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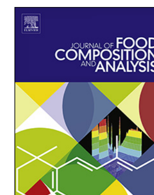
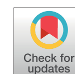


Original Research Article

# Riboflavin, niacin, folate and vitamin B12 in commercial microalgae powders



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ABSTRACT

This study aimed to investigate the riboflavin, niacin, folate and B12 content in microalgae powders. Riboflavin was determined with an ultra-high-performance liquid chromatographic (UHPLC) method after extraction and a two-enzyme treatment. Niacin analysis involved mild acidic hydrolysis, with niacin quantitated as the sum of nicotinic acid and nicotinamide using a UHPLC method. Both a microbiological (MBA) and a UHPLC method was used for vitamin B12 quantification as a cyanocobalamin (CNCbl) and UHPLC-mass spectrometry was used to confirm the vitamin B12 form. Total folate was determined both with MBA and as a sum of the vitamers with a UHPLC method after extraction and tri-enzyme treatment.

The riboflavin and niacin content varied from 21 to 41 μg/g and 0.13–0.28 mg/g, respectively, in *Chlorella* sp., *Spirulina* (*Arthrospira* sp.) and *Nannochloropsis gaditana* powders. *Chlorella* powders were, on average, richer in total folate (19.7 μg/g) than *Spirulina* powders were (3.5 μg/g). The sum of the folate vitamers determined with UHPLC matched better with the microbiological total folate content in *Chlorella* than in *Spirulina* powders. Pseudovitamin B12 was the predominant form over active vitamin B12 in *Spirulina* powders, whereas *Chlorella* sp. and *N. gaditana* powders solely contained active vitamin B12 up to 2.1 μg/g.

## 1. Introduction

Algae have been part of the human diet for thousands of years, especially among societies that lived near oceans or lakes (Wells et al., 2017a). However, only a few decades ago, the high protein content and favourable amino acid composition of microalgae raised interest among researchers who were searching for alternative protein sources due to the increasing world population. In addition to a high protein level, microalgae have been suggested to be a good source of lipids, polyunsaturated fatty acids, pigments and vitamins (Buono et al., 2014; Wells et al., 2017). Due to their excellent nutritional properties, microalgae are nowadays cultivated for health, food and cosmetic products. In addition, algal lipids are utilised in the production of biodiesel.

The freshwater green microalgae *Chlorella* sp. and the blue-green microalgae classified as *Arthrospira* sp. are among the most commercially cultivated genera. Actually, species of *Arthrospira* are classified as cyanobacteria due to their molecular phylogeny, yet they are still perceived as microalgae based on their phenotypic similarities with algae (Ramanan et al., 2016). Cultivation factors such as temperature, salinity, light and the availability of nutrients affect the chemical composition of the biomasses, which are produced either in closed bioreactors or in open ponds (Buono et al., 2014; Chen et al., 2011).

Microalgal biomasses are popular in the form of powders, tablets, capsules and liquids in the health food markets. In addition, the innovative inclusion of algal biomasses in different food products such as pasta, bread, puddings and ice cream has increased (Kovac et al., 2013). Algal supplements are particularly popular among vegetarians and vegans, who highlight their beneficial effects on vitality and immune function (Rzymski and Jaśkiewicz, 2017). Nowadays, health products

*Abbreviations:* CNCbl, cyanocobalamin; CHES, 2-cyclohexylamino-ethanesulfonic acid; DMBI, 5,6-dimethylbenzimidazole; FAD, flavin adenine dinucleotide disodium salt; FLR, fluorescence; FMN, riboflavin-5′-phosphate-mononatrium salt; HEPES, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid); LC–MS/MS, liquid chromatography-tandem mass spectrophotometry; MBA, microbiological method; METH or METE, methionine synthase; NA, nicotinic acid; NAM, nicotinamide; PDA, photo diode array; PGA, folic acid; QTOF, quadrupole time-of-flight; RDA, recommended daily allowance; TFA, trifluoroacetic acid; UHPLC, ultra-high-performance liquid chromatography; $H_4$folate, tetrahydrofolate; 10 − HCO-PGA, 10-formylfolic acid; 5, 10 − $CH^+$-$H_4$folate, 5,10-methenyltetrahydrofolate; 5 − $CH_3$-$H_4$folate, 5-methyltetrahydrofolate; 5 − HCO-$H_4$folate, 5-formyltetrahydrofolate; 5 − HCO-$H_4$folate, 5-formyltetrahydrofolate

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**Table 1**
Main information on the investigated microalgae powders.

| Powder | Abbreviation used in the text | Brand name | Production country | Strain | Food or feed grade |
|---|---|---|---|---|---|
| *Spirulina* | S1 | Duplaco | China | *A. platensis* | Feed |
| *Spirulina* | S2 | Puhdistamo | Taiwan | *A. platensis* | Food |
| *Spirulina* | S3 | CoCoVi | India | *A. platensis* | Food |
| *Spirulina* | S4 | CoCoVi | China | *A. maxima* | Food |
| *Spirulina* | S5 | Voimaruoka | United States | *A. platensis* | Food |
| *Chlorella* | C1 | Duplaco | South Korea | *C. vulgaris* | Feed |
| *Chlorella* | C2 | Puhdistamo | Taiwan | *C. pyrenoidosa* | Food |
| *Chlorella* | C3 | CoCoVi | India | *C. vulgaris* | Food |
| *Chlorella* | C4 | Voimaruoka | Japan | *C. vulgaris* | Food |
| *Nanno-chloropsis sp.* | N1 | Duplaco | unavailable | *N. gaditana* | Feed |

from *Chlorella* sp. and *Arthrospira* sp. are popular in the human nutrition markets as supplements with promising positive effects on health together with a high vitamin B content.

B vitamins are water-soluble coenzymes that play a central role in one-carbon metabolism, DNA repair, electron transfer and fatty acid synthesis in cells (Monteverde et al., 2017). In aquatic environments, phytoplankton are unable to grow only with light and inorganic nutrients as they also depend on the availability of some B vitamins. A high phytoplankton biomass is thus associated with high concentrations of B vitamins, which are excreted or released by bacteria or some other phytoplanktons (Monteverde et al., 2017; Sañudo-Wilhelmy et al., 2014). The availability of thiamin, biotin and vitamin B12 (hereafter, B12) may restrict the growth of microalgae (Croft et al., 2005). For example, over 50% of algae species are B12 auxotrophs. Microalgae that do not synthesise the vitamin *de novo* but still require it for metabolism may obtain it via a symbiotic relationship with bacteria (Croft et al., 2005).

Only some bacteria and archaea are able to synthesise B12 (Martens et al., 2002). Higher plants do not synthesise B12 and do not contain vitamin B12-dependent enzymes. In human diet, the main sources of B12 are foods of animal origin, where B12 is originated from feed or synthesised by bacteria in digestion system (Martens et al., 2002). Nevertheless, B12 has also been found in small amounts in some macro- and microalgae and plant-based foods (Watanabe et al., 2013). In algae, B12 mainly originates from a symbiotic relationship with B12 producing bacteria (Croft et al., 2005) and for instance, in mushrooms and fermented vegetables through contamination by certain bacteria (Watanabe et al., 2013).

