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Analytical Methods

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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1 **Ultra-high performance liquid chromatographic and mass spectrometric**
2 **analysis of active vitamin B12 in cells of *Propionibacterium* and fermented**
3 **cereal matrices**
4
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26 **ABSTRACT**

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

39

40

41 **Keywords**: Vitamin B12; *Propionibacterium freudenreichii*; fermented cereal
42 matrix; UHPLC; UPLC; immunoaffinity purification

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51 **1. Introduction**

52

53 Vitamin B12, one of the water-soluble vitamins, is needed in humans and
54 animals for the metabolic functions of two enzymes: methylmalonyl-CoA mutase
55 and methionine synthase. According to Institute of Medicine (1998), the
56 recommended dietary allowance (RDA) of vitamin B12 for adults is 2.4 μg/day. It
57 is only synthesized by a few bacteria and archeae (Martens, Barg, Warren, & Jahn,
58 2002). The structure of vitamin B12 consists of a corrin ring with a central cobalt
59 atom complexed to four pyrrole rings, a lower α-ligand and an upper β-ligand.
60 This vitamin includes three naturally occurring forms (adenosylcobalamin,
61 methylcobalamin and hydroxycobalamin) and one chemically transformed form
62 (cyanocobalamin), differing only in the upper ligand. Cyanocobalamin, the most
63 stable form, is obtained by reacting natural vitamers with cyanide and it is the
64 major vitamin B12 used in fortified foods, nutritional formula and pharmaceutical
65 preparations (Ball, 2006). The lower ligand of vitamin B12 is 5,6-
66 dimethylbenzimidazole (DMBI), which is essential for the binding of the vitamin
67 to the intrinsic factor for its absorption (Nielsen, Rasmussen, Andersen, Nexø, &
68 Moestrup, 2012). Corrinoids with lower ligands other than DMBI are found in
69 biological materials (Watanabe, Yabuta, Tanioka, & Bito, 2013). They are
70 biologically inactive in humans but have vitamin activity in microorganisms
71 (Herbert, 1988).

72 Animal-based foods such as meat, fish, eggs and dairy products are the
73 major contributors of vitamin B12 in the human diet. It is accumulated in animal
74 tissues or milk, primarily as a result of the gut microbial activities and feed
75 supplements. However, plant food materials are devoid of vitamin B12 (Ball,

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2006; Herbert, 1988). 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,

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101 Risse, & Hadorn, 2012), reflecting the poor specificity of MBA. Moreover, MBA
102 is labour intensive and time consuming.

103 HPLC based methods have been developed for the determination of vitamin
104 B12 in fortified foods, infant formula and vitamin supplements (Campos-Giménez,
105 Fontannaz, Trisconi, Kilinc, Gimenez, & Andrieux, 2008; Chen, Wolf,
106 Castanheira, & Sanches-Silva, 2010; Heudi, Kilinç, Fotannaz, & Marley, 2006;
107 Marley, Mackay, & Young, 2009). Some studies have also focused on the
108 quantification of vitamin B12 in non-fortified foods such as meat products
109 (Guggisberg et al., 2012), and milk-based fermented products (Van Wyk & Britz,
110 2010). These HPLC methods were based on UV detection. One HPLC method
111 reported by Pakin, Bergaentzlé, Aoudé-Werner and Hasselmann (2005) used
112 fluorescence detection after vitamin B12 was derivatised into a fluorescent
113 compound, α-ribazole. The latter method was sensitive, but it required lengthy
114 sample preparation time involving extensive sample derivatisation. The
115 introduction of an immunoaffinity sample purification technique has improved the
116 sensitivity of the HPLC measurement in fortified foods and supplements
117 (Campos-Giménez et al., 2008; Heudi et al., 2006; Marley et al., 2009).

118 However, the development of ultra-high performance liquid
119 chromatographic (UHPLC) technology that uses separation column made up of
120 sub-2 μm particles and improved instrumentation has considerably increased the
121 sensitivity and resolution of the chromatographic analysis (Swartz, 2005). To
122 date, one method based on UHPLC with mass spectrometric (MS) detection has
123 been reported for the analysis of vitamin B12 in milk and dairy products (Zironi,
124 Gazzotti, Barbarossa, Devicienti, Scardilli, & Pagliuca, 2013). The enhanced
125 sensitivity offered by the UHPLC technology and the sample clean-up and

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126 concentrating potential of vitamin B12-specific commercial immunoaffinity
127 columns have not yet been utilised for analysing vitamin B12 in natural or
128 microbiologically processed foods. Particularly, it is necessary to confirm that the
129 microbiologically synthesized forms are active for humans when biofortification
130 is applied. Up to now, cereal matrices, which account for a major portion of the
131 human diet but contain no vitamin B12, have not been considered for *in situ*
132 enrichment with vitamin B12 using food-grade microorganisms.

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

140

141

142 **2. Materials and methods**

143

144 *2.1. Chemicals, materials and reagents*

145 All chemicals and reagents used were of analytical grade. Sodium hydroxide,
146 acetic acid and the vitamin B12 assay medium were obtained from Merck
147 (Darmstadt, Germany); ethanol from Altia (Rajamäki, Finland); acetonitrile
148 (HPLC grade), trifluoroacetic acid (TFA) and sodium cyanide from Sigma-
149 Aldrich (Steinheim, Germany); cyanocobalamin from Supelco (Bellefonte, USA);
150 Tween 80 from Sigma (Aldrich, USA); and α-amylase (EC 232-588-1, A9857-

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151 5MU, *Aspergillus oryzae*) was from Sigma-Aldrich (Steinheim, Germany). A
152 certified reference material BCR 487 (lyophilised pig liver; Institute for Reference
153 Materials and Measurements, Geel, Belgium) was bought from Sigma-Aldrich.
154 Water (later, MilliQ water) used for the preparation of reagents and analyses was
155 obtained from a Milli-Q Plus system (0.22 μm, ≥ 18.2 MΩ cm, Millipore
156 Corporation, Bedford, MA, USA).

