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Analytical Methods

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Ultra-high performance liquid chromatographic and mass spectrometric analysis of active vitamin B12 in cells of Propionibacterium and fermented cereal matrices

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ABSTRACT

A sensitive and selective method is needed to analyse in situ produced vitamin B12 in plant-based materials, potential new dietary sources of vitamin B12. A UHPLC/UV method was developed and validated for the determination of human active vitamin B12 in cell extracts of Propionibacterium freudenreichii subsp. shermanii and after immunoaffinity purification in extracts of cereal matrices fermented by P. freudenreichii. An Acquity HSS T3 C18 column resulted in a baseline separation, a calibration curve of excellent linearity and a low limit of detection (0.075 ng/5 µL injection). As confirmed by UHPLC-MS, the active vitamin B12 could be separated from pseudovitamin B12. The recovery of vitamin B12 from purified spiked cereal matrices was good (> 90%; RSD < 5%). A nutritionally relevant amount of active vitamin B12 was produced by P. freudenreichii in cereal malt matrices (up to 1.9 µg/100 g) in 24 h at 28 ºC.

Keywords: Vitamin B12; Propionibacterium freudenreichii; fermented cereal matrix; UHPLC; UPLC; immunoaffinity purification
1. Introduction

Vitamin B12, one of the water-soluble vitamins, is needed in humans and animals for the metabolic functions of two enzymes: methylmalonyl-CoA mutase and methionine synthase. According to Institute of Medicine (1998), the recommended dietary allowance (RDA) of vitamin B12 for adults is 2.4 µg/day. It is only synthesized by a few bacteria and archaea (Martens, Barg, Warren, & Jahn, 2002). The structure of vitamin B12 consists of a corrin ring with a central cobalt atom complexed to four pyrrole rings, a lower α-ligand and an upper β-ligand. This vitamin includes three naturally occurring forms (adenosylcobalamin, methylcobalamin and hydroxycobalamin) and one chemically transformed form (cyanocobalamin), differing only in the upper ligand. Cyanocobalamin, the most stable form, is obtained by reacting natural vitamers with cyanide and it is the major vitamin B12 used in fortified foods, nutritional formula and pharmaceutical preparations (Ball, 2006). The lower ligand of vitamin B12 is 5,6-dimethylbenzimidazole (DMBI), which is essential for the binding of the vitamin to the intrinsic factor for its absorption (Nielsen, Rasmussen, Andersen, Nexø, & Moestrup, 2012). Corrinoids with lower ligands other than DMBI are found in biological materials (Watanabe, Yabuta, Tanioka, & Bito, 2013). They are biologically inactive in humans but have vitamin activity in microorganisms (Herbert, 1988).

Animal-based foods such as meat, fish, eggs and dairy products are the major contributors of vitamin B12 in the human diet. It is accumulated in animal tissues or milk, primarily as a result of the gut microbial activities and feed supplements. However, plant food materials are devoid of vitamin B12 (Ball,
A low level of vitamin B12 detected in some plant products and mushrooms probably originates from contaminating organisms or from vitamin uptake from soil residues (Koyyalamudi, Jeong, Cho, & Pang, 2009). Processed plant-based products (e.g. Tempe) have been found to contain a low level of vitamin B12 synthesized by the contaminating bacteria (Herbert, 1988; Keuth & Bisping, 1994; Mo et al., 2013). However, most of these data were obtained by a non-specific analytical method, i.e. microbiological assay (MBA), which does not differentiate between active forms of vitamin B12 and other corrinoids.

The analysis of vitamin B12 in non-fortified foods is challenging due to its low concentration. The typical contents in animal-based foods milk, cheese, meat and liver have been reported to be 0.9, 2.4, 1–2, and 83 µg/100 g, respectively (Ball, 2006). Natural forms of vitamin B12 are sensitive to light, therefore analytical methods utilise cyanide during the extraction to convert the natural forms into the more stable cyanocobalamin. Vitamin B12 in foods is traditionally determined by MBA using Lactobacillus delbrueckii ATCC 7830 as an assay organism, which is also the reference analytical method of AOAC (2006). Although it is a sensitive technique, MBA suffers from poor selectivity and often results in overestimation by 5–30% since the test organism can react to compounds other than vitamin B12 forms, such as analogues and nucleic acids (Ball, 2006). One such inactive corrinoid for humans has been identified as pseudovitamin B12, which is produced by Lactobacillus reuteri CRL1098 (Santos et al., 2007). This corrinoid has adenine instead of DMBI as the lower ligand. In meat products, vitamin B12 contents were up to 2.2-fold higher with MBA compared with high performance liquid chromatography (HPLC; Guggisberg,
Risse, & Hadorn, 2012), reflecting the poor specificity of MBA. Moreover, MBA
is labour intensive and time consuming.

