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The consistency of vitamin B_{12} in marketed microalgae powders

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A B S T R A C T

Vitamin B_{12} is a water nutrient that plays a key role, in DNA replication and the production of red blood cells as well as maintaining proper neuron function in the body system. Insufficient levels of this vitamin can result in health complications like megaloblastic anemia. At times, microalgal powders have surfaced as a source of vitamin B_{12}. The purpose of this study is to investigate the amount of active vitamin B_{12} in microalgae with a specific focus on commercially available strains, like *Chlorella* sp. and *Nannochloropsis gaditana*. The research discovered that *Chlorella* sp. and *N. gaditana* powders have vitamin B_{12} levels of, up to 2.1 μ/g whereas *Spirulina* powders contain pseudo vitamin B_{12} than active B_{12}. Collectively speaking *Chlorella* sp. and *N. gaditana* serve as good sources of active vitamin B_{12} while *Spirulina* seems to be less potent due, to its high pseudo-vitamin B_{12} content. This research highlights the promise of powders, like *Chlorella* sp. and *N. gaditana* as sources of accessible vitamin B_{12} that may contribute to addressing nutritional gaps in diets.

Keywords: *Chlorella* sp., *Nannochloropsis gaditana*, Cyanocobalamin (CNCbl), UHPLC, Powder analysis

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Introduction

Vitamin B_{12} popularly known as cobalamin is water soluble nutrient that plays multipurpose functions in the human body such as the synthesis of DNA red blood cell formation and neurological functioning (Madhubalaji *et al.*, 2021; Nef *et al.*, 2022). This molecule is very important in the cell metabolism and its main functions are related to one-carbon and fatty acid metabolism (Chandrasekaran and Karunasagar, 2014; Wells *et al.*, 2017). A deficiency in vitamin B_{12} results in dangerous conditions associated with anemia, and neurological disorders, and impacts the memory and cognition of a person (Ji *et al.*, 2021; Wells *et al.*, 2017). It is mainly found in animal foods because higher plants do not possess the enzymes which are necessary for the synthesis of this vitamin. But with the explosion of vegan diets as well as vegetarianism the world over, there has been a rise in the need for other sources of B_{12} from which the body can readily absorb (Ramanan *et al.*, 2016). Microalgae, particularly *Chlorella* sp. and *Spirulina*, are under discussion as a source of this enigmatic nutrient and that could be gotten from plants (Chen *et al.*, 2011; Martens *et al.*, 2002).

Several microalgal species including *Chlorella* sp. receive the capability of improving cognitive health. *Nannochloropsis oceanica* and *Nannochloropsis gaditana* contain active vitamin B_{12}. However, the existence of pseudo-vitamin B_{12} in some microalgal powders like *Spirulina* raises question marks over the efficacy and Bio availability of those supplements (Sandgruber *et al.*, 2021; Susanti *et al.*, 2022). Unfortunately, the currently available *Spirulina* powders contain pseudo-vitamin B_{12} which is not physiologically active as CN-Cbl (Del Bo *et al.*, 2019). To date, there is scanty information on the shy active vitamin B_{12} in microalgal products that are in the market and thus a problem with general public knowledge and labeling of products (Ganesan *et al.*, 2019). To fill these gaps, this study aims to determine the quantity of vitamin B_{12} in microalgal powders that are on the market (Araújo *et al.*, 2021; Gharibzahedi *et al.*, 2023).

This study seeks to identify the levels of active vitamin B_{12} in some microalgal powder products with particular reference to *Chlorella* sp. Among them there are, *N. gaditana,* and *Spirulina* (Jalilian *et al.*, 2019). This cross-sectional study uses MBA and UHPLC for bioassay to determine the concentration of active and the pseudo-B_{12} labelled in these powders (Bito *et al.,* 2016; Chamlagain *et al.,* 2015, 2018). The findings aim at shedding light on the nutritional value of these products, particularly to those who depend on them for B_{12} nutrient.

This study is relevant in today's world of the increasing popularity of plant-based diets for both consumers of the microalgal supplements and their manufacturers. In this way, it has given a much better understanding of the vitamin B_{12} content in these products, the labeling is, undoubtedly, more accurate the consumers enjoy better choices, and their diets are improved as well to help those at the edge of the risky B_{12} deficiency (Monteverde *et al.,* 2017; van den Oever and Mayer, 2022). Moreover, this study might help direct the future development in improving the levels of B_{12} in the microalgal products with the intention of making such products effective in providing nutrition solutions in the form of the said nutrient.