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

161

162 *2.2. Vitamin B12 extraction*

163

164 The protocol for vitamin B12 extraction (as cyanocobalamin) from sample
165 matrices was adopted from Kelleher and Broin (1991) for plasma samples with
166 some modifications. Cereal matrix (1–5 g), bacterial cell pellet (0.1–0.2 g) or pig
167 liver (0.1 g) weighed in an extraction tube was vortexed with 10 mL buffer (8.3
168 mM sodium hydroxide and 20.7 mM acetic acid; pH 4.5) containing 100 µL 1%
169 sodium cyanide and placed in a boiling water bath for 30 min. The samples were
170 then ice-cooled and centrifuged (Hermle, Wehingen, Germany) at 6900 × g for 10
171 min. The supernatant was collected in a fresh tube. The residue pellet was
172 vortexed once again with 5 mL buffer (pH 6.2, adjusted from the pH 4.5 buffer
173 with 3% sodium hydroxide) and centrifuged. The supernatants were combined
174 and the pH was adjusted to 6.2. The extract was then paper filtered (Ø 90 mm,
175 VWR, Leuven, Belgium) and the volume was adjusted to 25 mL with pH 6.2

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176 buffer. To ease the filtration of extracts for immunoaffinity purification, the
177 cereal-based samples were treated with 1 mL of α-amylase solution (50 mg/mL)
178 (37 °C; 30 min) to digest the starch before the boiling-water extraction (Marley et
179 al., 2009). All the analytical processes were carried out under subdued light or
180 were protected from direct light. The vitamin B12 content of the cereal matrices
181 was expressed in terms of fresh weight (ng/g matrix) and that of cells per unit
182 volume fermented medium (ng/mL).

183

184 *2.3. Immunoaffinity purification for UHPLC quantification*

185

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

196

197 *2.4. Ultra-high performance liquid chromatography (UHPLC)*

198

199 *2.4.1. UHPLC system*

200

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201 The chromatographic analysis was performed on a Waters Acquity UPLC
202 system (Milford, MA, USA) equipped with a photodiode array detector (PDA;
203 210–600 nm). In addition, it consisted of a binary solvent manager, autosampler
204 and column manager. The detection was performed at 361 nm and the absorption
205 spectra were recorded by the PDA. The autosampler was maintained at 4 °C and
206 the column was operated at 30 °C. The autosampler injected the sample solutions
207 (2–15 μL) onto a column via a 20-μL injection loop operated in a partial-loop
208 mode. Chromatographic data were collected and processed using the Waters
209 Empower 2 software.
210
211 *2.4.2. Method optimisation and validation*
212
213 Two reversed-phase C18 columns, high-strength silica T3 (HSS) and
214 ethylene bridged hybrid (BEH) from Waters (Milford, MA, USA), of similar
215 dimensions (2.1 x 100 mm; 1.8 μm and 1.7 μm particles, respectively) were
216 evaluated for the separation of vitamin B12 from sample extracts. A set of flow
217 rates (0.32□0.50 mL/min) was tested in a linear gradient mode of the mobile
218 phase consisting of MilliQ water (solvent A) and acetonitrile (solvent B); each
219 modified with 0.025% TFA. Finally, the following mobile phase gradients
220 (solvent A: solvent B) were maintained during a 10-min run: 0–0.5 min (95:5);
221 0.5–5 min (60:40); 5–6 min (60:40); and 6–10 min a linear gradient from 60:40 to
222 95:5 for equilibration. A calibration curve was produced in each sample set from
223 six cyanocobalamin standards with the vitamin concentration ranging from
224 0.015□0.75 ng/μL; each standard solution was injected twice. The
225 chromatographic method was assessed for the linearity of the calibration curve,

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226 limit of detection (LOD: signal-to-noise ratio, S/N= 3), and limit of quantitation
227 (LOQ: 3 times the LOD) of vitamin B12 using the cyanocobalamin standard
228 solutions. The resolution (calculated according to the formulae in the US
229 Pharmacopeia; USP) for the separation of cyanocobalamin in sample extracts and
230 the number of theoretical plates (USP) for each column were compared. LOD,
231 resolution and number of theoretical plates were obtained with the Waters
232 Empower 2 software.
233
234 *2.5. UHPLC-MS/MS analysis*
235
236 The mobile phase was modified with 0.5% formic acid instead of 0.025%
237 TFA for the UHPLC-MS study to minimise the ion suppression. The MS analysis
238 was carried out in a positive ion mode on an Esquire-LC quadrupole ion trap mass
239 spectrometer with an electrospray ionization (ESI) interface (Bruker Daltonics,
240 Bremen, Germany) and the data were analysed by the LC-MSD Trap version 5.2
241 (Bruker Daltonics). The scanning was carried out for a range of *m/z* 900□1400
242 and the tandem mass spectrometry (MS/MS) was performed for a particular *m/z*
243 according to the target molecule(s) using helium as the collision gas. The
244 following instrumental settings optimised for cyanocobalamin were used for the
245 MS analysis of cyanocobalamin ($[M+H]^+$ *m/z* 1356) and pseudovitamin B12
246 ($[M+H]^+$ *m/z* 1345) in sample extracts: nebulizer (nitrogen) 50.0 psi, dry gas
247 (nitrogen) 8.0 L/min, dry temperature 300 °C, capillary 4500 V, end plate offset
248 −250 V and trap drive 84.
249
250 *2.6. Microbiological assay*

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251

252 The MBA was performed on a 96–well microtiter plate (Corning, NY, USA)
253 based on Kelleher and Broin (1991) using *L. delbrueckii* ATCC 7830 as an assay
254 organism and cyanocobalamin as a calibrant. The sample extracts were
255 appropriately diluted at two levels with the extraction buffer (pH 6.2). A certified
256 reference material BCR 487 (pig liver) extract served as a reference sample in
257 each set of samples. A blank extract was used to obtain the actual readings of the
258 samples. Cyanocobalamin solutions of increasing concentration (0–8 pg/well) and
259 sample extracts, both 100 μL, were inoculated into the wells of the microtiter plate
260 (4 wells for each concentration), and 200 μL of freshly prepared sterile filtered
261 vitamin B12 assay medium (pH 6.2) inoculated with the cryopreserved assay
262 organism was introduced into each well. The plate was then incubated at an
263 optimised condition (35 °C; 19 h) and the turbidity was measured with a micro-
264 plate reader (Multiskan EX; Labsystems, Finland) at 595 nm. A calibration curve
265 of 8 concentration levels and the amount of vitamin B12 in each well were
266 obtained automatically by Ascent software version 2.6 (Labsystems).

