Food Chemistry 216 (2017) 301–308

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Isolation and analysis of vitamin B_{12} from plant samples

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ARTICLE INFO

Article history:
Received 21 March 2016
Received in revised form 10 July 2016
Accepted 13 August 2016
Available online 16 August 2016

Keywords:
Vitamin B_{12} (cobalamin)
Plant-based foods
Hippophae rhamnoides
Frankia alni
Immunoaffinity chromatography (IAC)
HPLC-UV
HPLC-MS/MS

ABSTRACT

Based on increased demands of strict vegetarians, an investigation of vitamin B_{12} content in plant sources, was carried out. The vitamin B_{12} concentration was determined by RP-HPLC with UV detection, after prior matrix isolation by immunoaffinity chromatography (IAC). Vitamin B_{12} was extracted in the presence of sodium cyanide, to transform all forms of cobalamin into cyanocobalamin. Diode array detector was used to monitor vitamin B_{12}, after its chromatographic separation under gradient elution with a mobile phase consisting of acetonitrile and trifluoroacetic acid 0.025% (w/v). The method demonstrated excellent linearity with a limit of detection 0.004 μg/ml. The method precision was evaluated for plant samples and it was below 0.7% (n = 6). Significant amounts of vitamin B_{12} in plants were detected in *Hippophae rhamnoides* (37 μg/100 g dry weight), in *Elymus* (26 μg/100 g dry weight) and in *Inula helenium* (11 μg/100 g dry weight).

1. Introduction

One of the most important groups of substances for normal cell function, growth and development are the vitamins. Lack of sufficient amounts of any of them can cause severe physiological problems. Vitamin B_{12} belongs to a group of compounds (corrinoids), which all contain a complex ring system with cobalt as a central atom (Guggisberg, Risse, & Hadorn, 2012); it is the only water-soluble vitamin that can be stored in the liver for many years (Carmel, 1996). The major forms of vitamin B_{12} (cobalamin) compounds are cyanocobalamin, adenosylcobalamin, methylcobalamin and hydroxycobalamin (Heudi, Kilinç, Fontannaz, & Marley, 2006); 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 (Marley, Mackay, & Young, 2009).

Vitamin B_{12} is acting as a co-enzyme and plays an important role in promoting carbohydrate and normal fat metabolism, it is essential in the formation of red blood cells, the normal functioning of the nervous system and in the translocation of the methyl group in DNA synthesis (Baker & Miller-Ihli, 2000; Szterk, Roszko, Małek, Czerwonka, & Waszkiewicz-Robak, 2012). Although vitamin B_{12} deficiency 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 (Pawlak, James, Raj, Dugan, & Lucas, 2012). According to the Institute of Medicine (National Academies, USA), the recommended daily allowance (RDA) for the vitamin B_{12} is 2.4 μg/d (Institute of Medicine, 1998).

Cobalamin is unique in 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 (Burgess, Smid, & van Sinderen, 2009). However, there is evidence that nitrogen fixing actinobacteria *Frankia alni* produce 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) (Wall, 2000). Owing to this symbiosis, content of vitamin B_{12} is possible

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http://dx.doi.org/10.1016/j.foodchem.2016.08.037
0308-8146/

to be found also in plants (Kysil, 2013). 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.

Major sources of vitamin 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} (Pawlak, Parrott, Raj, Cullum-Dugan, & Lucus, 2013; Kumar, Chouhan, & Thakur, 2010), 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} (Watanabe et al., 2012; Watanabe, Yabuta, Tanioka, & Bito, 2013). 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 (Watanabe, Katsura, et al., 1999). Moreover, widely consumed edible algae such as dried green (*Enteromorpha* sp.) and purple (*Porphyra* sp.) lavers (*Nori*) contain considerable amounts of vitamin B_{12} analogues (133 μg/100 g dry weight), however the biological activity of those algae-derived corrinoids in humans still remains unclear (Watanabe et al., 1999; Miyamoto, Yabuta, Kwak, Enomoto, & Watanabe, 2009; Watanabe, Takenaka, Kittaka-Katsura, Ebara, & Miyamoto, 2002). Actinorhizal plants such as *Hippophae rhamnoides* and *Myrica* which are symbiotic with actinobacteria *Frankia alni* are potential hosts for vitamin B_{12} corrinoids (Kysil, 2013; Kato, Kanayama, Ohkawa, & Kanahama, 2007).