Nevertheless, B12 has also been found in some macro- and microalgae (Watanabe et al., 2013). Because the main source of B12 in the human diet is foods of animal origin, algae have been considered as a potential source of B12 in vegan diets. However, for example, commercial *Spirulina* tablets were shown to mainly contain a corrinoid compound called pseudovitamin B12, which is not biologically active for humans (Watanabe et al., 1999). Information on other B-group vitamins in algal supplements or even in biomasses is scarce. Some data can be found in the literature, but the sampling and analytical methods are often insufficiently described. In addition, almost nothing is known about folate vitamers and the distribution of total niacin as nicotinic acid (NA) and nicotinamide (NAM).

The objective of this study was to investigate the riboflavin, niacin, folate and B12 content in selected commercial microalgae powders using UHPLC methods for quantification after suitable extraction, enzyme treatment and purification steps. In addition, the distribution of folate and niacin vitamers was studied. In the folate and B12 analyses, a UHPLC method was compared with a microbiological method (MBA).

## 2. Materials and methods

### 2.1. Samples

*Arthospira* sp. and *Chlorella* sp. are still the only microalgae species, which are approved as such for food use in EU. They had been consumed to a significant degree before May 1997 when the Novel Food Regulation (EC) No. 258/97 entered into force. Therefore, we concentrated in this study on these species and took samples from all their commercial dried biomass powder brands, which were available on the Finnish market in year 2016. Four different *Arthospira* sp. (hereafter, *Spirulina*) powder brands for food use (Puhdistamo, biomass from *A. platensis,* produced in Taiwan; CoCoVi, *A. maxima,* China; CoCoVi, *A. platensis*, India; Voimaruoka, *A. platensis* United States) and three different *Chlorella* sp. powder brands for food use (Puhdistamo, *C. pyrenoidosa*, Taiwan; CoCoVi, *C. vulgaris,* India; Voimaruoka, *C. vulgaris*, Japan) were obtained from local supermarkets or health food stores in the Helsinki area. According to the labels, all powder samples were prepared only from dried algal biomass without any other ingredients. From each brand, three consumer packets (150–200 g), each with a different production number, were purchased. A representative retail sample was prepared by pooling equal portions (50 g) of each of the three packets. In addition, one single packet of *C. vulgaris, Spirulina* (*A. platensis*) and *Nannochloropsis gaditana* powders for feed use were obtained from Duplaco (Hengelo, the Netherlands). Hereafter, the four *Chlorella* sp., five *Spirulina* and one *N. gaditana* powders are called C1–C4, S1–S5 and N1, respectively. The description of the samples used in this study is summarised in Table 1. The pooled powders were stored in the dark at −20 °C and the feed powders at +4 °C until vitamin analysis was undertaken (within 3 months).

### 2.2. Chemicals, standards and general instrumental conditions for the analysis

All reagents were of analytical grade and the solvents used in the UHPLC analysis were of HPLC or LC–MS grade. General laboratory reagents were purchased from VWR

International Oy (Helsinki, Finland). Milli-Q water (Millipore system; Bedford, MA, USA) was used to prepare reagents. Riboflavin (analytical standard), riboflavin-5′-phosphate-monosodium salt (purity ≥ 70%, FMN), flavin adenine dinucleotide disodium salt (≥ 95%, FAD), potassium dihydrogen phosphate (≥ 99%), sodium acetate, nicotinic acid (≥99.5%, NA), nicotinamide (≥99.5%, NAM), trifluoroacetic acid (99%, TFA), 2-cyclohexylamino-ethanesulfonic acid (≥99.0%, CHES), 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (≥99.5%, HEPES), sodium ascorbate (≥ 98%,), 2-mercaptoethanol (≥99.9%), sodium cyanide (≥97.0%, Na-cyanide) and cyanocobalamin (CNCbl, analytical standard) were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Ammonium acetate (≥ 96.0%), coppar

sulphate (≥ 99.0%) and hydrogen peroxide (30% for analysis) were obtained from Merck KGaA (Darmstad, Germany). In addition, the certified reference materials of the Institute for Reference Materials and Measurements (Geel, Belgium) BCR 487 (lyophilised pig liver powder), BCR 121 (wholemeal flour) and BCR 431 (Brussel sprouts) were obtained via Sigma-Aldrich (Steinheim, Germany).

The enzymes β-amylase (EC 3.2.1.2, A7130-10KU, type II-B, lyophilised from barley,) α-amylase (EC 3.2.1.1; A9857-5MU from *Aspergillus oryzae*) and protease (EC 232-909-5 from *Streptomyces cereus* P-5147, ≥3.5U/mg) were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Taka-Diastase (141.8 HUT/g) was obtained from Pfaltz and Bauer (Waterbury, CT, USA). Hog kidney conjugase was prepared in the laboratory and its activity was tested according to Kariluoto et al. (2004). Folate vitamer standards (6S)-tetrahydrofolate (97.3%, $H_4$folate, sodium salt), (S)-5-methyltetrahydrofolate (100%, 5-$CH_3$-$H_4$folate, calcium salt), (S)-5-formyltetrahydrofolate (97.3%, 5-HCO-$H_4$folate, sodium salt), 6R-5,10-methenyltetrahydrofolate hydrochloride (96.0%, 5,10-$CH^+$-$H_4$folate) and folic acid (96.4%, PGA) were obtained from Merck Eprova AG (Schaffhausen, Switzerland) and 10-formylfolic acid (99.5%, 10-HCO-PGA) was obtained from Schircks Laboratories (Jona, Switzerland). Folic acid casei medium (Difco, Becton Dickinson and Co, Sparks, MD) and vitamin B12 assay broth (*Lactobacillus*, Base; Merck, Darmstdat, Germany) were used in MBA for total folate and vitamin B12, respectively.

Each vitamin and their vitamers were analysed with the respective methods using a Waters Accquity UPLC system. The UPLC equipment consisted of a binary solvent manager, a sample manager, a column manager, a photo diode array (PDA) and fluorescence (FLR) detectors (Waters, Milford, MA). The autosampler injected the syringe filtered (0.2 μm) extract solutions (5–25 μL) via a 20-μL (riboflavin, niacin, B12) or a 50-μL (folate) injection loop operated in a partial-loop mode. The data was acquired and processed using Waters Empower 2 Software.

### 2.3. Quality control

All vitamin analyses were carried out as analytical triplicates, except the UHPLC analysis of B12, which was carried out in duplicate. Since enzymes were used during the extraction steps, a blank sample (water) was also included in each extraction batch for the riboflavin, folate and B12 analyses. All analytical steps were carried out under subdued lighting and especially in folate analysis, extracts and standard solutions were kept under nitrogen atmosphere whenever feasible. The concentration of each standard was confirmed spectrophotometrically (Lambda 25 UV/Vis, Perkin Elmer Inc., USA). The vitamin results are presented as the means of the analytical replicates. Certified reference materials were used as a quality control samples for each analyte: BCR 487 (pig liver) for riboflavin with the UHPLC method and vitamin B12 analysis with the MBA, BCR 121 (whole wheat flour) for total folate with the MBA, and BCR 431 (Brussels sprouts) for total niacin with the UHPLC method.