267

268 *2.7. Recovery study*

269

270 Since natural cereal matrices do not contain vitamin B12, the unfermented
271 rye malt matrix (from Section 2.8.) was used as a blank matrix for the recovery
272 study. The sample matrix (1.5 g) was spiked with cyanocobalamin at two levels
273 (17.7 ng and 53.1 ng), each level in triplicate, and vitamin B12 was extracted and
274 analysed by MBA directly and by UHPLC after immunoaffinity purification, as
275 explained earlier. The repeatability was shown by carrying out two independent

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276 experiments on two days. To test the effect of the sample matrix, the recovery was
277 also studied in the extraction buffer with the lower level of spiking (17.7 ng). The
278 recovery was calculated from the measured cyanocobalamin concentration in the
279 spiked samples to the concentration of added cyanocobalamin in the samples.
280
281 *2.8. Bacterial culture and fermentation of sample matrices*
282
283 Propionibacteria capable of vitamin B12 synthesis and allowed for use in
284 food production are mostly used in cheese manufacturing and in the industrial
285 production of vitamin B12 with genetically engineered strains (Thierry, Deutsch,
286 Falentin, Dalmasso, Cousin, & Jan, 2011). For this study, a strain of
287 *Propionibacterium freudenreichii* subsp. *shermanii (P. freudenreichii* ABM 5378)
288 isolated from a cheese starter culture was used for the production of vitamin B12
289 in the sample matrices.
290 Malt matrices: The fermented cereal samples were produced to study the
291 suitability of the developed method; the fermentation conditions used were not
292 optimised for vitamin B12 production. Rye and barley malt flours obtained by
293 milling malted grains (Laihian Mallas, Laihia, Finland) were separately mixed in
294 boiling MilliQ water (33% w/v) and cooked for 2 min. The gelatinised matrices
295 were transferred to glass flasks and then autoclaved at 121 °C for 15 min. The
296 culture preparation and fermentation of the matrices were carried out as described
297 by Kariluoto et al. (2010). Briefly, the cooled matrices were aseptically
298 transferred into pre-sterilised flasks and a culture of *P. freudenreichii* ABM 5378
299 was inoculated. The matrices were allowed to ferment at 28 °C for 24 h. After the
300 incubation, the cell counts (CFU/g) had increased by a log factor in both matrices

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301 while pH had decreased from 5.6 to 5.3 in the rye matrix and 5.4 to 5.2 in the
302 barley matrix. Likewise, an aqueous barley malt extract was fermented with
303 unidentified strains of bacteria producing lactic and propionic acids and then
304 concentrated.

305 Supplemented whey permeate (SWP): *P. freudenreichii* ABM 5378 was
306 grown in the SWP medium anaerobically for 72 h and then aerobically for 96 h at
307 30 °C (Hugenschmidt, Schwenninger, Gnehm, & Lacroix, 2010). Cheese whey
308 powder (Valio, Espoo, Finland) was used instead of whey permeate powder, as in
309 the study of Hugenschmidt et al. (2010), and the medium was supplemented with
310 5 mg/L cobalt(II) chloride (Sigma-Aldrich). The fermented broth was centrifuged
311 (12000 × g;10 min), the resulting cell pellet was re-suspended in 10 mL of pH 7.3
312 PBS buffer (Oxoid, Hampshire, England) and centrifuged again. The fresh cell
313 mass obtained was stored at −20 °C until the analysis.

314 *2.9. Statistical analysis*

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

320

321 3. Results and Discussion

322

323 *3. 1. UHPLC method optimisation and validation*

324

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

345

346 **Fig. 1**

347

348 The retention time of cyanocobalamin was stable during the analyses,
349 irrespective of the sample matrices. The 30-day average retention time was 3.27

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350 min with a narrow change from 3.25 to 3.29 min over the study period. The
351 UHPLC analysis was faster with a total run time of 10 min compared with over 30
352 min that was required for the HPLC methods (Heudi et al., 2006; Marley et al.,
353 2009). An external calibration curve of excellent linearity ($R^2 > 0.999$) was
354 achieved by injecting a series of cyanocobalamin standard solutions (0.015□0.75
355 ng/µL). The instrumental LOD and LOQ were 0.075 ng/*inj* and 0.225 ng/*inj*,
356 respectively, for a 5 µL injection. Previous HPLC/UV methods resulted in higher
357 LODs at the larger injection volumes: 1.5 ng/*inj* (50–100 µL) (Guggisberg et al.,
358 2012; Marley et al., 2009) and 3–3.3 ng/*inj* (100 µL) (Chen et al., 2010; Heudi et
359 al., 2006). The enhanced sensitivity allowed for the analysis with small samples
360 (< 5 g), unlike the bulk quantity needed for HPLC analysis (Campos-Giménez et
361 al., 2008; Guggisberg et al., 2012); thus minimising the matrix-related
362 interference. Moreover, HPLC methods have mostly been developed for fortified
363 foods and supplements for measuring added cyanocobalamin, at levels greater
364 than those normally found in foods.
365
366 **Table 1**
367
368 The accuracy of the UHPLC method was studied by determining the
369 recovery of spiked cyanocobalamin from a rye malt matrix. Low levels were
370 chosen to cover concentrations that are possible in the fermented cereal matrices.
371 The added levels were still too low to be detected with UHPLC without
372 purification of the extracts. On the other hand, the usefulness of the additional
373 purification and enrichment on the immunoaffinity columns was demonstrated by
374 the good recoveries at both levels. The mean inter-day recovery of

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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.

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400 However, Kelly, Gruner, Furlong and Sykes (2006) investigated corrinoids in
401 lamb tissues, including the liver, by HPLC. Contrary to previous reports that
402 corrinoids in the lamb liver were exclusively vitamin B12, these authors found
403 quite a marked fraction of the total corrinoids (up to 30%) as vitamin B12
404 analogues.
405
406 **Table 2**
407
408 *3.2. UHPLC analysis of vitamin B12 in cells and fermented cereal matrices*
409
410 Figure 2 shows example chromatograms with and without the
411 immunoaffinity clean-up for extracts of cells, pig liver, spiked rye malt matrix and
412 fermented rye malt matrix. Without the immunoaffinity purification, a higher
413 chromatographic background was obtained for the pig liver and the fermented
414 malt matrices while a cleaner chromatogram with a well-separated
415 cyanocobalamin peak (retaining at 3.27 min) was observed for the cell extract.
416 Besides the matrix effect on separation, another disadvantage was that the
417 concentration of cyanocobalamin in these extracts was low or below the LOD
418 (0.075 ng/5 µL injection). The immunoaffinity purified extracts produced a well-
419 separated peak with none or little background interference, demonstrating the
420 usefulness of the immunoaffinity clean-up for the analysis of low levels of
421 vitamin B12 in the complex matrices. Guggisberg et al. (2012) reported its
422 applicability for the analysis of vitamin B12 in meat and meat products.
423 Nevertheless, analysis of the matrices such as microbial cells, containing a higher
424 level of vitamin B12, did not require the immunoaffinity purification. Therefore,

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425 especially with the immunoaffinity purification this method could also be
426 applicable for other matrices. Separate validation for each material however is
427 needed.