HPLC based methods have been developed for the determination of vitamin
B12 in fortified foods, infant formula and vitamin supplements (Campos-Giménez,
Fontannaz, Trisconi, Kilinc, Gimenez, & Andrieux, 2008; Chen, Wolf,
Castanheira, & Sanches-Silva, 2010; Heudi, Kilinç, Fotannaz, & Marley, 2006;
Marley, Mackay, & Young, 2009). Some studies have also focused on the
quantification of vitamin B12 in non-fortified foods such as meat products
(Guggisberg et al., 2012), and milk-based fermented products (Van Wyk & Britz,
2010). These HPLC methods were based on UV detection. One HPLC method
reported by Pakin, Bergaentzlé, Aoudé-Werner and Hasselmann (2005) used
fluorescence detection after vitamin B12 was derivatised into a fluorescent
compound, α-ribazole. The latter method was sensitive, but it required lengthy
sample preparation time involving extensive sample derivatisation. The
introduction of an immunoaffinity sample purification technique has improved the
sensitivity of the HPLC measurement in fortified foods and supplements
(Campos-Giménez et al., 2008; Heudi et al., 2006; Marley et al., 2009).

However, the development of ultra-high performance liquid
chromatographic (UHPLC) technology that uses separation column made up of
sub-2 μm particles and improved instrumentation has considerably increased the
sensitivity and resolution of the chromatographic analysis (Swartz, 2005). To
date, one method based on UHPLC with mass spectrometric (MS) detection has
been reported for the analysis of vitamin B12 in milk and dairy products (Zironi,
Gazzotti, Barbarossa, Devicienti, Scardilli, & Pagliuca, 2013). The enhanced
sensitivity offered by the UHPLC technology and the sample clean-up and
concentrating potential of vitamin B12-specific commercial immunoaffinity columns have not yet been utilised for analysing vitamin B12 in natural or microbiologically processed foods. Particularly, it is necessary to confirm that the microbiologically synthesized forms are active for humans when biofortification is applied. Up to now, cereal matrices, which account for a major portion of the human diet but contain no vitamin B12, have not been considered for *in situ* enrichment with vitamin B12 using food-grade microorganisms.

We are studying *in situ* production of vitamin B12 in cereal-based matrices using food-grade propionibacteria. Therefore, the aim of this study was to develop a sensitive UHPLC/UV method for the analysis of vitamin B12 in cells of *Propionibacterium* and in fermented cereal matrices. The new method was compared with MBA, and the need for immunoaffinity-based sample purification and confirmation of the structure of the synthesized vitamer by UHPLC-MS were explored.

### 2. Materials and methods

#### 2.1. Chemicals, materials and reagents

All chemicals and reagents used were of analytical grade. Sodium hydroxide, acetic acid and the vitamin B12 assay medium were obtained from Merck (Darmstadt, Germany); ethanol from Altia (Rajamäki, Finland); acetonitrile (HPLC grade), trifluoroacetic acid (TFA) and sodium cyanide from Sigma-Aldrich (Steinheim, Germany); cyanocobalamin from Supelco (Bellefonte, USA); Tween 80 from Sigma (Aldrich, USA); and α-amylase (EC 232-588-1, A9857-
5MU, *Aspergillus oryzae*) was from Sigma-Aldrich (Steinheim, Germany). A certified reference material BCR 487 (lyophilised pig liver; Institute for Reference Materials and Measurements, Geel, Belgium) was bought from Sigma-Aldrich. Water (later, MilliQ water) used for the preparation of reagents and analyses was obtained from a Milli-Q Plus system (0.22 µm, ≥ 18.2 MΩ cm, Millipore Corporation, Bedford, MA, USA).

Cyanocobalamin stock solution (200 µg/mL) was prepared in 25% ethanol/MilliQ water and the concentration was measured by a spectrophotometer (Lambda 25 UV/Vis, Perkin Elmer Inc., USA) at 361 nm, as described by Indyk, Persson, Caselunghe, Moberg, Filonzi and Woollard (2002).

### 2.2. Vitamin B12 extraction

The protocol for vitamin B12 extraction (as cyanocobalamin) from sample matrices was adopted from Kelleher and Broin (1991) for plasma samples with some modifications. Cereal matrix (1–5 g), bacterial cell pellet (0.1–0.2 g) or pig liver (0.1 g) weighed in an extraction tube was vortexed with 10 mL buffer (8.3 mM sodium hydroxide and 20.7 mM acetic acid; pH 4.5) containing 100 µL 1% sodium cyanide and placed in a boiling water bath for 30 min. The samples were then ice-cooled and centrifuged (Hermle, Wehingen, Germany) at 6900 × g for 10 min. The supernatant was collected in a fresh tube. The residue pellet was vortexed once again with 5 mL buffer (pH 6.2, adjusted from the pH 4.5 buffer with 3% sodium hydroxide) and centrifuged. The supernatants were combined and the pH was adjusted to 6.2. The extract was then paper filtered (Ø 90 mm, VWR, Leuven, Belgium) and the volume was adjusted to 25 mL with pH 6.2
buffer. To ease the filtration of extracts for immunoaffinity purification, the cereal-based samples were treated with 1 mL of α-amylase solution (50 mg/mL) (37 °C; 30 min) to digest the starch before the boiling-water extraction (Marley et al., 2009). All the analytical processes were carried out under subdued light or were protected from direct light. The vitamin B12 content of the cereal matrices was expressed in terms of fresh weight (ng/g matrix) and that of cells per unit volume fermented medium (ng/mL).