### 2.4. Riboflavin analysis

Riboflavin was extracted and analysed with a UHPLC method described earlier by Chamlagain et al. (2016). The method, based on the European standard method for HPLC (EN 14152:2014, 2014), was optimised for a UHPLC. The limit of quantification (LOQ) was defined as a signal-to-noise ratio of 10:1. Thus, the instrumental LOQ was 30 pg/injection of 10 μl, which led to quantification level 0.010 μg/g of sample with the used sample weight (0.2 g) and dilution.

#### 2.4.1. Extraction

In brief, a sample (0.3 g) was extracted with 15 mL of 0.1 M hydrochloric acid in a boiling water bath for 60 min. After cooling, the pH was adjusted to 4.5 with 2.5 M sodium acetate followed by incubation (37 °C; 24 h) with Taka-Diastase (50 mg) and β-amylase (5 mg). The final volume (25 mL) of extract was obtained with weak acetic acid (0.02 M) and the filtered (0.2 μm) extract was analysed with UHPLC. A certified reference sample (0.5 g; BCR 487), and a blank sample (0.5 g, Milli-Q water) was included in each extraction set.

#### 2.4.2. UHPLC method

Riboflavin was analysed on a BEH C18 column (2.1 mm x 100 mm; 1.7 μm; Waters, Bedford, MA) with an FLR detector set at wavelengths of 444 nm (excitation) and 520 nm (emission). Separation was achieved with a linear gradient of 20 mM ammonium acetate dissolved in 30% aqueous methanol at a constant flow of 0.2 mL/min and at a temperature of 30 °C. Each extract was injected twice (10–15 μL). Quantification was based on an external standard method using a multilevel (n = 6) standard curve of riboflavin (0.01–1.0 ng/μL).

The concentration of riboflavin stock solution (0.1 mg/mL in 0.02 M acetic acid) was confirmed spectrophotometrically at 444 nm by using the molar absorption coefficient of 12,340 $l{\cdot}mol^{-1}\,cm^{-1}$ (EN 14152:2014, 2014). In addition, the activity of the enzymes in the extraction step was confirmed by using 20 μg of both FAD and FMN solutions as samples. In the control samples, FMN and FAD were added without enzymes. FAD and FMN were identified in the UHPLC run by using FMN and FAD standard solutions. Both FAD and FMN were completely converted to riboflavin in the samples with added enzymes. In this study, the obtained riboflavin content in the BCR 487 reference material was 129 ± 22.7 μg/g (n = 4). This is slightly higher than has been reported in the EN 14152, standard method (105 ± 23.5 μg/g dm).

### 2.5. Niacin analysis

The total niacin content was determined as a sum of NA and NAM with a European standard method (EN 15652, 2009) that was optimised for UHPLC. Mild acid extraction was used to release the bioavailable niacin, which includes the free niacin. In this study, the UHPLC assay of niacin had a LOQ of 0.06 ng and 0.03 ng/10 μL injection for NA and NAM, respectively, defined as a signal-to-noise ratio of 10:1. When sample weight (0.2 g), dilution, and injection volume were considered, the LOQ was estimated to be 0.00015 mg/g and 0.00010 mg/g for NA and NAM, respectively.

#### 2.5.1. Extraction

A sample (0.2 g) was extracted with 15 mL of mild acid (0.1 M HCl) in a boiling water bath for 60 min. After cooling on ice, the pH was adjusted to 4.5 with 2.5 M sodium acetate and the extract was centrifugated twice (10 000 rpm, 10 min, RT). The obtained supernatant was filled to 25 mL with water and analysed with UHPLC after filtering (0.2 μm). Each extraction batch also included the BCR 431 reference material (0.2 g).

#### 2.5.2. UHPLC method

A reversed-phase column (HSS T3 C18; 1.8 μm, 2.1 mm x 150 mm; Waters) at 30 °C was used for the chromatographic separation. The mobile phase was composed of potassium dihydrogen phosphate (70 mM) containing 150 mM of hydrogen peroxide and 5 μM of copper sulphate. NA and NAM were separated with an isocratic elution (0.3 mL/min) and detected with an FLR detector (wavelengths of 322 and 380 nm) after post-column derivatisation by UV irradiation (366 nm) in the presence of copper (II) ions and hydrogen peroxide. The post-column derivatisation was carried out on a knitted polytetrafluoroethylene reaction coil (1.59 mm o.d., 0.17 mm i.d., and 5 m length). The lamp power was 8 W.

Both forms of niacin were identified by their retention times and quantified based on an external standard method where peak areas were plotted against concentration (0.5–30 ng/inj.). The concentrations of the stock solutions (1 mg/mL in 0.1 M HCl) of NA and NAM were calculated using molar absorption coefficients of 5,170 and

5,007 l·mol$^{-1}$ cm$^{-1}$, respectively, after measuring the absorbance of the solutions at 260 nm with the spectrophotometer. The BCR 431 reference material was analysed in each extraction batch (n = 4) and had an average concentration of 40 ± 6.4 μg/g. The certified value for BCR 431 is 43 ± 3 μg/g analysed with an MBA or a chemical method using the König reaction.

### 2.6. Folate analysis

Total folate was determined both with an MBA and as a sum of vitamers ($H_4$folate, 5-$CH_3$-$H_4$folate, 5-HCO-$H_4$folate, PGA, 10-formyl-PGA and 5,10-$CH^+$-$H_4$folate) using a UHPLC method based on the descriptions by Edelmann et al. (2012). In both methods, the same sample extract obtained after extraction and enzyme treatment was used.

#### 2.6.1. Extraction and enzyme treatment

In brief, a sample (0.1–0.3 g) was heat-extracted with buffer (12 ml; 50 mM CHES, 50 mM HEPES, 2% sodium ascorbate and 10 mM mercaptoethanol in water; pH 7.85) followed by a tri-enzyme treatment with alpha-amylase, hog kidney conjugase and protease (Edelmann et al., 2012). After treatment and the inactivation of enzymes by boiling, the extract was filtered through a filter paper and filled to an exact volume (25 mL) with 0.5% sodium ascorbate (pH 6.1).

#### 2.6.2. Microbiological method

The total folate was determined according to the method described previously (Edelmann et al., 2012; Kariluoto et al., 2004). Briefly, the assay was carried out on microtiter plates, using *Lactobacillus rhamnosus* ATCC 7469 as the test organism and 5-HCO-$H_4$folate as the standard (eight levels, 0–80 pg/well). Two dilutions were made from each extract. The diluted extracts and standard solution (100 μL) were inoculated together with 200 μL of assay medium (Folic acid casei medium) containing the cryopreserved test organism into the wells of the microtiter plate. The turbidity was measured (595 nm) after incubation for 17 h at 35 °C with a micro-plate reader (Multiskan EX, Labsystems, Finland).

A certified reference material (BCR 121, wholemeal flour) was analysed for each incubation. The certified value for the BCR 121 reference material was 500 ± 70 ng/g dm when determined with an MBA. In this study, we obtained a content of 499 ± 33 ng/g dm (n = 4).