428 **Fig. 2**

429

430 The corrinoid in the cell extract, the immunoaffinity purified fermented malt
431 matrices and the pig liver eluted at the retention time of the cyanocobalamin
432 standard (Fig. 2) and had a PDA spectrum (210□600 nm) identical to that of
433 cyanocobalamin (Fig. 3). Furthermore, the UHPLC-MS/MS analysis revealed that
434 the fragmentation profile of the corrinoid for the *m/z* of 1356, $[M+H]^+$ of
435 cyanocobalamin, was similar to that of the cyanocobalamin standard, as shown in
436 the example mass spectra (Figs. 4A; 4B). The following major fragmentation
437 products of cyanocobalamin, as explained by Carkeet et al. (2006) and shown in
438 Figure 4A, were observed: *m/z* 1210 $[M+H−DMBI]^+$; *m/z* 1124
439 $[M+H−DMBI−CN−Co]^+$; *m/z* 997 $[M+H−DMBI−sugar−PO3]^+$ and *m/z* 912
440 $[M+H−DMBI−sugar−PO3−CN−Co]^+$. These observations confirmed that the
441 lower ligand of the corrinoid in the sample extracts was DMBI as in
442 cyanocobalamin, which is critical for the binding of the B12 vitamer to the
443 intrinsic factor to make it bioavailable for humans (Nielsen, Rasmussen, Andersen,
444 Nexø, & Moestrup, 2012).

445

446 **Fig. 3**

447

448 **Fig. 4**

449

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

458 To the best of our knowledge, we are the first to report vitamin B12 *in situ*
459 production in cereal matrices with propionibacteria and its analysis by UHPLC.
460 The vitamin B12 content of the *P. freudenreichii* fermented rye and barley malt
461 matrices measured with the UHPLC was 19.3 ng/g and 15.7 ng/g of fresh weight,
462 respectively (Table 2). On the other hand, the UHPLC analysis of the fermented
463 malt extract after the immunoaffinity purification revealed a peak in the
464 chromatogram at 3.22 min (Fig. 3A), i.e., before the retention time of
465 cyanocobalamin (3.27 min) from the standard solution and the immunoaffinity
466 purified pig liver extract (Figs. 3B; 3C). Its PDA spectrum (210□600 nm) was,
467 however, similar to that of cyanocobalamin. This peak, which was not seen in the
468 chromatograms of any other analysed samples, was suggested to be of
469 pseudovitamin B12 with adenine as its lower ligand. It is a corrinoid known to be
470 present in biological materials (Watanabe et al., 2013) and has been recently
471 characterised in *L. reuteri* CRL1098 (Santos et al., 2007). When the sample
472 extract was analysed by MS/MS for an *m/z* of 1356, $[M+H]^+$ of cyanocobalamin,
473 no fragment ions characteristic of cyanocobalamin were observed. However, a
474 fragmentation profile identical to the cyanocobalamin standard was obtained by

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475 the MS/MS for an *m/z* of 1345, $[M+H]^+$ of pseudovitamin B12 (Fig. 4C: *m/z* 1210
476 $[M+H−adenine]^+$; *m/z* 1124 $[M+H−adenine−CN−Co]^+$; *m/z* 997
477 $[M+H−adenine−sugar−PO3]^+$ and *m/z* 912 $[M+H−adenine−sugar−PO3−CN−Co]^+$.
478 The similar fragment ions obtained for the *m/z* of 1345 with that for the *m/z* of
479 1356 confirmed that the molecule had a structure otherwise similar to
480 cyanocobalamin, but a different lower ligand: adenine in place of DMBI.
481
482 *3.3. Comparison between UHPLC analysis and MBA*
483
484 The MBA results of the *P. freudenreichii* cells were similar to the UHPLC
485 derived results, whereas analysis of the certified reference pig liver (BCR 487)
486 resulted in a 17% higher vitamin B12 content by the MBA (Table 2). The vitamin
487 B12 contents determined by the UHPLC were 67% and 63% of the MBA results
488 for the fermented rye and barley matrices, respectively (Table 2). For the malt
489 extract fermented with bacteria producing lactic and propionic acids no detectable
490 level of cyanocobalamin was found by the UHPLC method in contrast to 1200
491 ng/g of vitamin B12 measured by the MBA. These sample-specific differences of
492 the vitamin B12 contents when measured by the MBA or UHPLC method could
493 be due to the inherent drawbacks associated with the MBA.
494 The MBA has been the golden standard method allowing for the
495 determination of low levels of vitamin B12 in foods and biological materials.
496 However, it has been reported to give higher vitamin B12 results than HPLC in
497 biological and fermented materials. The higher results are possible, as the assay
498 organism also responds to corrinoids that are inactive for humans, e.g.
499 pseudovitamin B12 and nucleic acids (Ball, 2006; Herbert, 1988; Indyk et al.,

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500 2002). These inactive corrinoids were reported in ovine tissues (Kelly et al., 2006)
501 and may also be synthesized by microorganisms (Herbert, 1988), including
502 propionibacteria. Quesada-Chanto et al. (1998) analysed vitamin B12 in the cells
503 of three strains of propionibacteria by an HPLC method and two
504 spectrophotometric methods. Vitamin B12 was only detected in cells of *P.*
505 *freudenreichii* with HPLC, whereas vitamin B12 analogues with retention time
506 different than authentic vitamin B12 were observed in the cells of other two
507 strains. However, one strain produced authentic vitamin B12 when the culture
508 medium was supplemented with DMBI. On the other hand, some bacteria produce
509 only inactive corrinoids even in the presence of DMBI. *L. reuteri* CRL1098
510 produced pseudovitamin B12 exclusively in a growth medium supplemented with
511 and without DMBI under anaerobic conditions (Santos et al., 2007).