2.3. Immunoaffinity purification for UHPLC quantification

The sample clean-up in the immunoaffinity column “Easy-Extract®” (R-Biopharma, Glasgow, Scotland) was carried out according to the manufacturer’s instructions. Briefly, the buffer in the immunoaffinity column was drained, after which 10–15 mL of the filtered (0.45 µm, Pall, Cornwall, UK) sample extract was loaded into the column. The column was washed with 10 mL of MilliQ water and 50 mL of air was applied. Vitamin B12 was then eluted with 3 mL of methanol and the elution was completed with an additional 0.5 mL of methanol. The eluate was evaporated at 50 °C under a stream of nitrogen and the residue was reconstituted in 300 µL of MilliQ water, which was then syringe filtered (0.2 µm, Pall, Cornwall, UK) to a Waters total recovery UPLC vial.

2.4. Ultra-high performance liquid chromatography (UHPLC)

2.4.1. UHPLC system
The chromatographic analysis was performed on a Waters Acquity UPLC system (Milford, MA, USA) equipped with a photodiode array detector (PDA; 210–600 nm). In addition, it consisted of a binary solvent manager, autosampler and column manager. The detection was performed at 361 nm and the absorption spectra were recorded by the PDA. The autosampler was maintained at 4 °C and the column was operated at 30 °C. The autosampler injected the sample solutions (2–15 µL) onto a column via a 20-µL injection loop operated in a partial-loop mode. Chromatographic data were collected and processed using the Waters Empower 2 software.

2.4.2. Method optimisation and validation

Two reversed-phase C18 columns, high-strength silica T3 (HSS) and ethylene bridged hybrid (BEH) from Waters (Milford, MA, USA), of similar dimensions (2.1 x 100 mm; 1.8 µm and 1.7 µm particles, respectively) were evaluated for the separation of vitamin B12 from sample extracts. A set of flow rates (0.32‒0.50 mL/min) was tested in a linear gradient mode of the mobile phase consisting of MilliQ water (solvent A) and acetonitrile (solvent B); each modified with 0.025% TFA. Finally, the following mobile phase gradients (solvent A: solvent B) were maintained during a 10-min run: 0–0.5 min (95:5); 0.5–5 min (60:40); 5–6 min (60:40); and 6–10 min a linear gradient from 60:40 to 95:5 for equilibration. A calibration curve was produced in each sample set from six cyanocobalamin standards with the vitamin concentration ranging from 0.015 to 0.75 ng/µL; each standard solution was injected twice. The chromatographic method was assessed for the linearity of the calibration curve,
limit of detection (LOD: signal-to-noise ratio, S/N = 3), and limit of quantitation (LOQ: 3 times the LOD) of vitamin B12 using the cyanocobalamin standard solutions. The resolution (calculated according to the formulae in the US Pharmacopeia; USP) for the separation of cyanocobalamin in sample extracts and the number of theoretical plates (USP) for each column were compared. LOD, resolution and number of theoretical plates were obtained with the Waters Empower 2 software.

2.5. UHPLC-MS/MS analysis

The mobile phase was modified with 0.5% formic acid instead of 0.025% TFA for the UHPLC-MS study to minimise the ion suppression. The MS analysis was carried out in a positive ion mode on an Esquire-LC quadrupole ion trap mass spectrometer with an electrospray ionization (ESI) interface (Bruker Daltonics, Bremen, Germany) and the data were analysed by the LC-MSD Trap version 5.2 (Bruker Daltonics). The scanning was carried out for a range of \( m/z \) 900‒1400 and the tandem mass spectrometry (MS/MS) was performed for a particular \( m/z \) according to the target molecule(s) using helium as the collision gas. The following instrumental settings optimised for cyanocobalamin were used for the MS analysis of cyanocobalamin ([M+H]\(^+\) \( m/z \) 1356) and pseudovitamin B12 ([M+H]\(^+\) \( m/z \) 1345) in sample extracts: nebulizer (nitrogen) 50.0 psi, dry gas (nitrogen) 8.0 L/min, dry temperature 300 °C, capillary 4500 V, end plate offset –250 V and trap drive 84.

