} content so that the vitamin B_{12} percentage remains constant for any measured quantity. However, each of the tested methods proved that alone could not fulfill the above conditions. They showed high deviations among replicate measurements (mortar & pestle - 47.1 % RSD and mill homogenizer - 12.3 – 14.0 % RSD) and low extraction efficiency (~ 5.4 % - use of mill homogenizer (Fig. 4.4)). The sample amount was proved one of the most important parameters (Fig. 4.3), as initially overloaded the immunoaffinity column by exceeding the capacity limit. Thus, the adjustment of the sample amount to the capacity limit of IAC and the combination of all grinding methods contributed significantly to overcome all these weaknesses. Therefore, the sample amount of 0.5 g with the combination of all grinding methods (Fig. 4.3 - method D) achieved to improve the % RSD among replicate measurements and to extract efficiently all the encased amount of vitamin B_{12} from the plant. It also significantly improved the precision of the assay in solid samples such as *Hippophae rhamnoides* berries from 47.1 % to 0.7 % RSD and in liquid samples from 9.0 % to 0.0 % RSD.

After the above experiments the “Sample Homogenization” procedure summarized as follows: Solid samples undergo an extended homogenization process. In the beginning, the sample is ground and homogenized to powder through a mixer mill (4 g sample in each container). The mixer mill operates at 30 Hz for 2.5 min, and the jars are filled with 55 – 60 ml liquid nitrogen. Afterwards, the sample is perfected into a fine powder by the use of mortar & pestle. Then 0.5 g of fine powder, (the sample amount is depending on the vitamin B_{12} content of the sample) are diluted in 10 ml buffer (in a 50 ml Falcon tube) and processed in a disperser with 24,000 rpm/ min, for 6 minutes. After the disperser, a fine homogenized suspension is prepared. The suspension (in the Falcon tube) sinks into an ultrasonic bath for 30 min. After the ultrasonic bath the suspension is ready for the “Extraction of Vitamin B_{12}” procedure. Liquid samples (10 – 30 ml) are stirred well to ensure the homogeneity of the sample, afterwards undergo the extended homogenization process from the disperser step until the ultrasonic bath. The “Sample Homogenization” procedure is depicted in Figure 4.6.

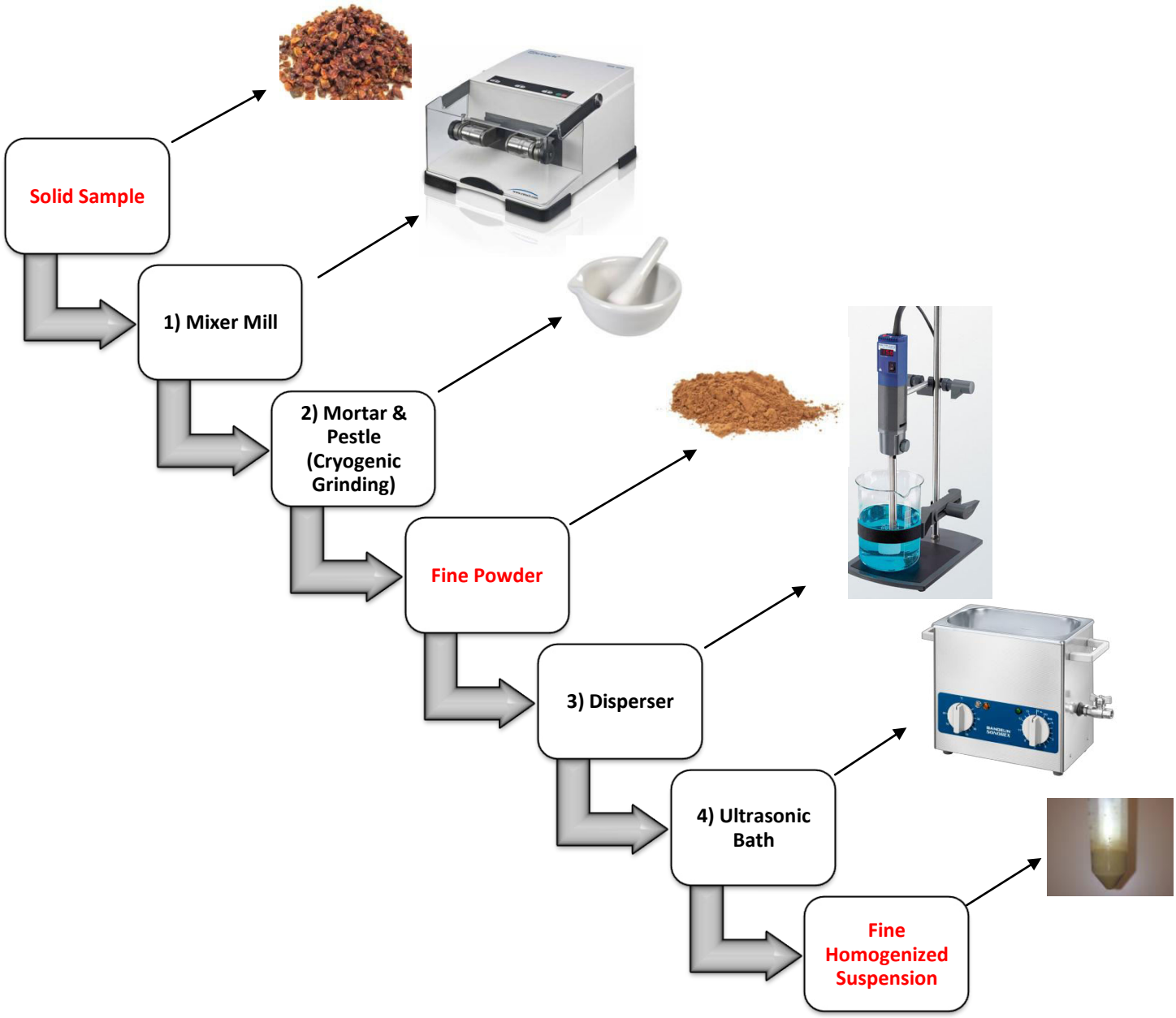


Figure 4.6: Flow chart of the optimized process "Sample Homogenization". A) Solid samples follow the flow chart from step 1 to 4, B) Liquid samples follow the process from step 3 to 4

4.2.2. Extraction of Vitamin B_{12}

After the "Sample Homogenization" step as described above, the extraction process was performed as it written in the initial protocol (Appendices 6.1.1). All forms of vitamin B_{12} were prior converted to the most stable form CN-cobalamin. This conversion was performed by heating the samples at 100 °C for at least 30 min in the presence of KCN. At this temperature (100 ^{o}C), enzymes/proteins such as pepsin and amylases, which have been added during the extraction process to improve vitamin B_{12} release from the plant matrix, are denatured prior to immunoaffinity step. The amylase treatment was also introduced to improve the filtration of the sample, by obtaining clear supernatants, particularly for plants containing starch. For high-fat-containing samples the filtration was performed twice, so as to obtain a less viscous and easier to handle solution for further processing.

For the release of vitamin B_{12} from the plant matrix, an incubation process of the enzymes (pepsin and α-amylase) at 37 ^{o}C, in which they have maximum activity, was necessary. However, the duration of the process had to be investigated. Thus, the effect of incubation time, of both enzymes, on the extraction efficiency was tested for 0.5 h, 1.5 h, 3.0 h, 6.0 h and 12.0 h. The results of the incubation times were examined statistically with Minitab 15 (statistical program) by one-way analysis of variance (ANOVA) (Appendices 6.2.1.). The highest concentration of vitamin B_{12} released from the samples was detected at incubation times of 1.5 h and 3.0 h (after the 3.0 h until the 12.0 h the released vitamin B_{12} remained stable). These two incubation times were investigated further in detail by the Fishers Least Significant Difference (LSD) test. The difference between the two incubation times in regard to cobalamin release did not differ statistically significantly. Both tests (ANOVA and LSD) showed *p* values less than 0.05, indicating that the results are statistically significant. Thus, the short incubation time of 1.5 h was chosen for the enzyme digestion in further tests.

Besides the optimum temperature, an important factor for the maximum activity of the enzymes was the pH. The optimum pH for pepsin ranges between pH 1.5 and pH 1.6, and the optimum pH for α-amylase ranges between pH 6.7 and pH 7.0. Due to this difference, an optimal pH (for the highest extraction efficiency) had to be found, through the use of a buffer solution. The effect of the buffer pH on the extraction efficiency was tested carefully. The samples were diluted with acetate buffer at pH 4 and after removal of all solid parts by filtration or centrifugation the pH of the supernatant was in the range of pH 3.3 – 4.6. In order to check the impact of the final pH on the analysis, samples were adjusted to different pH values in the range of pH 3 – 11. The samples were analyzed as described in the initial protocol (Appendices 6.1.1). The results are presented in Figure 4.7 and indicate that the best pH prior to the immunoaffinity step is pH 7. Here, the highest vitamin B_{12}

concentrations in the *Hippophae rhamnoides* samples were found. Thus, all further analyses were performed with a sample adjustment to pH 7 prior to the immunoaffinity step.

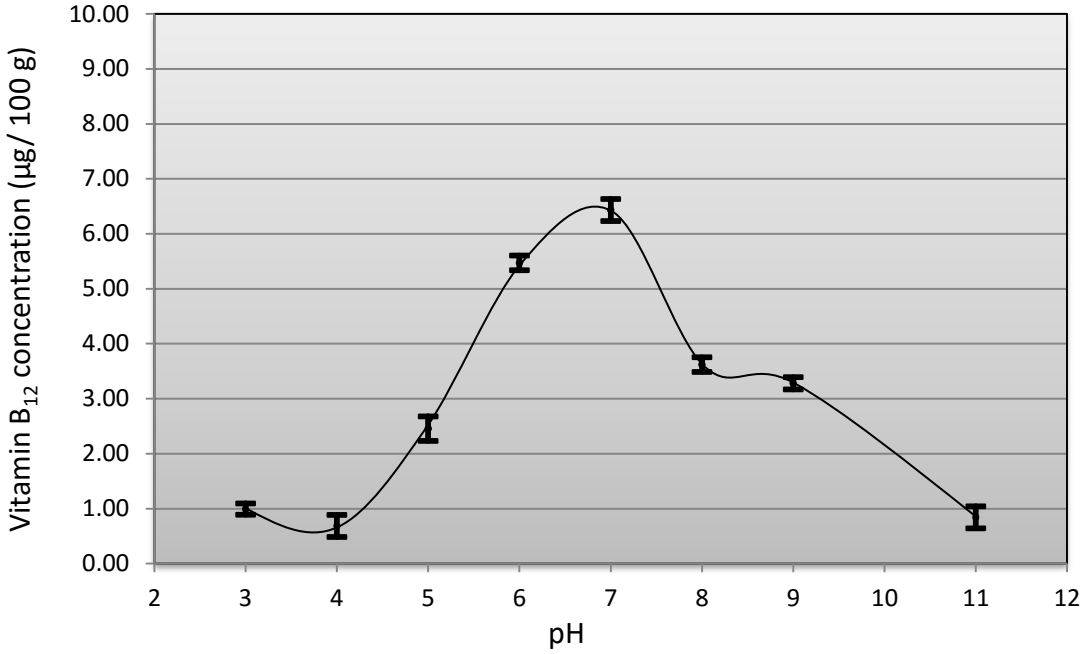


Figure 4.7: The effect of pH adjustment, before the IAC, to the determinable vitamins B_{12} concentration in *Hippophae rhamnoides* extract (mean and 95% confidence intervals, n=3)

As it is mentioned above, all forms of vitamin B_{12} were converted to the most stable CN-cobalamin prior to further analysis. According to the initial protocol (Appendices 6.1.1.) 1 ml of KCN (1% W/V) is added to the sample suspension. However, an experiment was conducted in order to investigate the influence of the added KCN amount on the final concentration of vitamin B_{12}, as well as the ability of the method to measure non-converted forms of cobalamins (methycobalamin (CH_3-Cbl), hydroxycobalamin (OH-Cbl) and adenosylcobalamin (adeno-Cbl)). The experiment followed the initial method (Appendices 6.1.1.) with berries of *Hippophae rhamnoides*.

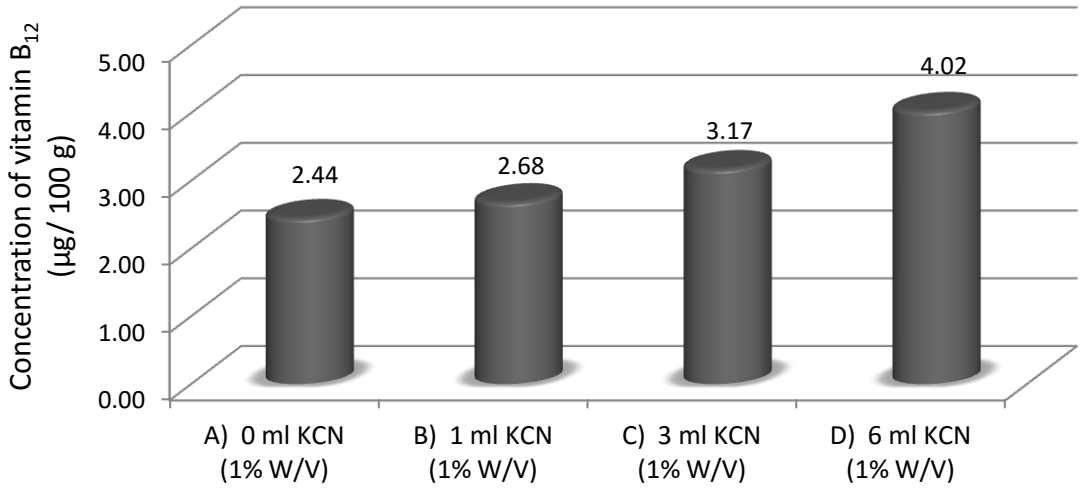


Figure 4.8: Effect of KCN (1% W/V) addition to the concentration of cobalamins in the analyzed sample (*Hippophae rhamnoides*). A) 0 ml KCN, B) 1 ml KCN, C) 3 ml KCN and D) 6 ml KCN

From the Figure 4.8 it is obvious that the increased amount of KCN disclosed higher amounts of vitamin B_{12}. This fact suggests that the recommended amount of 1 ml KCN 1 % (w/v) perhaps was not enough to convert all the existing cobalamins of the sample to CN-Cbl, and as a consequence the real amount of cobalamins might be higher. Another significant finding was also that without the addition of KCN, CN-cobalamin was found. In this case, two assumptions can be made, either the special antibody that captures the CN-Cbl in immunoaffinity column (IAC) can also react with other cobalamins such as OH-Cbl, adeno-Cbl etc. or there is CN-Cbl present in the sample of *Hippophae rhamoides* which is very unlikely, because CN-Cbl is an artificial form of vitamin B_{12} and does not exist in nature.

As it is already mentioned from other authors, for the conversion of cobalamins to CN-Cbl and the deactivation of the added enzymes/proteins, a heating step at 100 ^{o}C was necessary. An experiment was performed (initial protocol – see App. 6.1.1) to test the influence of the heating step to the liberation of the encased vitamin B_{12} from the sample matrix. The test materials were berries from two different companies, *Hippophae rhamnoides* (sample code 558) from Teutopharma and *Hippophae rhamnoides* from Naturix24. The samples of each *Hippophae rhamnoides* (kindly provided by Teutopharma or Naturix24) were divided in two groups, one followed the normal protocol (with a heating step) and the other followed the protocol without the heating step. Based on the results (see Fig. 4.9) the heating step yielded 15 - 28 % higher concentrations of vitamin B_{12}. That probably can be explained, due to facilitation of the enzyme activity to the liberation of the vitamin

B_{12}, from the analysed matrix, as well as due to the establishment of adequate conditions (100 ^{o}C) for the conversion of cobalamins to CN-Cobalamin.

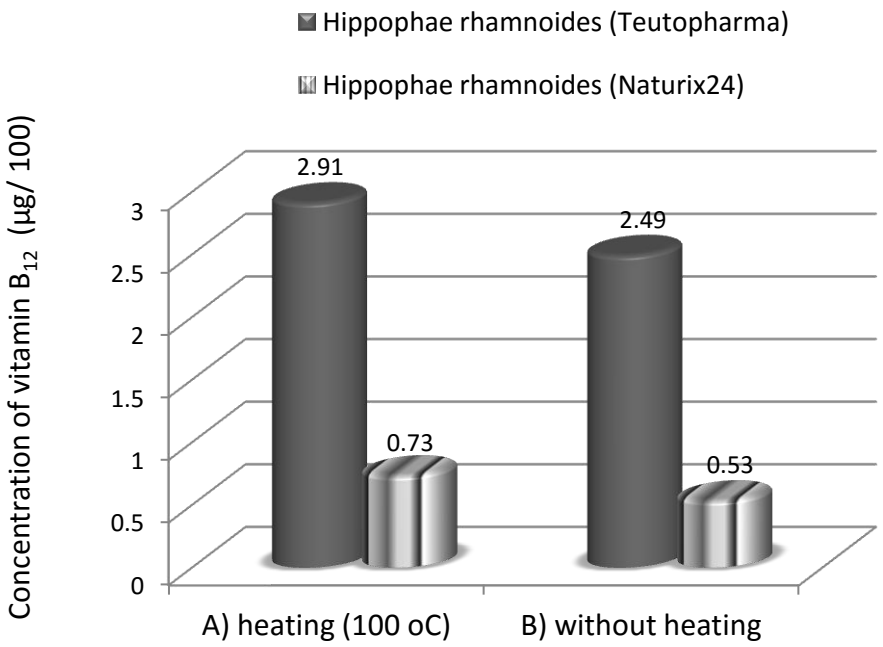


Figure 4.9: Effect of heating step (at 100 ^{o}C for 30 min) on the liberation of the encased vitamin B_{12} in *Hippophae rhamnoides* berries (bought from Teutopharm and Naturix24). A) Concentration of vitamin B_{12} including the heating step, B) Concentration of vitamin B_{12} without the heating step

The next step of the protocol was the cooling process of the samples to room temperature. In the initial protocol (6.1.1.) the samples were left to cool down at room temperature for 2 hours, without any further process. Owing to the time-consuming process of the assay, a cooling step with the use of an ice bath was decided to be used for 30 min.

In many cases, it was observed that during the centrifugation procedure the precipitate could not be separated totally from the supernatant. Hence, a higher speed (5,000 rpm) at a lower temperature 3 ^{o}C was tested (optical observation) and proved to be more efficient for clearer supernatant.

Summary – Discussion

The aim of the "Extraction of Vitamin B_{12}" part was after the homogenization of the sample, the fine powder to be processed in a way, so as to extract and liberate the encased cobalamins from the matrix (with the most efficient way), and convert them to CN-cobalamin which is the target substance that can be determined from the method. The majority of the steps of this part were tested and optimized, in order to reach the optimal conditions for the extraction of the highest values of vitamin B_{12} from the analysed matrix. Significant finding was the pH adjustment of the supernantant after the filtration step to pH 7, which yielded the highest concentrations of vitamin B_{12}. The heating step at 100 ^{o}C was proved important for the conversion of all cobalamins to cyanocobalamin, as well as to the denaturation of added enzymes (which were used prior, to improve vitamin B_{12} release from the plant matrix), in order to be removed and not interfere further with the analyte.

After all the above experiments the procedure of the method "Extraction of Vitamin B_{12}" has been optimized and summarized as follows: 1 ml of KCN (1% w/v), 1 g pepsin, and 300 μl α-amylase are added to a fine homogenized suspension (10 ml of 50 mM acetate buffer (pH 4.0) + 0.5 g fine powder), produced from the previous procedure "Sample Homogenization". Then the suspension is incubated at 37 ^{o}C for 1.5 h under agitation. The enzymatic reaction stops by incubating the sample in boiling water at 100 ^{o}C for 30 min. After cooling the sample in an ice bath for 30 min, the solution is centrifuged at 5,000 rpm for 25 min at 3 ^{o}C. The supernatant is collected and filtered through filter paper (589/2 Whatman, 90 mm). The pH is adjusted to pH 7 and the solution is quantitatively transferred to a volumetric flask and filled up to 100 ml with deionized H_20. The "Extraction of Vitamim B_{12}" procedure is depicted in the flow chart in Figure 4.10.

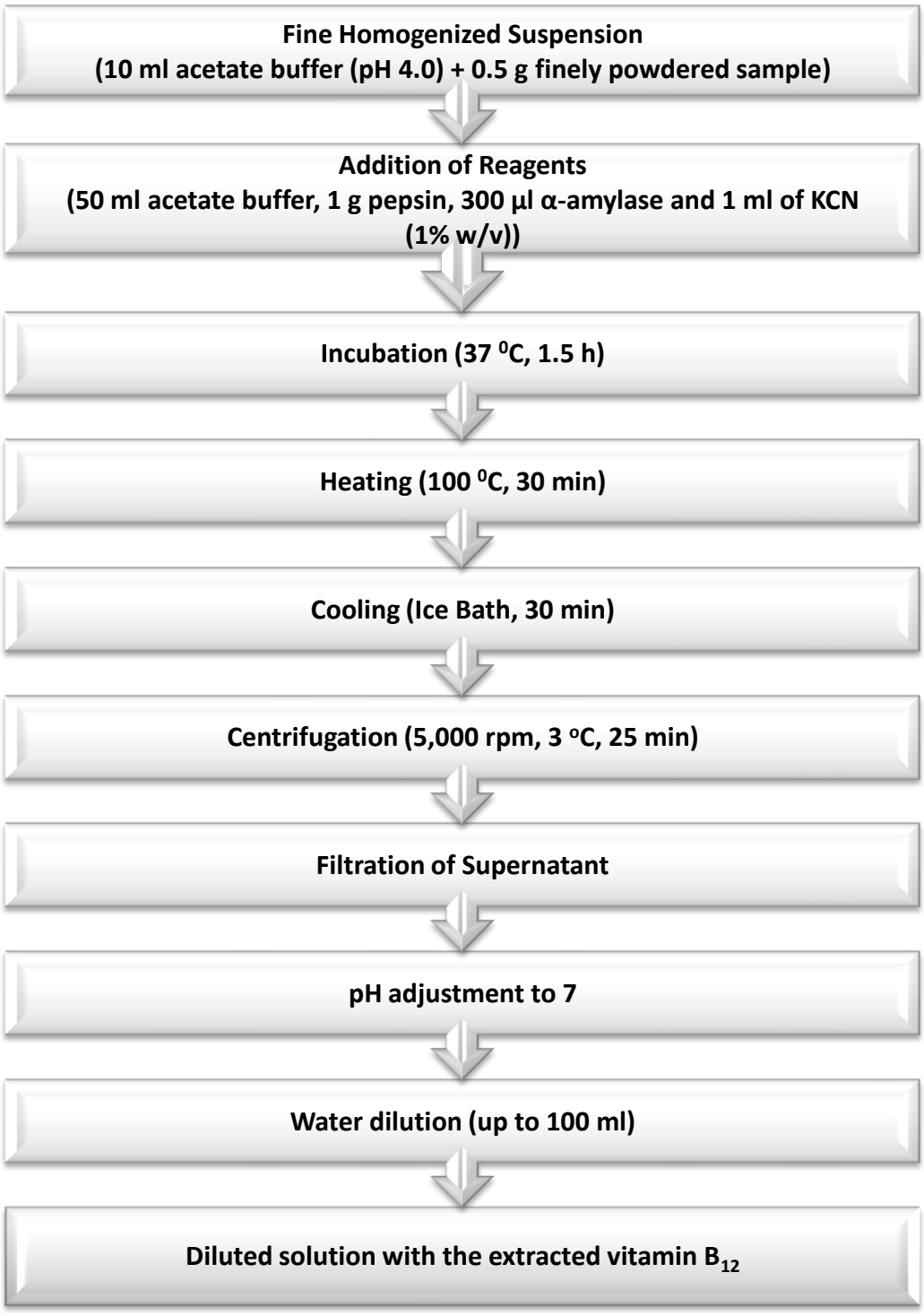


Figure 4.10: Flow chart of the optimized process "Extraction of Vitamin B_{12}"

4.2.3. Purification and Concentration of Vitamin B_{12}

After the optimization of the "Sample Homogenization" and "Extraction of Vitamin B_{12}" sections of the initial protocol, the next step "Purification and Concentration of Vitamin B_{12}" was followed as it was suggested by the manufacturer of the immunoaffinity columns (IAC) and the authors from previous publication such as Guggisberg et al.[37] with few adjustments.

Based on the results of a publication, the solid phase extraction (SPE) C_{18} for vitamin B_{12} purification in food sample was proved insufficient (C. Pakin et al., 2005[133]). Similar results were found by Heudi et al.(2006)[38], who found as well that the immunoaffinity column is clearly more specific for the determination of vitamin B_{12} in various food products compared to conventional SPE cartridges such as C_{18}. Thus, it was decided to use immunoaffinity columns (IAC) for the isolation of vitamin B_{12}. The IAC were highly selective and reproducible for a variety of samples (meat, vitamin tables, etc.) and for more complex samples such as plant-based products (*Hippophae rhamnoides*, *Inula helenium* etc.). This high selectivity of an immunoaffinity column was necessary because of the pepsin treatment of the samples and because of the very low concentrations of vitamin B_{12} in plant-based products. Pepsin enzymatically hydrolyses the proteins into a variety of very small fragments, and boiling the sample at 100 °C leads to an immense number of UV active components that increase the risk of chromatographic interferences with vitamin B_{12}.

In order to check the reliability of IAC and see if they meet all manufacturer's specifications, the IAC was tested in recovery studies. According to the manufacturer of IAC a 5 – 15 ml aliquot (corresponding to 0.1 – 1.0 µg vitamin B_{12}) of the sample extract has to pass through the column and then the column has to be washed by 10 ml water. The bounded vitamin B_{12} (with the antibody of the immunoaffinity column) had to be eluted with methanol. Then, 3 ml of methanol (100 %) elutes up to 100 % of the vitamin B_{12}.