#### 2.6.3. UHPLC method

The extract (5–15 mL) was cleaned up and concentrated before a UHPLC analysis with affinity chromatography (Edelmann et al., 2012). Prior to the UHPLC analysis, the extract was filtered (0.2 μm). The folate vitamers were determined as their monoglutamates according to the description by Edelmann et al. (2012). The vitamers were separated on an HSS T3 (1.8 μm, 2.1 × 150 mm; Waters) column (30 °C) by using a mobile phase composed of 30 mM potassium phosphate buffer at pH 2.2 and acetonitrile operated as a gradient at a constant flow rate of 0.4 mL/min. The folate vitamers were detected at the FLR and PDA detectors and quantified by using an external standard method, as described previously (Edelmann et al., 2012). A combined working solution prepared from five individual vitamer stock solutions ($H_4$folate, 5-$CH_3$-$H_4$folate, 5-HCO-$H_4$folate, PGA and 10-formyl-PGA) was used for the construction of the calibration curves.

The concentration of each stock solution was confirmed spectrophotometrically according to Kariluoto et al. (2004). In addition, a separate working solution for 5,10-$CH^+$-$H_4$folate was prepared. The working solutions were prepared from the stock solutions of each vitamer in 0.01 M acetate buffer containing 1% (w/v) sodium ascorbate and 10 mM 2-mercaptoethanol (pH 4.9).

The instrumental LOQ values for each folate vitamer based on the validation parameters according to Edelmann et al. (2012). LOQ values varied between vitamers and were for 15 μL injection 0.01 ng/inj for $H_4$folate, 5-$CH_3$-$H_4$folate and 10-HCO-PGA at a fluorescence detector and 0.16 ng/inj for 5-HCO-$H_4$folate, PGA and 5,10-$CH^+$-$H_4$folate at a UV detector. When sample weight (0.3 g), dilution, purification and injection volume were observed the LOQs were for $H_4$folate, 5-$CH_3$-$H_4$folate and 10-HCO-PGA and 5-HCO-$H_4$folate, PGA and 5,10-$CH^+$-$H_4$folate 0.012 μg/g and 0.217 μg/g, respectively.

### 2.7. Vitamin B12 analysis

Similar to the folate analysis, the total B12 content was determined both with an MBA and with a UHPLC method from the same sample extract. B12 was analysed as CNCbl. The concentration of the CNCbl stock solution was confirmed spectrophotometrically at 361 nm by using a molar absorption coefficient of 28,100 l·mol$^{-1}$ cm$^{-1}$.

#### 2.7.1. Extraction

Extraction was carried out as reported earlier (Chamlagain et al., 2015). In brief, the sample (0.2 g) was extracted for 30 min in a boiling water bath in 10 mL of extraction buffer (8.3 mmol/L sodium hydroxide/20.7 mmol/L acetic acid, pH 4.5) and l00 μL of Na-cyanide (1% w/v in water). After cooling, the extract was centrifugated twice and the supernatants were combined. The extract was filtered, the pH was adjusted to 6.2 and the volume was set to 25 mL.

#### 2.7.2. Microbiological method

The MBA of total B12 was carried out according to the method described by Chamlagain et al. (2015). In brief, two dilutions were made from each sample extract and total B12 was determined on microtiter plates using *Lactobacillus delbrueckii* ATCC 7830 as the growth indicator and CNCbl as the standard. The diluted extracts and CNCbl solutions (0–8 pg/well) were inoculated (100 μL) together with 200 μL of vitamin B12 assay broth containing the cryopreserved *L. delbrueckii* into the wells of the microtiter plate. The turbidity (595 nm) was measured after 19 -h incubation at 35 °C with a micro-plate reader.

The certified reference material BCR 487 was analysed for each incubation as a quality control sample. In this study, the MBA gave a concentration of 1008 ± 6 ng/g (n = 3) whereas the certified reference value determined with MBA is 1120 ± 90 ng/g dm.

#### 2.7.3. UHPLC method

The extract used for the MBA was purified and concentrated through an immunoaffinity column (Easi-Extract, R-Biopharma, Glasgow, Scotland) according to the manufacturer's instructions. Details on the purification were described previously (Chamlagain et al., 2015). The purified eluate was evaporated under a stream of nitrogen and the residue was reconstituted in 300 μL of water. CNCbl was detected at 361 nm on a reversed-phase C18 column (HSS T3, 2.1 × 100 mm, 1.8 μm; Waters). The resolution of the CNCbl was achieved with a linear gradient system of Milli-Q water and acetonitrile containing 0.025% of TFA at a constant flow rate of 0.32 ml/min and at a temperature of 30 °C. The quantification was based on an external standard method using a multilevel (n = 5) calibration curve (0.4–8 ng). If the extract contained pseudovitamin B12, the amount was quantified using the CNCbl calibration curve. In this study, a B12 content of 751 ng/g (n = 2) was obtained for the BCR 487 reference material with the UHPLC determination. The UHPLC result was lower than MBA result. The test organism *L. delbrueckii* used in MBA can utilize also incomplete corrinoids and analogues other than active B12 for its growth. Thus, MBA overestimated the vitamin B12 content in pig liver material as we noted also in our previous study (Chamlagain et al., 2015).

The instrumental LOQ value for cyanocobalamin based on the validation parameters according to Chamlagain et al. (2015) was 0.2 ng/inj (15 μL). When sample weight (0.2 g), purification step, and injection volume was observed, the LOQ was 0.035 μg/g of sample.

### 2.7.4. LC–MS method

The biologically active B12 form was confirmed with a mass spectrometric analysis performed on a high-resolution quadrupole time-of-flight (QTOF) mass spectrometer with an electrospray ionization interface (Synapt G2-Si, Waters, Milford, MA, USA) and operated in a positive ion mode, as reported by Chamlagain et al. (2018). Briefly, ions with an *m/z* range of 50–1500 were scanned, and the MS/MS was performed for ions of an *m/z* of 678.2882 ($[M+2H]^{2+}$ of CNCbl) and 672.7752 ($[M+2H]^{2+}$ of pseudovitamin B12) using argon as the collision gas. The 0.025% TFA in the mobile phase was replaced with formic acid (0.1%) for the MS analysis. The injection volume was 1–2 µL. The key MS settings were: a capillary voltage of 0.5 kV, a sampling cone voltage of 40 V, a source offset of 80 V, a source temperature of 150 °C, a desolvation temperature of 600 °C, a desolvation gas flow of 1000 L/h, a nebuliser gas flow of 6.5 bar, a cone gas flow of 50 L/h, a trap collision energy of 4 eV, a ramp trap collision energy of 15–90 eV, a trap gas flow of 2 mL/min and a scan time of 0.2 s. Further details can be found elsewhere (Chamlagain et al., 2018).

### 2.8. Statistical analysis

The vitamin and vitamer concentrations are presented as means of analytical replicates ± standard deviation (SD) (n = 3, except in UHPLC analysis of vitamin B12, n = 2). One-way analysis of variance (ANOVA) was applied (Microsoft Excel for Windows 10; Microsoft Corporation, Redmond, WA, USA) to compare vitamin contents in powders of different microalgae species and vitamin contents obtained by UHPLC and MBA. A p value $< 0.05$ was considered statistically significant.