2.6. Microbiological assay
The MBA was performed on a 96–well microtiter plate (Corning, NY, USA) based on Kelleher and Broin (1991) using *L. delbrueckii* ATCC 7830 as an assay organism and cyanocobalamin as a calibrant. The sample extracts were appropriately diluted at two levels with the extraction buffer (pH 6.2). A certified reference material BCR 487 (pig liver) extract served as a reference sample in each set of samples. A blank extract was used to obtain the actual readings of the samples. Cyanocobalamin solutions of increasing concentration (0–8 pg/well) and sample extracts, both 100 µL, were inoculated into the wells of the microtiter plate (4 wells for each concentration), and 200 µL of freshly prepared sterile filtered vitamin B12 assay medium (pH 6.2) inoculated with the cryopreserved assay organism was introduced into each well. The plate was then incubated at an optimised condition (35 °C; 19 h) and the turbidity was measured with a microplate reader (Multiskan EX; Labsystems, Finland) at 595 nm. A calibration curve of 8 concentration levels and the amount of vitamin B12 in each well were obtained automatically by Ascent software version 2.6 (Labsystems).

2.7. Recovery study

Since natural cereal matrices do not contain vitamin B12, the unfermented rye malt matrix (from Section 2.8.) was used as a blank matrix for the recovery study. The sample matrix (1.5 g) was spiked with cyanocobalamin at two levels (17.7 ng and 53.1 ng), each level in triplicate, and vitamin B12 was extracted and analysed by MBA directly and by UHPLC after immunoaffinity purification, as explained earlier. The repeatability was shown by carrying out two independent
experiments on two days. To test the effect of the sample matrix, the recovery was also studied in the extraction buffer with the lower level of spiking (17.7 ng). The recovery was calculated from the measured cyanocobalamin concentration in the spiked samples to the concentration of added cyanocobalamin in the samples.

2.8. Bacterial culture and fermentation of sample matrices

Propionibacteria capable of vitamin B12 synthesis and allowed for use in food production are mostly used in cheese manufacturing and in the industrial production of vitamin B12 with genetically engineered strains (Thierry, Deutsch, Falentin, Dalmasso, Cousin, & Jan, 2011). For this study, a strain of Propionibacterium freudenreichii subsp. shermanii (P. freudenreichii ABM 5378) isolated from a cheese starter culture was used for the production of vitamin B12 in the sample matrices.

Malt matrices: The fermented cereal samples were produced to study the suitability of the developed method; the fermentation conditions used were not optimised for vitamin B12 production. Rye and barley malt flours obtained by milling malted grains (Laihian Mallas, Laihia, Finland) were separately mixed in boiling MilliQ water (33% w/v) and cooked for 2 min. The gelatinised matrices were transferred to glass flasks and then autoclaved at 121 °C for 15 min. The culture preparation and fermentation of the matrices were carried out as described by Kariluoto et al. (2010). Briefly, the cooled matrices were aseptically transferred into pre-sterilised flasks and a culture of P. freudenreichii ABM 5378 was inoculated. The matrices were allowed to ferment at 28 °C for 24 h. After the incubation, the cell counts (CFU/g) had increased by a log factor in both matrices...
while pH had decreased from 5.6 to 5.3 in the rye matrix and 5.4 to 5.2 in the barley matrix. Likewise, an aqueous barley malt extract was fermented with unidentified strains of bacteria producing lactic and propionic acids and then concentrated.

Supplemented whey permeate (SWP): *P. freudenreichii* ABM 5378 was grown in the SWP medium anaerobically for 72 h and then aerobically for 96 h at 30 °C (Hugenschmidt, Schwenninger, Gnehm, & Lacroix, 2010). Cheese whey powder (Valio, Espoo, Finland) was used instead of whey permeate powder, as in the study of Hugenschmidt et al. (2010), and the medium was supplemented with 5 mg/L cobalt(II) chloride (Sigma-Aldrich). The fermented broth was centrifuged (12000 × g; 10 min), the resulting cell pellet was re-suspended in 10 mL of pH 7.3 PBS buffer (Oxoid, Hampshire, England) and centrifuged again. The fresh cell mass obtained was stored at −20 °C until the analysis.

2.9. Statistical analysis

All the statistical analyses were performed using IBM SPSS 20.0 (IBM Corporation, NY, USA). Average, standard deviation (SD) and relative standard deviation (RSD) are reported. One-way analysis of variance (ANOVA) and Tukey post hoc test were performed to compare between respective UHPLC and MBA results. A *p* value of < 0.05 was considered statistically significant different.