Some of the vitamin B_{12} analogues, apart from the fact of being biological inactive, can also block the vitamin B_{12} metabolism in mammalian cells (Kondo et al., 1982). 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, fermented foods have been tested. More specifically, fermented foods such as Tempeh (type of soybean-based product) contain 0.7–8 μg/100 g vitamin B_{12}, sauerkraut (7.2 μg/100 g) and fenugreek juice fermented with lactic acid bacteria (12.5 μg/100 ml). Thus, strict vegetarians are at higher risk for developing cobalamin deficiency than non-vegetarians, 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.

An investigation of the presence of vitamin B_{12} in natural plant matrices was conducted, so as to enable strict group of vegetarians (e.g. vegans) to ingest vitamin B_{12} from an appropriate food source. Because vitamin B_{12} exists in very low concentrations in plants, the sensitivity of the analytical method and the sample preparation are essential steps. 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 was applied. Heudi et al. (2006) have shown that this method is a good alternative to the standard microbiological assay (MBA) for cobalamin determination in food products such as milk-based infant formula powder. Other research groups have applied this immunoaffinity method for the analysis of vitamin B_{12} enriched products (Marley et al., 2009); or for determining the vitamin B_{12} content of different meat products and salami (Guggisberg et al., 2012). Watanabe et al. (2012) have implemented immunoaffinity columns after a solid phase extraction and a concentrating step in the determination of the vitamin B_{12} content of common edible mushrooms (Watanabe et al., 2012).

The aim of this study was to investigate and analyze vitamin B_{12} in natural plant matrices by developing a protocol based on established non-plant methods (Guggisberg et al., 2012; Heudi et al., 2006; Marley et al., 2009; Watanabe et al., 2012). This is the first time that IAC extraction in combination with HPLC-UV has been successfully applied for the analysis of berry samples and other plant matrices. Furthermore, our findings show the strengths of the optimized method which was tested in several plant matrices but also in meat samples and commercial vitamin B_{12} tablets. Special attention was paid to the homogenization procedure in which some samples needed an extra treatment in order to gain a very fine powder suitable for analysis.

2. Experimental section

2.1. Chemicals and reagents

Cyanocobalamin (vitamin B_{12}) (product code: V2876), sodium acetate trihydrate (product code: 71188), pepsin (product code: 77161), and trifluoroacetic acid (TFA) (product code: T6508) were all purchased from Sigma-Aldrich (Seelze, Germany). Methanol (gradient grade for HPLC) (catalog number 20864.320) and acetonitrile (gradient grade for HPLC) (catalog number 20060.320) were obtained from VWR (Darmstadt, Germany). Liquid nitrogen was obtained from Linde (Pullach, Germany). Stainzyme (α-amylase) (product code: NEN0019) was purchased from Novozymes (Bagsværd, Denmark). Potassium cyanide (product code: 31252) is available from Riedel-de Haën (Seelze, Germany).

2.2. Apparatus and materials

Deionized water was purified using an Arium 611 system based on a carbon-resin technology purchased from Sartorius (Gottingen, Germany) for the preparation of buffer solution (acetate buffer, pH 4) and for dilution; balances TE412, TE2145 and AC210S were purchased from Sartorius (Gottingen, Germany). Filter papers (589/2 Whatman, 90 mm) (product code. 10300109) were purchased from Whatman (Dassel, Germany). The vacuum glass syringe barrel (cod. No. 5-7044) was sourced from Supelco (Deisenhofen, Germany). The Mill MM400 was received from Retsch (Haan, Germany) and the Homogenizer (Ultra-Turrax T-25) was available from IKA (Staufen, Germany). The ultrasonic bath (Sonorex RK510H) was available from Bandelin (Berlin, Germany). The rotational vacuum concentrator (rotary evaporator) was purchased from Christ (Harz, Germany).

2.3. Samples

Sea buckthorn (*Hippophae rhamnoides*) berries and granulates, couch grass (*Elymus repens*), black salsify, parsnip (*Pastinaka sativa*), elecampane (*Inula helenium*), corn poppy (*Papaver rhoeas*), garlic mustard (*Alliaria petiolata*) were obtained from Teutopharma/Dr. Pandalis group (Glandorf, Germany). Sea buckthorn (*Hippophae rhamnoides*) berries purchased from Naturix24 (Dransfeld, Germany), sea buckthorn juice was obtained from Alnavit (Bickenbach, Germany) and bio-cultivations around Europe. Cranberry fruits were acquired from Seeberger (Ulm, Germany), liver (veal) was obtained from local food store (Wurst-Basar, Ronnenberg, Germany) and vitamin B_{12} tablets from Merz Pharma GmbH & Co. KGaA (Frankfurt am Main, Germany).

