Food Chemistry 204 (2016) 21–28

Contents lists available at ScienceDirect

Food Chemistry

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

Stability of added and *in situ*-produced vitamin B12 in breadmaking

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ARTICLE INFO

Article history:
Received 30 September 2015
Received in revised form 1 February 2016
Accepted 9 February 2016
Available online 10 February 2016

Keywords:
Breadmaking
Stability
Cyanocobalamin
Hydroxocobalamin
In situ-produced vitamin B12

ABSTRACT

Vitamin B12 exists naturally in foods of animal origin and is synthesised only by certain bacteria. New food sources are needed to ensure vitamin B12 intake in risk groups. This study aimed to investigate the stability of added cyanocobalamin (CNCbl, chemically modified form) and hydroxocobalamin (OHCbl, natural form) and *in situ*-synthesised vitamin B12 in breadmaking. Samples were analysed both with a microbiological (MBA) and a liquid chromatographic (UHPLC) method to test applicability of these two methods.

Proofing did not affect CNCbl and OHCbl levels. By contrast, 21% and 31% of OHCbl was lost in oven-baking steps in straight- and sponge-dough processes, respectively, whereas CNCbl remained almost stable. In sourdough baking, 23% of CNCbl and 44% of OHCbl were lost. *In situ*-produced vitamin B12 was almost as stable as added CNCbl and more stable than OHCbl. The UHPLC method showed its superiority to the MBA in determining the active vitamin B12.

1. Introduction

Vitamin B12 plays a crucial role in the methylation processes and in lipid and carbohydrate metabolism. Its structure can be considered one of the most complicated among biomolecules in nature. A molecule of vitamin B12 consists of a four-part corrin macrocycle that has cobalt as a central ion linked to various upper ligands, including cyano (cyanocobalamin, CNCbl), hydroxyl (hydroxocobalamin, OHCbl), methyl (methylcobalamin, MeCbl) or 5'deoxyadenosyl (deoxyadenosylcobalamin, AdoCbl) groups. In biologically active vitamin B12 forms (later active vitamin B12), the cobalt ion also joins to a lower ligand, 5,6-dimethylbenzimidazole (DMBI) glycosylated to a ribose phosphate group (Ball, 2006).

Only MeCbl and AdoCbl are co-enzyme forms. MeCbl is required for remethylation reaction of homocysteine to methionine by methionine synthase. Thus, it plays an important role in DNA synthesis, together with folate. Methylmalonyl-CoA mutase needs AdoCbl as a cofactor to catalyse the conversion of methylmalonyl-CoA to succinyl-CoA. This reaction is involved in metabolism of cholesterol, odd-chain fatty acids and several amino acids (Pawlak, James, Raj, Cullum-Dugan & Lucus, 2013). Cyanocobalamin is a chemically modified form of vitamin B12 widely used in various oral supplements, fortified foods and parenteral treatments. CNCbl and OHCbl are not known to have direct biological roles, and they are converted to MeCbl or AdoCbl in the cells (Kozyraki & Cases, 2013).

Vitamin B12 is synthesised only by certain microorganisms, and thus, the main source of the vitamin is food of animal origin. Meat, fish, milk and eggs contain vitamin B12, mainly in the form of AdoCbl, MeCbl and OHCbl, mostly bound to proteins (Pawlak et al., 2013). The recommended intake of vitamin B12 has been set at 2.0 μg/day for adults in the Nordic countries (Nordic nutrition recommendations 2012, 2014). Generally, dietary vitamin B12 intake is adequate among omnivores. However, concern about adequate intake is increasing. Vegans, vegetarians, elderly with impaired absorption, and people in countries with low intake of animal products are at high risk of vitamin B12 deficiency (Allen, 2009; Pawlak et al., 2013). The increased metabolic demands of pregnancy and lactation and faster growth in childhood increase risk of deficiency. In addition, several gastrointestinal disorders can lead to vitamin B12 malabsorption (Kozyraki & Cases, 2013; Pawlak et al., 2013).

Suboptimal intake of vitamin B12 is associated classically with megaloblastic anaemia and myelin deterioration in the nervous system (Pawlak et al., 2013). Insufficient B12 status of a mother in early pregnancy is considered a risk factor for neural tube defects in the foetus because it acts as a cofactor in folate metabolism (Kozyraki & Cases, 2013). In countries, where folic acid fortification is mandatory, there is a concern about folic acid's potential to mask vitamin B12 deficiency. Because of insufficient vitamin B12 intake among risk groups, many institutions recommend using vitamin B12 supplements or fortified foods. For example, in the

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http://dx.doi.org/10.1016/j.foodchem.2016.02.071
0308-8146/

United States, many cereals and soymilk are fortified with CNCbl. However, some vegans and elderly still avoid supplements or used doses too low to maintain vitamin B12 status (Pawlak et al., 2013).

New food sources are needed to ensure sufficient vitamin B12 intake by people who avoid animal products or vitamin B12 supplements. In addition, the recommended shift from animal-origin foods toward a more sustainable plant-based diet will increase the need to develop vitamin B12-containing plant products. Strains of *Propionibacterium freudenreichii* are generally recognised as safe (GRAS) and originally were used in fermented foods, especially as a starter in Emmental-type cheeses. Various functions of propionibacteria (PAB), including as producers of bacteriocins, organic acids, some B-group vitamins, conjugated linoleic acid and trehalose, have been noticed by the food industry. In particular, some species of PAB are known for their capability to synthesise vitamin B12 (Poonam, Pophaly, Tomar, De, & Singh, 2012). In our previous study, we showed that dairy-originated *P. freudenreichii* strain produced bioactive vitamin B12 in whey-based medium supplemented with cobalt. The produced amounts were notable (620 ng/mL) (Chamlagain, Edelmann, Kariluoto, Ollilainen, & Piironen, 2015). Microorganisms may also synthesise corrinoids with a different lower ligand than DMBI. These forms probably are not bioactive as cofactors in human metabolism (Watanabe, Yabuta, Bito, & Teng, 2014). Further, some PAB strains synthesise incomplete corrinoids or analogues other than active vitamin B12 under certain cultivation conditions (Vorobjeva, 1999). In addition, the test organism *Lactobacillus delbrueckii* used in microbiological assay (MBA) can use these analogues for its growth (Herbert, 1988). Thus, MBA may lead to overestimated vitamin B12 content, as we noted in our previous study (Chamlagain et al., 2015).