The study focuses on the analysis of three widely available microalgal powders: *Chlorella sp.* of green algae was used in the present study (Nef *et al.,* 2019). Superfoods in the group include *Chlorella* sp., *N. gaditana*, and *Spirulina*. In particular, it measures the levels of metabolically active vitamin B_{12}, as well as, pseudo-vitamin B_{12} using modern analytical techniques including the ultra-high-performance liquid chromatography UHPLC and microbead-based assay MBA (Edelmann *et al.,* 2019). The current study is restricted to commercial items, and hence the results derived are particular to the brands and samples used in the study. Microalgal powders obtained from *Chlorella* sp. and *Nannochloropsis gaditana* contain more active vitamin B_{12} as compared to *Spirulina* powders which are rich in pseudo-vitamin B_{12} (Martens *et al.,* 2002; Watanabe *et al.,* 1999). The microbiological assay (MBA) method provided a higher value of vitamin B_{12} content in microalgal powders than real value due to interference of pseudo-vitamin B_{12} with active vitamin B_{12} in MBA while UHPLC as a more precise method did not show this sort of interference hence giving near accurate results.

The specific objectives of this study are therefore to establish the density of native vitamin B_{12} in *Chlorella* sp. from commercial markets. The incorporation of and *N. gaditana* powders was performed using UHPLC and MBA techniques. Thus, the study aims to compare these techniques and identify their robustness to enhance the knowledge of the variation of B_{12} content in the marketed algal nutritional supplements hence its nutritional value for the consumers (Watanabe *et al.,* 2013).

Materials and Methods

Collection of samples

In the European Union (EU), only two species of microalgae, *Arthospira* sp. and *Chlorella* sp., have been designated as safe for human consumption. In this study, samples were taken from all of the online commercially available brands of dried biomass powder made from these species. There are currently four food-grade *Arthospira* sp. (henceforth, *Spirulina*) powder brands and three food-grade *Chlorella* sp. (Bito *et al.,* 2020; Edelmann *et al.,* 2019). All of the powders were purportedly made from 100% dried algal biomass, as stated on the packaging.

Quality control of vitamin B_{12}

Analytical duplicates were conducted for the vitamin B_{12} UHPLC analysis. Since enzymes were used in all steps of the extraction procedure, it was necessary to include a water control sample in the extraction mixture before testing for B_{12} (Madhubalaji *et al.,* 2021). Every stage of the analysis was performed in a dim light, and the folate analysis in particular required that the extracts as well as standard solutions be stored under an atmosphere of nitrogen whenever it was practical to do so. Spectrophotometric verification of each standard's concentration was performed (Shimadzu, UV-1800, 190-1100 nm). Spectrophotometry was used to confirm each standard's concentration. The results of the analytical replicates are shown as the means for the vitamins.

Vitamin B12 analysis

Both an MBA and a UHPLC method were used to determine the total B_{12} amount from the identical sample extract. The CNCbl was conducted to examine B_{12}. Using a spectrometer at the wavelength of 361 nm, the amount of CNCbl in the stock solution was determined by calculating the molar absorption value, which was found to be 28.01 lmol^{-1} cm^{-1}.

Extraction

Approximately 0.2g of dried biomass powder was extracted in a boiling water bath for 30 minutes using 100 L of a 1% (w/v) sodium cyanide solution, along with 10 mL of an extraction buffer containing 8.3 mmol/L NaOH and 20.7 mmol/L acetic acid was used to adjust the pH level up to 4.5. The extract was chilled, and subjected to two rounds of centrifugation, as well as the supernatants were then combined. Filtering was done on the extract, and pH and volume were maintained at 6.2 and 25 mL, respectively.

Microbiological method

According to the procedure outlined by (Chamlagain *et al.*, 2015) the MBA of total B_{12} was done. In brief, the overall B_{12} was quantified on µL plates with *Lactobacillus delbrueckii* ATCC 7830 as a growth signifier and CNCbl as a standard, with each sample extract diluted twice. In the microliter plate's wells, 100 µL of the compounded extracts as well as CNCbl solutions (0-8pg/well) were mixed with 200 µL of the vitamin B_{12} assay both which had the frozen *L. delbrueckii* in it. After a 19-hour incubation at 35°C, the turbidity (595 nm) was evaluated using a microplate reader. Quality control samples were evaluated against the approved reference material BCR 487 before every incubation. The concentration of MBA in this investigation was 1008.1 ± 6.01 ng/g (n = 3), while the reference value for MBA certification is 1120.01 ± 90.1 ng/g dm.