Based on this protocol, 1 ml of a standard solution of vitamin B_{12} with concentration 0.1 µg/ ml, which meets the optimal column performance (0.01 – 0.5 µg vitamin B_{12} onto the column), were passed through the IAC and the columns were subsequently washed with 10 ml water. At this point the IAC were eluted with 3 ml, 5 ml and 7 ml of methanol, respectively, so as to check the most efficient elution volume. The Figure 4.11 shows that 3 ml of methanol for the elution of the bound vitamin B_{12} are not enough to elute all the amount of the vitamin. In this case, the most efficient amount of methanol for elution of the vitamin B_{12} is 5 ml, which demonstrates 96 % recovery of the applied vitamin B_{12}. Larger volumes of methanol could not improve the elution efficiency.

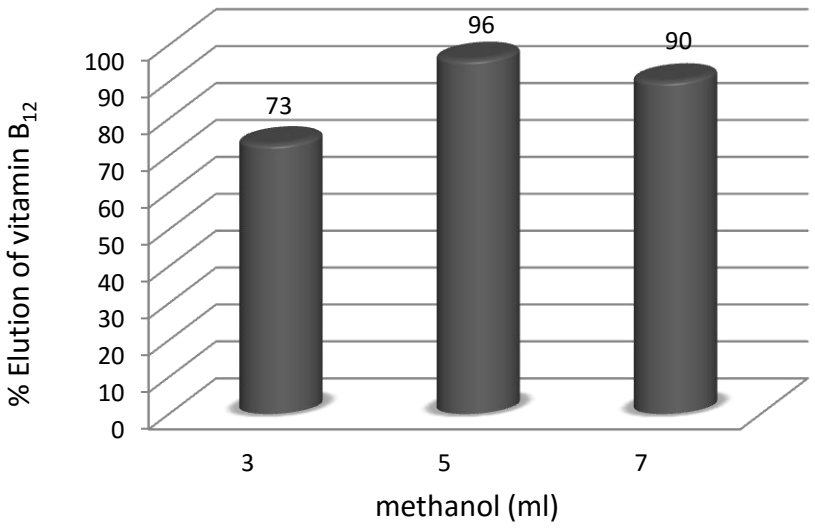


Figure 4.11: Elution efficiency of vitamin B_{12} eluted with 3 ml, 5 ml or 7 ml methanol from immunoaffinity columns

After the elution of the vitamin B_{12} from the immunoaffinity column, the methanolic extract followed an evaporation process to dryness, by drying the eluent at 60 ^{o}C under reduced pressure. An experiment was performed to check potential losses of vitamin B_{12} during the evaporation process. A series of standard solution of vitamin B_{12} were processed in the evaporation step to estimate potential losses of the vitamin during drying.

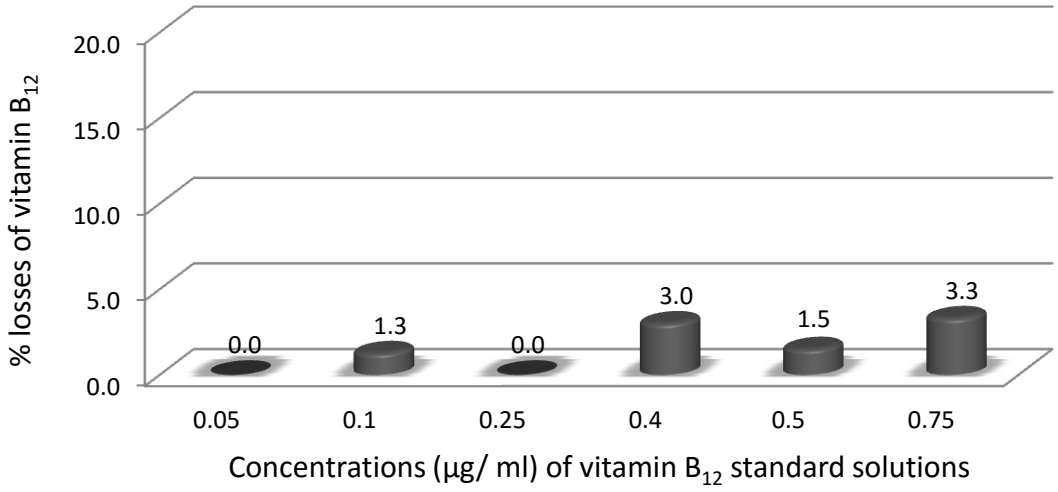


Figure 4.12: Losses of vitamin B_{12} during the evaporation step, measured by a series of vitamin B_{12} standard solutions (0.05 - 0.75 µg/ ml)

Based on the results (see Fig. 4.12) the losses of vitamin B_{12} passing through the evaporation step are very low and have no significant influence on the determinable amount of vitamin B_{12}.

Summary – Discussion

The initial protocol (App. 6.1.1.) was studied in a stepwise process. Each step of the process was investigated, in order to be optimized and reach the optimum performance levels, for the best possible determination of the vitamin B_{12} in the measured samples. The final flow chart of the optimal assay was formulated as it is illustrated in Figure 4.13.

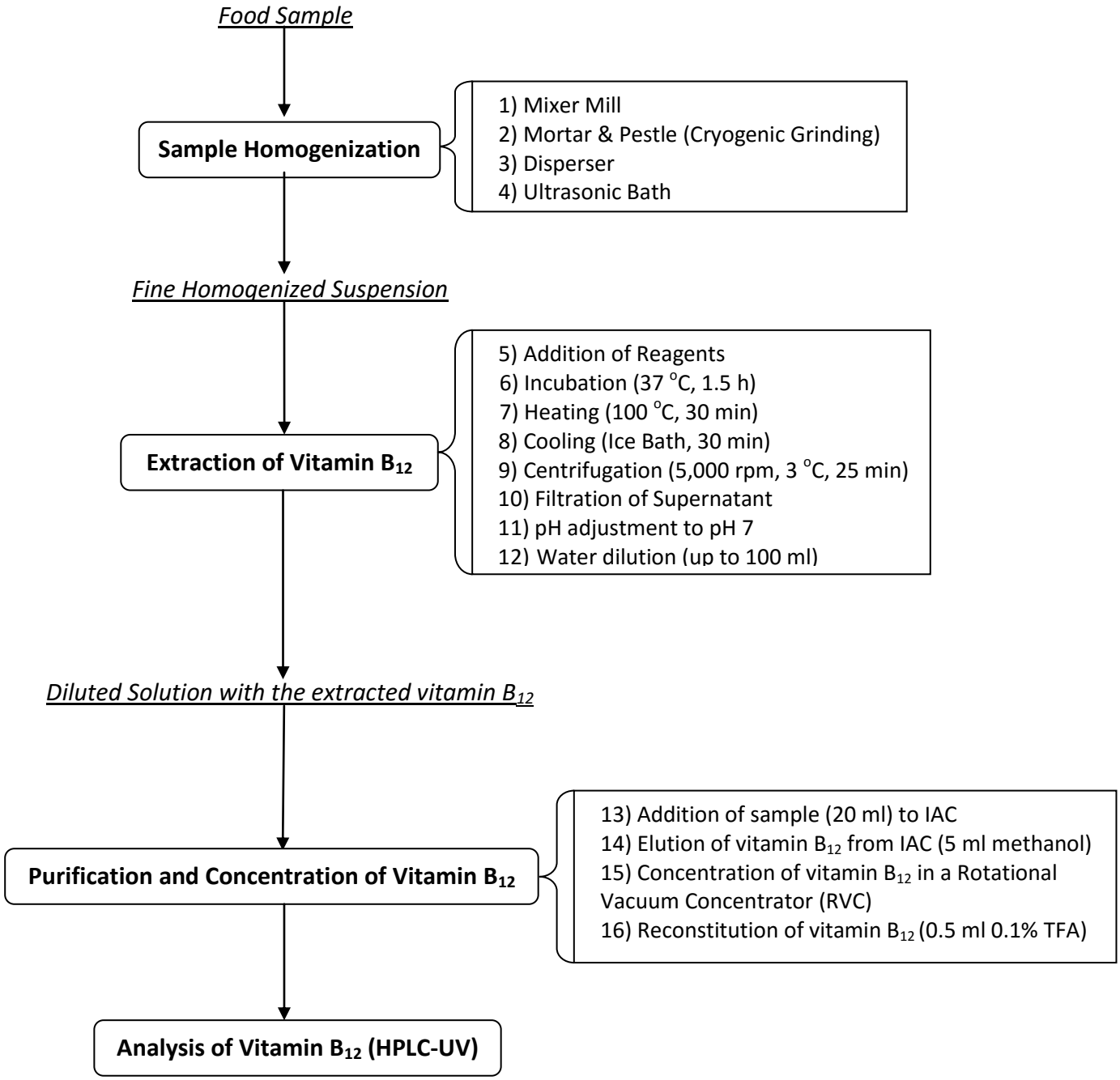


Figure 4.13: Optimized assay flow chart for the determination of vitamin B_{12} in various matrices

4.2.4. Analysis of Vitamin B_{12}

4.2.4.1. Detection of Vitamin B_{12} by HPLC-UV

All the samples that have been analysed were processed through the optimized assay protocol (see Figure 4.13) with few differences, depending on the nature of the sample (liquid or solid). With the completion of the process, the extracted vitamin B_{12} was isolated, purified from other co-eluted substances (which they were present although the IAC has a specific antibody only for CN-Cbl), and concentrated in order to facilitate the quantification of its content. A reversed-phase high performance liquid chromatography (HPLC) method for the quantification of vitamin B_{12} from plant matrices has been developed (Appendices 6.1.1.4.) based on previous publication such as Heudi et al.[38]. The analysis of the samples were performed with a diode-array detector (DAD), the vitamin B_{12} was monitored at 361 nm, a C_{18} column (100 mm x 4.6 mm, 2.6 µm) fitted with a C_{18} pre-column filter (4 mm x 3 mm). The separation of the analyte carried out with a preparation of mobile phase consisted of 0.025% (w/v) trifluoroacetic acid (TFA) in water solution and pure acetonitrile. The proportions of the eluents of the mobile phase have been optimized a few times, due to problems that appeared by co-eluted substances in the chromatograms or overlap of the analyte's peak (vitamin B_{12}) from other substances. The elution of the vitamin B_{12} was perfomed with a gradient HPLC elution profile A as it is shown in Figure 4.14 below.

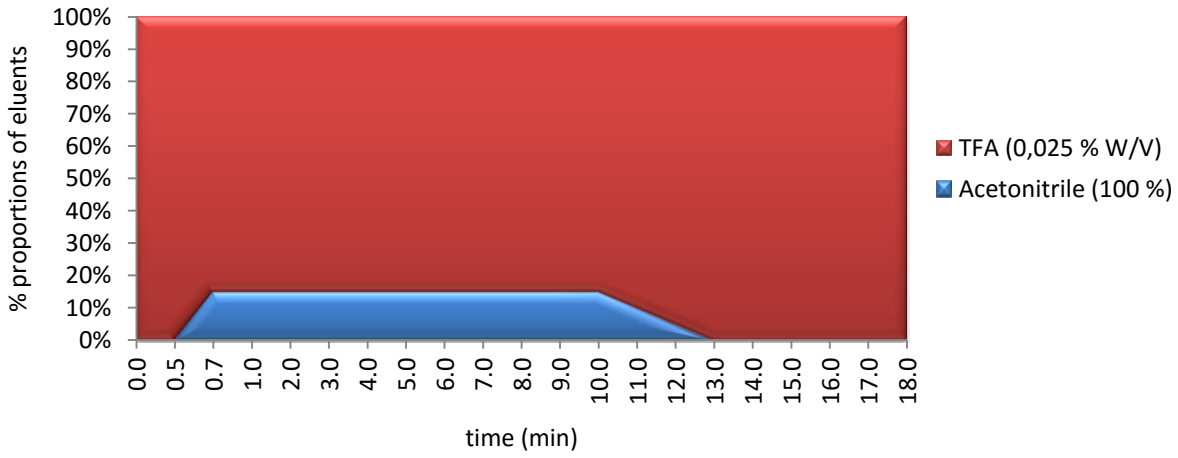


Figure 4.14: HPLC gradient profile (A): Gradient elution of vitamin B_{12} with 0.025% (w/v) trifluoroacetic acid (TFA) in water (mobile phase A) and pure acetonitrile (mobile phase B) at a flow rate of 0.5 ml/min

The development of the HPLC gradient profile was based on the sample of *Hippophae rhamnoides*, due to the complex nature of its matrix and the difficulty to separate the peak of vitamin B_{12} from the other co-eluted substances. After the application of the gradient HPLC elution profile (A) with samples from *Hippophae*

rhamnoides, the method could not separate completely the vitamin B_{12} (extracted from *Hippophae rhamnoides*) from other co-eluted substances and the pattern of replicate samples was different. Also, it is clear from the Figure 4.15 that between 7.0 min and 8.5 min the replicate chromatogram of the *Hippophae rhamnoides* had an impurity peak (Fig. 4.15 (C)). There are many causes for the presence of impurities in the column. The main reasons usually are either a dirty pre-column or column, or the elution of the left-overs (impurities) of the previous run during the next run. Consequently, odd peaks appear.

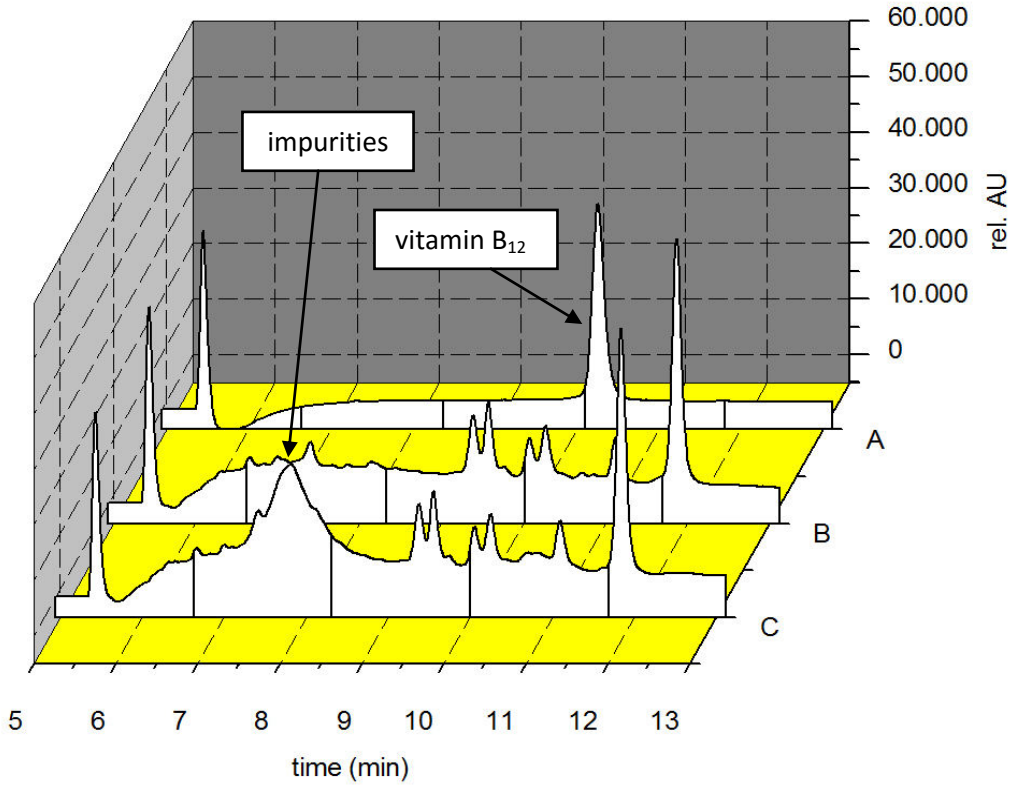


Figure 4.15: A) Chromatogram of a standard solution of vitamin B_{12}, B) + C) Chromatograms of the extracted vitamin B_{12} from *Hippophae rhamnoides* sample, after the application of the gradient HPLC elution profile (A)

For all the above reasons, a stepwise cleaning and regeneration program of the column were performed as recommended by the manufacturer (Kinetex Columns). The C_{18} reversed phase column was cleaned by flushing with 10 – 20 column volumes of the following steps:

1) 5:95 Acetonitrile / Water (or Methanol / Water) for buffer removal
2) 95:5 Acetonitrile / Water (or Methanol / Water)
3) THF (Tetrahydrofuran)
4) 95:5 Acetonitrile / Water (or Methanol / Water)
5) 5:95 Acetonitrile / Water (or Methanol / Water)
6) Equilibrate with mobile phase

The regeneration of the column was followed by a development of a new gradient HPLC elution profile (B) which included a washing step, so as to avoid remains of the previous sample to be eluted in the next run. The gradient elution of the new HPLC profile (B) is depicted in Figure 4.16.

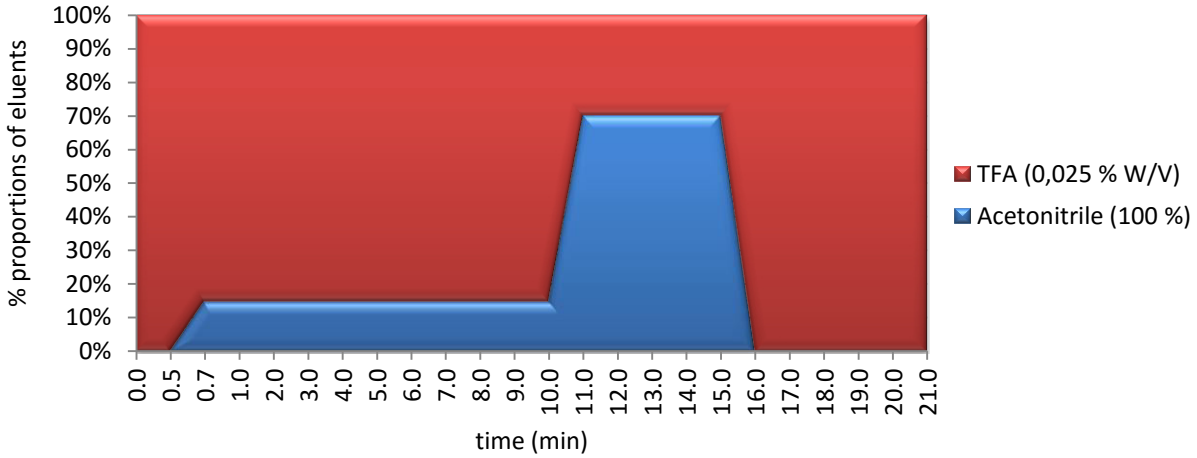


Figure 4.16: HPLC gradient profile (B): Gradient elution of vitamin B_{12} with 0.025% (w/v) trifluoroacetic acid (TFA) in water (mobile phase A) and pure acetonitrile (mobile phase B) at a flow rate of 0.5 ml/min

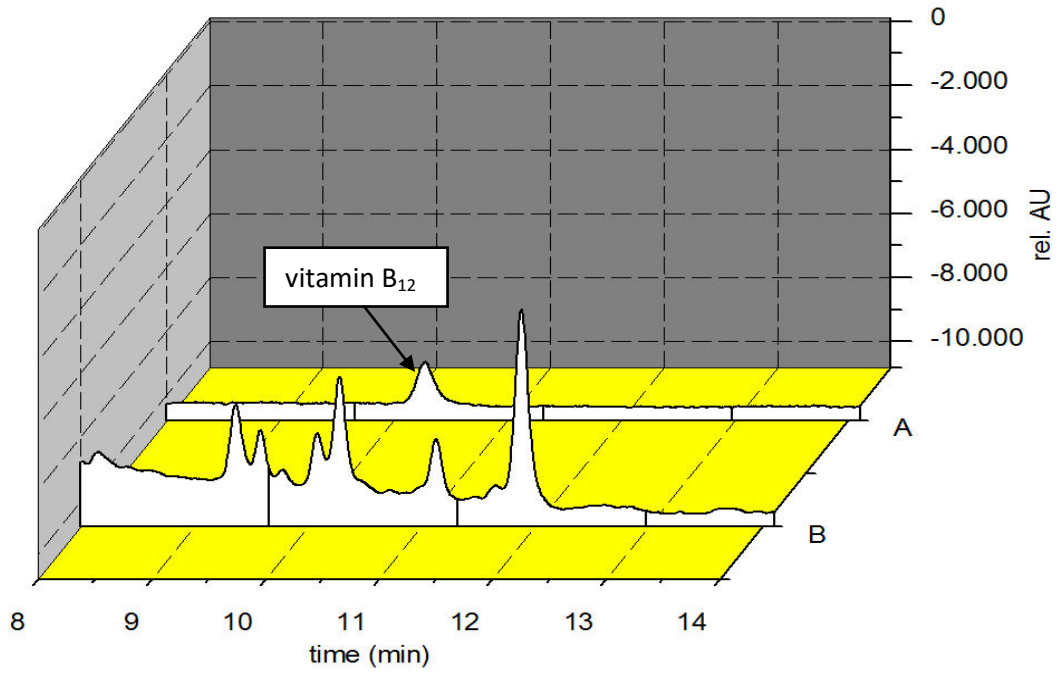


Figure 4.17: A) Chromatogram of a standard solution of vitamin B_{12} and B) Chromatogram of the extracted vitamin B_{12} from *Hippophae rhamnoides* sample, after the application of the HPLC gradient profile (B)

The HPLC gradient profile (B) was applied again in a *Hippophae rhamnoides* sample. The chromatograms disclosed improvements, concerning the appearance of impurities, which disappeared, as a result of an effective cleaning step of the modified method. Even though the chromatogram pattern of the standard solution of vitamin B_{12} and the vitamin B_{12} extracted from the sample (*Hippophae rhamnoides*) seemed similar, still the baseline separation of vitamin B_{12} was not complete, and the time of the run was longer (time consuming process). Thus, the HPLC profile was optimized for the third time and named HPLC gradient profile (C).

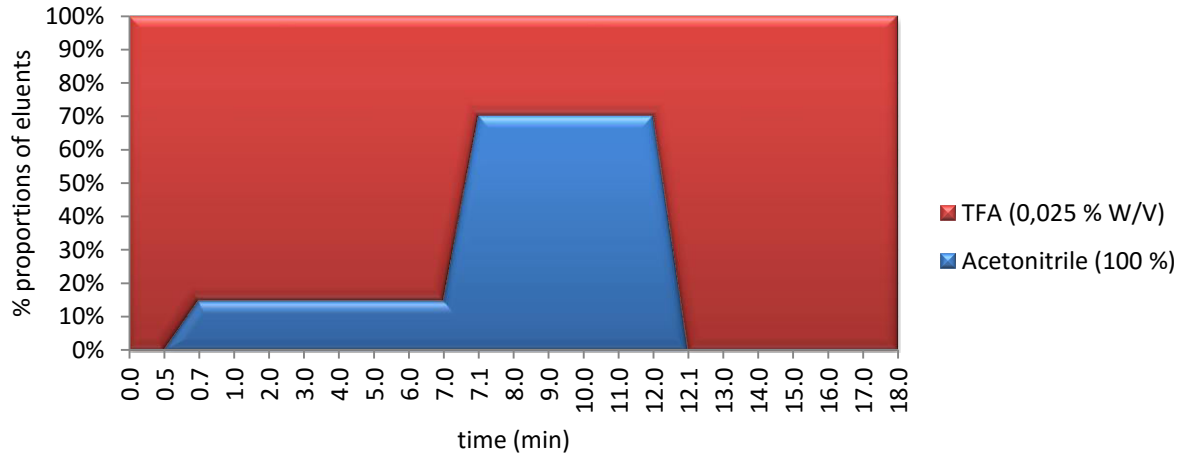


Figure 4.18: HPLC gradient profile (C): Gradient elution of vitamin B_{12} with 0.025% (w/v) trifluoroacetic acid (TFA) in water (mobile phase A) and pure acetonitrile (mobile phase B) at a flow rate of 1.0 ml/min

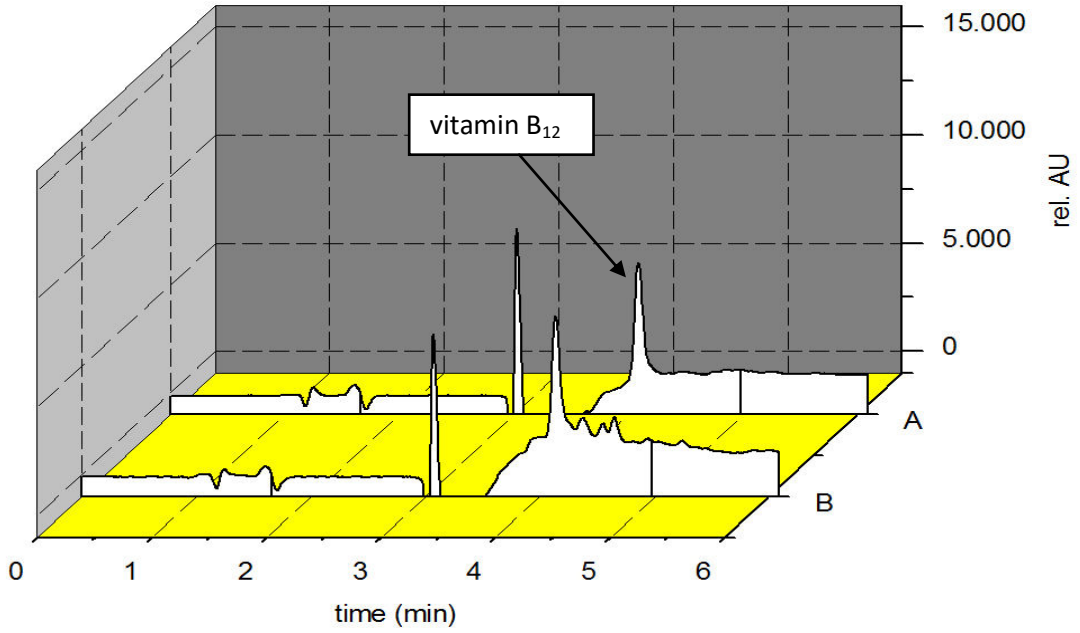


Figure 4.19: A) Chromatogram of a standard solution of vitamin B_{12} and B) Chromatogram of the extracted vitamin B_{12} from *Hippophae rhamnoides* sample, after the application of the HPLC gradient profile (C)

The purpose of the HPLC gradient profile (C) was to minimize the duration of the run, to keep vitamin B_{12} distinctly separated from other co-eluted substances, and at the end of the run to keep the column clear, with no remains from the previous runs. All the above parameters were achieved by the HPLC gradient profile (C) (Fig. 4.18) as it was applied to *Hippophae rhamnoides* sample (see Fig. 4.19). The HPLC gradient profile (C) (Appedices 6.1.2.4.) is the optimized gradient profile with the following characteristics. The total run time is 18.0 min, the vitamin B_{12} was detected with a retention time of t_R= 4.15 min and after the completion of the run the column contains no remains. The HPLC gradient profile has been applied in all the samples, from plant matrices and vitamin B_{12} tablets until meat and liquid samples (juices) with great success, separating the extracted vitamin B_{12} from other co-eluted substances effectively.