Among plant-based foods, bread would be a good product for fortification with synthetic or *in situ*-produced vitamin B12. However, to evaluate the potential of bread fortification, the stability of the vitamin throughout the baking process should be confirmed. Knowledge of the stability of added vitamin B12 in bread is limited and the retention of *in situ*-produced vitamin B12 in breadmaking has not been studied thus far to our best knowledge. Vitamin B12 contains several functional groups that are prone to various chemical reactions. The stability of natural food-bound B12 has been studied mainly in meat (Gille & Schmid, 2015), milk and fish (Watanabe, 2007), and reported losses have been 30–50%, depending on the cooking method and cooking time. In aqueous solutions, vitamin B12 is sensitive to light, oxidising and reducing agents and high temperatures. The presence of other vitamins also affects its stability (Lešková et al., 2006). CNCbl is considered the most stable form. AdoCbl and MeCbl are especially photosensitive and convert easily to OHCbl under ultraviolet radiation (Juzeniene & Nizauskaite, 2013).

The current research investigated the stability of vitamin B12 in baking processes. First, stability of added CNCbl and OHCbl was studied in the straight-, sponge- and sourdough processes. Next, the stability of vitamin B12 synthesised *in situ* by *P. freudenreichii* sp. *freudenreichii* was investigated in the straight- and sourdough processes. All baking samples were analysed with MBA and most with the UHPLC method also, since this study also aimed to compare these two methods for determining content of added and *in situ*-produced vitamin B12.

2. Materials and methods

2.1. Standards and reagents

CNCbl was obtained from Supelco (Bellefonte, PA, USA) and hydroxocobalamin hydrochloride (OHCbl) was purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Sodium hydroxide, acetic acid and vitamin B12 assay medium were from Merck (Darmstadt, Germany). In addition, the study used sodium cyanide, cobalt(II) chloride, trifluoroacetic acid (TFA), tryptone and lactate (60% w/w) from Sigma-Aldrich Chemie (Steinheim, Germany). All solvents were of HPLC grade (Rathburn Chemicals Ltd.; Walkburn, Scotland and Sigma-Aldrich Chemie; Steinheim, Germany). Milli-Q water (Millipore system; Bedford, MA, USA) was used to prepare reagents. For quantification, a stock solution of CNCbl was prepared in 25% ethanol, and the concentration was confirmed with a spectrophotometer at 361 nm (Chamlagain et al., 2015)

2.2. Breadmaking with added CNCbl and OHCbl

The effect of breadmaking on the stability of added CNCbl and OHCbl was studied using the straight-, sponge- and sourdough processes under normal baking conditions, that is, in daylight and at room temperature (RT).

2.2.1. Straight- and sponge-dough baking

The recipe (Table 1) for straight- and sponge-dough breads was based on white wheat flour (75% extraction rate). Optimal mixing time and water absorption capacity of the flours were determined with a farinograph (Brabender; Duisburg, Germany). The standard solutions (ca. 0.2–0.4 mg/ml) for CNCbl and OHCbl were prepared in water under reduced light immediately before each baking process.

The straight-dough process was repeated three times as control baking (without vitamin B12 addition) and three times with addition either of CNCbl or of OHCbl. Dough was prepared by mixing the total amount of flour, salt and sugar for 1 min at the lowest speed (No. 1) in a mixer (Hobart N50; ITW Food Equipment Group; USA). Fresh baker's yeast (Suomen Hiiva Oy; Lahti, Finland) dissolved in tap water (35 °C) was added to fat (fluid fat mixture, Keijuriini; Bunge Finland Oy; Finland) in a flour mixture. In fortified doughs, CNCbl (0.50 mg) or OHCbl (0.50 mg) were added directly into dough mix so that the total water amount stayed at 238 ml. The dough was kneaded for 4 min at the middle speed (No. 2). After resting for 10 min, the dough was divided in three pieces of 150 g each, which were moulded in the moulding unit of an extensigraph (type DM 90/40, Brabender; Dulsburg, Germany). Dough pieces were proofed in a proofing cabinet in pans (15 × 6.5 × 9 cm) for 90 min at 34–36 °C (RH 70–80%). Two of the proofed breads were baked in a convection oven at 180 °C for 20 min. The third proofed dough was halved and handled as sub-samples (see Section 2.4). After cooling for 60 min at RT, the breads were weighed and their volumes measured by the rapeseed-displacement method according to AACC 10-05.01 method to determine the specific volume (cm^3/g).

The sponge-dough process was repeated twice as control and twice with addition either of CNCbl or of OHCbl. The dough was begun by mixing half the amount of flour (200 g) with the total amount of water (35 °C) and yeast. In addition, CNCbl (0.50 mg) or OHCbl (0.50 mg) were added. Seed dough was mixed by hand for 1 min and allowed to ferment covered for 120 min at RT. After pre-proofing, the remaining ingredients were added and the dough

Table 1
Recipe for each dough replicates used for straight-dough and sponge-dough baking.