UHPLC analysis for vitamin B_{12}

An immunoaffinity column was used in accordance with the recommendations provided by the manufacturer in order to purify as well as concentrate the MBA extract (Easi-Extract, R-Biopharma, Scotland). Trifles of the cleansing process were discussed earlier (Chamlagain *et al.*, 2015). Water was used to evaporate the eluate after it was cleaned. At 30 degrees Celsius and a steady flow rate of 0.32 ml/min, Milli-Q water as well as acetonitrile containing 0.025% TFA was used in a linear gradient system to separate the CNCbl (Santos *et al.*, 2024). All measurements were taken with reference to an external standard and a multi-level (n = 5) calibration curve (0.40-7.999 ng) (Edelmann *et al.*, 2019). If the extract was found to contain pseudo-vitamin B_{12}, the quantity was discerned with the help of the CNCbl calibration curve (Chandra-Hioe *et al.*, 2020). For the BCR 487 reference material, the UHPLC determination yielded a B_{12} level of 751.1 ng/g (n = 2) in this investigation. The MBA result was superior to the UHPLC result. In besides active B_{12}, the test organism used in MBA, *L. delbrueckii*, can flourish on defective corrinoids and analogues. As we had discovered in a prior experiment, The B_{12} concentration in pig liver samples was overstated by MBA (Chamlagain *et al.*, 2015). According to the validation settings, found that the cyanocobalamin instrumental LOQ value was 0.2 ng/inj (15 µL). The LOQ was determined to be 0.035 g/g of sample after taking into account the sample magnitude (0.2 g), the purification stage, and the injection volume. Under a flow of nitrogen, after which the relic was restored in three hundred microliters of water. On a C18 column operating in a reversed phase, CNCbl was observed at 361 nm.

LC-MS method

The Bioavailability of B_{12} was confirmed using mass spectrometry, specifically a high-resolution quadrupole time-of-flight (QTOF) mass spectrometer with an electrospray ionization interface (Synapt G2-Si, Waters) operating in positive ion mode. In a nutshell, argon was used as the collision gas to scan ions with m/z values between 50 and 1500, and the MS/MS was carried out for ions with m/z values of 678.2882 ([M+2H]2+ of CNCbl) and 672.7752 ([M+2H]2+ of pseudo-vitamin B_{12}). For the MS analysis, formic acid (0.1%) was used in place of the TFA (0.025% of the mobile phase). The volume of the injection was 0.25 microliters. The main MS parameters were as follows (Table 1):

Table 1. Mass spectrometer (MS) parameters (Edelmann *et al.*, 2019).

Voltage of capillary	0.5 kV
Voltage of Sampling cone	39.90 V
Source offset	80.01 V
Source temperature	150°C
De-solvation temperature	601°C
De-solvation gas flow	1000 L/h
Flow rate of Nebulizer gas	6.51 L/h
Flow rate of Cone gas	50.05 L/h
Collision energy of trap	4.01 eV
Collision energy of ramp trap	1590 eV
Flow rate of Trap gas	2.00 mL/min
Scan time	0.20 s

Statistical analysis

Means and standard deviations (n=3, except for the B_{12} UHPLC analysis, which uses n=2) of analytical replicates are used to depict the vitamin and vitamin concentrations. Powders vitamin content was compared using an analysis of variance (Microsoft Excel) made from several microalgae species with those discovered using UHPLC and MBA. A statistically significant result was one with a p-value lower than 0.0.

Results and Discussion

Overall vitamin B_{12} Content

The MBA showed B_{12} content ranging from 0 to 2.4 µg/g in *Chlorella* and from 0.6 to 2.4 µg/g in *Spirulina* powders (Table 2).

Table 2. Vitamin B_{12} and pseudo vitamin B_{12} contents (µg/g) analysed with the UHPLC method and total vitamin B_{12} analysed with the microbiological method (MBA) in microalgae powder samples (Edelmann *et al.*, 2019).

Sample	Vitamin B_{12} with UHPLC	Pseudovitamin B_{12} with UHPLC	Vitamin B_{12} with MBA
S1, *Spirulina*, Duplaco	0.22 ± 0.02	0.77 ± 0.03	1.80 ± 0.036
S2, *Spirulina*; Puhdistamo	0.05 ± 0.01	0.62 ± 0.28	0.55 ± 0.021
S3, *Spirulina*; CoCoVi, India	0.39 ± 0.01	0.84 ± 0.05	1.94 ± 0.028
S4, *Spirulina*; CocoVi, China	0.24 ± 0.03	0.94 ± 0.23	1.88 ± 0.132
S5, *Spirulina*; Voimaruoka	0.52 ± 0.05	1.39 ± 0.01	2.35 ± 0.093
C1, *Chlorella*; Duplaco	< LOQ	nd	0.001 ± 0.0004
C2, *Chlorella*; Puhdistamo	0.69 ± 0.03	nd	0.71 ± 0.030
C3, *Chlorella*; Cocovi	0.25 ± 0.03	nd	0.29 ± 0.033
C4, *Chlorella*; Voimaruoka	2.11 ± 0.12	nd	2.43 ± 0.120
N1, *N. gaditana*; Duplaco	0.09 ± 0.01	nd	0.25 ± 0.010

