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

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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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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).