## 3. Results and discussion

### 3.1. Total riboflavin and niacin

The variation in riboflavin content among the *Spirulina* powders was low. The content ranged from 33.6 to 40.9 µg/g (Fig. 1, Table S1). In contrast, the content varied markedly among the *Chlorella* powders. The riboflavin content in the *Chlorella* powders varied from 20.7 to 33.6 µg/g and was significantly lower ($p < 0.05$) than that in the *Spirulina* powders. The content of riboflavin (22.1 µg/g) in the only studied *N. gaditana* powder was lower than the mean levels for *Spirulina* (36.3 µg/g) and *Chlorella* (28.0 µg/g).

In contrast to the riboflavin content, the total niacin content was, on average, higher in the *Chlorella* (0.24 mg/g) than in the *Spirulina* powders (0.16 mg/g) (Fig. 2, Table S1). However, no significant difference was observed ($p > 0.05$). The content of total niacin ranged from 0.14 mg/g to 0.28 mg/g in *Chlorella* and from 0.13 to 0.22 mg/g in *Spirulina*. The lowest level was observed in *N. gaditana* (0.11 mg/g). In all samples, NAM was the main vitamer. In the *Chlorella* samples, it contributed, on average, 82%, and in the *Spirulina* samples, 69% to the total niacin content. The separation of riboflavin and NA and NAM in S3 and C3 powders in UHPLC run is shown in Supplementary data Figure S1 and S2, respectively.

In the literature, the information on the analytical methods and the riboflavin content in the *Chlorella* sp. and *Spirulina* powders or biomasses is scattered and limited. In the present study, the riboflavin levels in the *Chlorella* and *Spirulina* powders were slightly lower than the levels reported in the books and earlier reviews. The riboflavin content in the *Chlorella* sp. has been shown to range from 43 to 57 µg/g dm (Andrade, 2018; Bishop and Zubeck, 2012; Liu and Hu, 2013; Safi et al., 2014) and in *Spirulina*, it ranges from 37 to 45 µg/g dm (Andrade, 2018; Belay, 2007; Bishop and Zubeck, 2012; Seyidoglu et al., 2017), respectively. An extremely low riboflavin content (2–9 µg/g) in *Spirulina* biomasses was reported by Babazhanov et al. (2004) when an HPLC method without any enzyme treatment in the extraction step was used.

Similarly, the data on niacin in microalgae is scarce and, to our knowledge, studies on the proportion of NA and NAM have not been published. Nevertheless, our total niacin results were in line with the previous literature. In the reviews by Liu and Hu (2013) and Safi et al. (2014), a total niacin content of 0.24–0.32 mg/g dm in *Chlorella* sp. biomasses is given. Belay (2007) reported a content of 0.149 mg/g in *Spirulina*. Babazhanov et al. (2004) determined only NAM content with an HPLC method in the dried biomasses of *Spirulina* and it varied from 0.006 to 0.050 mg/g. The highest NAM levels (0.050 mg/g) were found in samples cultivated in spring and summer and are in line with the NAM results in this study. In the present study, the distribution of NAM and NA in microalgae powders was similar to that in meat and yeast (Ndaw et al., 2002), whereas in cereal grains, NA was the predominant vitamer (Mihhalevski et al., 2013; Ndaw et al., 2002). Further, we used mild acidic (0.1 M HCl) hydrolysis to determine only bioavailable NAM and NA. Mild acid hydrolysis does not liberate chemically bound forms, which stay almost intact during digestion by gastric acids. For example, in cereal grains, NA is partly bound to polysaccharides, polypeptides or glycoproteins, and is mainly biologically unavailable (Wall and Carpenter, 1988). However, to the best of our knowledge, there are no previous studies on any complex of NA with macromolecules in microalgae cells.

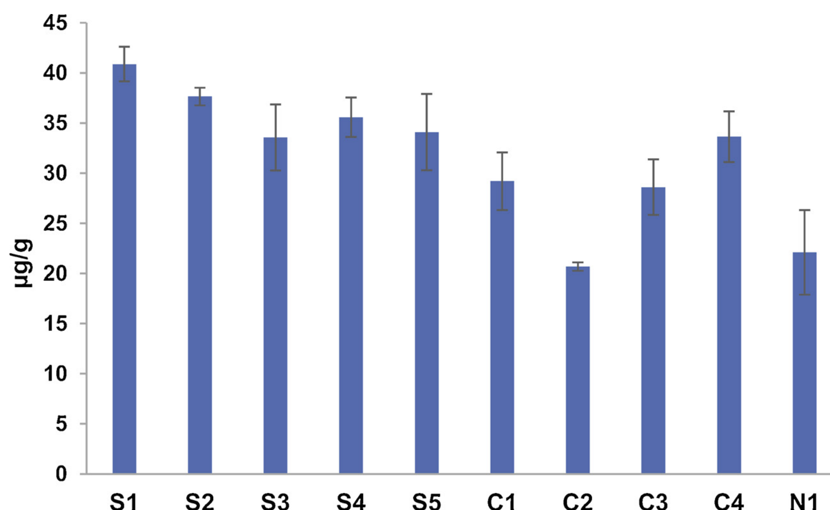


**Fig. 1.** The total riboflavin content in *Spirulina* powders (S1–S5, see Table 1), *Chlorella* sp. powders (C1–C4, see Table 1) and one *N. gaditana* powder (N1). The error bars represent the range of analytical replicates, n = 3.

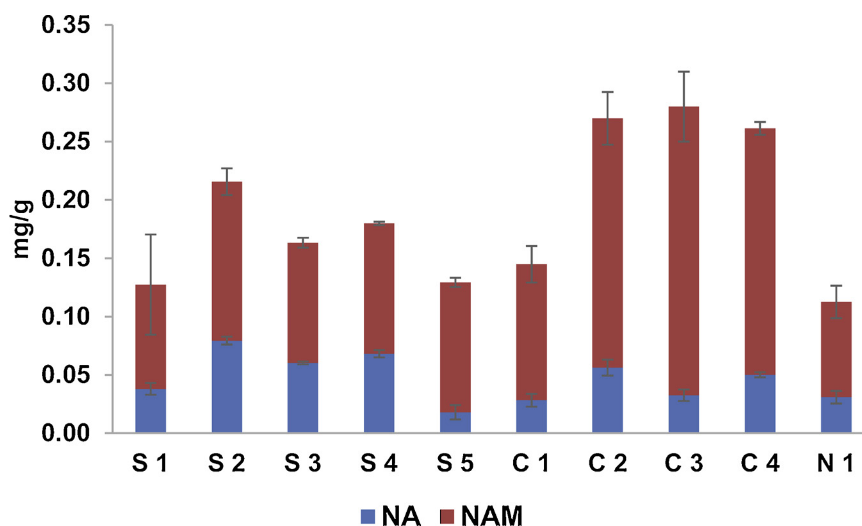


**Fig. 2.** The total niacin content consisted of the sum of nicotinic acid (NA) and nicotinamide (NAM) in *Spirulina* powders (S1–S5, see Table 1), *Chlorella* sp. powders (C1–C4, see Table 1) and one *N. gaditana* powder (N1, see Table 1). The error bars represent the range of analytical replicates, n = 3.