3. Results and Discussion

3.1. UHPLC method optimisation and validation
Gradient elution of the mobile phase consisting of water and acetonitrile modified with 0.025% TFA at a flow rate of 0.32 mL/min was found to be optimal for vitamin B12 separation on both the HSS T3 and BEH column. A higher flow rate (0.47 mL/min) resulted in a co-elution of interfering compounds in the sample extracts. Owen, Lee, and Grissom (2011) used the same flow rate and the mobile phase gradient for the separation of physiologic cobalamins from samples of cell culture media by a BEH C18 column. A sharper cyanocobalamin peak was produced by the HSS column than that by the BEH column both for the standard solution and for the sample extracts (Figure 1). The height of the cyanocobalamin peak for an equal volume of the injected sample was about 1.4-fold on the HSS column compared to that on the BEH column. The number of theoretical plates of the HSS column was greater by a factor of 2.5, as evidenced by a narrower and a sharper peak on the HSS column. Furthermore, a higher USP peak resolution (1.80) was obtained with the HSS column than with the BEH column (1.10) under identical elution conditions (Fig. 1). In addition, co-elution of an interfering compound was observed for the extracts of *P. freudenreichii* cells leading to the tailing of the peak on the BEH column, whereas the cyanocobalamin peak was well separated on the HSS column. Therefore, the HSS T3 C18 column was chosen for the validation and the analysis of vitamin B12 in sample matrices.

**Fig. 1**

The retention time of cyanocobalamin was stable during the analyses, irrespective of the sample matrices. The 30-day average retention time was 3.27
min with a narrow change from 3.25 to 3.29 min over the study period. The UHPLC analysis was faster with a total run time of 10 min compared with over 30 min that was required for the HPLC methods (Heudi et al., 2006; Marley et al., 2009). An external calibration curve of excellent linearity \((R^2 > 0.999)\) was achieved by injecting a series of cyanocobalamin standard solutions \((0.015\text{--}0.75 \text{ ng/\mu L})\). The instrumental LOD and LOQ were 0.075 ng/inj and 0.225 ng/inj, respectively, for a 5 µL injection. Previous HPLC/UV methods resulted in higher LODs at the larger injection volumes: 1.5 ng/inj \((50\text{--}100 \text{ µL})\) (Guggisberg et al., 2012; Marley et al., 2009) and 3--3.3 ng/inj \((100 \text{ µL})\) (Chen et al., 2010; Heudi et al., 2006). The enhanced sensitivity allowed for the analysis with small samples (< 5 g), unlike the bulk quantity needed for HPLC analysis (Campos-Giménez et al., 2008; Guggisberg et al., 2012); thus minimising the matrix-related interference. Moreover, HPLC methods have mostly been developed for fortified foods and supplements for measuring added cyanocobalamin, at levels greater than those normally found in foods.

**Table 1**

The accuracy of the UHPLC method was studied by determining the recovery of spiked cyanocobalamin from a rye malt matrix. Low levels were chosen to cover concentrations that are possible in the fermented cereal matrices. The added levels were still too low to be detected with UHPLC without purification of the extracts. On the other hand, the usefulness of the additional purification and enrichment on the immunoaffinity columns was demonstrated by the good recoveries at both levels. The mean inter-day recovery of
cyanocobalamin by UHPLC/UV was about 95% with a relative standard deviation (RSD) of < 5.0%, for both levels (Table 1). The cereal matrix did not affect the recovery of the cyanocobalamin, as evident from a similar recovery obtained in the extraction buffer. Equivalent recoveries of spiked concentrations were also obtained by the MBA (p > 0.05; Table 1). Recently, the HPLC determination of vitamin B12 in complex fortified foods was improved with the application of an immunoaffinity sample purification and enrichment column (Heudi et al., 2006), and then successfully validated in further studies (Campos-Giménez et al., 2008; Marley et al., 2009). In these earlier studies, a good recovery was also obtained for cyanocobalamin; however, much higher spiking concentrations were used. Interestingly, Marley et al. (2009) found that the affinity of other vitamin B12 forms to antibodies was lower; e.g. hydroxycobalamin was recovered only up to 75%, suggesting the benefit of converting natural forms into cyanocobalamin for the accurate determination of vitamin B12.

For BCR 487, a vitamin B12 content of 876 ng/g of dm (dry matter) was obtained by the UHPLC analysis, whereas the MBA resulted in a higher concentration (1056±80 ng/g of dm) (Table 2). The latter was in agreement with the certified reference value (1120±90 ng/g of dm). The UHPLC determined content was 83% of the MBA result (p < 0.05; Table 2); the difference could be due to the non-specificity of MBA. The certified vitamin B12 value of BCR 487 is based on the data obtained with the MBA. To our knowledge, this is the first report of the chromatographically analysed vitamin B12 content of BCR 487. A locally lyophilised pig liver sample analysed with HPLC after immunoaffinity purification had a vitamin B12 content of only 597–656 ng/g (Guggisberg et al., 2012). Distribution of corrinoids in pig liver has not been studied in detail.
However, Kelly, Gruner, Furlong and Sykes (2006) investigated corrinoids in lamb tissues, including the liver, by HPLC. Contrary to previous reports that corrinoids in the lamb liver were exclusively vitamin B12, these authors found quite a marked fraction of the total corrinoids (up to 30%) as vitamin B12 analogues.