4.2.4.2. Detection of Active Vitamin B_{12} by HPLC-MS/MS

After the collection of vitamin B_{12} fractions from *Hippophae rhamnoides* the sample with the concentrated vitamin B_{12} was dried and then reconstituted with 0.5 ml of 0.1 % formic acid (water solution). An HPLC gradient profile for the differentiation of vitamin B_{12} analogues were developed by a mass spectrometry (MS) detector. For the identification of vitamin B_{12} and the determination of its molar mass, a high performance liquid chromatography coupled to a triple quadrupole mass analyzer was used. The MS was conducted in positive ion mode, electospray ionization (ESI) with a scan range of m/z 600 – 1,600 (see Appendices 6.1.2.5.). The vitamin B_{12} was monitored at 361 nm, on a C_{18} column (250 mm x 4 mm, 5 μm) at 30 ^{o}C temperature. The separation of the analyte conducted with a gradient elution 0.1 % (V/V) formic acid in water (mobile phase A) and 0.1 % (V/V) formic acid in acetonitrile (mobile phase B) at a flow rate of 0.3 ml/ min. The gradient conditions are described in the Figure below (Fig. 4.20). The total run of the method was 38.0 min and the vitamin B_{12} was detected with a retention time of t_R= 15.1 min (Fig. 4.21). The chromatogram in Figure 4.21 of the 558 *Hippophae rhamnoides* sample contains inconsiderable peaks and “noise” at other retention times (e.g. 4 min), which may indicate the presence of other corrinoids in *Hippophae rhamnoides* sample. Due to low concentrations of vitamin B_{12} in the sample the analysis in MS was conducted in SIM (single ion mode) in order to increase the sensitivity of the method.

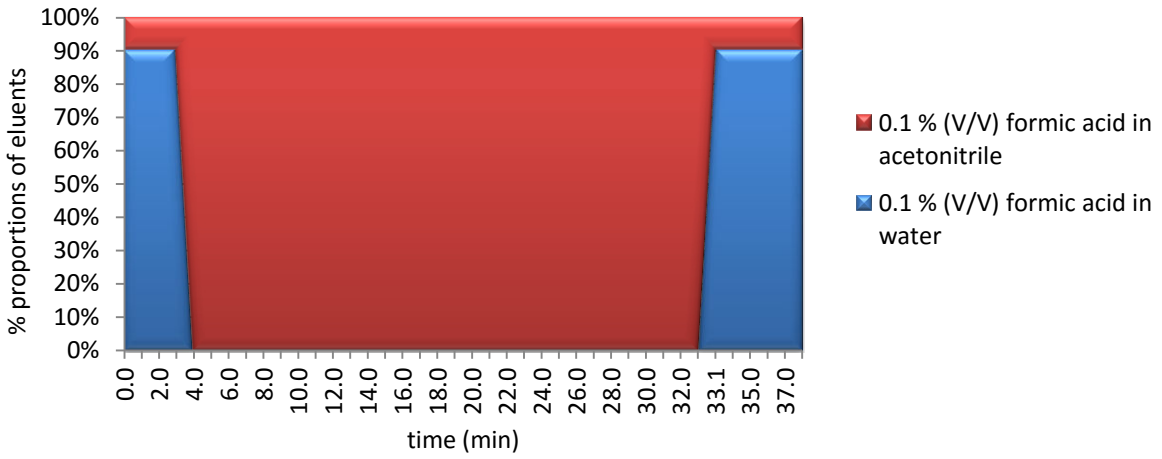


Figure 4.20: HPLC-MS/MS Gradient profile: Gradient elution of vitamin B_{12} with 0.1 % (V/V) formic acid in water (mobile phase A) and 0.1 % (V/V) formic acid in acetonitrile (mobile phase B) at a flow rate of 0.3 ml/ min

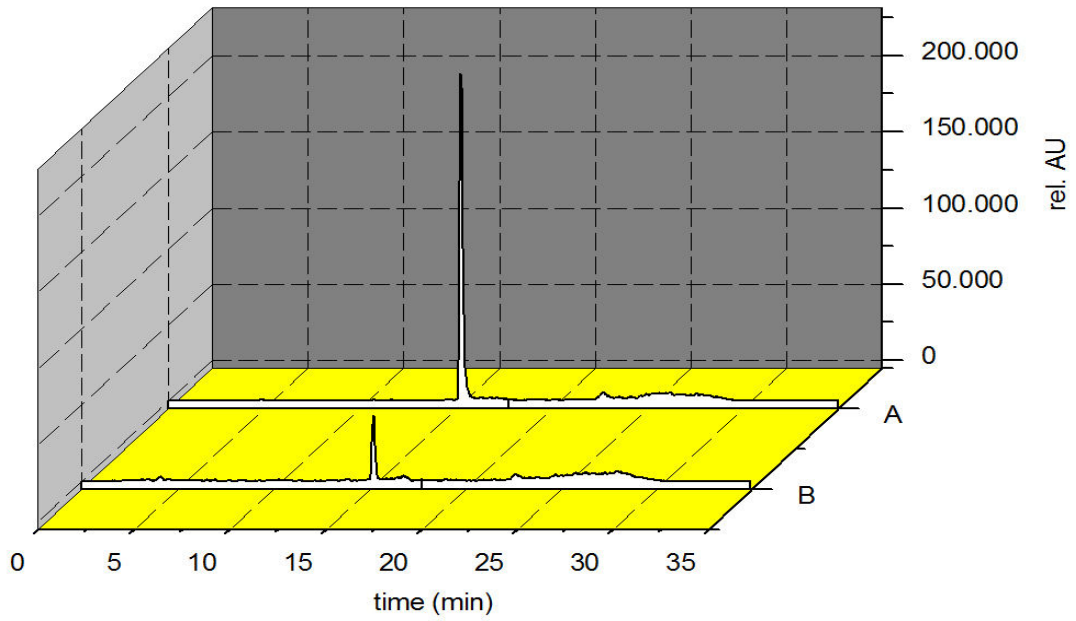


Figure 4.21: A) Chromatogram of cyanocobalamin (vitamin B_{12}) standard solution concentration 0.25 µg/ ml, and B) Chromatogram of immunoaffinity purified extract of 558 (*Hippophae rhamnoides*), after the application of HPLC-MS/MS gradient profile

Summary – Discussion

With the completion of the protocol of the method, a process for the qualification and quantification of vitamin B_{12} has been developed. For the detection of cyanocobalamin (vitamin B_{12}) an HPLC gradient elution profile with UV detector has been applied successfully, as well as an HPLC elution profile with a mass spectrometry (MS) detector for the investigation of cobalamins structural forms. Both HPLC elution profiles contribute to the complete determination of vitamin B_{12} in several matrices, with special interest in plant matrices. The developed HPLC profiles are depicted in Table 4.1.

Table 4.1: Gradient elutions of HPLC-UV and HPLC-MS/MS profiles

HPLC-UV *	
Time (min)	**A : B (%)**
0.0	100:0
0.5	100:0
0.7	85:15
7.0	85:15
7.1	30:70
12.0	30:70
12.1	100:0
18.0	100:0

HPLC-MS/MS **	
Time (min)	**A : B (%)**
0.0	90:10
3.0	90:10
23.0	0:100
33.0	0:100
38.0	90:10

*** HPLC-UV elution profile data:**

- **Eluent A: 0.025% (w/v) trifluoroacetic acid (TFA) in water**
- **Eluent B: pure acetonitrile**
- **Column temperature: 30 °C**
- **Flow rate: 1.0 ml/ min**
- **Wavelength of the UV detector: 361 nm**
- **Injection volume: 100 µl**

**** HPLC-MS/MS elution profile data:**

- **Eluent A: 0.1 % (w/v) formic acid (in water)**
- **Eluent B: 0.1 % (w/v) formic acid (in acetonitrile)**
- **Ion source: ESI (+)**
- **SIM width: 0.700 amu total**
- **The detector is set a fixed voltage: 1200.00 volt**
- **Syringe pump flow rate: 1.00 µl/ min**
- **ESI needle voltage positive: +5000.00**
- **ESI needle voltage negative: +600.00**
- **Drying gas temperature: 350.00**
- **API housing temperature: 50.00**
- **Nebulizer gas pressure: 55.00**
- **Drying gas pressure: 30.00**
- **Flow rate: 0.3 ml /min**

- **Wavelength of the UV detector: 361 nm**
- **Scan range of m/z: 600 – 1600**

4.3. Recovery Study

In the present study, the recovery studies were focused on plant matrices, such as *Hippophae rhamnoides*, which was the guide material of the present method. The plants were first analysed (concerning the vitamin B_{12}) by this assay, as there was no other available validated method to measure vitamin B_{12} in plant materials. The first measurements of different *Hippophae rhamnoides* samples confirmed the presence of vitamin B_{12}. The determination of the recovery was based on the technique of standard solution addition to the analysed matrix, in which there is presence of the analyte (vitamin B_{12}).

Different food samples (liver, vitamin B_{12} tablets, and plants) were spiked with a defined amount of vitamin B_{12}. All the samples were treated as described in the sample preparation procedures, as well as blank spikes (samples free of matrix spiked with known concentrations of vitamin B_{12}). Samples such as liver, vitamin B_{12} tablets, *Inula helenium* and blank spikes showed good recovery that ranged between 80 – 100 %. However the recovery from *Hippophae rhamnoides* berries was very low (4.6 %). That was the reason for a thorough investigation of the reduced recovery in *Hippophae rhamnoides*, Generally, a low recovery can occur for two main reasons. The first reason is matrix effects (Takenaka et al. (1997)) and the second is insufficient homogenization.

A series of experiments was performed based on the initial method (6.1.1), without performing the optimized homogenization method (see Fig. 4.6) with test sample *Hippophae rhamnoides*, in order to investigate the extensive losses of the spiked vitamin B_{12} amount. Spikes of vitamin B_{12} added in several steps of the process. The results showed that the deviation was very high between the replicate measurements and also among the values of the recovery in each step of the process. Thus, it was impossible to draw conclusions and specify the step of the extensive losses. The losses were investigated in the following categories:

4.3.1. Effect of Tested Material on Recovery

A first corrective action was to spike with vitamin B_{12} a blank spike and observe the losses of vitamin B_{12} during the process without the presence of the matrix. The results yielded recovery more than 95 % of the spiked vitamin B_{12}. The same experiment was repeated several times with spikes at different steps and confirmed that the blank spikes of vitamin B_{12} recovered up to 100 % in all steps of the process. First conclusion was that the reason of the losses was probably due to the matrix (in this case *Hippophae rhamnoides* (558)).

Another plant sample (cranberries) was investigated in terms of recovery. The cranberries were spiked with known amount of vitamin B_{12} in three different steps: 1^{st} spike, in the addition of the reagents. 2^{nd} spike, before the centrifugation process and 3^{rd} spike, during the sample addition at the immunoaffinity chromatography. Hence, the assay divided into 3 main parts. The 1^{st} spike of the vitamin B_{12} revealed recoveries between 60 – 70 %, the 2^{nd} 70 – 80 % and the 3^{rd} between 97 – 99 %. In this case, the results indicated that the losses of the spiked vitamin B_{12} was not only due to tested material, but probably resulted from steps contained in "Sample Homogenization" and/or "Extraction of Vitamin B_{12}" procedures, as the third part of "Purification and Concentration of Vitamin B_{12}" showed only minimal losses of vitamin B_{12}. However, the deviation between the replicate measurements was still high. Further experiments were performed again with *Hippophae rhamnoides* (558) berries, but this time with spikes at 7 different steps of the process, in order to specify the losses of vitamin B_{12} in further detail. Based on the flow chart of the process in correlation with the results of the recovery studies (Table 4.2) it was disclosed that the recovery starts from 46 % (at first spike) and at the last spike (7) reached values of 106 % of the spiked vitamin B_{12}. Similar results were obtained from another variety of *Hippophae rhamnoides* (Naturix24). It seems that the major losses took place until the filtration step of the process.

Table 4.2: Flow chart of the initial assay for the extraction of vitamin B_{12} from *Hippophae rhamnoides* berries with recovery data

Spike adding amount	Process step	Recovery
Spike 1	Sample Homogenization	**46 %**
	↓	
	Addition of reagents	
	↓	
	Incubation (37 ^{0}C, 3 h)	
Spike 2	↓	**43 %**
	Heating (100 ^{0}C, 35 min)	
	↓	
	Cooling (room temperature)	
Spike 3	↓	**30 %**
	Centrifugation (4,000 rpm, 4^{0}C for 25 min)	
Spike 4	↓	**70 %**
	Filtration	
Spike 5	↓	**80 %**
	pH adjustment to 7	
	↓	
Spike 6	Water dilution (up to 100 ml)	**99 %**
	↓	
Spike 7	Sample addition (IAC) (20 ml)	**106 %**
	↓	
	Concentration of B_{12} (RVC) (5 ml methanol)	
	↓	
	Reconstitution of B_{12} (0.5 ml 0.1% TFA solution)	
	↓	
	HPLC-UV	

4.3.2. Effect of Heating Step on Recovery

Based on the conclusions of the previous chapter (4.3.1.), it was decided to study the recovery of the added vitamin B_{12} with and without the heating step (100 ^{o}C for 30 minutes), so as to check if heating was the reason for the extensive losses of vitamin B_{12}. Samples from two different varieties of *Hippophae rhamnoides* berries (558 and Naturix24) were spiked at three different steps (3 replicate measurements) of the process, including the "heating step" and without the "heating step": 1st spike at the addition of the reagents, 2nd spike before the centrifugation process and 3rd spike before the addition of the sample at the immunoaffinity chromatography.

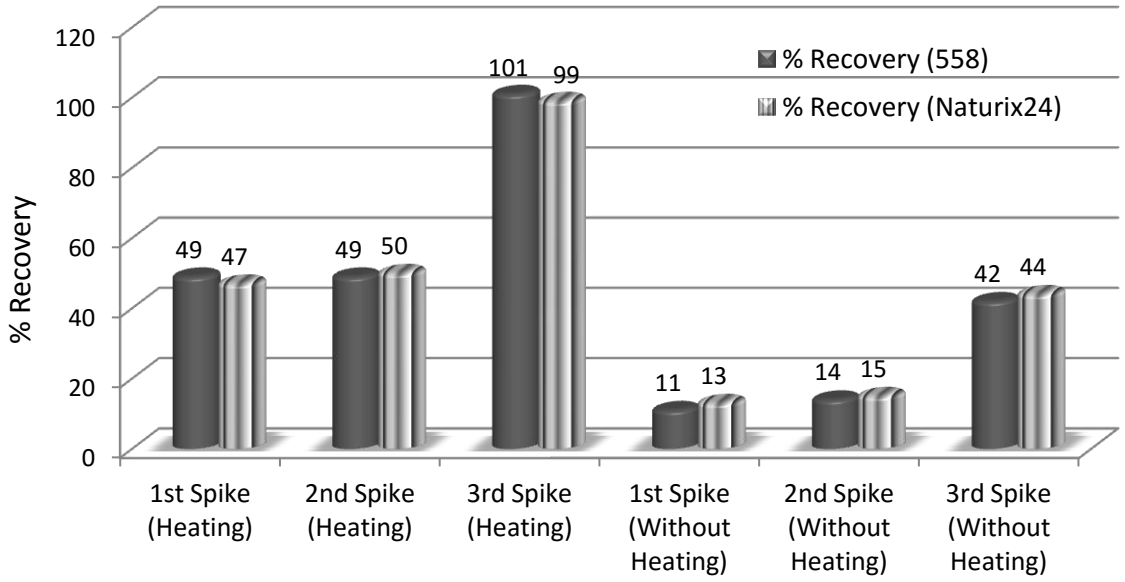


Figure 4.22: Effect of heating step on the recovery of the added vitamin B_{12}

The recovery data (Fig. 4.22) indicated that the absence of the heating step resulted in much lower recoveries in comparison with the presence of the heating step. Thus, it becomes obvious that the heating step had no negative influence on the recovery of the added vitamin B_{12}, and is in accordance with the results of chapter "Extraction of Vitamin B_{12}" (4.2.2.), in which the absence of the heating step also resulted in lower values of the extracted vitamin B_{12}. However, from the data of the normal process (presence of heating step) it was important to note that the recovery between the 1^{st} and 2^{nd} spike was approximately the same (47 – 50 %), which indicates that between those steps there were no further losses of the vitamin B_{12}. With the technique of indirect proof, it became clear that the losses were ranged between the "cooling step" and "water dilution step", this conclusion supported also from the Table 4.2 in which major losses of the spiked vitamin B_{12} disclosed in this area. After this experiment, it was decided to check the influence of the "filtration step" and "Centrifugation step".

4.3.3. Effect of Filtration Step on Recovery

In order to study the influence of the "Filtration Step" concerning the losses of the spiked vitamin B_{12}, 2 ml of standard solutions of vitamin B_{12} were prepared in three concentration levels (0.025, 0.050 and 0.100 μg/ ml), with the presence of 1 cm^2 filter paper (95 % cellulose) for 15 min and the same standard solutions without the presence of filter paper and they were measured directly by HPLC-UV. The time duration (15 min) was an estimation of the contact time between the sample and the filter paper in the filtration step.

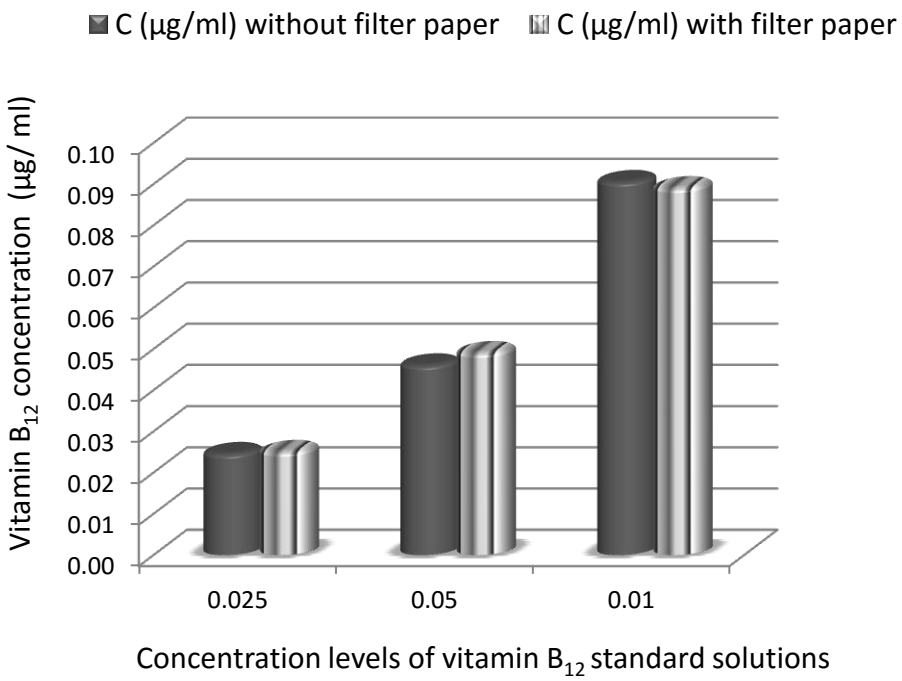


Figure 4.23: Interaction of filter paper (95 % cellulose) with standard solutions of vitamin B_{12} in three concentration levels (0.025, 0.050 and 0.100 μg/ ml)

Based on the results (Fig. 4.23) there are no significant differences between the values of concentrations of vitamin B_{12}, whether the solution contains filter paper or not. Thus, in the following chapter the effect of the "Centrifugation Step" to vitamin B_{12} will be studied.

4.3.4. Effect of Centrifugation Step on Recovery

The initial protocol (Appendices 6.1.1) was applied for the determination of vitamin B_{12} in *Hippophae rhamnoides* berries (Naturix24), with the only exception that the samples were treated in total with 50 ml buffer (50 mM acetate) (for concentration reasons), instead of 100 ml which was suggested in the protocol. After the "centrifugation step" the precipitate of the sample (~9 g) was taken for sequential centrifugations, through the process from "incubation step" (only by adding 50 ml buffer (50 mM acetate)) until the end of the process (HPLC-UV analysis). The purpose of the sequential centrifugations of the precipitate was to investigate the extraction efficiency and to estimate the real amount of vitamin B_{12} in the *Hippophae rhamnoides* sample (Naturix24). Based on the *Nernst distribution law* (1), the extraction coefficient of the process had to be stable in all sequential extractions. For the completion of the experiment the *Hippophae rhamnoides* sample (Naturix24) underwent seven sequential runs of the process (application of the overall protocol from the "incubation step" to the last step "HPLC-UV").

***Nernst distribution law*: $K_N = X_c^B / X_c^A$ (1)**

where the X_c^B denotes the fraction of C in phase B and X_c^A denotes the fraction of C in phase A, at constant temperature, respectively. By convention, the concentration extracted into phase B appears in the numerator of the equation. The equilibrium constant is independent of the rate at which it is achieved.

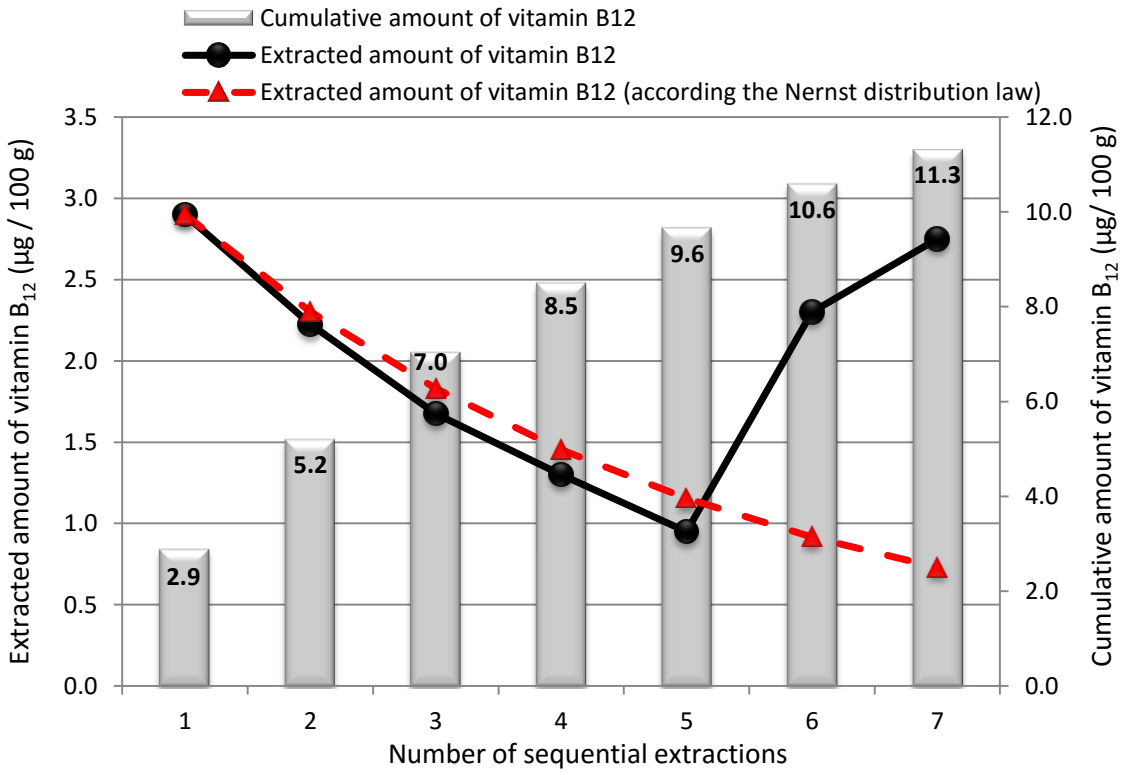


Figure 4.24: Sequential extraction of vitamin B_{12} from *Hippophae rhamnoides* (Naturix24). Black line: Actual values from extracted amount of vitamin B_{12}. Red dashed line: Extracted amount of vitamin B_{12} according the *Nernst distribution law.* Black bars: cumulative amount of vitamin B_{12} from the aggregate sequential extractions

The results of the sequential extractions followed the normal way until the 5th extraction and then the values were higher than expected (red dashed line – Fig. 4.24). Thus, the experiment stopped after the 7th sequential extraction. Nevertheless, the findings of the experiment were significant. The extraction efficiency of the method was proved to be insufficient, if it is considered that the final concentration of vitamin B_{12} in *Hippophae rhamnoides* (Naturix24) was at least 11.3 µg/ 100 g (Fig. 4.24), then the distribution coefficient of the process is approximately 0.332. However, the Figure 4.24 indicated that after the 7th extraction the precipitate still contains amounts of vitamin B_{12}, so the final concentration was expected to be much higher and the distribution coefficient < 0.332, respectively.

Based on these findings, the extraction procedure was insufficient and had to be optimized for better extraction efficiency. Probably, there was a correlation of the insufficient extraction of the analyte with the insufficient yield of the recovery studies. Moreover, another important reason of the insufficient recovery of the added vitamin B_{12}, as it was mentioned above (4.3.), could be matrix effects (Takenaka et al. (1997)). In the next chapter matrix effects will be investigated.

4.3.5. Effect of Matrix on Recovery

The matrix effects were investigated based on the assumption that plants have high contents of minerals (especially in *Hippophae rhamnoides*[134]) and high contents of vitamin C (both *Hippophae rhamnoides*[135] and cranberries[136]). Because of that, there is the possibility of complex formation among vitamin B_{12}, vitamin C and specific minerals such as copper that may lead to destruction of vitamin B_{12}. Under this hypothesis, possible interactions of vitamin B_{12} with the vitamin C, copper cations and their combinations were qualitatively investigated by UV/Vis spectroscopy.