Overall, the *Chlorella* and *Spirulina* powders were not shown to be good sources of riboflavin and niacin. The recommended daily allowances (RDAs) of riboflavin and niacin for adults are 1.3 and 16 mg, respectively (Institute of Medicine, 1998). A realistic daily portion of *Chlorella* and *Spirulina* powders, around 5 g (one tbs), would thus contribute 5–14% to the daily requirements of riboflavin and niacin.

### 3.2. Total folate with the MBA

The total folate content in the *Chlorella* and *N. gaditana* powders was around 6-fold higher ($p < 0.05$) than in the *Spirulina* powders (Fig. 3A and B). The total folate content ranged from 13.9 to 25.9 μg/g in *Chlorella* sp. and from 2.5 to 4.7 μg/g in *Spirulina* powders (Table S2). The amount of folate in *N. gaditana* (20.8 μg/g) was at the level of the *Chlorella* samples.

As for riboflavin and niacin, only scattered information on the total folate levels in microalgae is available in the literature, mainly reported in books without a proper description of the analytical methods. A few of the reviewed amounts ranging from 6 to 24 μg/g dm (Bishop and Zubeck, 2012; Liu and Hu, 2013) for *Chlorella* sp. agree with the total folate content in the *Chlorella* sp. powders in this study. Fujii et al. (2010) found only 2.4 μg/g dm of folate in *C. vulgaris* powder with an MBA by using only water without any enzyme treatment in the extraction phase. On the other hand, our results were close to the values of 17–26 μg/g determined with an MBA in four microalgae species (*Tetraselmis* sp., *N. gaditana, Pavlova pinguis* and *Stichococcus* sp.) when a similar tri-enzyme treatment was used as in our study (Brown et al., 1999). Similarly to the present study, previous publications have also reported lower folate content for *Spirulina* biomasses than for Chlorella biomasses. A total folate content of 0.1–0.26 μg/g (Bishop and Zubeck, 2012; Seyidoglu et al., 2017) and 2–6 μg/g (Babadzhanov et al., 2004) in *Spirulina* have been reported. Interestingly, the total folate content (0.64–5.4 μg/g dm) in dihé, an air-dried *Spirulina* product (Carcea et al., 2015), is in line with our total folate results for *Spirulina* powders. Carcea et al. (2015) used a similar extraction and tri-enzyme procedure in their MBA as in this study.

This study thus showed that *Chlorella* powders are better sources of folate than *Spirulina* powders are. The RDA for folate is 400 μg (Institute of Medicine, 1998). An adult consuming 5 g of *Chlorella* powder daily would ingest nearly a quarter of the RDA for folate. In contrast, *Spirulina* powders proved to be poor in folate. A daily 5-g portion of *Spirulina* powder would only provide 4% of the RDA for folate.

### 3.3. Folate vitamer distribution with the UHPLC method

5-$CH_3$-$H_4$folate was the main vitamer in both *Chlorella* and *Spirulina* powders contributing 35–86% to the sum of the identified vitamers (Fig. 3, Table S2). The next abundant vitamer was 5,10-$CH^+$-$H_4$folate in *Spirulina*, and in *Chlorella,* it was 5-HCO-$H_4$folate. Interestingly, 5-HCO-$H_4$folate was totally absent in the *Spirulina* samples. In addition, only one *Spirulina* powder (S5) contained $H_4$folate and none contained PGA. One *Chlorella* (C3) powder contained a high amount of PGA, around 40% of the sum of the vitamers. PGA is not a natural form synthesised by bacteria and plants. It is formed by oxidation during processing such as heating, and at a low pH from $H_4$folate and 5,10-$CH^+$-$H_4$folate through dihydrofolic acid (De Brouwer et al., 2007; Zhang et al., 2018). It could also be possible that if the cultivation medium contained added PGA as a nutrient, the *Chlorella* cells could have taken it up like they have been shown to accumulate exogenous B12 (Bito et al., 2016). In the supplementary material, Figure S3 illustrates the UHPLC separation of folate vitamers in C3 and S3 powders.

To our knowledge, there is no published folate vitamer data on microalgae, particularly on *Chlorella* sp. and *Spirulina* powders. Based on the results in this study, the vitamer profile of the *Chlorella* sp. powders and *N. gaditana* resembled that found in vegetables (Delchier et al., 2016) and in cereal grains such as wheat (Piironen et al., 2008). Although *Spirulina* is phylogenetically a bacterium, its vitamer profile differed from the profile reported for some endogenous bacteria (Kariluoto et al., 2010) and lactic acid bacteria (Gangadharan et al., 2010) because no $H_4$folate or 5-HCO-$H_4$folate were detected.

The total vitamer content in the *Chlorella* powders matched rather well with the MBA results and no significant difference ($p > 0.05$) was observed between these two methods. In contrast, the sum of the vitamers in the *Spirulina* powders was, on average, only 32% of the total folate content determined with the MBA ($p < 0.05$). *Spirulina* may have contained folate forms or other folate-like compounds that were not identified in this study. Several unknown peaks were detected, especially with FLR detection at 290/356 nm (Figure S3B) and one unknown peak was seen clearly at 360/460 nm but also at UV 290 nm and UV 360 nm (Figure S3A,C)

### 3.4. Total vitamin B12 with the MBA and with the UHPLC method

With the MBA, the B12 content varied from 0 to 2.4 μg/g and from 0.6 to 2.4 in the *Chlorella* and *Spirulina* powders, respectively (Fig. 4, Table S3). One *Chlorella* sample (C1) had no B12 and the content in *N.*