2.4. Preparation of stock and standard solutions

10 μg/ml vitamin B_{12} stock solution was prepared by weighing 1 mg vitamin B_{12} (cyanocobalamin) into a 100 ml amber volumetric flask. The vitamin B_{12} was diluted by filling up to 100 ml with deionized water, and the solution was placed in an ultrasonic bath for 15 min for complete dissolution. The stock solution is stable for 3 months at 4 °C. The stock solution was diluted to the following concentrations in order to prepare a six-point standard calibration curve: 0.01, 0.025, 0.05, 0.1, 0.2 and 0.3 μg/ml. The standard solutions series were measured in triplicate using the HPLC method as described below.

2.5. Sample preparation – extraction of vitamin B_{12}

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

I) *Solid samples*. The sample (0.5–10 g, depending on the content of vitamin B_{12} in the sample) was ground and homogenized to a fine powder in a Mill, operating at 30 Hz for 2.5 min, while filling the jars with 55–60 ml liquid nitrogen. 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 and 300 μl α-amylase were added, and the suspension was incubated at 37 °C for 1.5 h under agitation. The enzymatic reaction was stopped by incubating the sample in boiling water at 100 °C for 30–35 min. After cooling to room temperature, the solution was centrifuged at 4000 rpm for 25 min at 4 °C. The supernatant was collected and filtered twice 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 pieces in paper containers. The containers were placed in an incubator and dried at 90 °C for 24 h. The dry weights of the plant samples were derived by these procedures. All data also from fresh plant samples were corrected to this dry weight.

II) *Liquid samples*. An aliquot of each sample (10–30 ml) was stirred well to ensure the homogeneity of the sample, the rest of the process proceeded as described in I).

2.6. Purification of vitamin B_{12} via immunoaffinity column

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 $\geqslant$30 min before use. The IAC was attached to a vacuum glass syringe barrel and the column storage buffer was removed with a flow rate of 2 ml/min. 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. The column was washed with 10 ml deionized water (5 ml/min) and completely dried by using enhanced air pressure. After that, 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%, while 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 all of the vitamin present was eluted. The eluate was dried at 60 °C under reduced pressure and reconstituted in 500 μl of mobile phase (0.025% TFA) before HPLC-UV analysis was conducted.

2.7. HPLC – analysis

The analysis of the sample was performed using a Hitachi Chromaster HPLC system (VWR-Hitachi, Darmstadt, Germany) equipped with a DAD-UV detector (5430 Diode Array detector, VWR-Hitachi, Darmstadt, Germany). Sample injections of 100 μl were made from the Hitachi Chromaster 5210 auto-sampler. The chromatographic separations were achieved with a Kinetex C_{18} column (100 mm × 4.6 mm, 2.6 μm) fitted with a Kinetex C_{18} pre-column filter (4 mm × 3 mm ID, Phenomenex, Torrance, CA). The column oven temperature was 30 °C. Gradient elution was performed 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. Gradient conditions were 100% A at the first 0.5 min, from 0.7 min decreased to 85% A over 7.0 min, from 7.1 min decreased further to 30% A and held for 5 min, then returned to 100% A at 12.1 min and held for 5.9 min. The total run time was 18.0 min. The vitamin B_{12} was monitored at 361 nm and was detected with a retention time of t_R = 4.40 min.

2.8. Safety considerations

Potassium cyanide: Fatal if swallowed, inhaled, or comes in contact with skin. Potassium cyanide is a highly toxic, colorless crystalline compound. Consequently, solutions and extracts should be handled with extreme care under a fume hood. Gloves and other protective clothing must be worn as a safety precaution during the handling of this compound. Potassium cyanide residues can be decontaminated using 15% (w/v) sodium hypochlorite.

Trifluoroacetic acid: (TFA): Causes severe burns and eye damage. Wearing of protective gloves, clothing, eyewear, and face protection are necessary. Used only in an effective fume hood to remove vapors generated.

3. Results and discussion

An analytical assay was developed to isolate and determine the vitamin B_{12} content in plants quantitatively. For this purpose a sensitive and selective HPLC method with UV detection and an extraction protocol were established. A gradient elution of the mobile phase consisting of 0.025% (W/V) TFA and pure acetonitrile at a flow rate 1.0 ml/min was found to be optimal for the separation of vitamin B_{12}, as described in the section 2. 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. In the present work, the selectivity and the quantification limit were improved using immunoaffinity columns for vitamin B_{12} enrichment prior to analysis (see Supplementary Fig. 5).