Based on our HPLC findings and literature[134,135], a series of experiments took place in order to study all possible interactions, in conditions that could imitate (the best possible) the proportions of the above constituents (vitamin B_{12}, vitamin C and copper cations) in *Hippophae rhamnoides*. In order to check the sensitivity of UV/Vis spectroscopy, the signal density of the absorption bands of the examined constituents was tested by preparing water solutions of vitamin B_{12} in concentrations between 0.2 – 20 μg/ 100 ml, and water solutions of vitamin C and Cu^{+2} in concentrations between 10 – 100 mg/ 100 ml. After a compromise between the signal density of the absorption bands and the proportions of the constituents in order to be similar with *Hippophae rhamnoides*, it was decided to prepare mixtures containing vitamin B_{12} (10 μg/ 100 ml), vitamin C (100 mg/ 100 ml) and Cu^{2+} (10 mg/ 100 ml) in the following combinations:

- $V.B_{12,}$
- $V.B_{12}$ + V.C,
- $V.B_{12}$ + Cu^{2+}
- $V.B_{12}$ + V.C + Cu^{+2}

The mixtures were tested directly after the preparation (0 h) in pH 4 at room temperature.

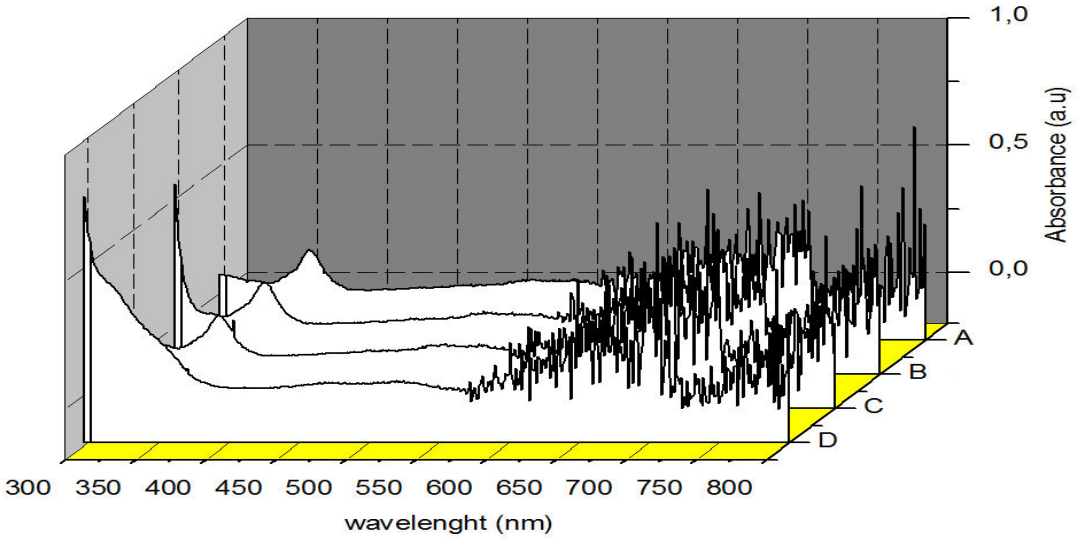


Figure 4.25: UV-Vis absorption spectra of water mixtures of vitamin B_{12} (10 µg/ 100 ml) at pH 4 (R.T) of A) vitamin B_{12}, B) vitamin B_{12} + vitamin C, C) vitamin B_{12} + Cu^{+2}, D) vitamin B_{12} + vitamin C + Cu^{+2}

The results from the UV-Vis spectra (Fig. 4.25) of different combinations of vitamin B_{12}, revealed that the combination of vitamin B_{12} with vitamin C and Cu^{+2} (Fig. 4.25 – D) introduced an effect on the characteristic absorbance band of vitamin B_{12} at 361 nm. As a first conclusion, the above combination of vitamin B_{12} + vitamin C + Cu^{+2} indicated that either the vitamin B_{12} was destroyed or made a complex with the other constituents.

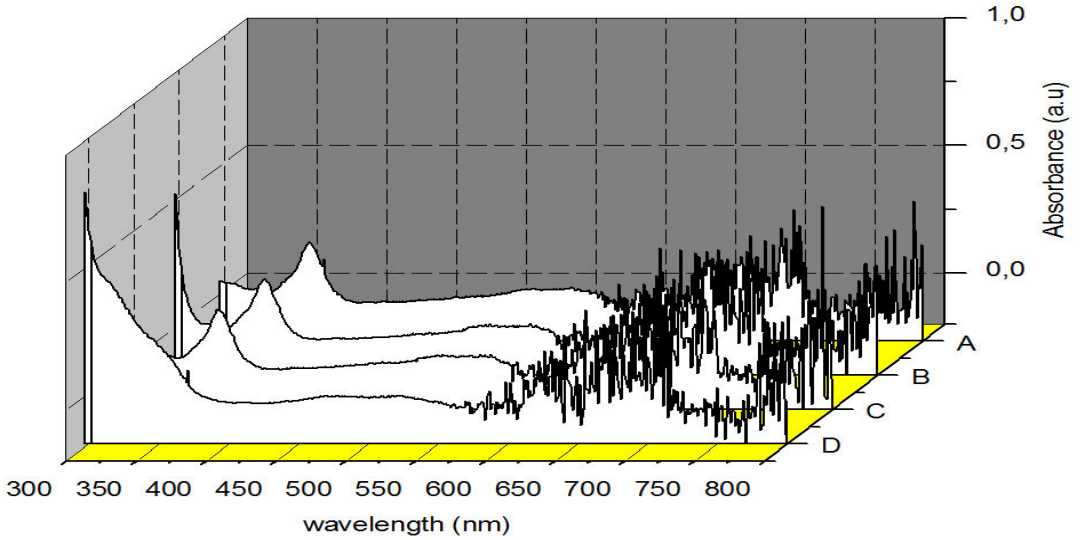


Figure 4.26: UV-Vis absorption spectra of water mixtures of vitamin B_{12} (15 µg/ 100 ml) at pH 4 (R.T) of A) vitamin B_{12}, B) vitamin B_{12} + vitamin C, C) vitamin B_{12} + Cu^{+2}, D) vitamin B_{12} + vitamin C + Cu^{+2}

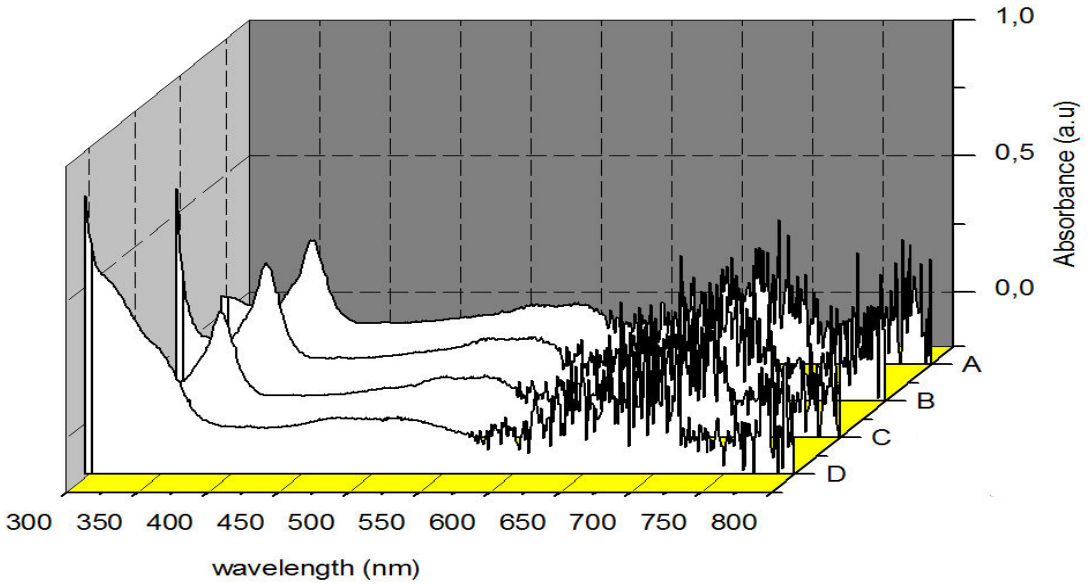


Figure 4.27: UV-Vis absorption spectra of water mixtures of vitamin B_{12} (20 µg/ 100 ml) at pH 4 (R.T) of A) vitamin B_{12}, B) vitamin B_{12} + vitamin C, C) vitamin B_{12} + Cu^{+2}, D) vitamin B_{12} + vitamin C + Cu^{+2}

Further investigations were conducted with higher concentrations of vitamin B_{12} whereas the concentrations of the other constituents were kept constant (Fig. 4.26 and Fig. 4.27), in order to find out whether there is an effect of vitamin B_{12} concentration on the formation of the different combinations. In Figure 4.26 the mixtures contained 15 µg/ 100 ml vitamin B_{12} and in Figure 4.27 20 µg/ 100 ml vitamin B_{12}, respectively. Both experiments (Fig. 4.26 and Fig 4.27) disclosed an overlap of vitamin B_{12} in relation with the combination of vitamin B_{12} + vitamin C + Cu^{+2}, similar with the Figure 4.25. Additionally, the combination of vitamin B_{12} + vitamin C + Cu^{+2} presented a change of color (optical observation of the mixture) to a brass from the initial pale pink color. However, after soft stirring of the solution the color returned to initial pale pink.

Figure 4.28 demonstrates data from Figure 4.26 measured again after 72 h at ambient temperature. The difference between Figure 4.28 and Figure 4.26 is shown in Figure 4.29. After 72 hours the absorption bands at 361 nm were increased in all combinations, especially in the combination of vitamin B_{12} + vitamin C and the combination of vitamin B_{12} + vitamin C + Cu^{+2}. In addition, a significant observation was that the stored solution of the combination vitamin B_{12} + vitamin C + Cu^{+2} after 72 hours had a stable brass color, before and after intensive stirring. Probably the complex of this combination was stabilized.

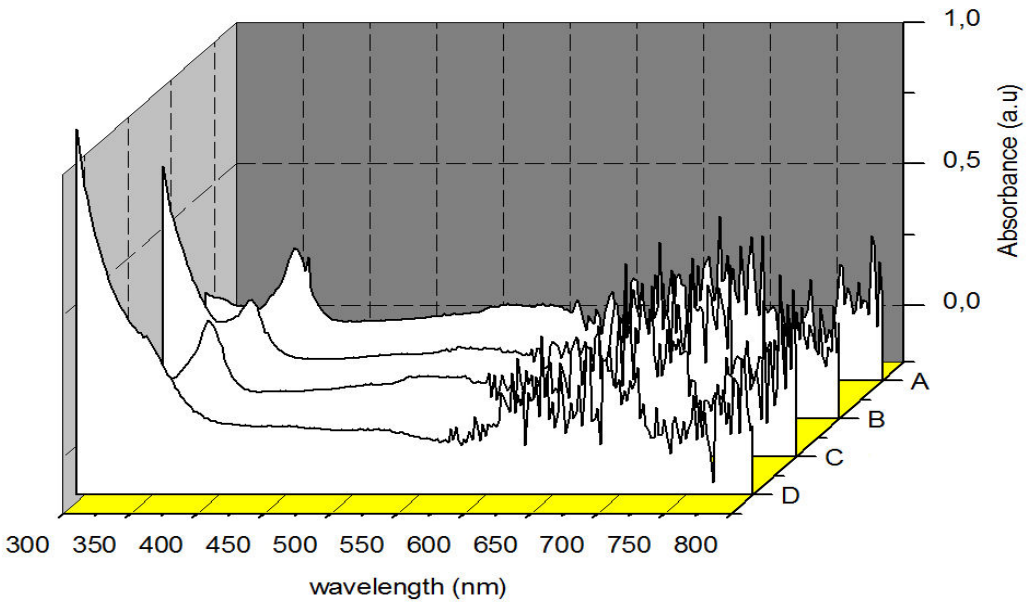


Figure 4.28: UV-Vis absorption spectra of water mixtures of vitamin B_{12} (15 µg/ 100 ml) at pH 4 (R.T) measured after 72 hours. A) vitamin B_{12}, B) vitamin B_{12} + vitamin C, C) vitamin B_{12} + Cu^{+2}, D) vitamin B_{12} + vitamin C + Cu^{+2}

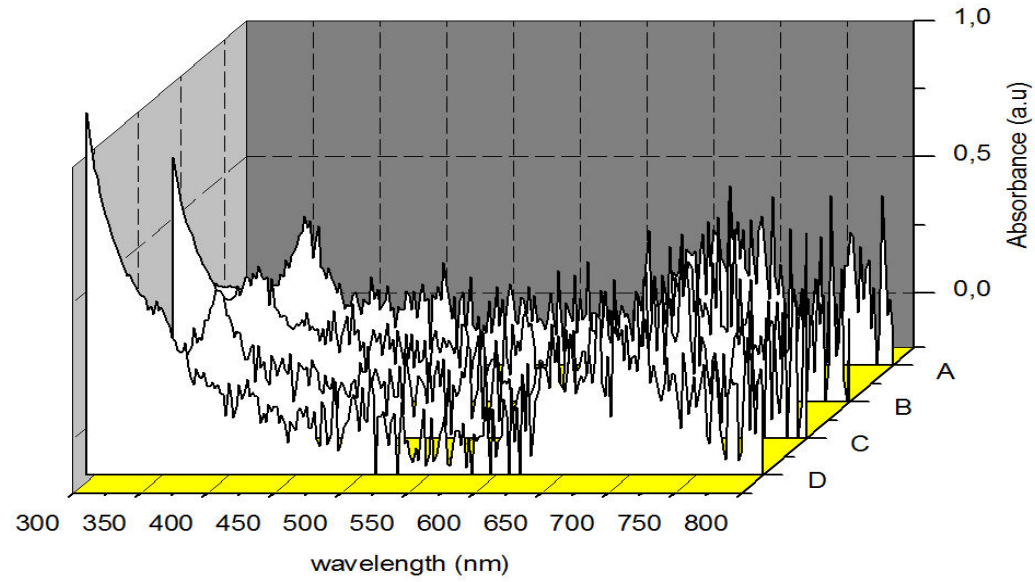


Figure 4.29: UV-Vis absorption spectra demonstrating the subtraction of Figure 4.26 from Figure 4.28

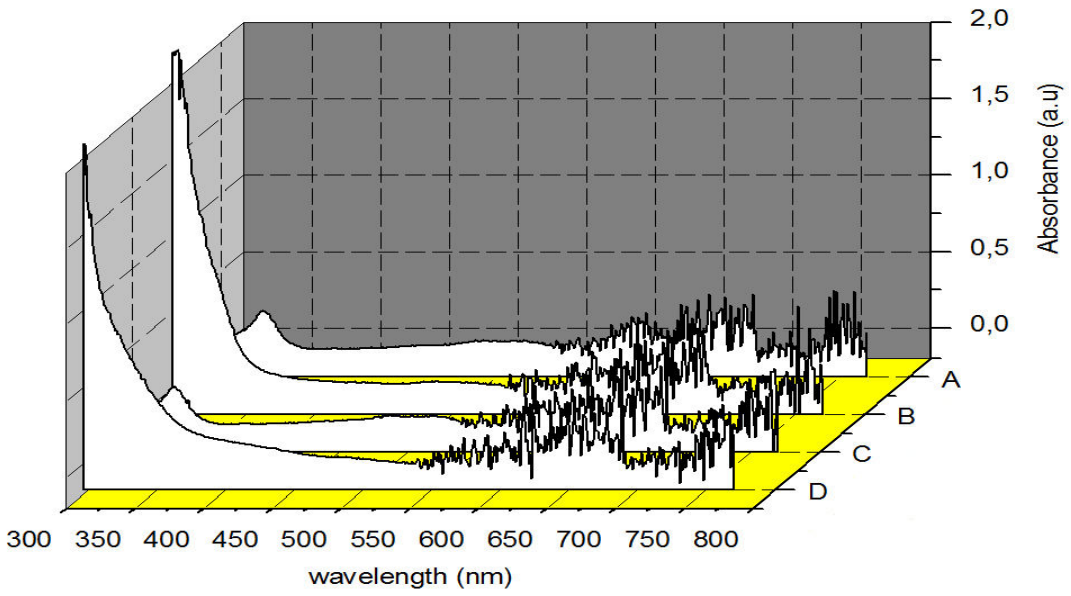


Figure 4.30: UV-Vis absorption spectra of water mixtures of vitamin B_{12} (15 µg/ 100 ml) at pH 4 after heating at 100 ^{0}C for 30 min and measurement when the samples were warm. A) vitamin B_{12}, B) vitamin B_{12} + vitamin C, C) vitamin B_{12} + Cu^{+2}, D) vitamin B_{12} + vitamin C + Cu^{+2}

Experiments were conducted to investigate the behavior of the same components (vitamin B_{12}, vitamin C and Cu^{+2}) during the heating step (100 ^{o}C for 30 min) of the assay. The same mixtures combinations containing 15 µg/ 100 ml vitamin B_{12} were divided in two groups, the first group was processed with a heating step at 100 ^{o}C for 30 min and measured directly in UV/Vis (warm samples) (Fig. 4.30) and the second group underwent the same process, but measured after the samples were cooled down (R.T) (Fig. 4.31). Both spectra (Fig. 4.30 and Fig. 4.31) depicted a greater effect on the characteristic absorbance band of vitamin B_{12}, in the combination of vitamin B_{12} + vitamin C + Cu^{+2}, and a new overlap of vitamin B_{12} from vitamin C.

To sum up, in ambient temperature conditions the previous spectra proved that only the combination of vitamin B_{12} + vitamin C + Cu^{+2} demonstrated an overlap of vitamin B_{12} absorbance band at 361 nm. However, with the addition of the heating step (100 ^{o}C for 30 min), a new overlap of vitamin B_{12} from vitamin C was revealed, in addition to the already known overlap among vitamin B_{12} + vitamin C + Cu^{+2}. The results of UV/Vis spectra confirmed that matrix effects take place when vitamin B_{12} co-exists with vitamin C and Cu^{+2}, as a consequence the vitamin B_{12} disappears (destroys or forms complexes).

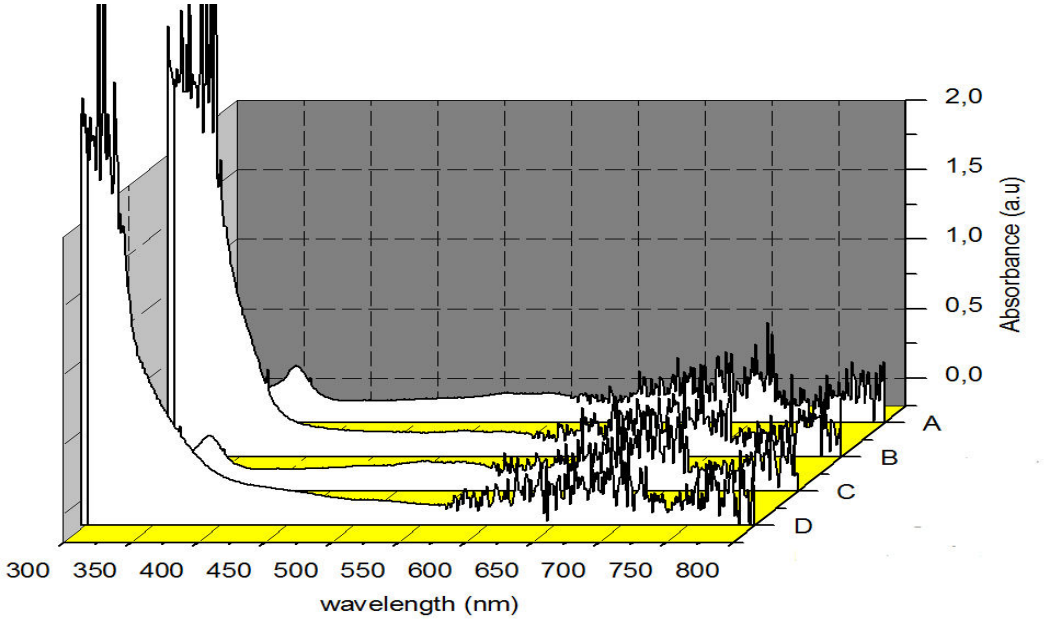


Figure 4.31: UV-Vis absorption spectra of water mixtures of vitamin B_{12} (15 μg/ 100 ml) at pH 4 after heating at 100 ^{0}C for 30 min and measurement when the samples were cooled down. A) vitamin B_{12}, B) vitamin B_{12} + vitamin C, C) vitamin B_{12} + Cu^{+2}, D) vitamin B_{12} + vitamin C + Cu^{+2}

After the above findings, and in order to confirm the results, similar experiments were performed quantitatively by the HPLC-UV method. Mixtures containing vitamin B_{12} (0.2 μg/ 100 ml), vitamin C (100 mg/ 100 ml) and Cu^{2+} (40 μg/ 100 ml) were prepared in different combinations. The mixtures were divided in two groups, the first group was processed under ambient temperature conditions and the second underwent a heating step at 100 ^{o}C for 30 min. Following the method protocol from the IAC step until the determination of vitamin B_{12} in HPLC, the results (Fig. 4.32) confirmed that the presence of vitamin C and Cu^{2+}, separately and combined, in a vitamin B_{12} solution leads to severe destruction of vitamin B_{12}. More specifically, the combination of vitamin B_{12}, vitamin C and Cu^{2+} caused almost total loss of vitamin B_{12} (89 %).

According to Takenaka et al.[137] the destruction of vitamin B_{12} is assumed to be concerned with radicals generated by vitamin C in the presence of copper. In order to prevent that, he suggested the carnosine as a natural water-soluble antioxidant, for the protection of vitamin B_{12} in the presence of vitamin C and copper. From another point of view, EDTA was also suggested as chelating agent due to its high efficiency in extracting many metals[138]. In order to prove that, mixtures of vitamin B_{12}, vitamin C and Cu^{2+} in concentrations similar to sea buckthorn berries were prepared (vitamin B_{12}: 10 μg/ 100 ml, vitamin C: 100 mg/ 100 ml and Cu^{2+} 300 μg/ 100 ml), carnosine and EDTA were added, separately, in the concentrations of 0.5

mM and 5 mM, respectively. The mixtures were measured at ambient conditions and after a heating step at 100 °C for 30 min. The results showed protection of vitamin B_{12} in the presence of vitamin C and Cu^{2+} with the addition of EDTA (0.5 mM and 5 mM) and carnosine (5 mM) (Fig. 4.33).

Thus, carnosine (5 mM), as the best additive protective, was used for the vitamin B_{12} assay or samples of *Hippophae rhamnoides* berries. Nevertheless, this addition did not cause any significant change (protection ability) in the analyzed concentrations, indicating that the immense loss of vitamin B_{12} in the assay was not only due to the vitamin C and Cu^{2+} effect.

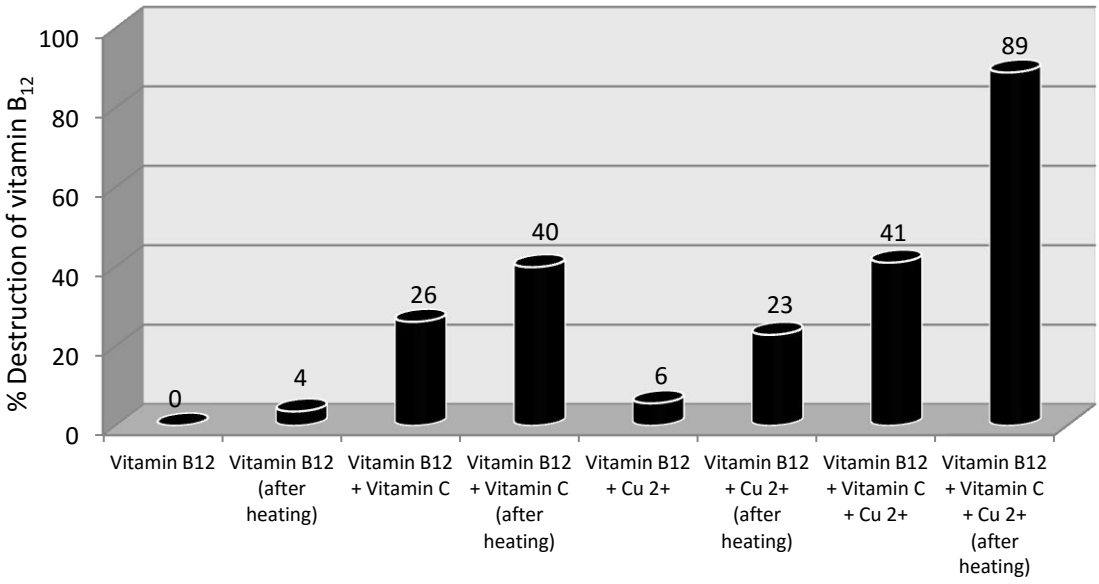


Figure 4.32: The % destruction of the vitamin B_{12} in the presence of vitamin C and Cu^{2+}, at ambient temperature and after a heating step (100 °C for 30 min)

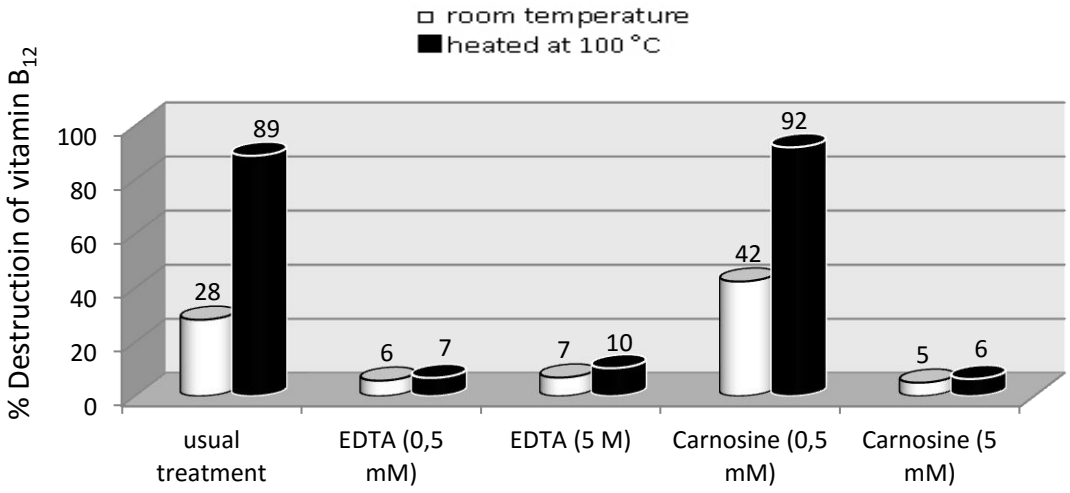


Figure 4.33: Protection of vitamin B_{12} by adding EDTA or carnosine, in a mixture with vitamin C and Cu^{2+}

4.3.6. Effect of Homogenization Process on Recovery

The homogenization process has already investigated in chapter 4.2.1 in relation with its impact on the extracted amount of vitamin B_{12} from the sample, as well as its impact on % RSD among replicate measurements (Fig. 4.3). However, the impact of the grinding methods in combination with the sample amount on the recovery studies has not yet been investigated. The following combinations of grinding methods were applied: A) mill homogenizer, B) mill homogenizer + mortar and pestle, C) mill homogenizer + mortar and pestle + disperser, D) mill homogenizer + mortar and pestle + disperser + ultrasound bath, parallel with the reduction of the sample amount from 10 g to 0.5 g following the direction A to D (Fig. 4.34). The outcomes of these combined experiments were that every new added grinding method contributed to gradually:

i) increase of vitamin B_{12} concentration, whereas the combination of all grinding methods (method D) extracted the highest amounts of vitamin B_{12} (37.01 μg/ 100 g), due to better homogenization and significant size reduction of the sample, that led to better liberation of the bound vitamin B_{12} (see Fig 4.3).
ii) increase of the recovery values (following the direction A to D) of the spiking analyte (vitamin B_{12}) up to 84 % (Fig 4.34). Similar levels of recovery were achieved with the other samples such as liver, vitamin B_{12} tablets, *Inula helenium* and blank spikes.
iii) reduction of the matrix effects (complexation reactions of vitamin B_{12} with vitamin C and Cu^{+2}), due to significantly lower quantity of the processed amount of the sample (0.5 g).
iv) significant improvement of the precision of the assay from 14 to 0.7 % RSD, due to perfect homogenization of the sample (see Figure 4.3).