Ingredient	Weight (g)	The mass ratio to flour mass (%)
Wheat flour	400	100
Tap water (35 °C)	238	59.5
Fresh yeast	16	4
Sugar	12	3
Margarine	12	3
Salt	4	1

mixed for 1 min at the lowest speed in a mixer followed by mixing for 3 min at the middle speed. After mixing, the dough was moulded, proofed (90 min), baked, cooled and weighed, and bread volumes were measured as in the straight-dough process.

2.2.2. Sourdough baking

Fortified sourdough baking was studied in two ways: CNCbl or OHCbl was added either before (Test 1) or after (Test 2) the 16-h-fermentation. Both baking tests were performed once either with CNCbl or with OHCbl, and once without (control dough) vitamin addition. In the following description of processes, amounts that differed in Test 2 are given in parenthesis. A rye-dough starter was originated in a local bakery (Pirjon Pakari; Nurmijärvi, Finland) and stored at +4 °C. The seed contained about 50–100 times more unspecified lactic acid bacteria (LAB) (larger rods 10^6 cfu/g and smaller rods 10^9 cfu/g) than yeast cells (10^6 cfu/g), which is typical for rye-dough seeds. 200 g (160 g) of seed was reactivated by fermenting it with 125 g (100 g) tap water and 75 g (60 g) whole-rye flour for 5 h at 30 °C. Then, 26 g (50 g) of reactivated seed was mixed with 320 g (619 g) of tap water and 194 g (375 g) of rye flour at RT by hand. At that point in Test 1, 0.54 mg of CNCbl or of OHCbl was added. The dough was left to ferment, covered, for 16 h at 30 °C.

After 16-h fermentation, 410 g (806 g) of the dough was mixed by hand with 134 g (252 g) of rye flour and 5.1 g (9.5 g) of salt. At that point in Test 2, 0.8 mg of CNCbl and 0.8 mg of OHCbl were added. The dough was mixed by hand for 1 min and finally in the mixer for 3 min (for 2 min at the lowest speed and 1 min at the middle speed). After a floor time (60 min at 30 °C), the dough was divided into three (four) pieces of 150 g (200 g) that were moulded by hand and proofed in pans for 30 min at 30 °C. Two (three) of the breads were baked in a convection oven for 70 min at 180 °C, cooled for 60 min at RT and weighed immediately. The third (fourth) unbaked, proofed dough was halved and handled as sub-samples (Section 2.4).

2.3. Baking with fermented malt extract containing in situ-produced vitamin B12

2.3.1. Preparation of malt medium and its fermentation by Propionibacterium freudenreichii

Fermented malt medium was prepared three times: twice for straight-dough baking (Trial 1 and Trial 2) and once for sourdough baking. Barley malt extract (ME; Laihian Mallas Ltd.; Laihia, Finland) and lactate (60% w/w) were mixed (ME-lactate) in Milli-Q water followed by pH adjustment to 6.4 with potassium phosphate buffer. The solution was centrifuged and the supernatant filtered. A stock solution of tryptone (0.2 g/ml in water) and the ME-lactate solution were autoclaved separately. Fermentation medium (ME-medium) was prepared by mixing ME-lactate, tryptone and sterile-filtered $CoCl_2$ solution just prior to fermentation, so that final concentrations were: ME 100 g/L, lactate 8 g/L, tryptone 5 g/L and $CoCl_2$ 5 mg/L.

P. freudenreichii sp. *freudenreichii* was sub-cultured by inoculating colonies from a propionic agar plate in triplicate into ME-medium and incubating for 3 days at 30 °C anaerobically. Sub-culturing was repeated twice before the final three cultures (1% v/v) were inoculated into 300 ml (in straight-dough baking) or 350 ml (in sourdough baking) of prepared ME-medium. An anaerobic fermentation at 30 °C for 72 h was followed by incubation under mild aerobic conditions until 168 h with shaking (150 rpm). Vitamin B12 contents in each medium were determined with the MBA and UHPLC methods.

2.3.2. Straight-dough baking

Straight-dough baking was carried out three times in two independent trials (Trial 1 and Trial 2) using ME-medium (238 ml) as a liquid instead of tap water. ME-media were used immediately after the 168-h fermentation. From each separately fermented media (n = 3 in both trials) a straight dough was made based on the recipe in Table 1 in Section 2.2.1. Generally, the active vitamin B12 content in the media used in Trial 2 was higher (on average, 690 ng/ml, n = 3) than in Trial 1 (on average, 190 ng/ml, n = 3).

2.3.3. Sourdough baking

Fermented ME media also were tested in sourdough baking (n = 3). Fermentation, dough-making and baking were carried out according to the sourdough recipe used in Test 1, as described in Section 2.2.2., but after regeneration of the seed, tap water (320 ml) was replaced with a fermented ME-medium (active vitamin B12 content, on average, 370 ng/ml, n = 3).

2.4. Sampling of doughs and breads

Two analytical sub-samples (25–50 g) were taken from each step of each dough-making process. In addition, baked breads (two from each trial) were halved crosswise into four pieces, and two of the pieces from both breads were combined, cut in small pieces, and divided in two plastic bags (about 50 g) before freezing. Sub-samples were frozen (−20 °C) immediately as a thin layer. One frozen sub-sample from each baking step was freeze-dried (Christ Alpha 2-4 LD Plus; Osterode, Germany) within one week at a chamber pressure of less than 1 mbar for approximately 24 h. Immediately after freeze-drying, the sample was milled (Grindomix GM 200; Retsch GmbH; Haan, Germany) until homogeneity (about 20–30 s, 10,000 rpm). The milled, freeze-dried sample was packed in vacuum bags (polyamide–polyethylene) under vacuum (Multivac; Brand; Düsseldorf, Germany) and stored at −20 °C until vitamin B12 and residual moisture content analysis.