512 Campos-Giménez et al. (2008) noticed a 20–30% higher vitamin B12
513 content in milk-based infant cereals and soy-based fortified formula by MBA
514 compared to HPLC. Poor selectivity of the MBA was further shown in the
515 analysis of spirulina tablets and shellfish where the MBA results were up to 6–8-
516 fold higher than concentrations determined by a specific intrinsic factor based
517 chemiluminescence method (Watanabe, Takenaka, Abe, Tamura, & Nakano,
518 1998). The majority of the measured vitamin B12 may thus have been inactive
519 corrinoids.
520

521 **4. Conclusion**

522

523 The developed UHPLC/UV method enabled sensitive and specific
524 quantitation of the vitamin B12 content in microbial cells, and in fermented cereal

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525 matrices after purification on immunoaffinity columns. This method allowed for
526 the selective determination of bioactive vitamin B12, thus eliminating the
527 possibility of measuring inactive corrinoids as in the MBA. One such analogue,
528 pseudovitamin B12, was identified and confirmed in a malt extract fermented by
529 bacteria producing lactic and propionic acids. A nutritionally relevant amount of
530 active vitamin B12 was produced by *P. freudenreichii* in rye and barley malt
531 matrices. The LC-MS is a useful tool for the identification of *in situ* produced
532 bioactive vitamin B12 from inactive corrinoids in food matrices or microbial
533 materials.

534

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542 *in situ* synthesis of vitamin B12 and folate in cereal matrix).

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650 **Figure captions:**

651

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

659

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

666

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

671

672 **Fig. 4.** UHPLC-MS/MS spectra of an *m/z* $[M+H]^+$ of 1356 for the
673 cyanocobalamin standard (A) and the corrinoid in the immunoaffinity purified
674 extract of the fermented barley malt matrix (B), and of an *m/z* $[M+H]^+$ of 1345

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675 (pseudovitamin B12) for immunoaffinity purified malt extract fermented with
676 bacteria producing lactic and propionic acids (C).

677

678

679 **Table captions:**

680

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

685

686 **Table 2.** Vitamin B12 contents of certified reference material BCR 487
687 (lyophilised pig liver), *P. freudenreichii* fermented cereal matrices (28 °C; 24 h),
688 cell mass from *P. freudenreichii* fermented supplemented whey permeate (SWP)
689 (30 °C; 168 h) and concentrated malt extract fermented with bacteria producing
690 lactic and propionic acids determined by UHPLC and MBA.

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Sample	Spiked amount (ng)	Recovery, % (RSD, %)			
		Day 1		Day 2	
		UHPLC	MBA	UHPLC	MBA
	0.0	ND	ND	ND	ND
Rye malt matrix	17.7	97.2 (3.1)[a]	95.0 (0.9)[a]	93.8 (5.3)[a]	98.2 (4.3)[a]
	53.1	96.8 (2.4)[a]	94.8 (3.7)[a]	91.2 (2.5)[a]	96.1 (5.7)[a]
Extraction Buffer	17.7	94.6 (1.0)[a]	92.7 (5.7)[a]	97.4 (1.9)	NA

ND = Not detected
NA = Not analysed
Values with dissimilar superscript letters (a and b) along each row indicate significant difference ($p < 0.05$).

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Sample	UHPLC (ng/g)	MBA (ng/g)	UHPLC/MBA, %
BCR 487 pig liver (n = 8)	876 (±49)[a]*	1056 (±80)[b]*	82.9
Fermented rye malt matrix ($n = 2 \times 2$)[‡]	19.3 (±1.5)[a]	28.8 (±2.4)[b]	67.0
Fermented barley malt matrix ($n = 2 \times 2$)[‡]	15.7 (±2.4)[a]	25.0 (±2.3)[b]	62.8
Cell mass (from fermented SWP) (n = 3)	620 (±90)[a#] (ng/mL)	650 (±60)[a#] (ng/mL)	95.4
Concentrated fermented malt extract (n = 3)	ND	1196 ± 56	-

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.

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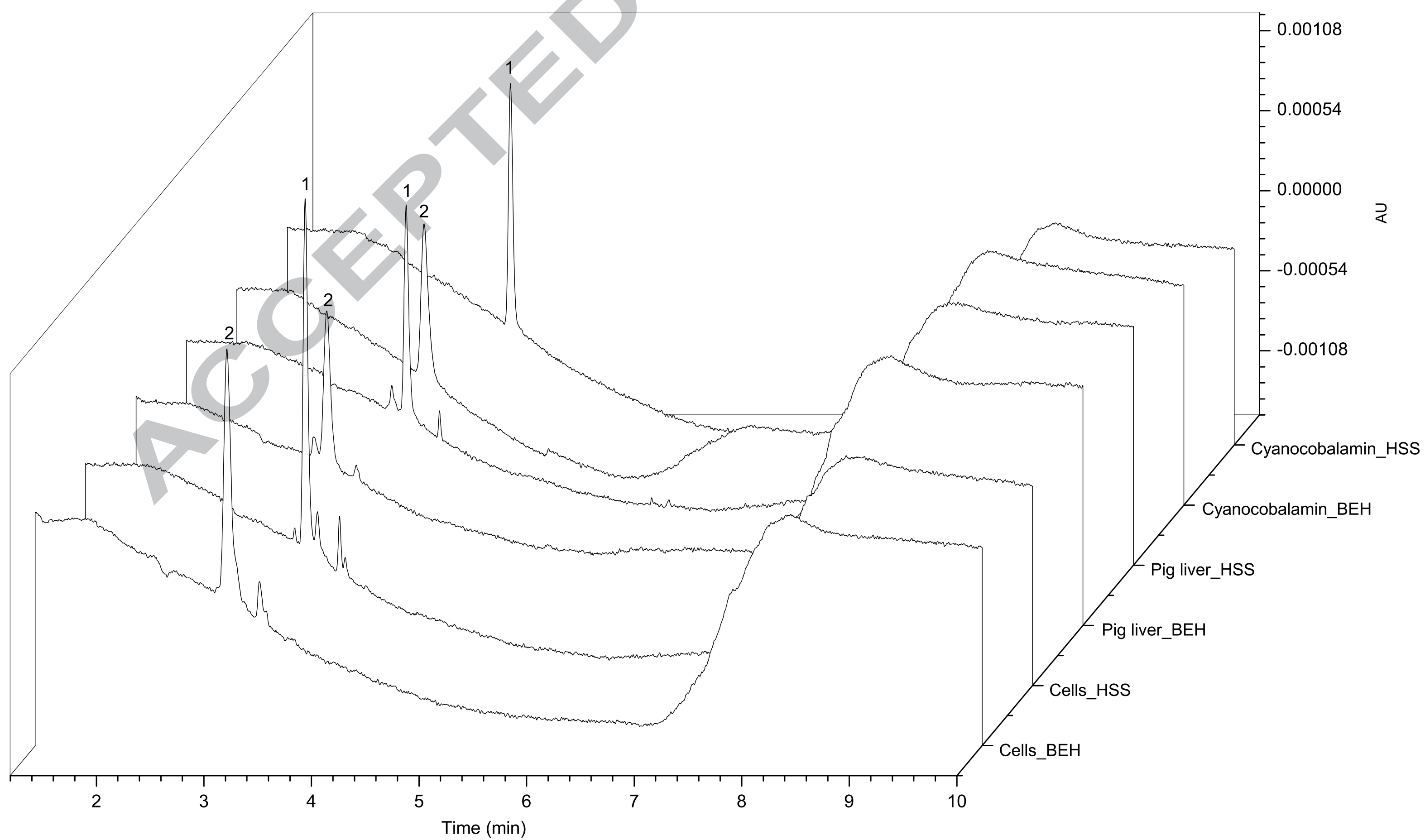