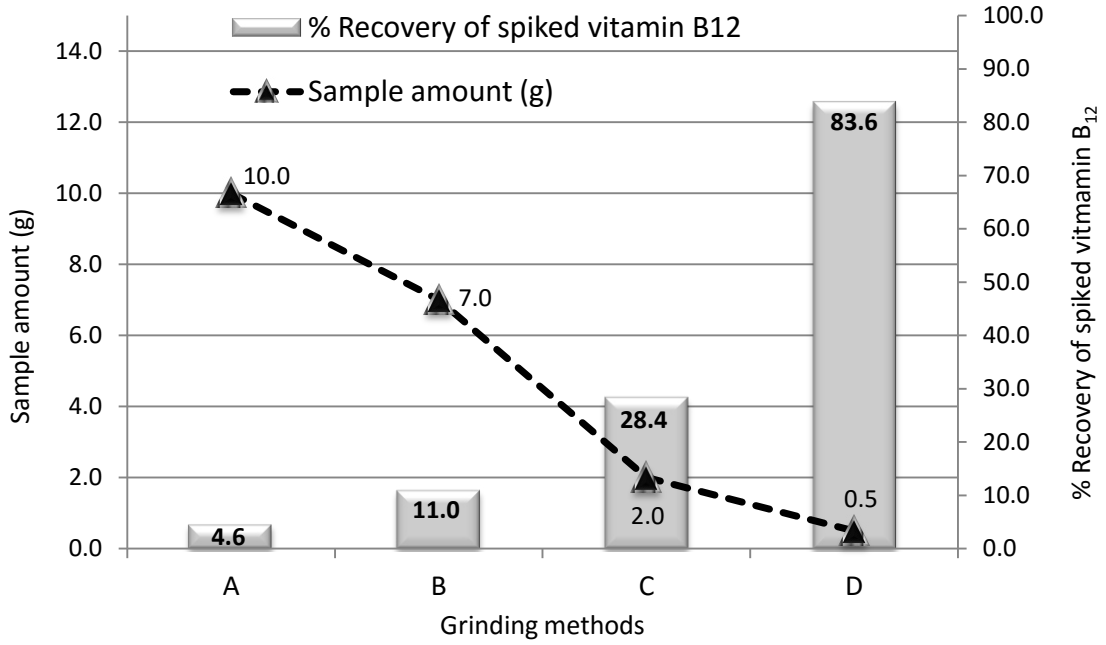


Figure 4.34: Influence of grinding method and sample amount on the recovery of the spiked amount of vitamin B_{12} in *Hippophae rhamnoides*. Grinding methods: A) mill homogenizer, B) mill homogenizer + mortar and pestle, C) mill homogenizer + mortar and pestle + disperser, D) mill homogenizer + mortar and pestle + disperser + ultrasonic bath

Summary

An extensive investigation carried out in order to determine the low recoveries, which appeared during the measurement of *Hippophae rhamnoides*. Several steps of the assay investigated in order to find out the source of vitamin B_{12} losses. After the previous investigations, we came to the conclusion that the main reasons for the extensive losses of vitamin B_{12} were the insufficient homogenization process, the sample amount and the matrix effects caused by the presence of vitamin C and copper cations. The optimized homogenization procedure overcame all these problems, and as a result the recovery of vitamin B_{12} in *Hippophae rhamnoides* returned to levels similar to the other samples (82 – 90 %).

4.4. Stability of Vitamin B_{12}

Previous publications have highlighted the instability of vitamin B_{12} during ambient light conditions and thermal treatment processes, respectively[11]. Major attention was given to the stability of the analyte during sample preparation and analysis. The stability of vitamin B_{12} was tested with a series of standard solutions in the range of the method concentrations. The standards solutions of the analyte (vitamin B_{12}) should be tested over at least a 48 hour period (depends on intended use), and the quantitation of components should be determined by comparison to freshly prepared standards[139]. For assay methods a stability criterion is that the sample, the standard solutions and the mobile phase will be stable for 48 h under defined storage conditions. The stability is considered to be acceptable when the change in the standard or sample response is within 2% relative to freshly prepared standards.

Hence, fresh standard solutions were measured directly after preparation; the same standard solutions stored in a capped volumetric flask on a laboratory bench under normal daylight conditions at RT and were measured after 24 h and 48 h. Extensive losses were disclosed in concentrations at LOQ levels (10.80 % after 24 h and 21.33 % after 48 h). In the area of the target concentration of the method (C=0.1 µg/ ml) the standard solutions showed relatively good stability, with losses of vitamin B_{12} within 2.9 – 4.4 % for the first 48 hours. Although, the resulting values were not that close to the limit of 2 %, concentrations higher than 0.5 µg/ml remained stable for more than 2 – 3 days. Generally, as the concentration increased, the vitamin losses decreased in an inversely proportional manner (see Fig 4.35).

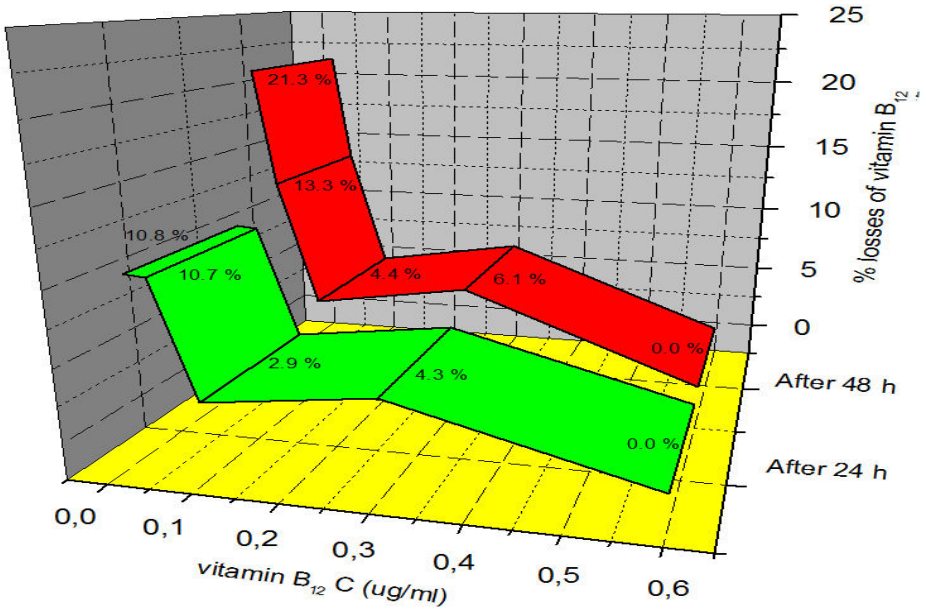


Figure 4.35: Effect of ambient conditions (light and temperature) on the stability of vitamin B_{12} standard solutions (C= 0.025 – 1.000 µg/ ml) after 24h and 48h

Apart from the short-term stability of vitamin B_{12} in ambient conditions, significant interest had the stability of vitamin B_{12} in long-term duration for the determination of the ideal concentration of a stock solution. An experiment was carried out in order to investigate the effect of ambient light and temperature conditions on the stability of vitamin B_{12} after 4 days and 7 days (Fig. 4.36).

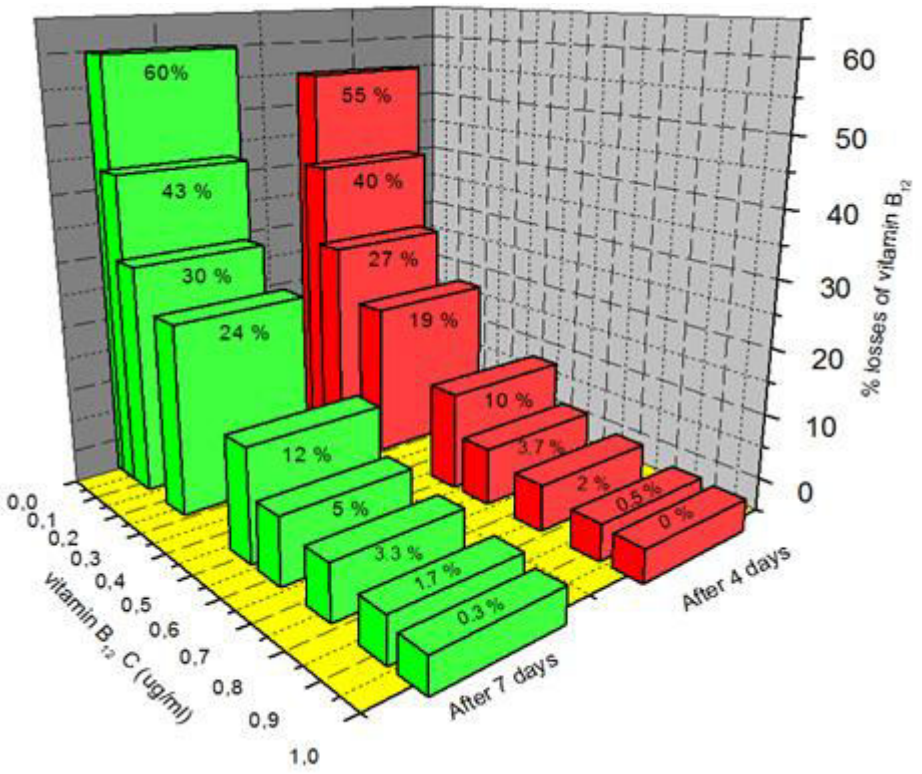


Figure 4.36: Effect of ambient conditions (light and temperature) on the stability of vitamin B_{12} standard solutions (C= 0.025 – 1.000 µg/ ml) in a time period of 4 and 7 days

The graph in Figure 4.36 demonstrated that the concentrations of vitamin B_{12} standard solutions decreased in an inversely proportional manner, both after 4 and 7 days, respectively. The losses in each concentration level are higher after storing vitamin B_{12} for 7 days in ambient conditions compared to 4 days. Major losses occurred in a range of concentrations (0.025 – 0.600 µg/ ml) and the losses are gradually reduced as the concentrations reach higher levels from 0.7 till 1.0 µg/ ml. Concentrations higher than 1.0 µg/ ml are expected to be stable more than a week in ambient conditions. By combining the above results (Fig 4.36) with several relative publications, a stock solution of cyanocobalamin (vitamin B_{12}) with higher concentration, such as 10 µg/ ml, under light protection at lower temperatures (4 ^{o}C), can be stable for 2 months.

One more experiment was conducted concerning the ambient light and temperature conditions, in order to identify the effect of ambient conditions to the stability of

vitamin B_{12}. Fresh standard solutions of vitamin B_{12} were prepared in replicates in the concentration of 0.5 μg/ ml and divided in two groups, one exposed to ambient light, and the second one kept in the dark. Both groups left in those conditions for 60 h and then were measured by HPLC-UV. The results showed average 4.7 % losses of vitamin B_{12} of the first group (light-protected) and for the second group (light-exposed) an average of 12.0 % losses of vitamin B_{12} (Fig. 4.37). Remarkable was that the concentration of vitamin B_{12} in the level of 0.5 μg/ ml, demonstrated 12 % losses in 2 ½ days, in contrast with the previous experiment (Fig. 4.36) in which the same concentration of vitamin B_{12} (0.5 μg/ ml) showed losses 10 % in 4 days. In spite of that, the results indicate that the long-term stay of vitamin B_{12} in unprotected light ambient conditions has a considerable impact to the stability of vitamin B_{12}. It is strongly suggested that samples with vitamin B_{12} have to be handled under subdued light, as well as to use amber glassware or covered tubes.

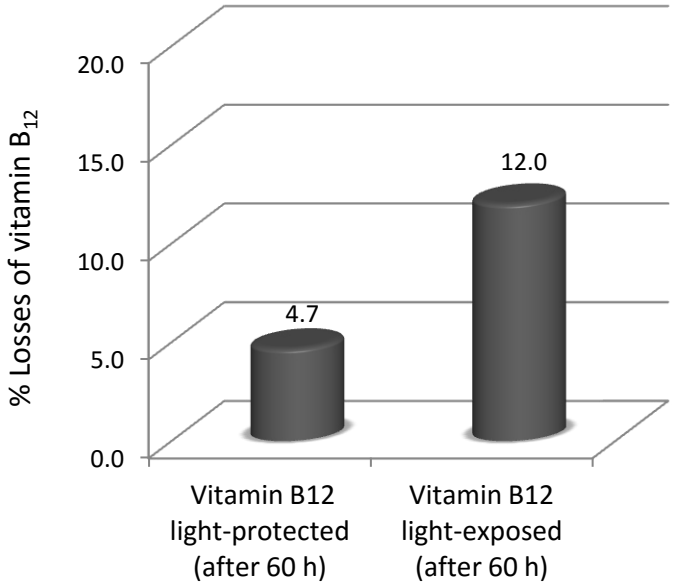


Figure 4.37: Effect of ambient light to stability of vitamin B_{12} (C=0.5 μg/ml) after 60 h exposure to ambient conditions

The stability of vitamin B_{12} in a thermal treatment at 100 ^{o}C was investigated, as 100 ^{o}C represents the highest temperature in which the analyte will undergo through the method protocol. The results showed that after 15 min duration the vitamin B_{12} content was constant without losses, but after 30 - 45 min the losses increased to 8.33 % of the initial vitamin B_{12} content (tested with 0.1 μg/ ml vitamin B_{12} standard solution). Therefore, samples during the thermal treatment stayed no longer than 30 min at 100 ^{o}C, in order to avoid losses of the vitamin B_{12}, and standard solutions of vitamin B_{12}, which were used for studies (calibration plot, recovery, validation etc.), were freshly prepared and used on a daily basis.

4.5. Determination of Moisture Content

Due to different moisture content of each one of the samples, before the application of the assay moisture content determinations were performed, in order to incorporate suitable weight corrections. The water molecules in a plant or meat product may be present in a variety of different molecular environments. Depending on their interaction with the surrounding molecules the water molecules in these different environments normally have different physicochemical properties.

- Bulk water: - Bulk water is free from any other constituent so that each water molecule is surrounded only by other water molecules.
- Capillary or trapped water: - Capillary water is held in narrow channels by capillary forces. Trapped water is held within spaces in a food which are surrounded by a physical barrier that prevents the water molecules from easily escaping e.g, an emulsion droplet or a biological cell.
- Physically bound water: - A significant fraction of the water molecules in many foods is not completely surrounded by other water molecules, but is in molecular contact with other food constituent e.g. protein, carbohydrate or minerals.
- Chemically bound water: - some of the water molecules present in a food may be chemically bound to other molecules as water of crystallization is as hydrate e.g $NaS0_4.10H_20$.

Prior to moisture content analysis, it was important to prevent any loss or gain of water, as well as the selection of a representative sample. For this reason, the samples in order to avoid exposure to the atmosphere and to the fluctuation of the temperature, were stored in dark light-protected sealed containers. The moisture content of all samples was determined gravimetrically based on an oven-drying method (Appendice 6.1.3.). The samples were chopped into small size parts so as to extend the surface area of material exposed to the environment, and to remove the moisture with faster rate. Weighed samples were placed in an oven for 24 h at 90 ^{o}C until they reached a constant weight. The calculation of the moisture content could be expressed on a fresh weight (FW) basis or on a dry weight (DW) basis.

$$\% \text{ moisture}_{(FW \text{ basis})} = \frac{FW-DW}{FW} \times 100$$

$$\% \text{ moisture}_{(DW \text{ basis})} = \frac{FW-DW}{DW} \times 100$$

However, the fresh weight (FW) basis is preferred, because it tends to minimize changes in water content, whereas the dry weight (DW) can change seasonally giving unsatisfactory results. The results of the examined samples are listed in Appendices 6.1.3. Table 6.5.

4.6. Validation Study for Vitamin B_{12} Assay

The validation of analytical methods is an important regulatory requirement in pharmaceutical analysis. A valid analytical method has to provide documented evidence, and a high degree of assurance, so as the employed analytical method for a specific test, will be suitable for its intended purpose[139]. Regulatory authorities, over recent years, have become increasingly aware of the necessity of ensuring that the data submitted to them in applications for marketing authorizations have been acquired using validated analytical methodology. The International Conference on Harmonization (ICH) has introduced guidelines for analytical methods validation[140,141]. The U.S. Food and Drug Administration (FDA) methods validation draft guidance document[142,143,144], as well as United States Pharmacopoeia (USP)[145] both refer to ICH guidelines. These draft guidances define regulatory and alternative analytical procedures and stability-indicating assays. The FDA has proposed to add a section from the Code of Federal Regulations (CFR) 211.222, of analytical methods validation, to the current Good Manufacturing Practice (cGMP) regulations[146]. This would obligate pharmaceutical manufacturers to establish and document the accuracy, sensitivity, specificity, reproducibility, and any other necessary attribute (e.g., system suitability, stability of solutions) for the validation of methods. The first step in method validation is the preparation of a protocol.

The overall assay for the quantification of vitamin B_{12} in plants was carefully validated, prior to a final LC-MS/MS assay of the vitamin B_{12} from *Hippophae rhamnoides*.

4.6.1. Linearity and Range

4.6.1.1. Linearity

Accoording the International Conference on Harmonization (ICH) linearity of an analytical procedure is the ability (within a given range) to obtain test results that are directly proportional to the concentration (amount) of analyte in the sample[140]. The linearity has to specify a minimum of five concentration levels, along with certain minimum specified ranges. The minimum specified range is from 80 – 120 % of the target concentration. Acceptability of linearity data is often judged by examining the correlation coefficient and y-intercept of the linear regression line for the response versus concentration plot. Generally the regression coefficient (r^2) > 0.998 is considered as evidence of acceptable fit of the data to the regression line. The y-intercept should be less than a few percent of the response obtained for the analyte at the target level. The Percent Relative Standard Deviation (RSD), intercept, and slope should be calculated[139].

In the present assay development, the target concentration is 0.1 µg/ ml vitamin B_{12}. The 80 – 120 % range in our method includes concentrations from 0.08 – 0.12 µg/ ml vitamin B_{12}. However, linearity was first investigated in a broaden range of vitamin B_{12} concentrations from 0.01 – 0.7 µg/ ml (10 – 700 % of nominal concentration, n=6) in order to specify the linear range of the method with statistical approaches. Fresh prepared standard solutions of vitamin B_{12} in ten levels (n=6) (0.01, 0.025, 0.05, 0.10, 0.20, 0.30, 0.40, 0.50, 0.60 and 0.70 µg/ ml) were analysed directly by HPLC-UV. Usually, this relationship can be represented by the equation y = a + bx, where a is the y-intercept and b is the slope. The slope indicates the steepness of a line and the intercept indicates the location where it intersects an axis. The following regression equation was found by plotting the peak area (y) versus the vitamin B_{12} concentration (x) expressed in µg/ ml:

peak area = 69 + 333,814 vitamin B_{12} (µg/ ml) with **R^2 (adj) = 99.41 %**.

Whereas the intercept of the equation is 69 and the slope is 333,814. The value of the y-intercept (69) indicates the average peak area when the concentration of vitamin B_{12} is 0 µg/ ml. The value of the slope (333,814) indicates that for each concentration level, the peak area increases, on average, by 333,814 arbitrary units (mAU).

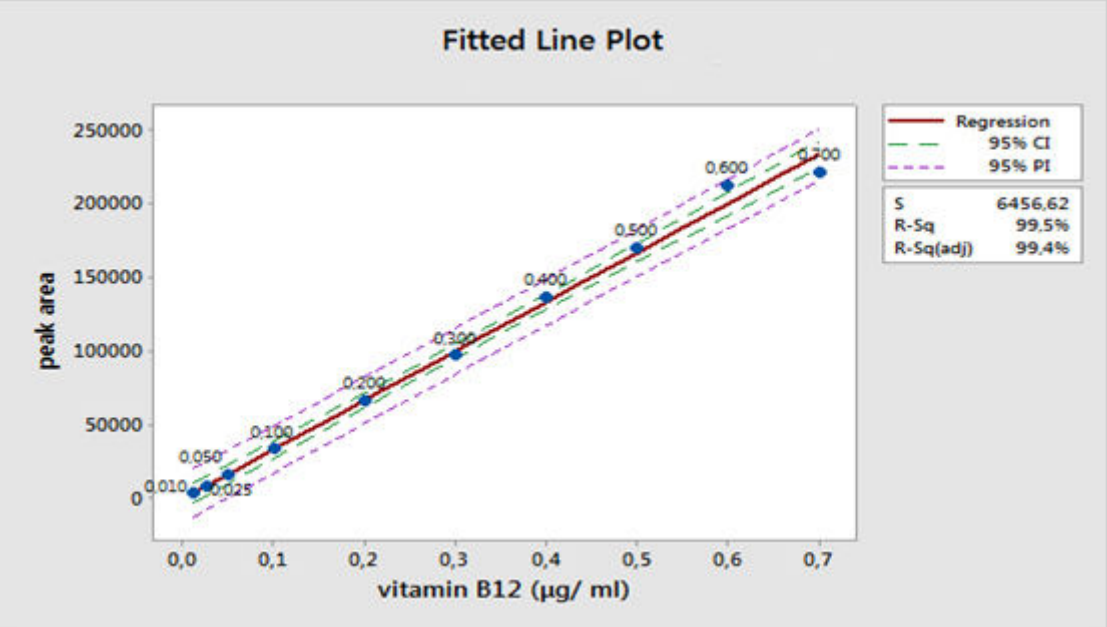


Figure 4.38: Graphical presentation of the linear regression line (0.01 – 0.70 µg/ ml) with the 95 % confidence interval and prediction interval

The demonstration of the coefficient R^2 obtained from the regression line demonstrates a satisfactory relationship between peak area and concentration of vitamin B_{12} (Fig. 4.38). The data obtained from linearity experiments are presented in Table 4.3.

Table 4.3: Assessment results of the linearity (range from 0.01 – 0.70 µg / ml vitamin B_{12}) of the HPLC method, for the assay of vitamin B_{12} employing the analytical working standard dissolved in the mobile phase

Concentration (µg/ ml)	Concentration as percent of 0,1 µg/ ml	peak area as mean of 6 injections	Peak area RSD (%)
0.010	10	3435	7.37
0.025	25	8077	7.36
0.050	50	15619	1.63
0.100	100	33126	0.34
0.200	200	66212	0.80
0.300	300	97068	0.53
0.400	400	136549	10.64
0.500	500	170088	0.32
0.600	600	212407	1.22
0.700	700	221165	9.33

Before further examination of the regression, it was necessary to obtain a diagnosis of the validity of the regression through the graph of Figure 4.39.

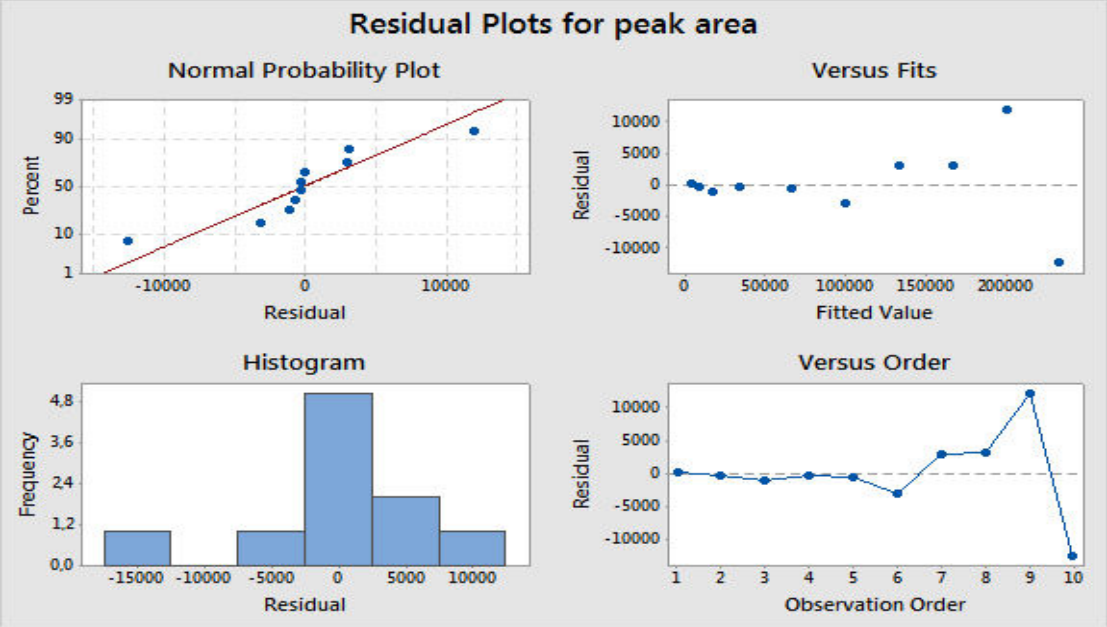


Figure 4.39: Graphs for the estimation of the normality of residuals (produced by statistical program MINITAB 17)

Although, the results presented in the Table 4.4 demonstrated P value for the analysis of variance lower than 0.05, meaning that there is statistical significant linear regression (Fig. 4.38) between the measured peak area and the concentration levels of vitamin B_{12}, the Figure 4.39 shows that the graphs of the normal plot (graphical technique for assessing whether or not a data set is

approximately normally distributed) and the histogram of the residuals (shows the distribution of the residuals for all observations) are following the normal distribution but not in excellent way. It is observed that the graph of residuals versus the fitted values (estimated responses), are not dispersed uniformly. So the regression is not independent of the actions of x values and the fitted values. Additionally, by examining the results of Table 4.4 (*the abbreviations of Table 4.4 are explained below), there are 2 observations (9 and 10) with a relative high value of standard residual. Thus, these two observations have to be examined further in order to ascertain whether their absence improve the adjustment of the regression line or not.