**Table 2**

### 3.2. UHPLC analysis of vitamin B12 in cells and fermented cereal matrices

Figure 2 shows example chromatograms with and without the immunoaffinity clean-up for extracts of cells, pig liver, spiked rye malt matrix and fermented rye malt matrix. Without the immunoaffinity purification, a higher chromatographic background was obtained for the pig liver and the fermented malt matrices while a cleaner chromatogram with a well-separated cyanocobalamin peak (retaining at 3.27 min) was observed for the cell extract. Besides the matrix effect on separation, another disadvantage was that the concentration of cyanocobalamin in these extracts was low or below the LOD (0.075 ng/5 µL injection). The immunoaffinity purified extracts produced a well-separated peak with none or little background interference, demonstrating the usefulness of the immunoaffinity clean-up for the analysis of low levels of vitamin B12 in the complex matrices. Guggisberg et al. (2012) reported its applicability for the analysis of vitamin B12 in meat and meat products. Nevertheless, analysis of the matrices such as microbial cells, containing a higher level of vitamin B12, did not require the immunoaffinity purification. Therefore,
especially with the immunoaffinity purification this method could also be applicable for other matrices. Separate validation for each material however is needed.

**Fig. 2**

The corrinoid in the cell extract, the immunoaffinity purified fermented malt matrices and the pig liver eluted at the retention time of the cyanocobalamin standard (Fig. 2) and had a PDA spectrum (210–600 nm) identical to that of cyanocobalamin (Fig. 3). Furthermore, the UHPLC-MS/MS analysis revealed that the fragmentation profile of the corrinoid for the m/z of 1356, [M+H]⁺ of cyanocobalamin, was similar to that of the cyanocobalamin standard, as shown in the example mass spectra (Figs. 4A; 4B). The following major fragmentation products of cyanocobalamin, as explained by Carkeet et al. (2006) and shown in Figure 4A, were observed: m/z 1210 [M+H–DMBI]⁺; m/z 1124 [M+H–DMBI–CN–Co]⁺; m/z 997 [M+H–DMBI–sugar–PO3]⁺ and m/z 912 [M+H–DMBI–sugar–PO3–CN–Co]⁺. These observations confirmed that the lower ligand of the corrinoid in the sample extracts was DMBI as in cyanocobalamin, which is critical for the binding of the B12 vitamer to the intrinsic factor to make it bioavailable for humans (Nielsen, Rasmussen, Andersen, Nexø, & Moestrup, 2012).

**Fig. 3**

**Fig. 4**
The UHPLC analysis of *P. freudenreichii* cells obtained from the fermented SWP accounted for a vitamin B12 production of 620 ng/mL. In a study by Hugenschmidt et al. (2010), 37 screened *P. freudenreichii* strains produced vitamin B12 of approximately 100 to 2500 ng/mL in SWP with added cobalt and DMBI, showing an inter-strain diversity of vitamin B12 synthesis. In our study, the medium was not supplemented with DMBI. Thus, DMBI had to be synthesized by the bacteria from the substrate in the SWP, which might have limited the vitamin B12 production.

To the best of our knowledge, we are the first to report vitamin B12 *in situ* production in cereal matrices with propionibacteria and its analysis by UHPLC. The vitamin B12 content of the *P. freudenreichii* fermented rye and barley malt matrices measured with the UHPLC was 19.3 ng/g and 15.7 ng/g of fresh weight, respectively (Table 2). On the other hand, the UHPLC analysis of the fermented malt extract after the immunoaffinity purification revealed a peak in the chromatogram at 3.22 min (Fig. 3A), i.e., before the retention time of cyanocobalamin (3.27 min) from the standard solution and the immunoaffinity purified pig liver extract (Figs. 3B; 3C). Its PDA spectrum (210–600 nm) was, however, similar to that of cyanocobalamin. This peak, which was not seen in the chromatograms of any other analysed samples, was suggested to be of pseudovitamin B12 with adenine as its lower ligand. It is a corrinoid known to be present in biological materials (Watanabe et al., 2013) and has been recently characterised in *L. reuteri* CRL1098 (Santos et al., 2007). When the sample extract was analysed by MS/MS for an *m/z* of 1356, [M+H]+ of cyanocobalamin, no fragment ions characteristic of cyanocobalamin were observed. However, a fragmentation profile identical to the cyanocobalamin standard was obtained by
the MS/MS for an m/z of 1345, [M+H]⁺ of pseudovitamin B12 (Fig. 4C: m/z 1210
[M+H−adenine]⁺; m/z 1124 [M+H−adenine−CN−Co]⁺; m/z 997
[M+H−adenine−sugar−PO3]⁺ and m/z 912 [M+H−adenine−sugar−PO3−CN−Co]⁺.
The similar fragment ions obtained for the m/z of 1345 with that for the m/z of
1356 confirmed that the molecule had a structure otherwise similar to
cyanocobalamin, but a different lower ligand: adenine in place of DMBI.