3.1. Effect of sample preparation on cobalamin recovery

Due to the different nature of the samples used for the evaluation of the method, the effectiveness of the sample preparation was tested by recovery studies. 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 samples of cobalamin. Samples such as liver, vitamin B_{12} tablets, *Inula helenium* and blank samples showed good recovery that ranged between 80 and 100%. However, the recovery from *Hippophae rhamnoides* berries was very low (4.6%). A low recovery can occur for two reasons. The first reason is matrix effects (Takenaka et al., 1997) and the second is

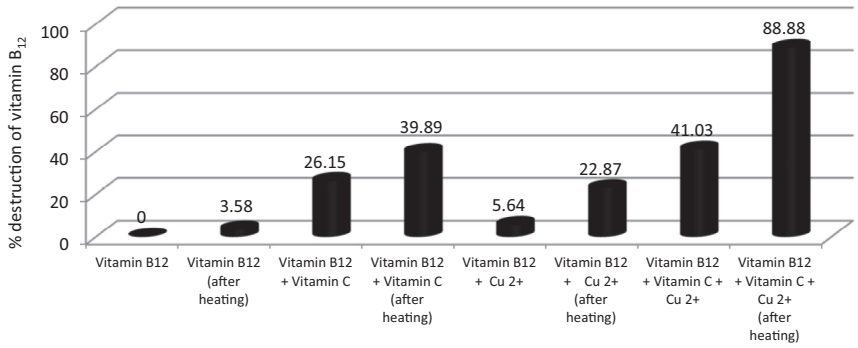

Fig. 1. The % destruction of the vitamin B_{12} in the presence of vitamin C and Cu^{2+}, at ambient temperature and after a heating step (100 °C for 30 min).

insufficient homogenization. The influence of these effects was tested with the following experiments.

I) *Matrix effects*. The matrix effects were investigated based on the assumption that plants have high contents of minerals (especially in *Hippophae rhamnoides* (Skuridin et al., 2013)) and high contents of vitamin C (both *Hippophae rhamnoides* (Gutzeit, Baleanu, Winterhalter, & Jerz, 2008) and cranberries (Viskelis et al., 2009)). 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 masking or destruction of vitamin B_{12}. Under this hypothesis, possible interactions of vitamin B_{12} with the vitamin C, copper cations and their combinations were investigated. 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 °C for 30 min. Following the method protocol from the IAC step until the determination of vitamin B_{12} in HPLC, the results (Fig. 1) 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. (1997), carnosine (a natural water-soluble antioxidant) can prevent the destruction of vitamin B_{12} in the presence of vitamin C and copper. In order to prove that, mixtures of vitamin B_{12}, vitamin C and Cu^{2+} in concentrations similar to that of 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 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) (see Supplementary Fig. 6).

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

II) *Insufficient homogenization*. The case of insufficient homogenization of the sample (*Hippophae rhamnoides*) was tested by adding two more grinding methods (Homogenizer and ultrasonic bath). Each grinding instrument has different size reduction mechanism. The mortar and pestle technique uses friction by sandwiching the sample between two hard surfaces that slide against each other. The Mill uses attrition and impact (capable of cell disruption), while the Homogenizer uses high shear. The ultrasonic bath disrupt a sample by pressure, created by a probe that rapidly expands and contracts at high frequencies.

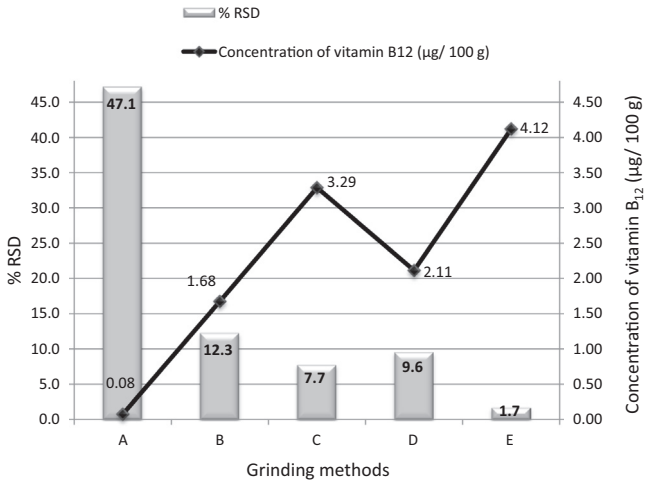

Fig. 2. The influence of the grinding methods on the concentration (μg/100 g) of the extracted vitamin B_{12} from 9 g *Hippophae rhamnoides* berries and on the % RSD between replicate measurements (n = 3). Grinding methods: A) mortar and pestle – cryogenic grinding, B) Mill, C) Mill + Homogenizer, D) Mill + ultrasonic bath, E) Mill + Homogenizer + ultrasonic bath.