Table 4.4: Regression Analysis: Peak area versus vitamin B_{12} C (μg/ml)

Analysis of Variance					
Source	**DF**	**Adjusted sums of squares**	**Adjusted mean squares**	**F-Value**	**P-Value**
Regression	1	63616656749	63616656749	1526,02	0,000
vitamin B_{12} (μg/ ml)	1	63616656749	63616656749	1526,02	0,000
Error	8	333503777	41687972		
Total	9	63950160526			

Model Summary	
S	6456,62
R-sq	99,48 %
R-sq(adj)	99,41 %
R-sq(pred)	98,83 %

Coefficients					
Term	**Coefficient**	**Standard error coefficient**	**T-Value**	**P-Value**	**VIF**
Constant	69	3201	0,02	0,983	
vitamin B_{12} (μg/ ml)	333814	8545	39,06	0,000	1,00

Fits and Diagnostics for All Observations					
Observations	**Peak area**	**Fit**	**Residuals**	**Standardized residuals**	
1	3435	3407	28	0,00	
2	8077	8415	-338	-0,06	
3	15619	16760	-1141	-0,20	
4	33126	33451	-325	-0,05	
5	66212	66832	-620	-0,10	
6	97068	100213	-3145	-0,51	
7	136549	133595	2954	0,49	
8	170088	166976	3112	0,53	
9	**212407**	**200358**	**12049**	**2,18**	**R**
10	**221165**	**233739**	**-12574**	**-2,51**	**R**

*****

S = the square root of the mean square error
R-sq = estimated R-square

R-sq(adj) = estimated adjusted R-square
F-value = the test statistic used to determine whether the term is associated with the response
p-value = determines whether the results are statistically significant
t-value = is a test statistic for t-tests that measures the difference between an observed sample statistic and its hypothesized population parameter in units of standard error
VIF = Variance inflation factors measure how much the variance of the estimated regression coefficients are inflated as compared to when the predictor variables are not linearly related

As a consequence, a further investigation of the regression was necessary. Because the deviations from linearity are sometimes difficult to detect, two additional graphical procedures were used for the right estimation of linearity:

i) The first procedure plotted the residuals from the regression line versus the concentrations of vitamin B_{12} standard solutions (Fig. 4.40).

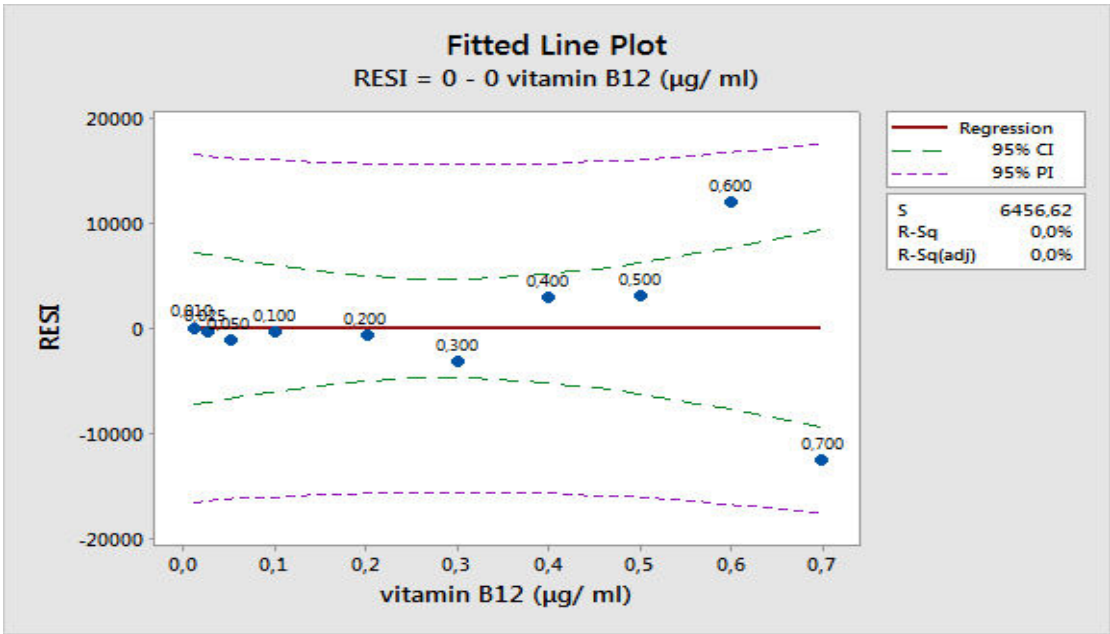


Figure 4.40: Plot of residuals (from regression analysis) versus the concentrations of vitamin B_{12} (processed by statistical program MINITAB 17)

ii) The second procedure plotted the residuals from the regression line versus the logarithm of the concentrations of vitamin B_{12} standard solutions (Fig. 4.41).

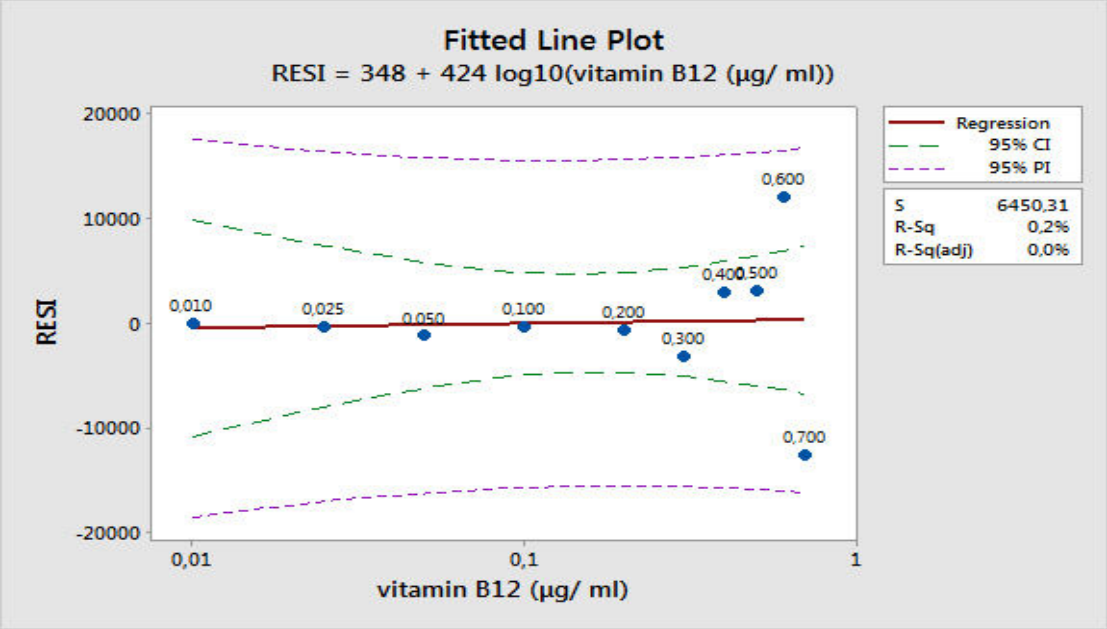


Figure 4.41: Plot of residuals (from regression analysis) versus the logarithm of the concentrations of vitamin B_{12} (processed by statistical program MINITAB 17)

Based on the regulations of International Conference on Harmonization (ICH) in correlation with the two graphs above (Fig. 4.40 and Fig. 4.41), the residuals are not equally distributed between positive and negative values, from the concentration level of 0.4 – 0.7 µg/ ml, and the results of the regression analysis (Table 4.4 and Figure 4.41) indicated that concentration levels of 0.6 µg/ ml and 0.7 µg/ ml influenced negatively the adjustment of the linear regression line.

Thus, it was decided to create a new calibration plot, including the concentrations of vitamin B_{12} from 0.01 – 0.30 µg/ ml. A six-point regression line was created from the following concentrations of the standard solutions of vitamin B_{12}: 0.01, 0.025, 0.05, 0.10, 0.20 and 0.30 µg/ ml (n=6). The following regression equation was found by plotting the peak area (y) versus the vitamin B_{12} concentration (x) expressed in µg/ ml:

peak area = 81 + 325,621 vitamin B_{12} (µg/ ml) with **R^2 (adj) = 99.96 %**.

Whereas the intercept of the equation is 81 and the slope is 325,621. The demonstration of the coefficient R^2 obtained from the regression line and demonstrates a satisfactory relationship between peak area and concentration of vitamin B_{12} (Fig. 4.42). The data obtained from linearity experiments are presented in Table 4.5 (from 0.010 – 0.300 µg/ ml vitamin B_{12}).

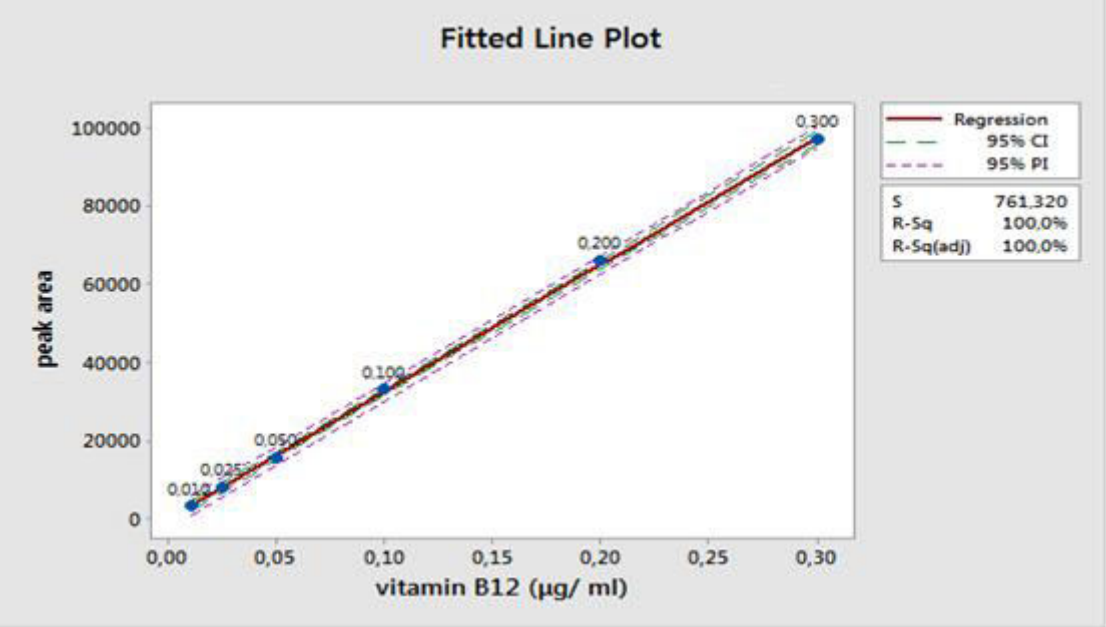


Figure 4.42: Graphical presentation of the linear regression line (0.01 – 0.30 µg/ ml) with the 95 % confidence interval and prediction interval

Before a further examination of the regression, necessary was to obtain a diagnosis of the validity of the regression through the graph of Figure 4.43.

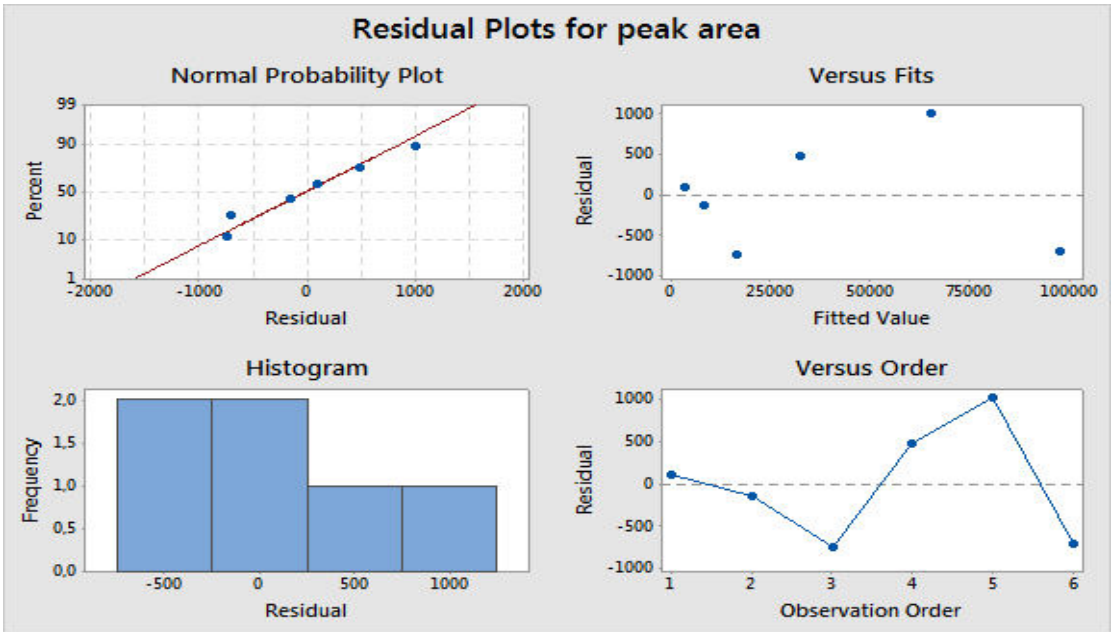


Figure 4.43: Graphs for the estimation of the normality of residuals (produced by statistical program MINITAB 17)

The Figure 4.43 shows that the graphs of normal plot and the histogram of the residuals are following the normal distribution in excellent way. It is observed that the graph of residuals versus the fitted values, are dispersed uniformly. So the

regression is independent of the actions of x values (concentration of vitamin B_{12}) and the fitted values.

By examining the results of Table 4.5 it is shown that with the absence of the concentrations 0.4 μg/ ml, 0.5 μg/ ml, 0.6 μg/ ml and 0.7 μg/ ml the accuracy of the regression line has improved significantly.

Table 4.5: Regression analysis: Peak area versus vitamin B_{12} C (μg/ml)

Analysis of Variance					
Source	**DF**	**Adjusted sums of squares**	**Adjusted mean squares**	**F-Value**	**P-Value**
Regression	1	6894097598	6894097598	11894,41	0,000
vitamin B_{12} (μg/ ml)	1	6894097598	6894097598	11894,41	0,000
Error	4	2318433	579608		
Total	5	6896416031			

Model Summary	
S	761,320
R-sq	99,97 %
R-sq(adj)	99,96 %
R-sq(pred)	98,87 %

Coefficients					
Term	**Coefficient**	**Standard error coefficient**	**T-Value**	**P-Value**	**VIF**
Constant	81	461	0,18	0,869	
vitamin B_{12} (μg/ ml)	325621	2986	109,06	0,000	1,00

Fits and Diagnostics for All Observations				
Observations	**Peak area**	**Fit**	**Residuals**	**Standardized residuals**
1	3435	3407	28	0,00
2	8077	8415	-338	-0,06
3	15619	16760	-1141	-0,20
4	33126	33451	-325	-0,05
5	66212	66832	-620	-0,10
6	97068	100213	-3145	-0,51

The results presented in Table 4.5 demonstrated *P* value for the analysis of variance lower than 0.05, meaning that there is statistical significant linear regression (Fig. 4.42) between the measured peak area and the concentration levels of vitamin B_{12}. Thus, the R^2 (adj) (adjusted= n<10) was improved to 99.97 %, meaning that the adjustment of the regression line was excellent. Thus, the method has an excellent linear range of concentrations from 0.01 μg/ ml until 0.30 μg/ ml, which covers from 10 – 300 % of the target concentration.

4.6.1.2. Range

ICH defines the range of an analytical procedure as the interval from the upper to the lower concentration (amounts) of analyte in the sample (including these concentrations), in which has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity. For assay tests, ICH requires the minimum specified range to be 80 – 120 % of the test concentration[147].

In this study, the data obtained during the linearity and recovery studies were used to assess the range of the assay method. The precision data for this assessment was the precision of the three replicate samples, analyzed at tree different levels in the recovery studies. The valid analytical range of the method was the range of concentrations, which passed the linearity and accuracy criteria, and yields an RSD of < 2%. The linearity data described earlier demonstrates acceptable linearity for vitamin B_{12} over the range of 10 – 300 % of the target concentration. The RSD values obtained for the recovery of vitamin B_{12} at 30, 100, and 300 % of target are 0.85, 0.70, and 0.72 %, respectively (Table 4.6). Each value was the result of three individual sample preparations and analysis. These data support a method range of 30 – 300 % of the target concentration.

4.6.2. Accuracy

ICH defines the accuracy of an analytical procedure as the closeness of agreement between the conventional true value or an accepted reference value and the value found. Accuracy can also be described as the extent to which test results generated by the method and the true value agree[147]. Accuracy usually can be determined in one of four ways. 1st, accuracy can be assessed by analyzing a sample of known concentration (reference materials), and comparing the measured value with the true value. The 2nd approach is to compare test results from the new method with results from an existing alternate well-characterized procedure that is known to be accurate[139]. The 3rd approach is based on the recovery of known amounts of analyte. This is performed by spiking analyte in blank matrices. For assay methods, spiked samples are prepared in triplicate at three levels over a range of 50-150% of the target concentration. The % recovery should then be calculated. Another technique which can also be used to determine the recovery of the spiked analyte is the 4th approach, the technique of standard additions. This approach is used if it is not possible to prepare a blank spike matrix without the presence of the analyte[139]. It recommended from ICF[141] to collect data from a minimum of nine determinations over a minimum of three concentration levels covering the specified range (e.g., three concentrations, three replicates each).

4.6.2.1. Estimation of Accuracy

Taking all the conclusions from the recovery study (Chapter 4.3) under consideration and integrating the new optimized homogenization method to the assay protocol, a

matrix spiking method with defined analyte concentration (standard solution) was used for estimation of the recovery. Vitamin B_{12} was spiked to *Hippophae rhamnoides* powder samples, to vitamin B_{12} tablets, to liver (veal) and to *Inula helenium*, an amount of analyte underwent the same procedures without matrix (blank spike). The amounts of added analyte at the level of the target concentration (0.1 µg/ml) of our method. *Hippophae rhamnoides* samples were spiked at three concentration levels, the 1st at the limit of quantification (LOQ), the 2nd in the target concentration (0.1 µg/ml) and the 3rd at the upper limit of the linear range (0.3 µg/ ml). The following equation was used for the estimation of recovery from spiked samples:

$$\textbf{Recovery of spiked sample (\%)} = \frac{(Q_{found} - Q_{original\ sample})}{Q_{spiked}} * 100\%$$

Where, Q_{found} is the concentration in the sample with the addition of vitamin B_{12}, $Q_{original\ sample}$ is the concentration of a sample without any addition and Q_{spiked} is the concentration of the added vitamin B_{12}. The results of the recovery rate and the relative standard deviations of the measured samples are listed in Table 4.6. Blank spikes performed with the highest recovery (100 %) due to absence of matrix, the liver (veal) sample performed with 89 % recovery, whereas complex samples such as *Hippophae rhamnoides* and *Inula helenium* performed lower recoveries between 82 – 90 %, due to matrix effects and the influence of homogenization process.

Table 4.6: Precision and recovery data

Samples	Added amount of Vitamin B_{12} (µg/ ml)	Recovery of spiked sample (%)	RSD (%)	n
Hippophae rhamnoides	0.03	80	0.85	3
	0.10	90	0.70	3
	0.30	82	0.72	3
Blank spike	0.17	100	0.67	3
Elecampane (*Inula helenium*)	0.03	84	6.73	3
Vitamin B_{12} tablets	0.12	80	0.89	3
Liver (veal)	0.12	89	0.79	3

4.6.3. Specificity / Selectivity

For the analysis of an active component of a pharmaceutical product, it is important to have a good knowledge of assay interference by possible degradants or synthesis precursors and assay interference by chemicals employed in sample preparation.

In the present dissertation, the specificity of the method was studied with the comparison of the HPLC chromatogram of a blank sample, a spiked cranberry sample and the cranberry sample without spike (Fig. 4.44). From Figure 4.44 it becomes obvious that the vitamin B_{12} is well separated from any potential interference. Therefore, this method was specific for vitamin B_{12}.

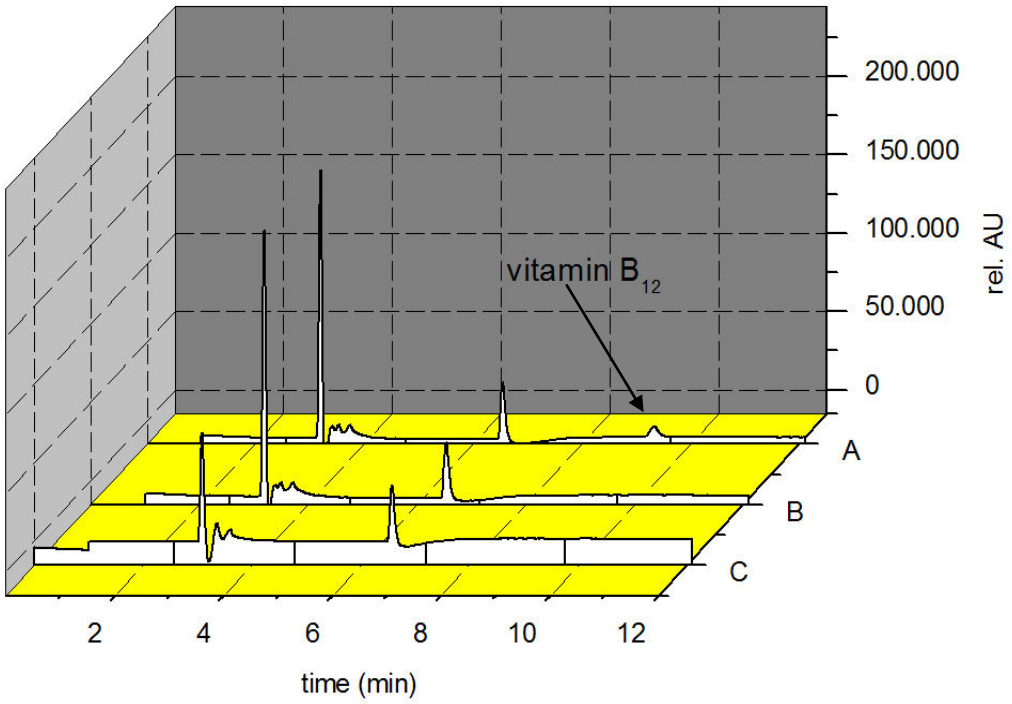


Figure 4.44: HPLC-UV chromatograms of spiked cranberries sample (A), of cranberries sample (B) and a chromatogram of mobile phase (TFA 0.01% w/v) (C). The chromatographic conditions are described in chapter 4.2.4.1.

4.6.4. Precision (System Repeatability, Method Repeatability and Intermediate Repeatability)

Precision is the measure of the degree of repeatability of an analytical method under normal operation, and is normally expressed as the % RSD for a statistically significant number of samples. Precision may be performed at three different levels: system repeatability, method repeatability, and intermediate repeatability.

4.6.4.1. System Repeatability

The system repeatability studies the deviations of measurements under the same conditions and in the same measuring instrument. A precision criterion for an assay method is that the system repeatability (RSD) will be ≤ 1 %, and for the impurity assay, at the limit of quantitation, the instrument precision (repeatability) will be ≤ 5 %[139]. In this work, the system repeatability of the method was estimated by 36 determinations with 6 replicate injections of a standard solution. Six different concentration levels of a standard solution were tested (0.010, 0.025, 0.050, 0.100, 0.200 and 0.300 µg/ml) during the same day, under the same experimental conditions. The relative standard deviation (% RSD) of the analytes ranges from 7.37 % (for lowest standard elution concentration) to 0.53 % (for the highest standard elution concentration) (Fig. 4.45).

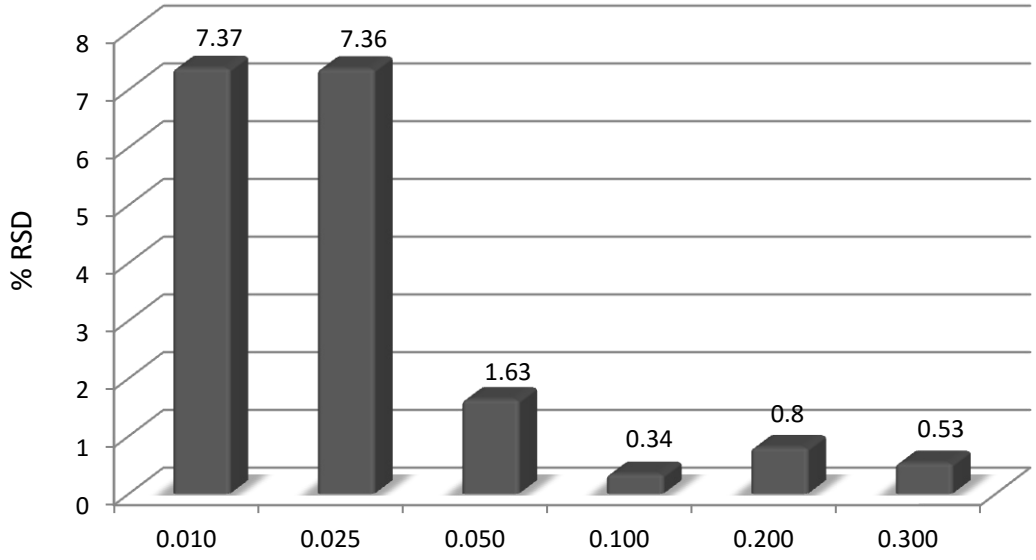


Figure 4.45: % Relative standard deviation (% RSD) of standard solution of cyanocobalamin in the concentration range of 0.01-2.00 µg/ml

4.6.4.2. Method Repeatability

Method repeatability (intra-day assay precision) is the results of the method operating over a short time interval under the same conditions (intra-assay precision). It should be determined from a minimum of six determinations at 100 % of the target concentration[139].