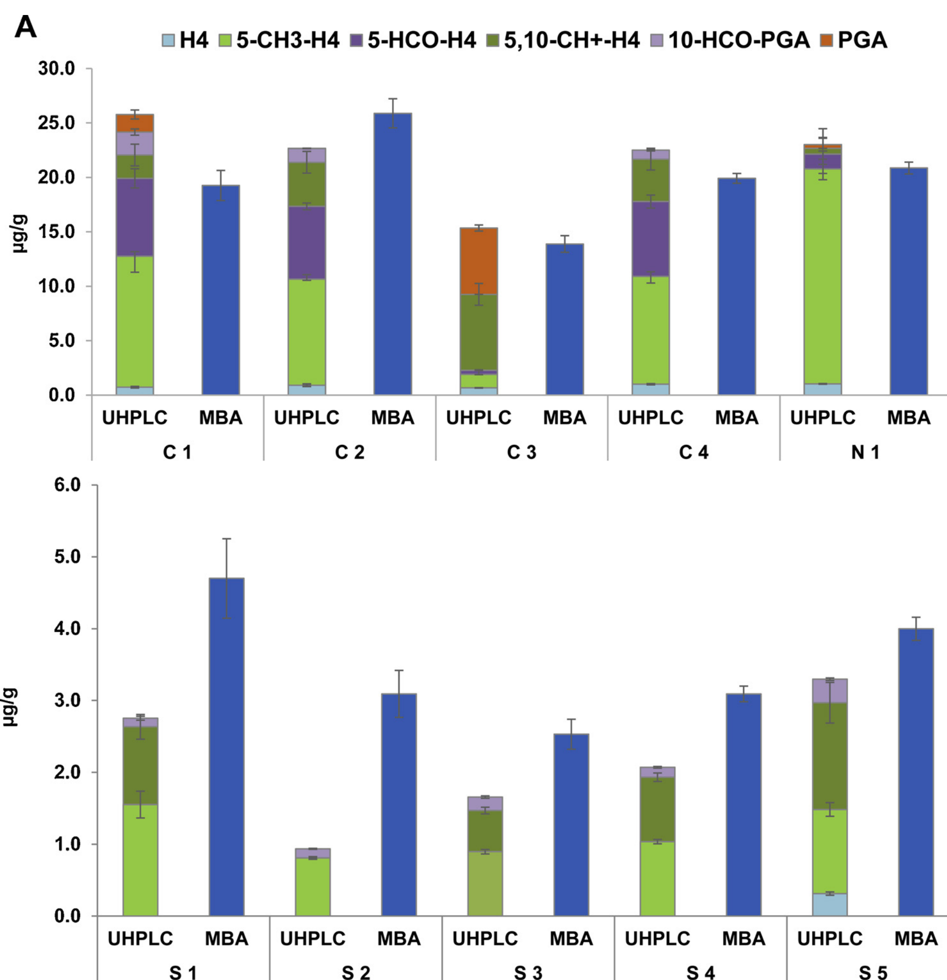


**Fig. 3.** The total folate content determined with the microbiological method (MBA) and folate vitamer content analysed with the ultra-high-performance liquid chromatography (UHPLC) method A. in *Chlorella* sp (C1–C4, see Table 1.), *N. gaditana* (N1, see Table 1) and B. in *Spirulina* powders (S1–S5, see Table 1). The error bars represent the range of analytical replicates, n = 3.

*gaditana* was only 0.25 µg/g. The B12 content of *Chlorella* was within the range reported by Bito et al. (2016) for *Chlorella* health supplements (i.e., tablets), varying from a trace amount to 4.5 µg/g dm determined with the MBA. In addition, Kittaka-Katsura et al. (2002) reported a range of 2.0–2.9 µg/g dm for B12 in commercial *Chlorella* tablets analysed both with MBA and with a chemiluminescence assay. Our MBA results were in line with values reported in the books and reviews for *Chlorella* sp. biomasses at 0.001–0.8 µg/g (Andrade, 2018; Bishop and Zubeck, 2012; Liu and Hu, 2013) and for *Spirulina* at 1.6–3.2 µg/g dm (Belay, 2007; Bishop and Zubeck, 2012; Seyidoglu et al., 2017). Further, a B12 content of 1.3–2.4 µg/g analysed in *Spirulina* tablets with the MBA method (Watanabe et al., 1999) was also in accordance with the results in this study.

Our B12 results obtained both by MBA and UHPLC were close to each other for all *Chlorella* powders ($p < 0.05$). In the chromatograms, only a peak eluting at the retention time of CNCbl was detected (Fig. 5A). This peak had a PDA spectrum identical to that of CNCbl. In addition, the UHPLC-MS/MS analysis showed that the corrinoid in the *Chlorella* extracts produced ions with an $m/z$ of 678.2882 $[M+2H]^{2+}$, identical to positively doubly charged ions produced when the CNCbl standard was analysed (Fig. 5C). Upon fragmentation, the major fragment ions with an $m/z$ of 1209.4858, 997.4926, 912.4362, 359.1023

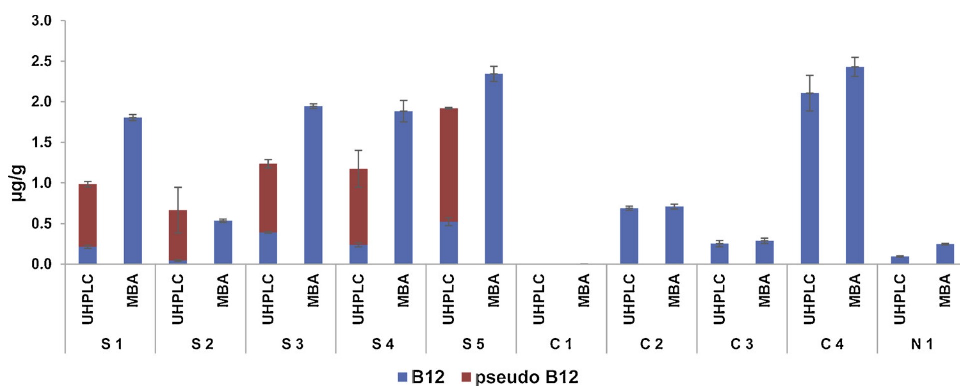


**Fig. 4.** The total vitamin B12 and pseudovitamin B12 content determined with the microbiological (MBA) and ultra-high-performance liquid chromatography (UHPLC) methods in *Spirulina* powders (S1–S5, see Table 1), *Chlorella* sp. powders (C1–C4, see Table 1) and in *N. gaditana* (N1, see Table 1) powder. The error bars represent the range of analytical replicates (n = 3, MBA; n = 2, UHPLC).

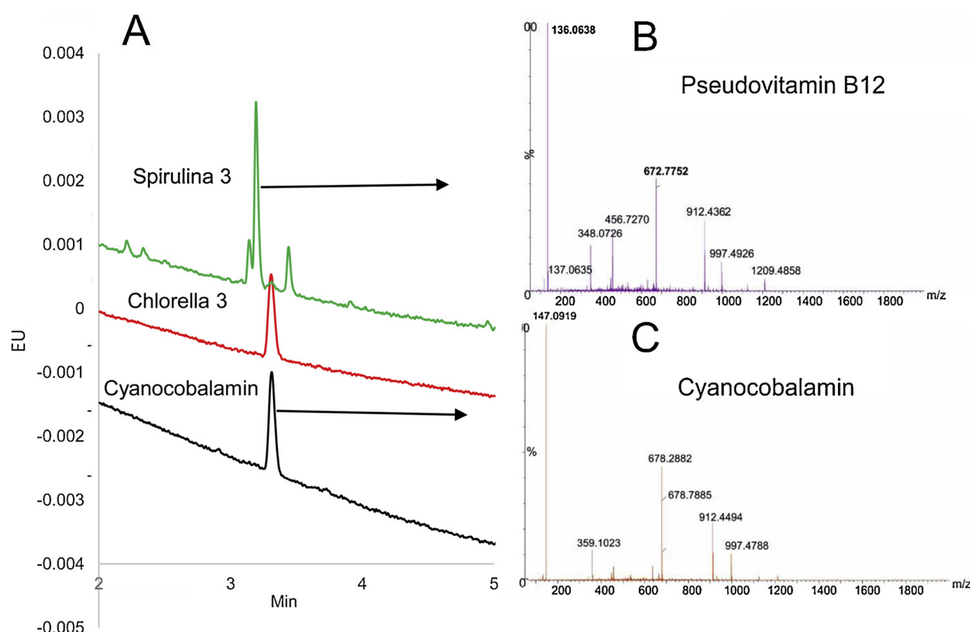


**Fig. 5. A**. Ultra-high-performance liquid chromatography (UHPLC) chromatograms of *Spirulina* (S3, see Table 1) extract showing a pseudovitamin B12 peak and of *Chlorella* sp. (C3, see Table 1) extract and of cyanocobalamin standard showing a vitamin B12 peak (RT = 3.4), respectively; **B**. quadrupole time-of flight (QTOF) tandem mass spectrometry (MS/MS) spectra of the pseudovitamin B12 peak from S3 and **C**. of the vitamin B12 peak from C3 and cyanocobalamin standard.

and 147.0919 were detected in both the *Chlorella* extract and the CNCbl standard. The fragmentation profile confirms that the corrinoid in the *Chlorella* extract contained 5,6-dimethylbenzimidazole (DMBI) (*m/z* 147.0919[DMBI + H]$^{+}$) as a lower ligand, as is found in the active form of B12.