2.5. Vitamin B12 analysis

2.5.1. Extraction and microbiological assay

Vitamin B12 content was determined as cyanocobalamin in all freeze-dried sub-samples and in selected media samples as described previously, with minor modifications (Chamlagain et al., 2015). Briefly, samples (freeze-dried, of 0.2–0.5 g) were heat-extracted (30 min in boiling water bath) in duplicate using extraction buffer (8.3 mmol/l sodium hydroxide/20.7 mmol/l acetic acid, pH 4.5) and 100 µl Na-cyanide (1% w/v in water). To make handling of the starch-rich samples easier, 0.5 ml of alpha-amylase (50 mg/l, EC 3.4.24.31; St Louis, MO, USA) was added and the sample incubated in a water bath (30 min, 37 °C). After centrifugation, the pH was adjusted to 6.2. The extract was filtered, and the volume was adjusted to 25 ml with pH 6.2 buffer. Samples were stored at −20 °C until MBA or UHPLC analysis. In all samples, total CNCbl or total OHCbl as CNCbl content was determined by MBA using *L. delbrueckii* (ATCC 7830) as the growth indicator organism and CNCbl as the calibrant. Certified reference material CRM 487 (pig liver, obtained from the institute for Reference Materials and Measurements; Geel, Belgium) was analysed in each incubation as a quality-control sample. Action limits in the control chart were 1079 ± 148 ng/fw. The certified value of CRM 487 is 1120 ± 90 ng/g dm. In addition, vitamin B12 content of the duplicated samples was not allowed to differ by more than 10%.

2.5.2. Purification and UHPLC analysis

Selected dough, bread and medium samples also were analysed by UHPLC. The same sample extract used for MBA was purified and concentrated through the immuno-affinity column (Easy-Extract; R-Biopharma; Glasgow, Scotland). Details of the purification were described previously (Chamlagain et al., 2015). Purified eluate (ca. 3.5 ml) was evaporated under a stream of nitrogen, and the

residue was reconstituted in 500 µl of Milli-Q water. A syringe-filtered sample was stored at −20 °C until UHPLC determination.

UHPLC analysis has been described elsewhere (Chamlagain et al., 2015). Briefly, cyanocobalamin was separated on a reversed-phase C18 column (HSS T3, 2.1 × 100 mm, 1.8 µm; Waters; Bedford, MA, USA) at 361 nm using the Waters Acquity UPLC separation system (PDA detector, binary solvent manager, sample manager, column manager). Resolution of cyanocobalamin was achieved with a linear gradient system of Milli-Q water and acetonitrile, containing 0.025% TFA, at a constant flow rate 0.32 ml/min. Quantification was based on an external standard method using a multilevel ($n = 5$) calibration curve (0.4 –8 ng). Each sample was injected twice (10–15 µl).

2.6. Moisture content and acidity of doughs and breads

All freeze-dried samples were analysed for residual moisture content in duplicate with the AACC 44-15A method so that vitamin B12 content could be reported on a dry matter basis. In addition, to evaluate vitamin content on a fresh-weight basis, moisture content of selected fresh samples was determined.

Acidity (pH) of doughs and breads was measured in frozen sub-samples after thawing (30 min at RT). Then, 10 g of dough or ground bread was suspended in 100 ml Milli-Q water by mixing for 1 min with a blender (Bamix M122; Mettlen, Switzerland). The sample was allowed to rest for 15 min at RT before pH measurement with a potentiometer (PHM220; Meter Lab; Lyon, France). During measurement, the sample was kept under magnetic stirring.

2.7. Data analyses

Statistical analysis (one-way ANOVA; SPSS version 22.0, IBM SPSS Statistics; Chicago, IL) was performed to test for significant differences in vitamin B12 content between each baking step or between the MBA and the UHPLC results. Differences were considered statistically significant at $p < 0.05$.

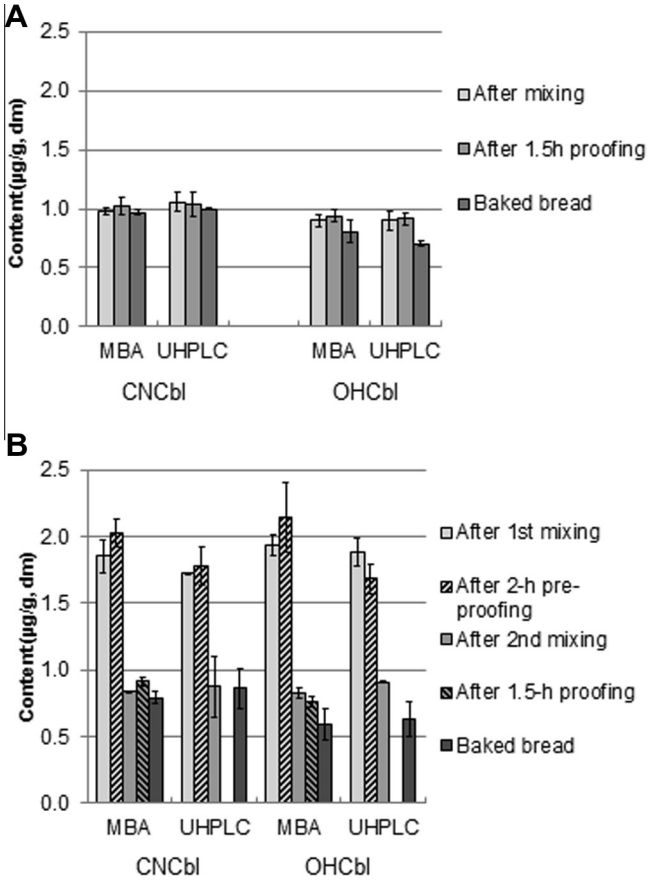

Fig. 1. Content of added CNCbl and OHCbl (µg/g, dm) in straight-dough samples (A) and in sponge-dough samples (B) analysed by the microbiological assay (MBA) and ultra-high performance liquid chromatography (UHPLC) methods. Error bars represent standard deviations of three independent baking trials (A) or the range of two independent trials (B). In sponge-dough baking (B) sub-samples after 1.5-h proofing were not determined by the UHPLC method.