Insufficient homogenization of the sample can 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 sample amount (9 g) and the use of various combinations of different grinding methods (Fig. 2). In order to compare the concentration of the extracted vitamin B_{12} in the different method combinations (A, B, C, D and E), a one-way ANOVA (Minitab 16) with one factor (concentration of vitamin B_{12}) with five levels (A, B, C, D and E) was conducted. The analysis of variance showed that the average differences between the different grinding methods and the concentration of vitamin B_{12} was statistically significant, $F(4,10) = 229.3$, $p = 0.00$ (p-value less than 0.05). 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 results indicated that the average concentration of the extracted vitamin B_{12} was significantly different in method E ($M = 4.10$, $SD = 0.68$) than in the other four methods (A, B, C and D) and between the methods B and D the differences were not significant, as there

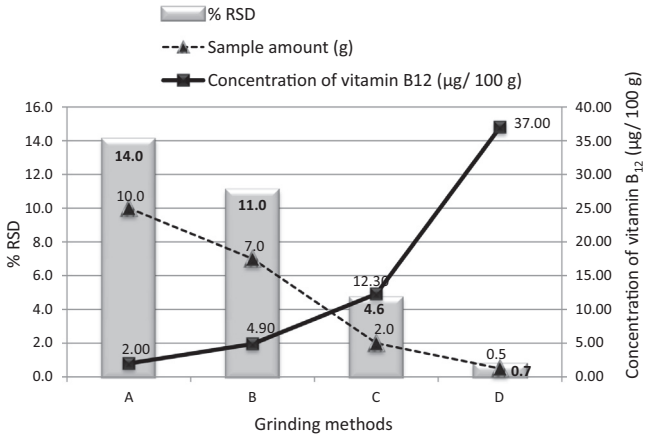

Fig. 3. Influence of grinding method and sample amount (g) on the determinable concentration (µg/100 g) of vitamin B_{12} of *Hippophae rhamnoides* and on the % RSD among replicate measurements (n = 3). Grinding methods: A) Mill (4.6% Recovery), B) Mill + mortar and pestle (11.0% Recovery), C) Mill + mortar and pestle + Homogenizer (28.4% Recovery), D) Mill + mortar and pestle + Homogenizer + ultrasonic bath (83.6% Recovery).

was an overlap of their confidence intervals. The results showed that the extra grinding methods had a positive impact on the concentration of the vitamin B_{12} extracted from the sample. Every new additional grinding method contributed to the increase of vitamin B_{12} concentration, with the combination of all three resulting in the highest vitamin B_{12} value (4.1 µg/100 g).

Based on these results, the application of different grinding methods resulting in the increase in vitamin B_{12} concentration indicated that the actual content of vitamin B_{12} in *Hippophae rhamnoides* possibly had not been determined yet. Consequently it is possible the actual vitamin B_{12} content may be higher than the levels measured in this study (Fig. 2). Therefore, a second step in the investigation was to establish the influence of sample size (in g) (Fig. 3). Further experiments involved an additional homogenization procedure and adjustment of the sample size, according to the immunoaffinity column capacity. The following combination of grinding methods was applied: A) Mill, B) Mill + mortar and pestle, C) Mill + mortar and pestle + Homogenizer, D) Mill + mortar and pestle + Homogenizer + ultrasound bath, in conjunction with the reduction in the sample size from 10 g to 0.5 g following the direction A to D (Fig. 3). The analysis of variance showed that the effect of the different grinding method in combination with the reduction of the sample size was significant, $F(3,8) = 4387.97$, $p = 0.000$. Post hoc comparisons using the Tukey HSD test indicated that the mean score of vitamin B_{12} concentration in all four methods (A, B, C and D) are significantly different. Taken together, these results suggest that the method D really do have an effect on the vitamin B_{12} concentration levels. The outcome of these combined experiments was that the lowest sample amount (0.5 g) in combination with all four grinding methods (D) resulted in the highest concentration of vitamin B_{12} (Fig. 3).