In this study, the method repeatability (intra-assay precision) was estimated by assaying 6 replicate samples from homogenous powder mixtures of *Hippophae rhamnoides* plants which were analyzed according to the described assay, during the

same day, under the same experimental conditions. The relative standard deviation (% RSD) of the analyte (peak area) was 0.7 % and the relative standard deviation of the retention time of the analyte was 0.4 % as presented in Table 4.7.

Table 4.7: Demonstration of method repeatability of the HPLC assay for vitamin B_{12} as shown by the results of 6 replicate injections of *Hippophae rhamnoides* mixtures

Injection number	R.T (min)	Peak area (mV s)
1	4.157	29,122
2	4.127	29,008
3	4.153	29,105
4	4.160	29,016
5	4.113	28,900
6	4.137	29,501
Mean	4.141	29,108
% RSD	**0.452**	**0.714**

4.6.4.3. Intermediate Repeatability

Intermediate precision (inter-day variation) is the results from within lab variations, due to random events, such as different days, analysts, equipment, etc. In determining intermediate repeatability, experimental design should be employed, so that the effects (if any) of the individual variables can be monitored. Precision criteria for an assay method is that the intra-assay precision will be ≤ 2 %, and for impurity assay, at the limit of quantitation, the instrument precision will be ≤ 5 %, and the intra-assay precision will be ≤ 10 %[139].

In this study, intermediate precision (within-laboratory variation) was demonstrated by two operators, by analyzing the same powder mixtures of *Hippophae rhamnoides*, in the same HPLC system. The RSD values from all operators were in the range of 0.8 % (n=2), less than 1%, and illustrated the good precision of the analytical method.

4.6.5. Limit of Detection and Quantification

The limit of detection (LOD) and limit of quantitation (LOQ) tests for the procedure are performed on samples containing very low concentrations of analyte. LOD is defined as the lowest amount of analyte that can be detected above baseline noise; typically, three times the noise level. LOQ is defined as the lowest amount of analyte

which can be reproducibly quantitated above the baseline noise, that gives S/N = 10^{139}.

The limit of detection (LOD) and limit of the quantification (LOQ) of the method were calculated with the linear calibration curve method. This method is based on the standard deviation of response and slope. Hence LOD and LOQ can be expressed as LOD= 3.3 σ/b, and LOQ=10 σ/b, where σ is the standard deviation of the response and b is the slope of the calibration curve (y=a+bx). The LOD of the method was 0.004 µg/ ml and LOQ was 0.014 µg/ ml (Appendices 6.2.2.).

Summary

An HPLC assay for determining active ingredients in pharmaceutical products, and subsequent method validation, can be complex and time-consuming. However, a well-defined protocol and documented validation plan simplifies and shortens the process, while at the same time provides the regulatory agencies evidence that the analytical system and method is suitable for its intended use. This dissertation was intended to perform a method validation study, for the determination of vitamin B_{12} in plant matrices, by generating both useful and meaningful data that meet all FDA, USP, and ICH validation requirements for pharmaceutical analysis. Vitamin B_{12} was quantified by an external 6-point calibration curve using cyanocobalamin solutions with concentrations between 0.01 – 0.3 µg/ ml. The calibration curve of pure solutions of vitamin B_{12} was linear (r^2= 0.999), and the range of the analytical method included values between 30 – 300 % of the target concentration. The limit of detection was 0.004 µg/ ml and the limit of quantification was 0.014 µg/ ml, both were calculated with the linear calibration curve method. The repeatability of the method (0.7 % RSD) was determined by measuring vitamin B_{12} in *Hippophae rhamnoides* berries (n=6) that underwent the method protocol. The accuracy, which ranged between 80 – 100 %, was determined by adding vitamin B_{12} to homogeneous samples of *Hippophae rhamnoides*, blank spike, *Elecampane* (*Inula helenium*), vitamin B_{12} tablets and Liver (veal). For the sample of *Hippophae rhamnoides* three levels of vitamin B_{12} (0.03 µg/ml, 0.10 µg/ml and 0.30 µg/ml) were added and quantified performing a sample preparation, extraction, purification and concentration at each level three times.

4.7. Results and Discussion

The vitamin B_{12} exists in free and bound form in food products. However, vitamin B_{12} in plant samples can be found only in bound form. The optimized method caused the release of all bound vitamin B_{12} and enabled the quantitative chromatographic isolation of the vitamin B_{12}. The extensive homogenization step, achieved to perfect the powder of solid samples such as *Hippophae rhamnoides* that due to insufficient homogenization, introduced significant deviations in vitamin B_{12} measurements and enhanced matrix effects. In addition, the application of the extensive homogenization step had a positive effect on vitamin B_{12} release in liquid samples (such as black mustard extract, *Hippophaes rhamnoides* juice, plant drugs 818, 819, 822 and 823 from Teutopharma), contributing to a better stirring and homogenization of the sample. The choice of pH 7 was crucial for obtaining the highest values of vitamin B_{12}. The results of HPLC showed significant amounts of vitamin B_{12} in several plant-based sources, with a maximum value of *Hippophae rhamnoides* plant containing 37.01 μg vitamin B_{12} / 100 g dry weight (Table 4.8). Some of the plants such as corn poppy (*Papaver rhoeas*) revealed traces of vitamin B_{12}, however, the quantification was impossible because the signal of the peak was below the detection limit of the method.

Table 4.8: Vitamin B_{12} concentration in different food sources*

Plant-Based Solid Samples	Vitamin B_{12} content (μg/ 100g)
Hippophae rhamnoides	37.01
Hippophae rhamnoides **(analyzed in an external lab with AOAC 952.20/ 45.2.02 Method)**	0.11
Hippophae rhamnoides **(analyzed in an external lab with Biacore Method)**	0.08
Hippophae rhamnoides **Granulate**	3.10
Hippophae rhamnoides **Extract (Bio-cultivation)**	0.00 (Below LOD)
Hippophae rhamnoides **(Bio-cultivation)**	4.58
Elymus **(dry extract)**	23.10
Elymus **(grinded)**	25.83
Elecampane (*Inula helenium*)	10.62

Tab. 4.9 continued

Cranberry	0.00
Black salsify	0.00
Parsnip (*Pastinaka sativa*)	0.00
Corn poppy (*Papaver Rhoeas*)	0.00
Garlic mustard (*Alliaria petiolata*)	0.00
vitamin B_{12} tablets	9,583.79 (49.03 µg/ Tablet)
Liver (veal)	65.59
Extracts	**Vitamin B_{12} content (µg/ 100g)**
***Hippophae rhamnoides* juice**	0.10
Black mustard (fluid)	1.52
Plant drug 818 Teutopharma	26.28
Plant drug 819 Teutopharma	25.06
Plant drug 822 Teutopharma	17.98
Plant drug 823 Teutopharma	13.26

*** all the values that are listed above (Table 4.8) are the measured amount of vitamin B_{12} by HPLC-UV without the correction from the recovery studies.**

4.7.1. Vitamin B_{12} Tablets – Release Study

The objective of this study was to evaluate retard tablets intended to release vitamin B_{12} (CN-Cobalamin) slowly, ideally within 4 h to release linear the total amount of the table. Two types of retard tablets were evaluated, 30 µg vitamin B_{12} / tablet and 50 µg vitamin B_{12} / tablet. Both were diluted in various proportions of deionized water solutions at pH 2, mimicking stomach conditions and measured in various durations (0h, 1h, 2h, 4h and 8h) by the HPLC-UV gradient elution profile (C).

The first experiment investigated the release time (h) of vitamin B_{12} from the tablet (30 µg vitamin B_{12} / tablet), diluted in 10 ml deionized water at pH 2. Replicate samples were measured and the results are plotted in Figure 4.46. Based on this dilution (V=10 ml) the expected final concentration is 3 µg/ ml vitamin B_{12}. The results demonstrated a linear regression ($y = 0{,}2978\ x + 0{,}112$, $R^2 = 0{,}958$) between the vitamin B_{12} and the release time during the first 4 hours. According to Figure 4.46 the concentration of vitamin B_{12} remains stable after 4 h at 1.23 µg/ ml. Hence, the release of the vitamin B_{12} content was incomplete and only 41 % of the vitamin B_{12} content of the table was released.

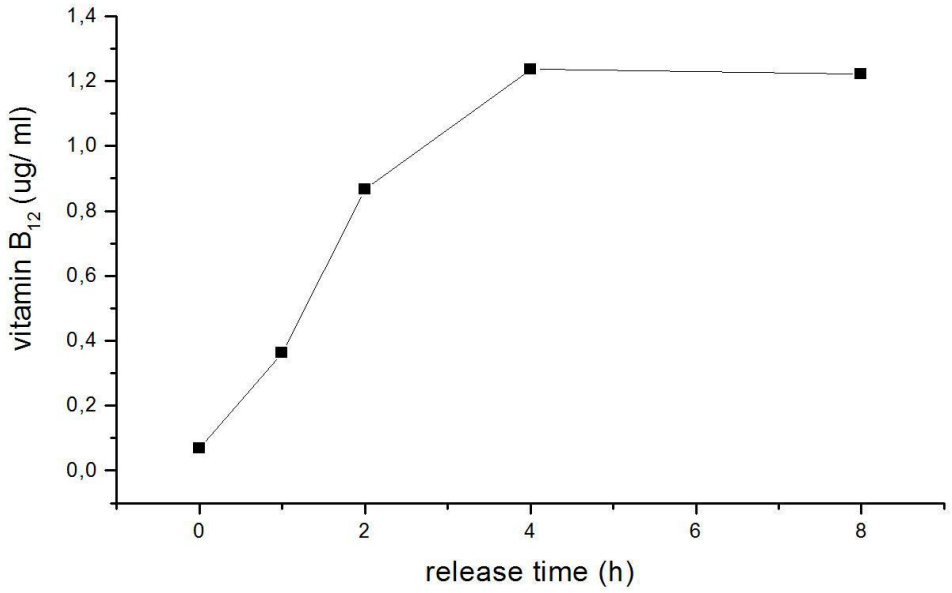


Figure 4.46: Release kinetics plot of vitamin B_{12} tablets at 5 different durations (0h, 1h, 2h, 4h and 8h), diluted in 10 ml deionized water (pH 2)

The second study investigated the release of vitamin B_{12} from the tablets containing 50 µg vitamin B_{12}, in different dilution volumes:

A) 20 ml
B) 35 ml
C) 50 ml

in the same five durations (0h, 1h, 2h, 4h and 8h) in deionized water (pH 2) (Fig. 4.47). The expected concentrations were A) 2.50 µg/ ml, B) 1.43 µg/ ml and C) 1.00 µg/ ml, respectively.

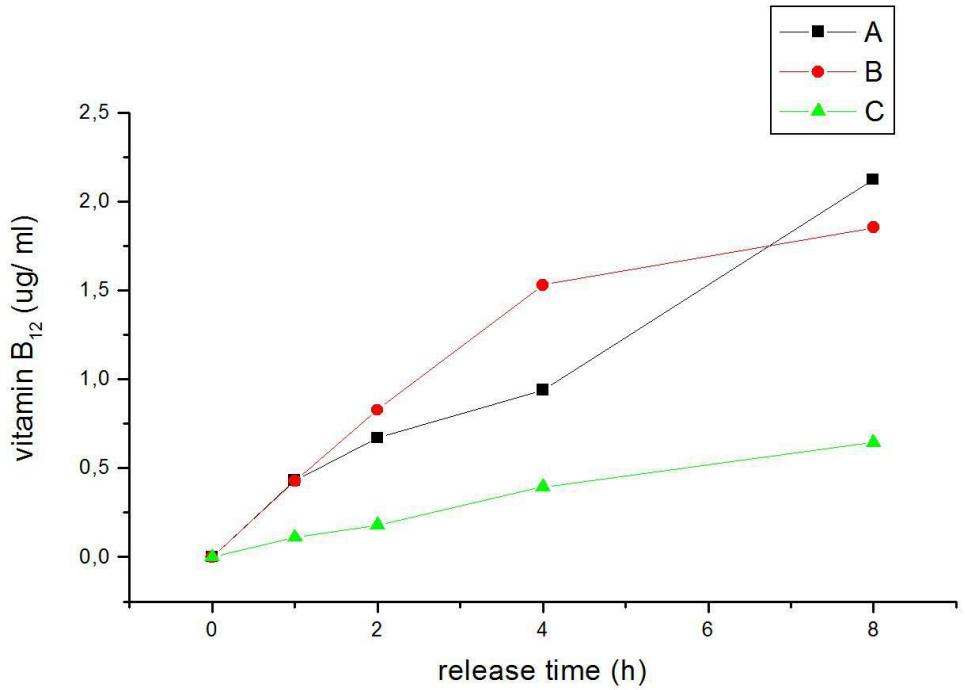


Figure 4.47: Release kinetics plot of vitamin B_{12} tablets at 5 different durations (0h, 1h, 2h, 4h and 8h), diluted in A) 20 ml deionized water, B) 35 ml deionized water and C) 50 ml deionized water, all at pH 2

According to Figure 4.47 the tablet diluted in 20 ml deionized water (A) released vitamin B_{12} content of 2.12 µg/ ml, meaning 85 % release of the total vitamin B_{12} content. Even though, the plot (A) follows a linear curve for the first 4 h, after 8 h there is an unexpected increase of the vitamin B_{12} release. In 35 ml deionized water (B) the released vitamin B_{12} content reached 1.85 µg/ ml, which is in contrast to the expected final concentration of 1.43 µg/ ml. Probably the dilution was between 25 – 30 ml deionized water. In 50 ml deionized water (C) the vitamin B_{12} release was at 0.65 µg/ ml, namely 65 % of the total vitamin B_{12} amount.

In conclusion, the dilution of the tablet in 20 ml deionized water (pH 2) contributed to the highest release of the vitamin B_{12}, approaching 85 % of the total amount.

4.7.2. HPLC-MS/MS – Investigation of Vitamin B_{12}

Due to the fact that high amounts of biological inactive analogues of vitamin B_{12} have been reported in other plant-based sources such as *Spirulina*, that can block vitamin B_{12} metabolism, it is essential to distinguish the real vitamin B_{12} from other vitamin B_{12} analogues (such as pseudo-vitamin B_{12}) in the plant samples. The lower ligand of real vitamin B_{12}, 5,6-dimethylbenzimidazole, is essential for the binding of the vitamin to the intrinsic factor for its absorption. Other inactive analogues of vitamin B_{12} have different lower ligands, e.g. pseudo-vitamin B_{12} has adenine as lower ligand. Microbiological assay (MBA), does not differentiate between active forms of vitamin B_{12} and other corrinoids, thus, an HPLC-MS/MS method was developed in order to measure the exact molecular mass of the measured vitamin B_{12} and distinguish the vitamin B_{12} forms.

Before the development of the new gradient elution profile by HPLC-MS/MS, the basic forms of vitamin B_{12}: CN-cobalamin, OH-cobalamin, adenosyl-cobalamin and methyl-cobalamin, were investigated relative to the retention time characteristics of their peak (Fig. 4.48), with fresh standard solutions by the optimized HPLC-UV gradient elution profile (C).

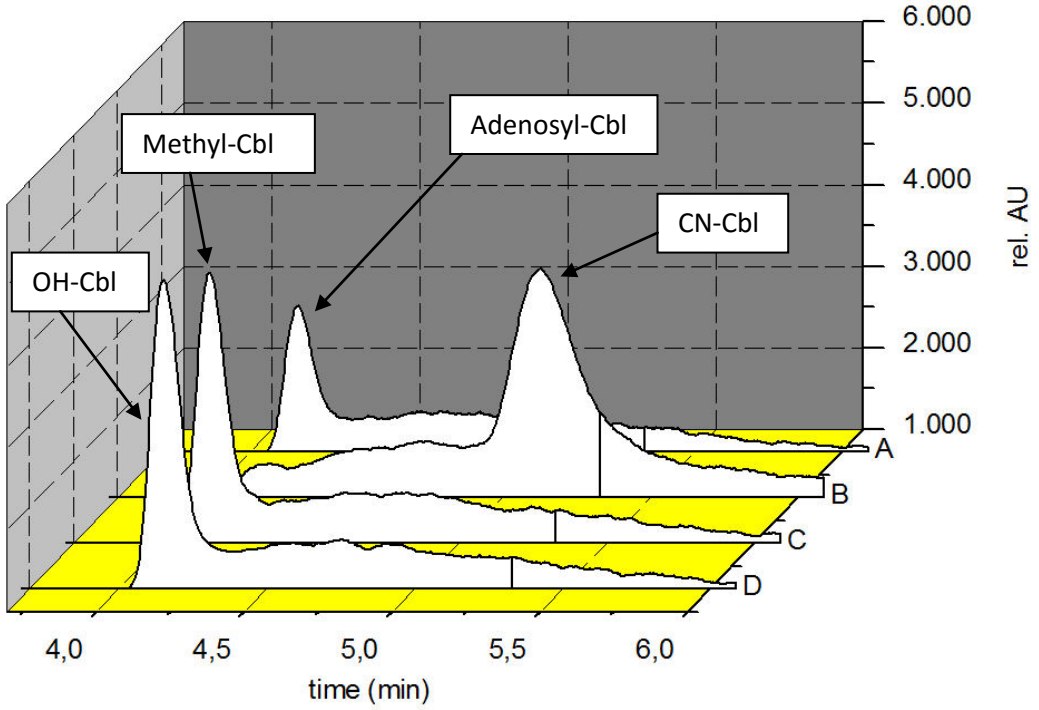


Figure 4.48: A) Chromatogram of adenosyl-cobalamin standard solution, B) Chromatogram of cyano-cobalamin (vitamin B_{12}) standard solution, C) Chromatogram of methyl-cobalamin standard solution, and D) Chromatogram of hydroxo-cobalamin standard solution

The gradient HPLC-UV profile (C) could separate the cyano-cobalamin (vitamin B_{12}) from the other forms of vitamin B_{12}, however, the same did not happen between the methy-cobalamin, adenosyl-cobalamin and hydroxo-cobalamin which disclosed the same retention time. An important observation was that all the standards had the same concentration (0.2 µg/ ml) but the size (peak area) and the shape of the peaks were different. That was expected, as the other forms of vitamin B_{12}, apart from CN-cobalamin, are sensitive to light and the losses of the vitamin came sooner. After using all the potentials of the HPLC-UV method for the characterization of vitamin B_{12} forms, it was decided to develop a more powerful detection profile with the use of mass spectrometry.

Prior to HPLC-MS/MS method, a fraction collector was connected with the HPLC-UV apparatus, using the optimized elution profile (C), and gathered the vitamin B_{12} fractions from *Hippophae rhamnoides*. Special attention was paid to gather only the peak of vitamin B_{12} without any of the co-eluted substances. A lot of fractions were gathered accumulative together and were kept in the freezer (- 80 ^{o}C) until further process. Then the fractions of vitamin B_{12} were dried under vacuum and reconstituted with 0.5 ml of 0.1 % formic acid (water solution). The final sample was analyzed by a new elution profile by HPLC-MS/MS (Appendices 6.1.2.5.). Based on the MS/MS spectra (Fig. 4.49), the corrinoid peak in *Hippophae rhamnoides* sample was confirmed as cyanocobalamin (vitamin B_{12}), as the molecular mass of the corrinoid matches with vitamin B_{12} (m/z: 1,355.37). The *Hippophae rhamnoides* sample and standard solution of vitamin B_{12} have the same fragmentation profile (the ratio of m/z 678.5 to m/z 1,355.6 is equal in both spectra). Due to low concentrations of vitamin B_{12} in the present samples the analysis in MS was conducted in SIM (single ion mode) in order to increase the sensitivity of the method. The chromatogram in Figure 4.49 of the *Hippophae rhamnoides* sample contains also inconsiderable peaks and “noise” at other retention times which may indicate the presence of other corrinoids in *Hippophae rhamnoides*. To the best of our knowledge, the main form of cobalamins in *Hippophae rhamnoides* is vitamin B_{12}.

In conclusion, with 6.5 g dried *Hippophae rhamnoides* berries an individual can reach the recommended daily amount of vitamin B_{12} (2.4 µg/day). In addition, the concentration of *Hippophae rhamnoides* in vitamin B_{12} approaches nearly 74 % of vitamin B_{12} content of one of the richest natural sources of vitamin B_{12} such as pig liver (59.7 µg/ 100 g)[37]. The same sample was analysed via the commercial Biacore assay and only 0.08 µg/ 100 g were found.

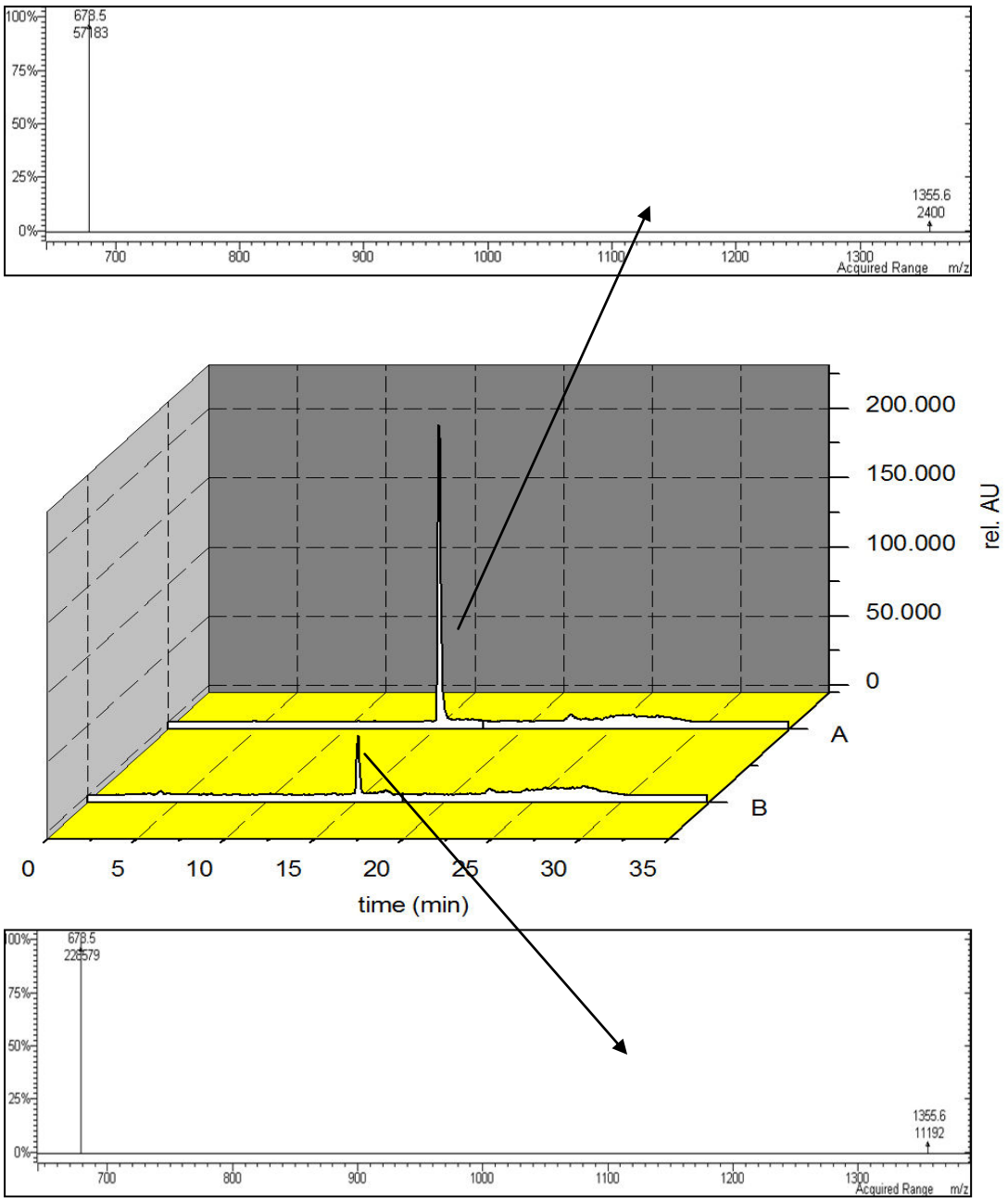


Figure 4.49: A) Chromatogram of cyanocobalamin (vitamin B_{12}) standard solution (C=0.25 µg/ml), and HPLC-MS/MS spectra of an m/z $[M+H]^+$ of 1,355 of cyanocobalamin (vitamin B_{12}), B) Chromatogram of immunoaffinity purified extract of 558 (*Hippophae rhamnoides*), and HPLC-MS/MS spectra of an m/z $[M+H]^+$ of 1,355 for the corrinoids in the immunoaffinity purified extract of the 558 *Hippophae rhamnoides*

5. Conclusions and Outlooks

A new assay procedure was developed, evaluated and applied to determine the vitamin B_{12} content in a variety of plant samples. After the implementation of an extended homogenization procedure (mill homogenizer, morter and pestle, disperser and ultrasonic bath), the method was validated in-house and demonstrated good selectivity, good recovery rates of 80 - 100 %, and good repeatability (0.7 % RSD) for the accurate determination of vitamin B_{12} in complex matrices with a limit of detection of 0.004 µg/ ml. The immunoaffinity columns in conjunction with HPLC-UV gives the opportunity to quantify vitamin B_{12} in a range of matrices, from the simplest matrices such as vitamin tablets, to a juice to more complex food matrices and plant samples. Despite the general agreement that plants can not synthesize vitamin B_{12}, actinorhizal plants such as *Hippophae rhammnoides* which are symbiotic with actinobacteria *Frankia alni* can cumulate amounts of vitamin B_{12}. Taking into account that *Frankia alni* has a symbiotic relationship with many plants, the chances of finding vitamin B_{12} in other plants are significant. The development of the new HPLC-UV assay for the isolation and analysis of vitamin B_{12} in a variety of samples (without depending on food origin, meaning animal-based source or plant-based source) in solid or liquid phase, in combination with the developed HPLC-MS/MS detection method, gives the opportunity to researchers not only to isolate and quantify vitamin B_{12} in a range of foods but also to identify the vitamin B_{12} forms. The forms can be investigated in terms of the nature of vitamin B_{12}, namely, whether the sample contains real vitamin B_{12} or inactive analogues of cobalamin such as pseudo-vitamin B_{12} which are inactive in human beings. Based on our recent results *Hippophae rhammnoides*, *Inula helenium*, *Elymus,* Black Mustard *(Brassica nigra),* have shown satisfactory amounts of vitamin B_{12}, and that raises hope for vegetarians to intake vitamin B_{12} from plant-based sources.