3. Results and discussion

3.1. Stability of added CNCbl and OHCbl in breadmaking

The stability of added CNCbl and OHCbl was studied in the straight-, sponge- and sourdough processes. The MBA and UHPLC methods gave equal results in samples of straight- and sponge-dough processes ($p > 0.05$) (Fig. 1) but in sourdough baking, results obtained by MBA were higher than UHPLC results in some cases (Fig. 2). Next, effects of baking steps are evaluated based on the UHPLC results, followed by more detailed discussion of these two analytical methods.

The proofing steps (1.5-h in straight-dough and two separate proofings in sponge-dough) did not affect CNCbl or OHCbl levels significantly ($p > 0.05$). In contrast, baking decreased OHCbl content, whereas CNCbl stayed almost stable. In straight-dough baking, the total loss of added OHCbl was 21 ± 5% from the dough-mixing to baked-bread stages, and in sponge-dough baking it was on average 31% (range 24–36%) from the second-mixing to baked bread stages (Fig. 1). In the sponge-dough process, more flour and other ingredients were added after the first proofing, diluting vitamin B12 content (Fig. 1B). Therefore, losses can be calculated only separately for the pre-proofing stage and for the stages from the second mixing to baked bread.

Sourdough making was more destructive to added CNCbl and OHCbl than the straight- or sponge-dough processes. In addition, OHCbl losses were higher than those of CNCbl (Fig. 2). Because of the dilution effect of flour addition after 16-h fermentation, the loss of CNCbl and OHCbl could be observed only between the first mixing and 16-h fermentation stages and between the second mixing and baked bread stages. The 16-h fermentation and the 1.5-h proofing showed no reduction of added CNCbl and OHCbl, but losses occurred mainly during the baking step. Total baking losses from the second mixing to baked sourdough bread stages were almost equal in Tests 1 and 2 for CNCbl (23% and 26%, respectively) and for OHCbl (44% and 55%, respectively).

In both Tests 1 and 2, MBA and UHPLC results for samples from the second mixing to baked bread stages were in line with each other (Fig. 2). However, in Test 1, with added OHCbl, MBA yielded higher results than the UHPLC method after first mixing and after 16-h fermentation (Fig. 2A). In sourdough baking, LAB may produce vitamin B12-like compounds that have a positive effect on growth of *L. delbrueckii* in MBA (Herbert, 1988). This might have led to the discrepancy between UHPLC and MBA results.

To the best of our knowledge, this study is the first to investigate stability of added CNCbl and OHCbl in wheat baking in detail, including sourdough baking. On the whole, available data on the loss of added vitamin B12 in baking is extremely limited. A pilot programme of B-vitamin enrichment in wheat flours in France (Czernichow, Blacher, & Ducimetiere, 2003) reported 45% loss of CNCbl added to flour in wheat-dough baking. In folic acid and CNCbl cofortified bread leavened with baking powder, baking loss of CNCbl averaged 19% (Winkels, Brouwer, Clarke, Katan, & Verhoef, 2008). In contrast, more data is available on the retention

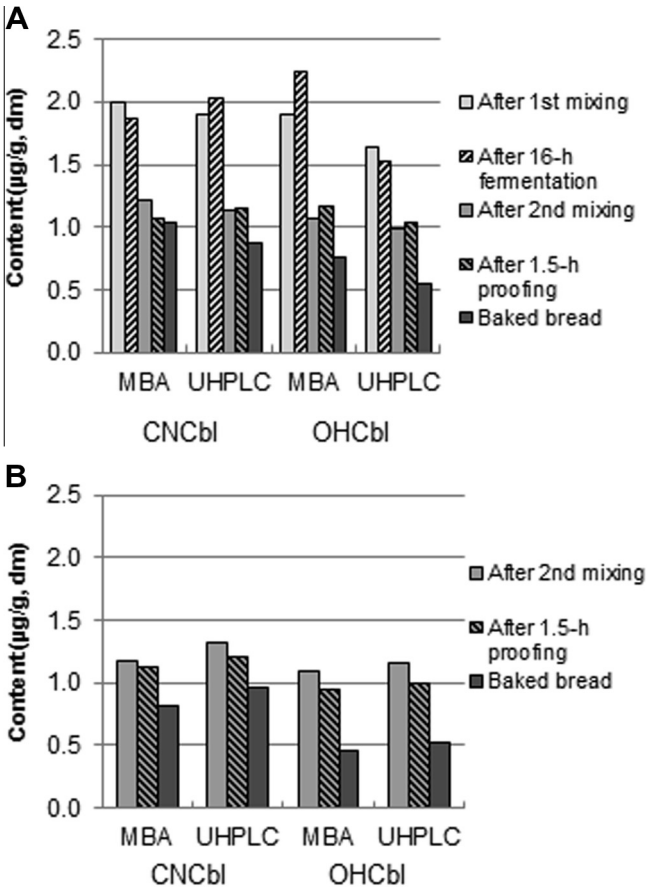

Fig. 2. Content of added CNCbl and OHCbl (µg/g, dm) in sourdough samples analysed by the microbiological assay (MBA) and ultra-high performance liquid chromatography (UHPLC) methods. In Test 1 (A), CNCbl and OHCbl were added before 16-h fermentation, and in Test 2 (B), they were added after 16-h fermentation. Tests 1 and 2 were performed once and values are means of two analytical replicates, which did not differ by more than 10%.