Therefore, by the improved sample preparation procedure the matrix effects (complexation reactions of vitamin B_{12} with vitamin C and Cu^{+2}) were reduced. Furthermore, the extracted amount of vitamin B_{12} increased through the use of the appropriate sample size (by not exceeding the capacity limit of the immunoaffinity column – 1.0 µg vitamin B_{12} per column), while the precision of the assay was also improved from 14.6 to 0.7% RSD, and the recovery of the spiking analyte (vitamin B_{12}) was increased to 84%. Similar levels of recovery were achieved with the other samples such as liver, vitamin B_{12} tablets, *Inula helenium* and blank samples (samples free of matrix, spiked with known concentrations of vitamin B_{12}) (Table 1).

3.2. Extraction of vitamin B_{12}

The extraction process followed the sample preparation. All forms of vitamin B_{12} were converted to the most stable CN-Cobalamin by heating the samples at 100 °C in the presence of KCN (as described under "Sample Preparation – Extraction of Vitamin B_{12}"). This conversion performed for at least 30 min. At this temperature (100 °C), enzymes/proteins such as pepsin and amylase, which have been added during the extraction process to improve vitamin B_{12} release from the plant matrix (proteins and polysaccharides, respectively), are denatured prior to the immunoaffinity step (Heudi et al., 2006). 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 (*Hippophae rhamnoides*) the filtration was performed twice, so as to obtain a less viscous and easier to handle solution for further processing.

The effect of incubation time of both enzymes on the extraction efficiency was tested. The incubation times were 0.5 h, 1.5 h and 3.0 h. The results of the incubation times were examined statistically with Minitab (statistical program) by one-way analysis of variance (ANOVA). The analysis of variance showed that the effect of incubation time was significant, $F(2, 3) = 10.19$, $p = 0.046$. The highest concentration of vitamin B_{12} released from the samples was detected for incubation times of 1.5 h and 3.0 h. These two incubation times were investigated in detail by the Fisher's Least Significant Difference (LSD) test. The difference between the two incubation times in regard to cobalamin release did not differ statistically significantly ($p < 0.05$). Hence, the short incubation time of 1.5 h was chosen for the enzyme digestion in further tests.

The effect of the buffer pH on the sample dilution 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 to pH 4.6. In order to check the impact of the final pH on the analysis, samples were adjusted to different pH values between pH 3 and pH 11. The samples were analyzed as described in the Section 2. The results indicated that the best pH prior to the immunoaffinity step is pH 7. At this pH, the highest vitamin B_{12} concentrations in the *Hippophae rhamnoides* samples were found. Thus, all further analysis was performed with a sample adjustment to pH 7 prior to the immunoaffinity step (see Supplementary Fig. 7).

3.3. Stability of vitamin B_{12}

Previous publications have highlighted the instability of vitamin B_{12} during ambient light conditions and thermal treatment processes, respectively (Kesava Raju, Yu, Schiel, & Long, 2013). Major attention was given to the stability of the analyte during sample preparation and analysis. Thus, the stability of vitamin B_{12} was tested with a series of standard solutions in the range of the concentrations coinciding with the linearity range of the method. First, the degradation of vitamin B_{12} under ambient light

Table 1
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 sample	0.17	100	0.67	3
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

conditions at room temperature was tested. Therefore, the fresh standard solution was measured directly after preparation; and after 24 h and 48 h under ambient light conditions at room temperature. The results disclosed extensive losses in concentrations at LOQ levels (10.80% after 24 h and 21.33% after 48 h) and as the concentration increased, the vitamin losses decreased in an inversely proportional manner. Concentrations higher than 0.5 μg/ml remained stable for more than 2–3 days (see Supplementary Fig. 8).

Second, an experiment regarding thermal degradation stability of vitamin B_{12} was performed. Therefore, the thermal stability of vitamin B_{12} in a thermal treatment at 100 °C was investigated, namely at the highest temperature applied during the method. 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 standard solution). Therefore, the thermal treatment was limited to a maximum of 30 min at 100 °C, in order to avoid losses of the vitamin B_{12}. All standard solutions used in the study were freshly prepared and used on a daily basis.

After the optimization of the "Sample Preparation" and the "Extraction of Vitamin B_{12}" from different sources, especially plant samples, the final vitamin B_{12} content was analyzed via HPLC as described in the Section 2.

4. Validation of vitamin B_{12} quantification in plants

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

4.1. Linearity – limit of detection (LOD) and limit of quantification (LOQ)

Vitamin B_{12} was quantified under optimal analytical conditions by means of an external calibration curve. Six concentrations points (n = 6) were used for the preparation of the calibration curve and the linearity was determined in the range of 0.01–0.30 μg/ml. The calibration curve of pure solutions of vitamin B_{12} was linear (r^2 = 0.9997). The limit of detection (LOD) and limit of the quantification (LOQ) of the method were calculated with the linear calibration curve. This method is based on the standard deviation of response and slope. Therefore 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 (see Supplementary Table 3 and Fig. 9).