3.3. Comparison between UHPLC analysis and MBA

The MBA results of the *P. freudenreichii* cells were similar to the UHPLC
derived results, whereas analysis of the certified reference pig liver (BCR 487)
resulted in a 17% higher vitamin B12 content by the MBA (Table 2). The vitamin
B12 contents determined by the UHPLC were 67% and 63% of the MBA results
for the fermented rye and barley matrices, respectively (Table 2). For the malt
extract fermented with bacteria producing lactic and propionic acids no detectable
level of cyanocobalamin was found by the UHPLC method in contrast to 1200
ng/g of vitamin B12 measured by the MBA. These sample-specific differences of
the vitamin B12 contents when measured by the MBA or UHPLC method could
be due to the inherent drawbacks associated with the MBA.

The MBA has been the golden standard method allowing for the
determination of low levels of vitamin B12 in foods and biological materials.
However, it has been reported to give higher vitamin B12 results than HPLC in
biological and fermented materials. The higher results are possible, as the assay
organism also responds to corrinoids that are inactive for humans, e.g.
pseudovitamin B12 and nucleic acids (Ball, 2006; Herbert, 1988; Indyk et al.,
2002). These inactive corrinoids were reported in ovine tissues (Kelly et al., 2006) and may also be synthesized by microorganisms (Herbert, 1988), including propionibacteria. Quesada-Chanto et al. (1998) analysed vitamin B12 in the cells of three strains of propionibacteria by an HPLC method and two spectrophotometric methods. Vitamin B12 was only detected in cells of *P. freudenreichii* with HPLC, whereas vitamin B12 analogues with retention time different than authentic vitamin B12 were observed in the cells of other two strains. However, one strain produced authentic vitamin B12 when the culture medium was supplemented with DMBI. On the other hand, some bacteria produce only inactive corrinoids even in the presence of DMBI. *L. reuteri* CRL1098 produced pseudovitamin B12 exclusively in a growth medium supplemented with and without DMBI under anaerobic conditions (Santos et al., 2007).

Campos-Giménez et al. (2008) noticed a 20–30% higher vitamin B12 content in milk-based infant cereals and soy-based fortified formula by MBA compared to HPLC. Poor selectivity of the MBA was further shown in the analysis of spirulina tablets and shellfish where the MBA results were up to 6–8-fold higher than concentrations determined by a specific intrinsic factor based chemiluminescence method (Watanabe, Takenaka, Abe, Tamura, & Nakano, 1998). The majority of the measured vitamin B12 may thus have been inactive corrinoids.

4. Conclusion

The developed UHPLC/UV method enabled sensitive and specific quantitation of the vitamin B12 content in microbial cells, and in fermented cereal
matrices after purification on immunoaffinity columns. This method allowed for
the selective determination of bioactive vitamin B12, thus eliminating the
possibility of measuring inactive corrinoids as in the MBA. One such analogue,
pseudovitamin B12, was identified and confirmed in a malt extract fermented by
bacteria producing lactic and propionic acids. A nutritionally relevant amount of
active vitamin B12 was produced by *P. freudenreichii* in rye and barley malt
matrices. The LC-MS is a useful tool for the identification of *in situ* produced
bioactive vitamin B12 from inactive corrinoids in food matrices or microbial
materials.

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Academy of Finland Project No. 257333 (Natural fortification of foods: Microbial
in situ synthesis of vitamin B12 and folate in cereal matrix).

References

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Keuth, S., & Bisping, B. (1994). Vitamin B12 production by *Citrobacter freundii* or *Klebsiella pneumoniae* during tempeh fermentation and proof of
enterotoxin absence by PCR. *Applied and Environmental Microbiology*, 60(5), 1495-1499.


Figure captions:

**Fig. 1.** Chromatograms showing the separation characteristics of the HSS T3 and BEH C18 columns (2.1 mm x 100 mm, 1.8 and 1.7 µm particles) for cyanocobalamin from standard solution (0.15 ng/µL), an immunoaffinity purified extract of BCR 487 (lyophilised pig liver) and an extract of *P. freudenreichii* cells. The injection volume, flow rate, column temperature and mobile phase were identical. 1 = cyanocobalamin peak on HSS column, 2 = cyanocobalamin peak on BEH column.

**Fig. 2.** Chromatograms of unpurified and immunoaffinity purified extracts of BCR 487 pig liver, cyanocobalamin-spiked control rye malt matrix, fermented rye malt matrix and cells of *P. freudenreichii*. The cyanocobalamin concentration (ng/µL) of the purified extract of pig liver, spiked matrix, fermented rye malt matrix and cells were 0.166, 0.034, 0.037, and 0.180, respectively. The injection volume was 10 µL. 1 = cyanocobalamin peak (retention time: 3.27 min).