of another synthetic B vitamin, folic acid, in breadmaking. Reported losses for added folic acid in various wheat-bread making processes have varied from 10% to 32% (Anderson, Slaughter, Laffey, & Lardner, 2010; Gujska & Majewska, 2005; Osseyi, Wehling, & Albrecht, 2001; Tomiuk et al., 2012; Öhrvik, Öhrvik, Tallkvist, & Witthöft, 2010). In the present study, the loss of added OHCbl in wheat-baking processes agreed with these reports, although the mechanism of degradation in folic acid is different than in vitamin B12. Further, added folic acid remained nearly stable throughout the fermentation processes (Gujska & Majewska, 2005), as did added CNCbl and OHCbl in this study.

The loss of added OHCbl in this study was of the same order as the losses of natural vitamin B12 reported for animal-derived products. However, in most of those studies, vitamin B12 content was determined only with MBA. In beef, losses were 28–49% after various cooking, roasting or grilling treatments (Bennink & Ono, 1982; Czerwonka, Szterk, & Waszkiewicz-Robak, 2014). Microwave heating caused 30–40% losses for natural vitamin B12 in beef, pork and milk (Watanabe et al., 1998). Nishioka, Kanosue, Yabuta, and Watanabe (2011) studied cooking losses in herring using MBA, taking into account the overestimation effect of deoxyribosides and deoxynucleotides on MBA results, and reported high vitamin B12 losses (41–70%) in normal grilling, boiling, steaming and microwaving.

The present study showed that more added CNCbl and OHCbl were lost in sourdough baking than in wheat baking. Sourdough breads were baked longer (70 min) than wheat breads (20 min). A recent study indicated that prolonged heat-treatments accelerated the degradation of OHCbl in its aqueous solution buffered at pH 7 (Nishioka et al., 2011). According to other previous publications, higher losses of food-bound vitamin B12 in meat, fish and milk also were caused by longer duration and/or higher temperature of cooking (Gille & Schmid, 2015; Nishioka et al., 2011; Watanabe, 2007). In contrast, heating below 100 °C did not destroy vitamin B12 in milk (Gille & Schmid, 2015), and vacuum-packed pouch cooking of fish prevented B12 loss (Nishioka et al., 2011).

In addition to heat sensitivity, cobalamins are sensitive to pH changes and light. The lower pH might partly be responsible for the lower retention of CNCbl and OHCbl in the sourdough process compared with sponge- and straight-dough making. The pH stayed almost stable throughout the straight- and sponge-dough processes, being near to pH 5.7 (Table 2). In contrast, during the 16-h fermentation, the pH in sourdough decreased from the initial 6.4 to 3.8, as it does in typical sourdough fermentation due to the action of lactic and acetic acid produced by LAB. In addition, the contribution of oxidising and reducing agents, including other vitamins, to stability of added cobalamins in dough systems must be considered. However, in the current study, their concentrations in doughs were remarkably lower than those in reported stability studies. Riboflavin (Ahmad, 2012; Juzeniene & Nizauskaite, 2013) and nicotinamide (Ahmad, Ansari, & Ismail, 2003) promoted the photo-degradation of CNCbl to OHCbl, especially at low pH. Furthermore, thiamine and vitamin B6 had a decomposition effect on CNCbl in water solution (Monajjemzadeh, Ebrahimi, Zakeri-Milani, & Valizadeh, 2014). In the presence of other reactive compounds, OHCbl may further degrade to oxidation products. Ascorbic acid as a reducing agent degraded CNCbl first to OHCbl, which in turn, degraded further to oxidation products, especially at pH 5. In addition, OHCbl was destroyed more rapidly by ascorbic acid than was CNCbl (Ahmad et al., 2014). Ahmad et al. (2014) explained the degradation of CNCbl and OHCbl by reduction of Co^{3+} to Co^{2+}, which leads to release of cobalt from the molecule, cleavage of the corrin ring and formation of oxidation products.

In this study, added CNCbl was more stable than OHCbl. However, we cannot judge how much CNCbl possibly had been converted to OHCbl because in the extraction step, OHCbl was converted back to CNCbl. If CNCbl converts to OHCbl, but no further, it still is available in the bread. If the OHCbl initially formed from CNCbl degrades further to the corrin ring and oxidation products, added CNCbl is lost completely, as obviously happened in sourdough baking. In conclusion, this study showed that interactions of cobalamins with other compounds connected to longer baking time and longer exposure to light at low pH may decrease stability of both CNCbl and OHCbl in baking processes.

3.2. Stability of vitamin B12 in baking with malt extract fermented by P. freudenreichii

Straight- and sourdough baking processes also were carried out using malt extract containing *in situ*-produced vitamin B12 instead of water. The presence of active vitamin B12 form was confirmed by the UHPLC method (Chamlagain et al., 2015). Fermented malt media and baking samples resulted after extraction and purification, with a peak eluting at the retention time of CNCbl (UV361 nm at 3.27 min) and showing a PDA spectra (210–600 nm) identical to that of CNCbl. Because MBA is not able to measure only active B12 in fermented matrix, we based the stability evaluation on UHPLC results.