6. Appendices

6.1. Methods and Protocols

6.1.1. Initial Vitamin B_{12} Assay (protocol)

Note: Vitamin B_{12} is sensitive to light. All operations were conducted under subdued light, use of amber glassware or covered tubes.

6.1.1.1. Sample Preparation

9-10 g of the sample was ground and homogenized to a fine powder with the use of mortar and pestle, combined with the use of liquid nitrogen until the powder of the sample to be homogenized (the best possible).

6.1.1.2. Extraction of Vitamin B_{12}

Afterwards, an aliquot was accurately weighed and transferred into a flask.

- 60 ml of 50 mM acetate buffer (pH 4.0),
- 1 ml of KCN (1% w/v),
- 1 g pepsin
- 300 µl α-amylase

were added, and the suspension was incubated at 37 ^{o}C for 3 h under agitation. The enzymatic reaction was stopped by incubating the sample in boiling water at 100 ^{o}C for 30-35 min. After cooling to room temperature, the solution was centrifuged at 4,000 rpm for 25 min at 4 ^{o}C. The supernatant was collected and filtered through filter paper (589/2 Whatman, 90 mm). The solution was quantitatively transferred to a volumetric flask.

Plant samples with high moisture content were cut into small particles in paper containers. The containers were placed in an incubator and dried at 90 ^{o}C for 24 h. The dry weights of the plant samples were derived by this procedures. All data also from fresh plant samples were corrected to dry weight.

6.1.1.3. Purification and Concentration of Vitamin B_{12}

- **Column preparation**

The immunoaffinity columns (IAC) (EASI-EXTRACT Vitamin B_{12}, product code: R80B, R-BiopharmRhone Ltd., Darmstadt, Germany) were acclimated to ambient conditions by removing them from the refrigerator ≥ 30 min before use. The IAC was attached to a vacuum glass syringe barrel, which was connected to a 15 ml falcon tube and the column storage buffer was removed with a flow rate of 2 ml/ min.

- **IAC purification**

After the removal of the buffer, 35-40 ml of the sample were applied to the column. The drain valve was set so as the flow rate to be approximately 2 ml/ min, a constant vacuum is guaranteed by the oil rotary pump. The application under pressure is important for the complete binding of the cyanocobalamin to the antibodies.

- **Column washing**

The column was washed with 10 ml deionized water in 2 ml portions, and then was completely dried by using enhanced air pressure.

- **Elution of vitamin B_{12}**

The vitamin was eluted from the column, at a flow rate of 1 drop per second using 3 ml of methanol (100 % HPLC grade) and collected. At that point backflushing is recommended, so as to increase contact time of solvent with the antibody gel, ensuring that the entire vitamin was eluted.

- **Evaporation of methanol**

The eluate was dried at 60 ^{o}C under reduced pressure and reconstituted in 500 µl of mobile phase (0.025% TFA) before HPLC-UV analysis was conducted.

6.1.1.4. Analysis of Vitamin B_{12} (HPLC-UV) – Gradient profile (A)

-Column: Kinetex C_{18} column (100 mm x 4.6 mm, 2.6 µm)
-Pre-column: Kinetex C_{18} pre-column filter (4 mm x 3 mm ID)
-Eluents:

- Eluent A: 0.025% (w/v) trifluoroacetic acid (TFA) in water
- Eluent B: pure acetonitrile

Table 6.1: HPLC-UV gradient elution profile (A)

Time (min)	A: B (%)
0.0	100:0
0.5	100:0
0.7	85:15
10.0	85:15
13.0	100:0
18.0	100:0

- Column temperature: 30 °C
- Flow rate: 0.5 ml /min
- Wavelength of the UV detector: 361 nm
- Injection volume: 100 µl

6.1.1.5. Analysis of Vitamin B_{12} (HPLC-UV) – Gradient profile (B)

-Column: Kinetex C_{18} column (100 mm x 4.6 mm, 2.6 µm)

-Pre-column: Kinetex C_{18} pre-column filter (4 mm x 3 mm ID)

-Eluents:

- Eluent A: 0.025% (w/v) trifluoroacetic acid (TFA) in water
- Eluent B: pure acetonitrile

Table 6.2: HPLC-UV gradient elution profile (B)

Time (min)	A: B (%)
0.0	100:0
0.5	100:0
0.7	85:15
10.0	85:15
10.1	85:15
11.0	30:70
15.1	30:70
16.0	100:0
21.0	100:0

- Column temperature: 30 °C
- Flow rate: 0.5 ml /min
- Wavelength of the UV detector: 361 nm
- Injection volume: 100 µl

6.1.2. Optimized Vitamin B_{12} Assay (protocol)

Note: Vitamin B_{12} is sensitive to light. All operations were conducted under subdued light, use of amber glassware or covered tubes.

6.1.2.1. Sample Preparation

I) ***Solid samples***. The sample (0.5-10 g, depending on the content of vitamin B_{12} in the sample) underwent an extended homogenization process:

a. Mixer Mill, the sample (4 g in each container) was ground and homogenized to a fine powder, operating at 30 Hz for 2.5 min, and filling the jars with 55-60 ml liquid nitrogen.
b. Mortar and pestle (to perfect the fine sample).
c. Disperser: the sample from (b) was diluted in 10 ml buffer (in a 50 ml Falcon tube) and processed with 24,000 rpm/ min, for 6 minutes.
d. Ultrasonic bath: the Falcon tube with the dispersion after step C, was sunk into the ultrasonic bath for 30 min.

II) ***Liquid samples***. An aliquot of each sample (10-30 ml) was stirred well to ensure the homogeneity of the sample, and underwent only the steps C) and D) from the extended homogenization process, the rest of the process was proceeded as described in I).

6.1.2.2. Extraction of Vitamin B_{12}

Afterwards, the aliquot of 10 ml was accurately weighed and transferred into a flask.

- 50 ml of 50 mM acetate buffer (pH 4.0),
- 1 ml of KCN (1% w/v),
- 1 g pepsin
- 300 µl α-amylase

were added, and the suspension was incubated at 37 ^{o}C for 1.5 h under agitation. The enzymatic reaction was stopped by incubating the sample in boiling water at 100 ^{o}C for 30 min. After cooling in an ice bath for 30 min, the solution was centrifuged at 5,000 rpm for 25 min at 3 ^{o}C. The supernatant was collected and filtered through filter paper (589/2 Whatman, 90 mm). The pH was adjusted to pH 7 and the solution was quantitatively transferred to a volumetric flask and filled up to 100 ml with deionized H_20.

Plant samples with high moisture content were cut into small particles in paper containers. The containers were placed in an incubator and dried at 90 ^{o}C for 24 h. The dry weights of the plant samples were derived by this procedure. All data also from fresh plant samples were corrected to this dry weight.

6.1.2.3. Purification and Concentration of Vitamin B_{12}

- **Column preparation**

The immunoaffinity columns (IAC) (EASI-EXTRACT Vitamin B_{12}, product code: R80B, R-BiopharmRhone Ltd., Darmstadt, Germany) were acclimated to ambient conditions by removing them from the refrigerator ≥ 30 min before use. The IAC was attached to a vacuum glass syringe barrel, which was connected to a 15 ml falcon tube and the column storage buffer was removed with a flow rate of 2 ml/ min.

- **IAC purification**

A slow and steady flow rate is essential for the capture of the vitamin by the antibody. 20 ml of the sample were applied to the column (for optimal column performance the sample must be loaded onto the column containing a quantity of 0.01 -0.05 µg vitamin B_{12}. Do not exceed a quantity of 1.0 µg, as this is close to the capacity.)

- **Column washing**

The column was washed with 10 ml deionized water (5 ml/ min) and completely dried by using enhanced air pressure.

- **Elution of vitamin B_{12}**

The vitamin was eluted from the column, at a flow rate of 1 drop per second using 5 ml of methanol (100 % HPLC grade) and collected. The elution efficiency of 5 ml methanol is above 96 %, larger volumes of methanol could not improve the elution efficiency. At that point backflushing is recommended, so as to increase contact time of solvent with the antibody gel, ensuring that the entire vitamin was eluted.

- **Evaporation of methanol**

The eluate was dried at 60 ^{o}C under reduced pressure and reconstituted in 500 µl of mobile phase (0.025% TFA) before HPLC-UV analysis was conducted.

6.1.2.4. Analysis of Vitamin B_{12} (HPLC-UV) – Gradient profile (C)

-Column: Kinetex C_{18} column (100 mm x 4.6 mm, 2.6 µm)
-Pre-column: Kinetex C_{18} pre-column filter (4 mm x 3 mm ID)
-Eluents:

- Eluent A: 0.025% (w/v) trifluoroacetic acid (TFA) in water
- Eluent B: pure acetonitrile

Table 6.3: HPLC-UV gradient elution profile (C)

Time (min)	A: B (%)
0.0	100:0
0.5	100:0
0.7	85:15
7.0	85:15
7.1	30:70
12.0	30:70
12.1	100:0
18.0	100:0

- Column temperature: 30 °C
- Flow rate: 1.0 ml/ min
- Wavelength of the UV detector: 361 nm
- Injection volume: 100 µl

6.1.2.5. Analysis of Active forms of Vitamin B_{12} (HPLC-MS/MS gradient profile)

-Column: C_{18} Pyramin (250 mm x 4 mm, 5 µm)
-Eluents:

- Eluent A: 0.1 % (w/v) formic acid (in water)
- Eluent B: 0.1 % (w/v) formic acid (in acetonitrile)

Table 6.4: HPLC-MS/MS gradient elution profile

Time (min)	A: B (%)
0.0	90:10
3.0	90:10
23.0	0:100
33.0	0:100
38.0	90:10

- Ion source: ESI (+)
- SIM width: 0.700 amu total
- Detector is set a fixed voltage: 1200.00 volt
- Syringe pump flow rate: 1.00 µl/ min
- ESI needle voltage positive: +5000.00
- ESI needle voltage negative: +600.00
- Drying gas temperature: 350.00
- API housing temperature: 50.00
- Nebulizer gas pressure: 55.00
- Drying gas pressure: 30.00
- Flow rate: 0.3 ml /min
- Wavelength of the UV detector: 361 nm
- Scan range of m/z: 600 – 1600

6.1.3. Determination of Moisture Content (protocol) – Gravimetric Method

The determination of the moisture content of solid samples conducted following the above protocol:

1. Labeling of the vials with a code for each sample.
2. Drying of vials at 90 ^{o}C for 4 hours.
3. Cool down the vials (in a desiccator) at room temperature for 1 hour.
4. Careful weighing of the vials (4-digit analytical balance).
5. Addition of sample to vials and weighing (sample + vials).
6. Drying the samples+vials in an oven at 90 ^{o}C for 24 hours.
7. Cool down samples+vials (in a desiccator) at room temperature for 1 hour.
8. Careful weighing of samples+vials (4-digit analytical balance).
9. Calculation of moisture content.

The calculation of the moisture content was expressed in a fresh weight (FW) basis:

$$\% \text{ moisture}_{(\text{FW basis})} = \frac{\text{FW-DW}}{\text{FW}} \times 100$$

However, the fresh weight (FW) basis was preferred, because tends to minimize changes in water content. The results from the analysed samples are listed below in Table 6.5.

Table 6.5: % Moisture content of samples

Sample codes	Names	% moisture content
482 / 476 / 558	*Hippophae rhamnoides*	07.72
483 / 477	*Hippophae rhamnoides*	06.63
484	*Hippophae rhamnoides*	07.87
485	-	11.61
541	Celery powder	10.15
543	Black salsify	8.16
544	Parsnip (*Pastinaka sativa*) powder	11.22
545	Elecampane (*Inula helenium*)	6.68
546	Corn poppy (*Papaver rhoeas*)	12.59
547	Garlic mustard (*Alliaria petiolata*)	09.04
548	Sea buckthorn berries, organic farming Europe	13.66
549	Sea buckthorn berries, organic farming Europe	08.65
550	Sea buckthorn granule, Dr. Pandalis	10.11
551	Sea buckthorn extract, organic farming Europe	08.73
554	Sea buckthorn berries, organic farming Europe	12.82
556	-	06.13
493	-	03.79
818	-	0.00
819		02.74
820	Dry extract of European sort of couch grass radix (*Agropyron repens*)	02.48
821	Grinded European sort of couch grass radix (*Agropyron repens*)	07.86
823	-	0.63
	Cranberries	13.29
584	-	12.82

6.2. Statistical Determinations

6.2.1. Statistical Determination of the Incubation Time

In order to compare the concentration of vitamin B_{12} in 3 three incubation times, two samples of each incubation time were applied to the method (6.1.1). Then each

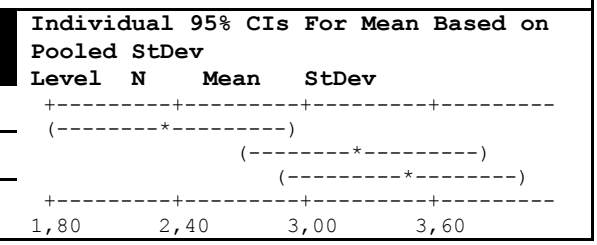
sample was measured for the determination of vitamin B_{12} concentration. In order to test for the equality of means and to assess the differences between pairs of means, it was used one-way ANOVA with one factor (incubation time) with three levels (0.5, 1.5 and 3.0 h). The results of the command ANOVA are shown in Table 6.6.

Table 6.6: One-way ANOVA: Vitamin B_{12} concentration (µg/ 100 g) versus Incubation time (h)

Source	DF	SS	MS	F	P
Incubation time (h)	2	1,3052	0,6526	10,19	0,046
Error	3	0,1922	0,0641		
Total	5	1,4975			

S	R-Sq	R-Sq(adj)
0,2531	87,16%	78,61%

Level	N	Mean	StDev
0,5	2	2,3695	0,0827
1,5	2	3,1815	0,3995
3,0	2	3,4715	0,1605



The p-value for the incubation time ANOVA is less than 0.05. This result indicates that the average differences between the incubation time and the concentration of vitamin B_{12} is statistically significant. Thus, some of the group means are different.

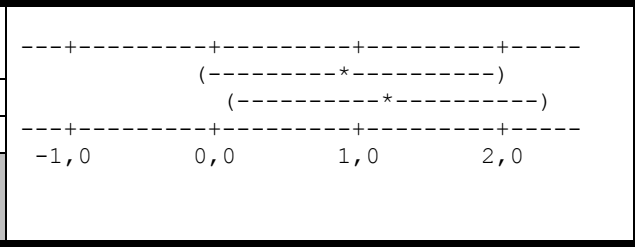
For further investigation the Tukey comparison results to formally test whether the difference between a pair of groups is statistically significant. The Table 6.7 that includes the Tukey simultaneous confidence intervals show that the confidence interval for the difference between the incubation time 1.5 h and 3.0 h were overlapped. This indicates that the differences are not significant.

Table 6.7: Grouping information using the Tukey method

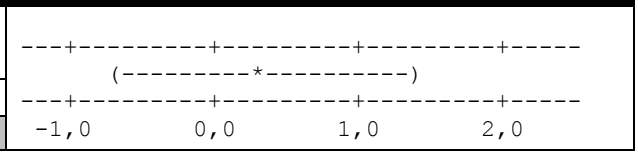
Incubation time (h)	N	Mean	Grouping
3,0	2	3,4715	A
1,5	2	3,1815	A B
0,5	2	2,3695	B
Means that do not share a letter are significantly different.			
Tukey 95% Simultaneous Confidence Intervals All Pairwise Comparisons among Levels of Incubation time (h)			
Individual confidence level = 97,50%			

Tab. 6.8: continued

Incubation time (h) = 0,5 subtracted from:				
Incubation time (h)	Lower	Center	Upper	
1,5	-0,2458	0,8120	1,8698	
3,0	0,0442	1,1020	2,1598	



Incubation time (h) = 1,5 subtracted from:				
Incubation time (h)	Lower	Center	Upper	
3,0	-0,7678	0,2900	1,3478	



6.2.2. Statistical Determination of LOD and LOQ

For the determination of the limit of detection (LOD) and the limit of quantification (LOQ), and in order to avoid subjective estimations such as the use of visual evaluation methods or not so accurate methods such as methods based on signal to noise (S/N) approach, the LOD and LOQ were calculated with the linear calibration curve method. According to the formula: LOD= 3.3 σ/b, and LOQ=10 σ/b, where σ is the standard deviation of the response and b is the slope of the calibration curve (y=a+bx). However the σ can be expressed as

I. The standard deviation of blank spike
II. The residual standard deviation of the regression line
III. The standard deviation of the y-intercepts of regression lines

Based on fresh standard solutions of vitamin B_{12} a regression line was produced (see chapter 4.5.1.1. – Table 4.3 and Table 4.5) with the statistical program MINITAB 17 resulting the following data:

peak area = 81 + 325621 vitamin B_{12} (µg/ ml)

slope = 325621

standard deviation of y-intercept = 461

The LOD and LOQ were calculated based on the (iii) approach:

LOD= (3.3 * 461)/ 325621=0.00467 => **LOD = 0.004 µg/ ml**

LOQ= (10 * 461)/ 325621=0.01415 => **LOQ = 0.014 µg/ ml**

6.3. Samples - Ingredient List

6.3.1. Vitamin B_{12} Tablets Ingredients (per 100 g)

Microcrystalline cellulose, maltodextrin, L-glutamine (11.8%)
Coating compositions: hydroxypropylmethylcellulose, sodium carboxymethylcellulose, hydroxypropylcellulose, L-serine (7.8%)
Separating agent: magnesium salts of fatty acids, cyanocobalamin (vitamin B_{12} 0.006%)
Excipient: mannitol, vegetable oil (coconut)
Caking agent: talc, fatty acids
Dyes: titanium dioxide, crimson, yellow iron oxide

6.4. Materials

6.4.1. Reagents and Buffers

Name	Company, country
Cyanocobalamin (vitamin B_{12})	pr. code: V2876, Sigma-Aldrich (Seelze, Germany)
Hydroxocobalamin	pr. code: H1428000, Sigma-Aldrich (Seelze, Germany)
Methylcobalamin	pr. code: M9756, Sigma-Aldrich (Seelze, Germany)
Coenzyme B_{12}	pr. code: C0884, Sigma-Aldrich (Seelze, Germany)
Sodium acetate trihydrate	pr. code: 71188, Sigma-Aldrich (Seelze, Germany)
Pepsin	pr. code: 77161, Sigma-Aldrich (Seelze, Germany)
Trifluoroacetic acid (TFA)	pr. code: T6508 Sigma-Aldrich (Seelze, Germany)
EDTA	pr. code: 03682 Sigma-Aldrich (Seelze, Germany)
L-carnosine	pr. code: C9625 Sigma-Aldrich (Seelze, Germany)
Copper(II) sulfate pentahydrate	pr. code: C3036 Sigma-Aldrich (Seelze, Germany)
Methanol (gradient grade for HPLC)	catalog. number 20864.320, VWR (Darmstadt, Germany)
Acetonitrile (gradient grade for HPLC)	catalog number 20060.320 VWR (Darmstadt, Germany)
Liquid nitrogen	- , Linde (Pullach, Germany)
Immunoaffinity columns	pr. code: R80B, R-BiopharmRhone Ltd., Darmstadt, Germany)
Stainzyme (α-amylase)	pr. code: NEN0019, Novozymes (Bagsværd, Denmark)
Potassium cyanide	pr. code: 31252, Riedel-de Haën (Seelze, Germany)
Ascorbic acid	Fluka Chemie GmbH (Buchs, Switzerland)
Acetic acid	Sigma-Aldrich (Seelze, Germany)
Sea buckthorn (*Hippophae rhamnoides*)	Teutopharma (Glandorf, Germany)
Black salsify	Teutopharma (Glandorf, Germany)
Parsnip (*Pastinaka sativa*)	Teutopharma (Glandorf, Germany)

Elecampane (*Inula helenium*)	Teutopharma (Glandorf, Germany)
Corn poppy (*Papaver rhoeas*)	Teutopharma (Glandorf, Germany)
Garlic mustard (*Alliaria petiolata*)	Teutopharma (Glandorf, Germany)
Sea buckthorn (*Hippophae rhamnoides*)	Naturix24 (Dransfeld, Germany)
Sea buckthorn juice	Alnavit (Bickenbach, Germany)
Cranberry fruits	Seeberger (Ulm, Germany)
Liver (veal)	Wurst-Basar (Ronnenberg, Germany)
Vitamin B_{12} tablets	Merz Pharma GmbH & Co. KGaA (Frankfurt am Main, Germany).

6.4.2. Equipment

Apparatus	Name, company
Water purification system	Arium 611 system, Sartorius (Gottingen, Germany)
Analytical balance	TE412, Sartorius (Gottingen, Germany)
Analytical balance	TE2145, Sartorius (Gottingen, Germany)
Analytical balance	AC210S, Sartorius (Gottingen, Germany)
Ice machine	Ziegra Eismaschinen GmbH, (Isernhagen, Germany)
Filter papers	pr. code. 10300109, Whatman (Dassel, Germany)
Pipettes	Eppendorf AG, (Hamburg, Germany)
Centrifuge Tubes	Corning BV Life Sciences, (Amsterdam, Netherland)
Vacuum glass syringe barrel	pr. code. No. 5-7044, Supelco (Deisenhofen, Germany)
pH meter	766 Knick Elektronische Messgeräte GmbH & Co.KG, (Berlin, Germany)
Freeze Dryer	GAMMA 1-16 LSC plus Martin Christ GmbH, (Osterode, Germany)
Mixer Mill	MM400, Retsch (Haan, Germany)
Dispersion homogenizer	Ultra-Turrax T-25, IKA (Staufen, Germany)
Rotational vacuum concentrator system	RVC 2-18, Christ (Harz, Germany)
HPLC system	Hitachi Chromaster, VWR-Hitachi, (Darmstadt, Germany)
DAD-UV detector	5430 Diode Array detector, VWR-Hitachi, (Darmstadt, Germany)
Auto-sampler	Hitachi Chromaster 5210, VWR-Hitachi, (Darmstadt, Germany)
Pump	Varian 212 LC, (Darmstadt, Germany)
UV-Vis detector	Varian Pro Star 325, (Darmstadt, Germany)
TQ-MS mass spectrometer	Varian 320, (Darmstadt, Germany)
Column	Eurospher 100-C18-5-HD, Macherey-Nagel, (Düren, Germany)
Kinetex C_{18} column	Phenomenex, (Torrance, CA, USA)
Kinetex C_{18} pre-column filter	Phenomenex, (Torrance, CA, USA)

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Curriculum vitae

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EDUCATION

2012 – 2016	Doctor of Natural Sciences (Dr. rer. nat.) at the Institute of Technical Chemistry, Leibniz University of Hanover, Germany Supervisor: Prof. Dr. Thomas. Scheper Doctoral thesis title: "Quantitative Determination of Vitamin B_{12} in Plants"
2010 – 2012	Master of Science (M.Sc.) in Quality Management and Production Organization Systems for the Food Industry Department of Food Technology, Alexander Technological Educational Institute, Thessaloniki, Greece Master thesis title: "Extraction and Characterization of Hydrocoloids from Plant Extracts (Okra and Salep)" Graduation grade: "Excellent"
2000 – 2008	Diploma in Chemistry Department of Chemistry, University of Ioannina, Greece Principal subjects: Industrial Chemistry and Food Chemistry Diploma thesis title: "The energy future of humanity after oil depletion" Graduation grade: "Good"

PROFESSIONAL EXPERIENCE

2012 – 2016	Researcher and Doctoral Candidate Institute for Technical Chemistry, Leibniz University of Hanover Research: Development of Analytical Methods for the Isolation and Purification of Biomolecules from Natural Matrices and Characterization of Active Forms Teaching: Practical Courses ("Phytopharmaceuticals", "Basic Operation for the Production of Active Pharmaceutical Ingredients" and "Carnitine Analytics")
2010 – 2012	Research Assistant Department of Food Technology, Alexander Technological Educational Institute, Thessaloniki, Greece Approved Project: "Isolation and characterization of Polysaccharides and oligosaccharides from okra (Abelmoschus esculentus)", (Archimedes III action, GSRT, Greece) (budget 75,000 Euros)
2004 – 2012	Self Employed Private Tutor, Thessaloniki, Greece Private Tutoring in Chemistry and Physics 09/2007 – 09/2008: Military Service
2004	Chemist-Analyst (Practicum) General Chemical State Laboratory, Department of Fuels and Oils, Thessaloniki, Greece Analysis of: density, flash point, CFPP, viscosity, distillation characteristics, flow point, moisture, sulfur content, PAHs, tracers (kinizarini, SY124)

RESEARCH INTERESTS

Determination of active biomolecules from natural matrices, development and validation of analytical methods, extractions and isolations of biomolecules, physicochemical characterization of colloids and emulsions, nanotechnology, polymer and interfacial chemistry

Publications

2016 **Isolation and Analysis of vitamin B_{12} from Plant Samples**
M. Nakos, I. Pepelanova, S. Beutel, U. Krings, R. Berger, T. Scheper.
Article, *Food Chemistry*. Submitted

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