**Fig. 3.** Chromatograms of immunoaffinity purified extracts of malt extract fermented with bacteria producing lactic and propionic acids (A), *P. freudenreichii* fermented rye malt matrix (B) and cyanocobalamin standard solution (C). tR = retention time.

**Fig. 4.** UHPLC-MS/MS spectra of an m/z [M+H]⁺ of 1356 for the cyanocobalamin standard (A) and the corrinoid in the immunoaffinity purified extract of the fermented barley malt matrix (B), and of an m/z [M+H]⁺ of 1345
(pseudovitamin B12) for immunoaffinity purified malt extract fermented with bacteria producing lactic and propionic acids (C).

**Table captions:**

**Table 1.** Recovery of cyanocobalamin by UHPLC/UV from immunoaffinity purified extracts of the spiked unfermented rye malt matrix (1.5 g) and the microbiological assay (MBA) of the extracts without purification (n = 3; for both days).

**Table 2.** Vitamin B12 contents of certified reference material BCR 487 (lyophilised pig liver), *P. freudenreichii* fermented cereal matrices (28 °C; 24 h), cell mass from *P. freudenreichii* fermented supplemented whey permeate (SWP) (30 °C; 168 h) and concentrated malt extract fermented with bacteria producing lactic and propionic acids determined by UHPLC and MBA.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Spiked amount (ng)</th>
<th>Recovery, % (RSD, %)</th>
<th>Day 1</th>
<th>Day 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>UHPLC</td>
<td>MBA</td>
</tr>
<tr>
<td></td>
<td>0.0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Rye malt matrix</td>
<td>17.7</td>
<td>97.2 (3.1)a</td>
<td>95.0 (0.9)a</td>
<td>93.8 (5.3)a</td>
</tr>
<tr>
<td></td>
<td>53.1</td>
<td>96.8 (2.4)a</td>
<td>94.8 (3.7)a</td>
<td>91.2 (2.5)a</td>
</tr>
<tr>
<td>Extraction Buffer</td>
<td>17.7</td>
<td>94.6 (1.0)a</td>
<td>92.7 (5.7)a</td>
<td>97.4 (1.9)</td>
</tr>
</tbody>
</table>

ND = Not detected
NA = Not analysed
Values with dissimilar superscript letters (a and b) along each row indicate significant difference ($p < 0.05$).
<table>
<thead>
<tr>
<th>Sample</th>
<th>UHPLC (ng/g)</th>
<th>MBA (ng/g)</th>
<th>UHPLC/MBA, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCR 487 pig liver (n = 8)</td>
<td>876 (±49)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1056 (±80)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>82.9</td>
</tr>
<tr>
<td>Fermented rye malt matrix (n = 2 × 2)&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>19.3 (±1.5)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28.8 (±2.4)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>67.0</td>
</tr>
<tr>
<td>Fermented barley malt matrix (n = 2 × 2)&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>15.7 (±2.4)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.0 (±2.3)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>62.8</td>
</tr>
<tr>
<td>Cell mass (from fermented SWP)</td>
<td>620 (±90)&lt;sup&gt;a&lt;/sup&gt;&lt;sup&gt;#&lt;/sup&gt;</td>
<td>650 (±60)&lt;sup&gt;a&lt;/sup&gt;&lt;sup&gt;#&lt;/sup&gt;</td>
<td>95.4</td>
</tr>
<tr>
<td>(n = 3)</td>
<td>(ng/mL)</td>
<td>(ng/mL)</td>
<td></td>
</tr>
<tr>
<td>Concentrated fermented malt extract</td>
<td>ND</td>
<td>1196 ± 56</td>
<td>-</td>
</tr>
<tr>
<td>(n = 3)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SD
Values with different superscript letters (a and b) along the row indicate significant difference (p < 0.05)
*Dry matter basis
†Biological replicates = 2 and analytical replicates = 2
‡Expressed per unit volume fermented medium
ND = Not detected
SWP = supplemented whey permeate
The certified value for BCR 487 was 1120 ± 90 ng/g of dry matter.
Absorption spectrum of pseudovitamin B12

Absorption spectrum of cyanocobalamin

Pseudovitamin B12, $t_R = 3.22$ min

Cyanocobalamin, $t_R = 3.27$ min
A sensitive UHPLC/UV method was developed for the analysis of vitamin B12.

The method was suitable for analysing microbial cells and fermented cereal matrices.

Immunoadsorption purification enabled analysis of vitamin B12 in fermented cereal matrices.

Active vitamin B12 was produced in cells and cereal matrices by *P. freudenreichii*.

Pseudovitamin B12 could be separated and further identified with UHPLC-MS.