The straight-dough baking process did not destroy *in situ*-produced, active vitamin B12 significantly ($p > 0.05$, $n = 6$), either when low (Trial 1) or high (Trial 2) vitamin B12 content ME-media were used in baking (Fig. 3). By contrast, as in breadmaking with added CNCbl and OHCbl, the sourdough baking process

Table 2
Acidity (pH) of sub-samples from different baking processes, moisture losses of breads during the baking step, and specific volumes of baked breads. Values are means of at least two analytical replicates from one to six baking trials (n = 1–6).

Baking process		Control dough	Added CNCbl	Added OHCbl	*In situ*-produced vitamin B12
Straight-dough		n = 3	n = 3	n = 3	n = 6
	pH				
	After mixing	5.7	5.7	5.7	5.4
	After 1.5-h proofing	5.8	5.8	5.8	5.4
	Baked bread	5.7	5.7	5.7	5.6
	Moisture loss of the baked bread, %	15.1 ± 0.2	14.2 ± 0.2	14.7 ± 0.2	11.3 ± 0.2
	Specific volume of the baked bread, cm^3/g	3.6 ± 0.1	3.4 ± 0.1	3.5 ± 0.2	2.4 ± 0.2
Sponge-dough		n = 2	n = 2	n = 2	
	pH				
	After 1st mixing	5.8	5.8	5.9	
	After 2-h pre-proofing	5.9	5.9	5.9	
	After 2nd mixing	5.8	5.8	5.8	
	After 1.5-h proofing	5.6	5.6	5.6	
	Baked bread	5.6	5.6	5.6	
	Moisture loss of the baked bread, %	13	15	16	
	Specific volume of the baked bread, cm^3/g	3.4	4.0	4.1	
Sourdough Test 1		n = 1	n = 1	n = 1	n = 3
	pH				
	After 1st mixing	6.4	6.4	6.4	5.5
	After 16-h fermentation	3.8	3.9	3.8	4.9
	After 2nd mixing	4.1	4.2	4.1	5.1
	After 1.5-h proofing	4.1	4.2	4.1	5.1
	Baked bread	4.2	4.2	4.2	5.2
	Moisture loss of the baked bread, %	26	28	28	26
Sourdough Test 2		n = 1	n = 1	n = 1	
	pH				
	After 2nd mixing	4.1	4.1	4.2	
	After 1.5-h proofing	4.1	4.1	4.2	
	Baked bread	4.2	4.2	4.3	
	Moisture loss of the baked bread, %	26	29	25	

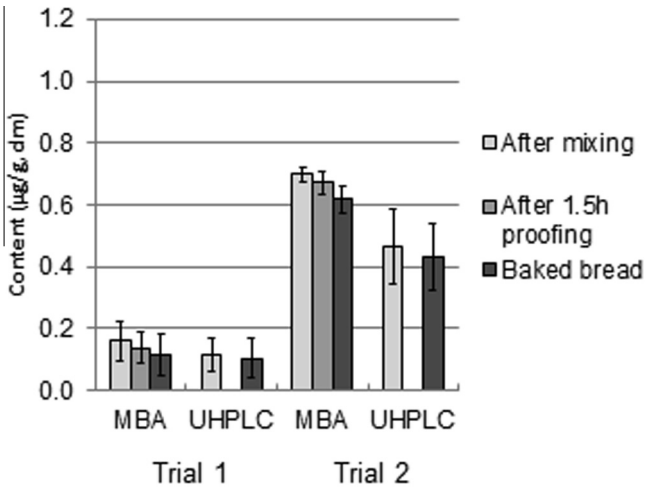

Fig. 3. Vitamin B12 content of straight-dough samples (µg/g, dm) baked in fermented malt extract medium in Trial 1 (low-vitamin content) and in Trial 2 (high-vitamin content) determined by the microbiological assay (MBA) and the ultra-high performance liquid chromatography (UHPLC) methods. Error bars represent standard deviations of three separate baking processes with three biological replicates of fermented media. Sub-samples after 1.5-h proofing were not determined by the UHPLC method.

decreased the content of in *in situ*-produced vitamin B12 during the baking step (Fig. 4). From the second mixing to baked bread stages, vitamin loss was 29 ± 4% ($p < 0.05$, n = 3) when determined by the UHPLC method (Fig. 4). During the 16-h fermentation and 1.5-h proofing steps, the amount of active B12 increased only slightly based on the UHPLC results. However, MBA results showed a significant increase of 24 ± 14% ($p < 0.05$). It might be possible that synthesis of B12 continued during fermentation. In addition, lactic acid produced by LAB might have supported the synthesis, because lactate is a favourable carbon source for PAB (Piveteau, 1999). Furthermore, LAB in sourdough might synthesise compounds other than active vitamin B12 that might stimulate growth of the test organism (Herbert, 1988).

To our best knowledge, the present study is the first in which active vitamin B12 produced *in situ* was used in breadmaking. In baking, active B12 was more stable than added OHCbl and almost as stable as added CNCbl. Cofactor enzyme forms AdoCbl and MeCbl synthesised by *P. freudenreichii* are accumulated inside the cells bound to proteins (Martens, Barg, Warren, & Jahn, 2002; Miyano, Ye, & Shimizu, 2000). These natural forms probably are protected to some extent from light, heat and pH, which may explain the better retention of the vitamin produced *in situ*. However, in the sourdough process, conditions were destructive even to protein-bound, active vitamin forms, and degradation of *in situ*-produced vitamin probably was caused by the same factors that caused degradation of added CNCbl and OHCbl, namely long baking time and low pH. However, in the sourdough process with malt extract, pH did not decrease as low as in normal sourdough baking, being near to pH 5 (Table 2).