In contrast to the *Chlorella* powders, the B12 content determined with UHPLC in the *Spirulina* powders was significantly lower ($p < 0.05$) than that obtained with the MBA, on average, only ca. 25% of the total B12 content given by the MBA. In addition to a small peak matching the retention time of the CNCbl standard (ca. 3.4 min), the chromatograms of the *Spirulina* extracts revealed a major peak eluting just before the CNCbl peak (Fig. 5A). The LC–MS/MS analysis confirmed that the peak eluting at 3.3 min was in fact pseudovitamin B12, an analogue of B12 with adenine as the lower ligand (*m/z* 136.0638) instead of 5,6-dimethylbenzimidazole (DMBI) as in CNCbl (Fig. 5). This peak produced doubly charged ions with an *m/z* of 672.7762 [M + 2 H]$^{2+}$, which, on fragmentation, produced the fragment ions characteristic of pseudovitamin B12 (Fig. 5B). When the pseudovitamin B12 content of the *Spirulina* powders was quantified using the CNCbl calibration curve and added to the content of active B12, the UHPLC results matched better with the results of the MBA (Fig. 4.) Previously, *Spirulina* tablets were shown to mainly contain the pseudo form (Watanabe et al., 1999) and its proportion was around 83% of the total content. In addition, other edible cyanobacteria, for example, *Aphanizomenonflos-aquae, Aphanothece sacrum, Nostoc commune, Nostoc flagelliforme and Nostochopsis* sp. have also been noted to be richer in pseudovitamin B12 than in active B12 (Watanabe et al., 2013).

The vast majority of cyanobacteria, and thus also *Spirulina*, synthesise and utilise pseudovitamin B12 as a cofactor for specialised methionine synthase (METH; EC 2.1.1.14), a structure that prefers an adenine instead of DMBI as a lower ligand in the B12 structure (Helliwell et al., 2016; Tanioka et al., 2010). Pseudovitamin B12 exhibits a ca. 500-fold lower binding capacity to human intrinsic factor than B12 with DMBI; thus, supplements made from *Spirulina* biomass cannot be regarded as a good source of bioavailable B12 (Helliwell et al., 2016). The reason for the presence of active B12 in *Spirulina* powders is not clear. *Spirulina* possibly absorbs B12 from the culture medium like the *Chlorella* sp. does (Bito et al., 2016). However, Watanabe et al. (2001) showed that *Spirulina platensis* did not accumulate exogenous B12 when it was cultivated in a synthetic medium with CNCbl. In contrast, Teng et al. (2014) analysed both forms in the edible cyanobacterium *Nostoc flagelliforme* with a LC–MS/MS method and suggested that N. *flagelliforme* might synthesise both pseudovitamin B12 and active B12.

*Spirulina* only uses pseudovitamin B12 in its one-carbon metabolism, whereas most of the *Chlorella* species, as well as plants, do not need B12 as a co-enzyme for METH at all (Bito et al., 2016; Croft et al., 2005; Helliwell et al., 2011). B12-independent algae have methionine synthase, so-called METE (EC 2.1.1.13), which functions without B12. However, there are also algae that encode both isoforms of methionine synthase. If extracellular B12 is available, these species use METH, otherwise they use METE (Helliwell et al., 2011).

In this study, the *Chlorella* sp. powders were produced either from *C. pyrenoidosa* or *C. vulgaris* according to the manufactures. It has been shown that B12 is not essential for *C. pyrenoidosa* or *C. vulgaris* (Croft et al., 2005). However, they are able to accumulate or absorb extracellular CNCbl and even convert added CNCbl into B12 coenzymes (Bito et al., 2016). Based on the observations by Bito et al. (2016), the active B12 of *Chlorella* powders in the current study probably originated from B12 added in the culture medium or from B12-synthesising bacteria. Furthermore, one of the *Chlorella* powders in this study had no B12. Bito et al (2016) also noticed that the B12 content in some *Chlorella* supplements was extremely low. Some *Chlorella* species have a rigid trilaminar layer as outer components of the cell wall instead of one microfibrillar layer. This may hinder compounds with a high molecular weight such as B12 from penetrating inside the cells (Liu and Hu, 2013; Maruyama et al., 1989).

To sum up the significance of *Chlorella* and *Spirulina* powders as a source of B12, *Chlorella* sp. powders are generally better sources of B12 than *Spirulina* powders are. However, the variation in the B12 content of *Chlorella* products may be high, as was shown in this study, probably depending on the available external B12 in the cultivation process. A daily portion (5 g) of *Chlorella* sp. powder with 2 μg/g or with 0.25 μg/g active B12 will offer a 5-fold or at least half, respectively, of the recommended daily intake of 2.4 μg (Institute of Medicine, 1998). On the other hand, although most of the B12 in the *Spirulina* powders is in a non-active pseudo form, the daily requirement can be met by consuming 4 g of *Spirulina* powder.

## 4. Conclusion

This study demonstrated that both *Chlorell*a sp. and *Spirulina*

powders were found to be a source of riboflavin and niacin. In terms of total niacin, NAM contributed more than NA both in the *Chlorella* and *Spirulina* powders. In addition, the *Chlorella* sp. powders were shown to be good sources of folate. The total folate content analysed with the MBA method was, on average, six-fold higher in the *Chlorella* sp. than in the *Spirulina* powders. The predominant folate vitamer in both powders was 5-$CH_3$-$H_4$folate. The *Chlorella* sp. powders were also rich in 5-HCO-$H_4$folate and 5,10-$CH^+$-$H_4$folate. In contrast, 5-HCO-$H_4$folate and $H_4$folate were absent from the *Spirulina* powders. The MBA results for the *Chlorella* sp. and *N. gaditana* samples matched well with the UHPLC results, whereas for the *Spirulina* powders, the MBA gave a higher folate content than the UHPLC did.

The *Chlorella* sp. powders and *N. gaditana* powder contained solely active B12 with varying levels. The active B12 content in one powder out of the four analysed was so high that one portion (5 g) would provide more than the RDA. On the contrary, all the *Spirulina* powders contained high amounts of pseudovitamin B12 and only a minor amount of active B12. The MBA overestimated B12 content, since it was not able to separate the active B12 form from non-active forms. Overall, this study has updated the information on the B vitamin content in commercial microalgae and has demonstrated the importance of the extraction and quantification methods in the analysis of B vitamins.

## Conflict of interest

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jfca.2019.05.009.

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