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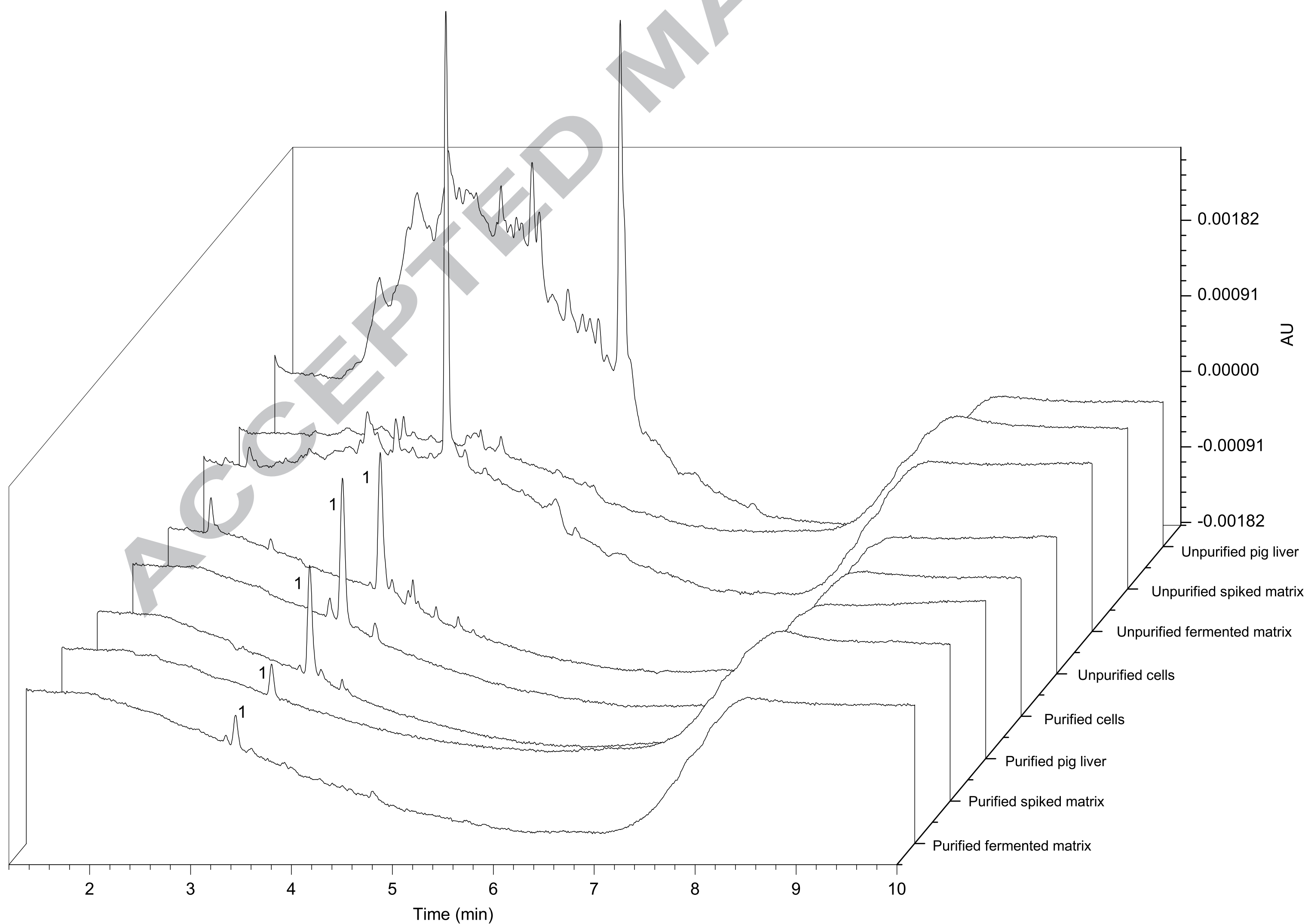