In this study, we also observed an apparent discrepancy between MBA and UHPLC results of samples baked in fermented malt extract (Figs. 3 and 4). In fermented malt media used in baking, MBA returned 33 ± 8% (n = 9) higher vitamin B12 content than did the UHPLC method. The test organism (*L. delbrueckii*) used in MBA utilises also incomplete corrinoid compounds, deoxyribosidies and deoxyribonucleotides for its growth, and this may explain why MBA yields higher contents than UHPLC (Watanabe, 2007). Incomplete corrinoids may arise from incomplete B12 synthesis due to the insufficient *de novo* synthesis of DMBI or lack of exogenous supplementation (Moore & Warren, 2012). In addition to active vitamin B12, fermented media probably also contained variable amounts of other corrinoids due to lack of endogenous DMBI

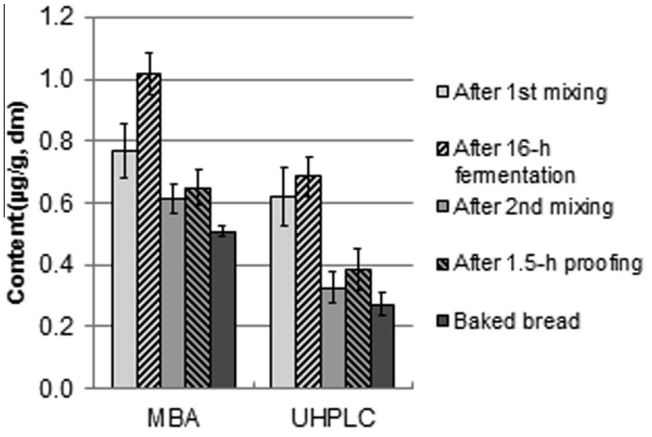

Fig. 4. Vitamin B12 content of sourdough samples (µg/g, dm) baked in fermented malt extract media as determined by the microbiological assay (MBA) and ultra-high performance liquid chromatography (UHPLC) methods. Error bars represent standard deviations of three separate baking processes with three biological replicates of fermented media.

or/and unfavorable fermentation conditions. This might be another reason for variation between biological replicates of fermented media and, hence, for variation of vitamin B12 content in baking samples (Figs. 3 and 4).

3.3. General quality of baking with added and in situ-produced active vitamin B12

Addition of CNCbl and OHCbl affected neither moisture loss nor specific volume of breads baked by various processes (Table 2). Furthermore, pH values in CNCbl and OHCbl bakings adhered to pH values of control bakings. Based on subjective observations, breads baked in fermented malt medium seemed to have a mild cheese-like taste combined with a pleasant, sweet, malty taste. Specific volumes of these malt breads were slightly lower (on average 2.4 cm^3/g) than those of control or fortified breads (on average 3.5 cm^3/g) (Table 2). Probably, propionic and acetic acids produced by *P. freudenreichii* in fermented malt media inhibited the growth of *Saccharomyces cerevisiae*. Weak acids have been shown to have antifungal properties when they are able to enter into the cell as undissociated forms. An acidification effect inside the cell prevents the essential metabolic functions (Ullah, Orij, Brul, & Smits, 2012). On the other hand, acids could be used as preservatives to extend the shelf-life of bread.

Because the main objective of the present study was to investigate stability of vitamin B12 in breadmaking, the baking and fermenting processes were not optimised for *in situ* production of vitamin B12. However, our results were encouraging to further research to obtain bread products with adequate *in situ*-produced vitamin B12 content combined with acceptable baking and sensory quality. This could be achieved by optimising both cultivation conditions in the fermentation process and the amount of vitamin B12-rich malt medium used in baking. In this study, the content of active vitamin B12 in straight-dough bread was at the highest on average 0.23 µg/g fw (in Trial 2). A person consuming 30 g (one portion of bread) of this bread would ingest 7.5 µg of active vitamin B12, three times the daily recommendation (Nordic nutrition recommendations 2012, 2014).

4. Conclusion

Added CNCbl was stable, whereas 20–30% of added OHCbl was lost during the baking steps in straight- and sponge-dough processes. In the sourdough process, baking losses of OHCbl were even higher, and nearly 25% of added CNCbl was lost as well. Malt medium containing active vitamin B12 produced *in situ* by *P. freudenreichii* was successfully used in the straight- and sourdough processes. *In situ*-produced vitamin B12 was nearly as stable as added CNCbl and more stable than OHCbl in the straight- and sourdough processes. In the straight-dough process, *in situ*-produced vitamin B12 was stable, but in the baking step of the sourdough process, it was lost, similar to added CNCbl.

Results obtained by both the MBA and UHPLC methods matched when the contents of added CNCbl and OHCbl were determined in samples produced by the straight- and sponge-dough processes. By contrast, MBA was not accurate when analysing added vitamin B12 in sourdough samples. Furthermore, MBA was not suitable to determine *in situ*-produced active vitamin B12. The fermented malt extract may have contained corrinoid-like compounds that stimulated the growth of the test organism used in MBA. This study demonstrated that *in situ* synthesis of active vitamin B12 is a promising option to fortify breads. The results encourage further research to obtain bread products with adequate vitamin B12 content as well as acceptable baking and sensory quality.

Acknowledgements

The study was financed by the Academy of Finland Project No. 257333 (Natural fortification of foods: Microbial *in situ* synthesis of vitamin B12 and folate in cereal matrix). The authors wish to thank Laurie Martin for her assistance in baking and Miikka Olin for his technical support during UHPLC analyses.

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