4.2. Specificity (selectivity)

A comparison among the HPLC chromatogram of the mobile phase (TFA 0.025% w/v), a spiked cranberry sample and the cranberry sample without spiking gave the selectivity. It was clear that the vitamin B_{12} peak did not interfere with other substances. Under the chromatographic conditions of this experiment, the retention time of the vitamin B_{12} was t_R = 9.80 min (see Supplementary Fig. 10).

4.3. Precision (system repeatability, method repeatability and intermediate repeatability)

The system repeatability of the method was estimated by 36 determinations with 6 replicate injections of a standard solution. Six different concentrations of a standard solution were tested (0.010, 0.025, 0.050, 0.100, 0.200 and 0.300 μg/ml). The relative standard deviation (% RSD) of the analytes ranges from 7.37% (for the lowest standard elution concentration) to 0.53% (for the highest standard elution concentration) (see Supplementary Fig. 11). The assay repeatability was estimated with 6 replicate samples from homogenous powder mixtures of *Hippophae rhamnoides* plants which were analyzed according to the described assay. The relative standard deviation (% RSD) of the analyte was 0.7%. The intermediate repeatability was determined by three independent analysts, by analyzing the same powder mixtures of *Hippophae rhamnoides*, and the % RSD was low and the method precision therefore acceptable.

4.4. Accuracy (recovery)

A matrix spiking method with defined analyte concentration (standard solution) was used for estimation of the recovery. Pure vitamin B_{12} powder was added to *Hippophae*, to vitamin B_{12} tablets, to liver (veal) and to *Inula helenium*, while the same procedures were applied to the analyte without matrix (blank sample). The

Table 2
Vitamin B_{12} concentration in different food sources*.

Plant-Based Solid Samples	Vitamin B_{12} content (μg/100g)
Hippophae rhamnoides (from research garden Teutopharma GmbH/Dr. Pandalis group, Glandorf Germany)	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
European varieties of Elymus repens (dry extract) (Sidea couch grass)	23.10
European varieties of Elymus repens (grinded) (Sidea couch grass)	25.83
Inula helenium (from research garden Teutopharma GmbH/Dr. Pandalis group, Glandorf Germany)	10.62
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	9583.79 (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 extract (from research garden Teutopharma GmbH/Dr. Pandalis group, Glandorf Germany)	26.28

* All the values that listed above (Table 2) are the measured amount of vitamin B_{12} by HPLC-UV without the correction from the recovery studies.