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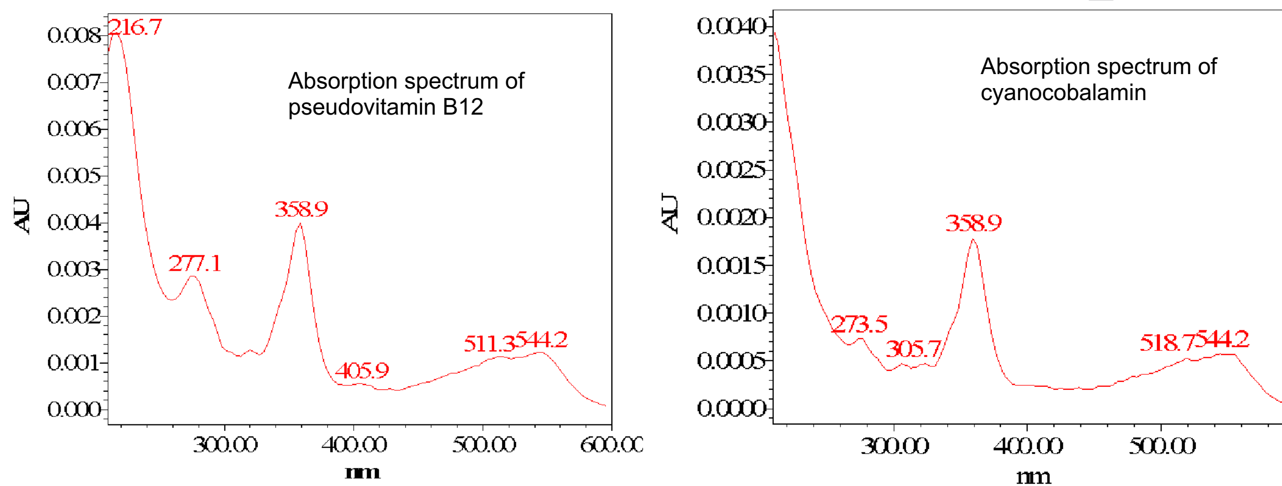

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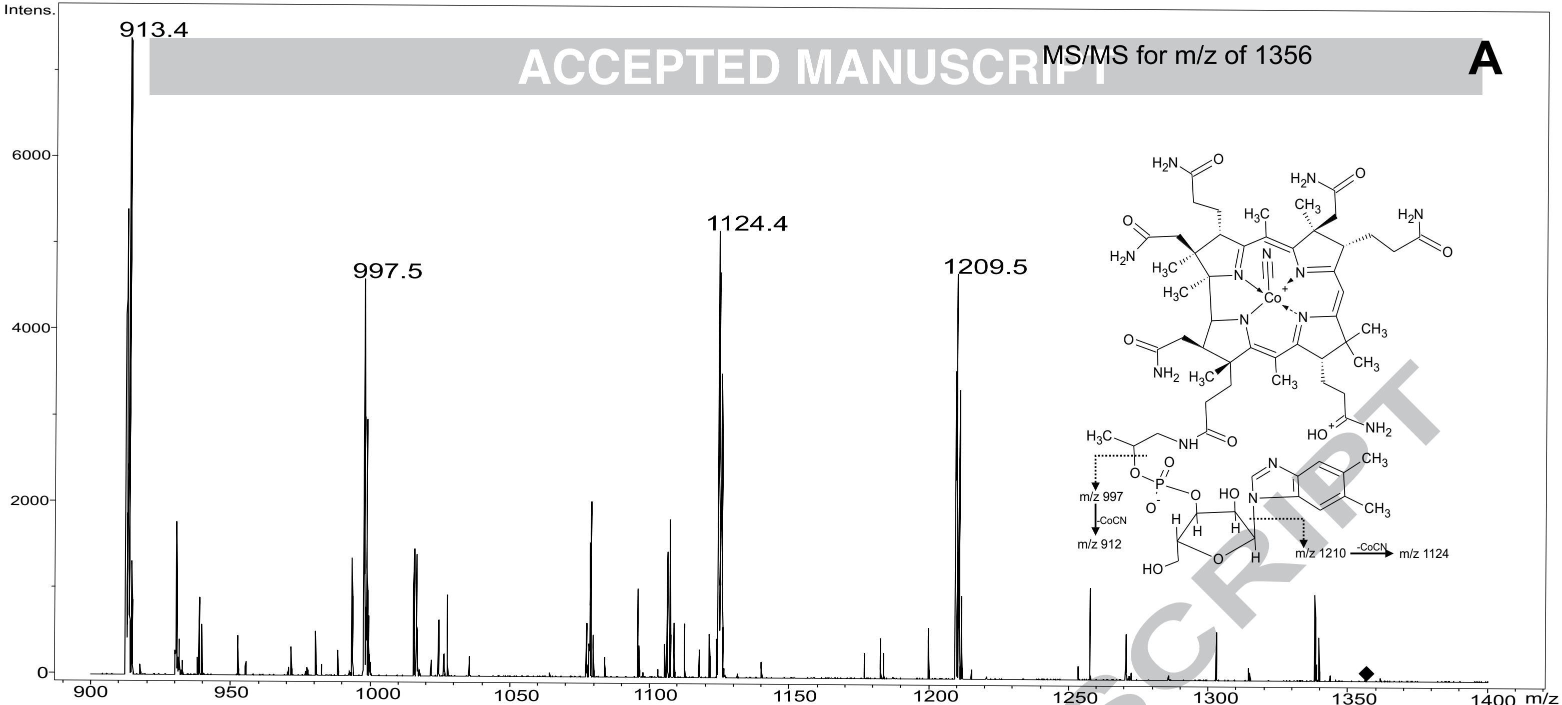