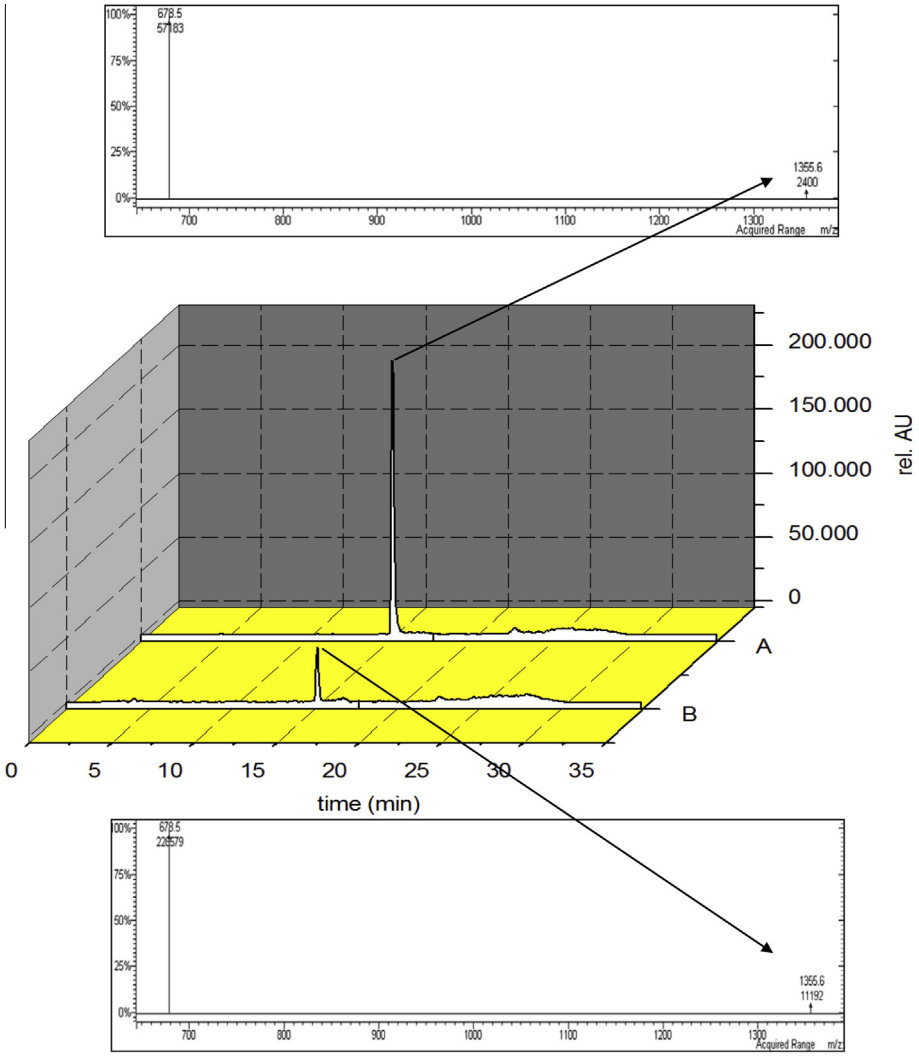

Fig. 4. A) Chromatogram of cyanocobalamin (vitamin B_{12}) standard solution concentration 0.25 μg/ml, and HPLC-MS/MS spectra of a *m/z* $[M+H]^+$ of 1355 of cyanocobalamin (vitamin B_{12}), B) chromatogram of immunoaffinity purified extract of 558 (*Hippophae rhamnoides*), and HPLC-MS/MS spectra of a *m/z* $[M+H]^+$ of 1355 for the corrinoids in the immunoaffinity purified extract of the 558 *Hippophae rhamnoides*.

amounts of added analyte were at the level of the target concentration (0.1 μg/ml) of our method. *Hippophae rhamnoides* samples were spiked at three concentration levels, the first at the limit of quantification (LOQ), the second in the target concentration (0.1 μg/ml) and the third at the upper limit of our linear range (0.3 μg/ml). The following equation was used for the estimation of recovery from spiked samples:

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

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 1. *Hippophae rhamnoides* was analyzed via the commercial Biacore assay and only 0.08 μg/100 g were found. The recovery of vitamin B_{12} with the optimized method from liver (veal) was 89%, the recovery from *Hippophae rhamnoides* 84%, the recovery from the vitamin B_{12} tablet was 80% and the blank samples showed 100% recovery.

5. Quantification of total vitamin B_{12} in plants/(sample analysis)

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 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 maximum value for *Hippophae rhamnoides* plant containing 37.01 μg vitamin B_{12}/100 g dry weight (Table 2). That means, 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) (Guggisberg et al., 2012).

5.1. HPLC-MS/MS – investigation of vitamin B_{12}

Due to the fact that high amounts of biological inactive analogues of vitamin B_{12} that can block vitamin B_{12} metabolism

have been reported in other natural sources, such as *Spirulina*, it is essential to distinguish the real vitamin B_{12} from other vitamin B_{12} analogues (such as pseudo-vitamin B_{12}). The lower ligand of real vitamin B_{12} is 5,6-dimethylbenzimidazole, which 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. The microbiological assay (MBA) does not differentiate between active forms of vitamin B_{12} and other corrinoids, thus, an HPLC-MS/MS method was performed in order to measure the exact molecular mass of the measured vitamin B_{12} and distinguish the vitamin B_{12} forms.

Based on in the MS/MS spectra (Fig. 4), 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} (1355.37). 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 1355.6 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 Fig. 4 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 cobalamin in *Hippophae rhamnoides* is mainly vitamin B_{12} (above 98%).

6. Conclusions

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, mortar and pestle, Homogenizer 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 quantification of 0.014 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 cannot synthesize vitamin B_{12}, actinorhizal plants such as *Hippophae rhamnoides* which are symbiotic with actinobacteria *Frankia alni* can accumulate 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. Based on our recent results European varieties of *Elymus* (Sidea couch grass), of *Hippophae rhamnoides* and of *Inula helenium* as well as *Brassica nigra* (black mustard), have shown satisfactory high amounts of vitamin B_{12}, raising the hope for vegans to intake vitamin B_{12} from plant-based sources.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.08.037.

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