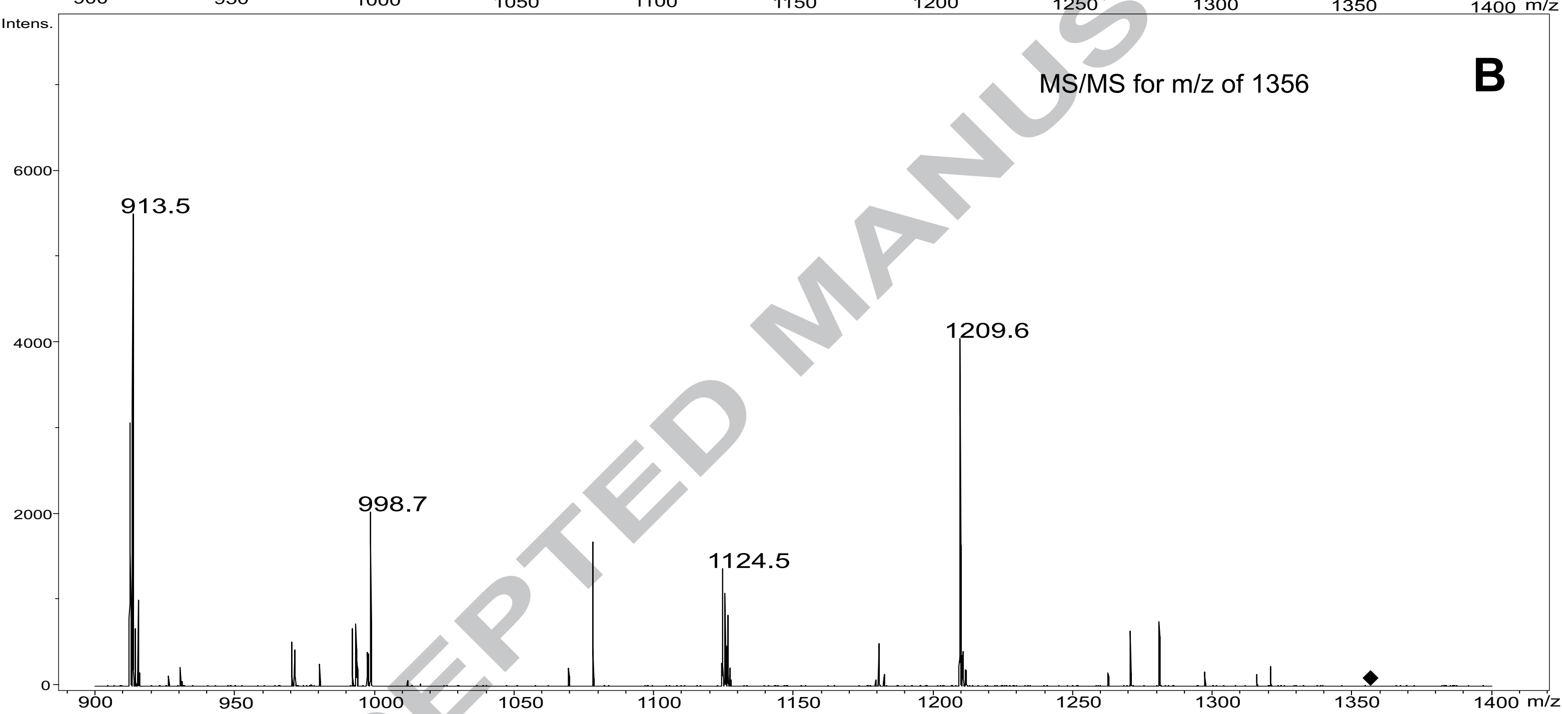

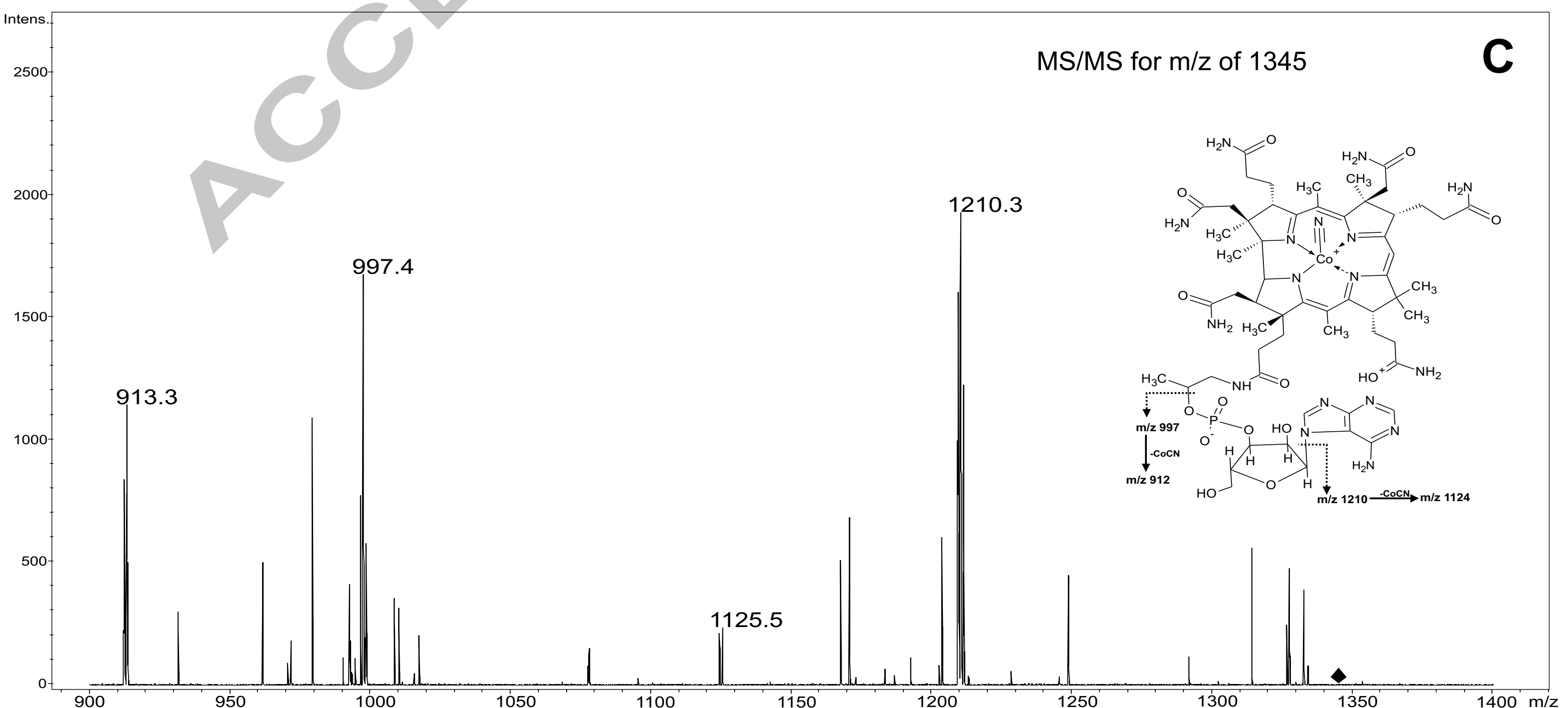

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- A sensitive UHPLC/UV method was developed for the analysis of vitamin B12.
- The method was suitable for analysing microbial cells and fermented cereal matrices.
- Immunoaffinity purification enabled analysis of vitamin B12 in fermented cereal matrices.
- Active vitamin B12 was produced in cells and cereal matrices by *P. freudenreichii*.
- Pseudovitamin B12 could be separated and further identified with UHPLC-MS.

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