The values are introduced as means ± SD of three analytical replicates (n=3) in MBA or means ± range (n=2) in UHPLC method.
nd = not detected, under the limit of detection; < LOQ = under the limit of quantification (0.035 µg/g).

Only 0.249 µg/g of B_{12} was present in *N. gaditana*, and none was present in one *Chlorella* sample (C1). Chlorella's B_{12} level was amidst the normal scale illustrated by (Bito *et al.*, 2016) for *Chlorella* health supplements (i.e., tablets), ranging in concentration from a small quantity to 4.5 µg/g evaluated by the MBA. The total content of vitamin B_{12} and pseudo-vitamin B_{12} was determined using microbiological analysis (MBA) and ultra-high-performance liquid chromatography (UHPLC) in the *Spirulina* powders (S1–S5, see Table 2), *Chlorella* species (C1–C4, see Table 2), and *N. gaditana* (N1, see Table 2) respectively. The range of results from the analytical replicates (n = 3 MBA and n = 2 UHPLC) is depicted by the error bars (Fig. 1).

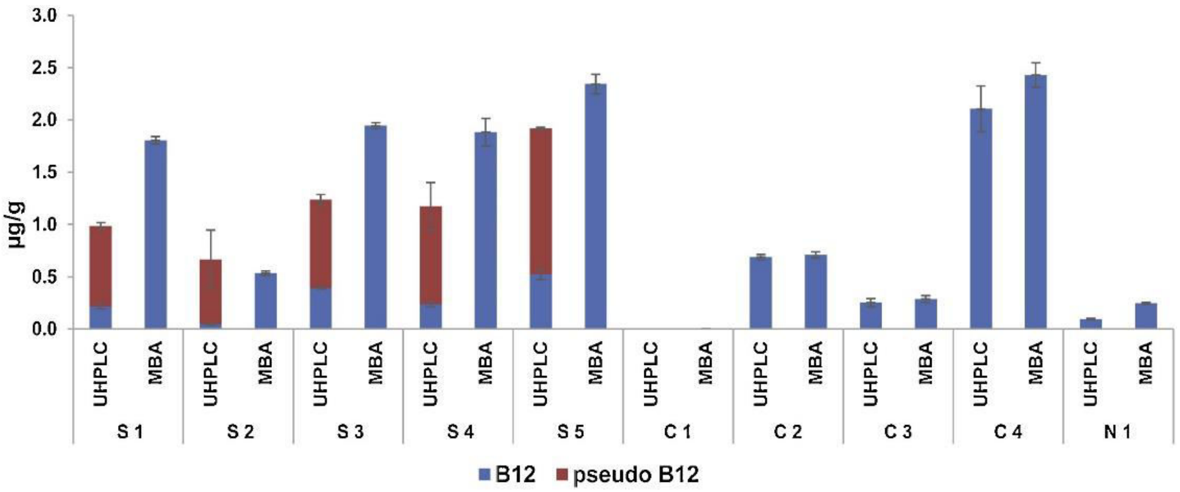

Fig. 1. The total vitamin B_{12} and pseudo-vitamin B_{12} content (Kittaka-Katsura *et al.*, 2002).

In addition, Kittaka-Katsura *et al.* (2002) revealed a B_{12} concentration variation of 2.01–2.92 µg/g dm in markets of available *Chlorella* pills that were examined using MBA as well as a chemiluminescence test. Our MBA results for *Chlorella* sp. biomasses at 0.001-0.8 µg/g (Chamlagain *et al.*, 2015; Maruyama *et al.*, 1989) also for *Spirulina* at 1.6-3.2 µg/g dm were consistent with values given review papers (Bishop and Zubeck, 2012). Additionally, the results of this analysis were consistent with a B_{12} concentration of 1.3–2.4 µg/g that was determined by the MBA method in *Spirulina* tablets (Bito *et al.*, 2016; Edelmann *et al.*, 2019). The B_{12} concentrations that we measured with MBA and UHPLC were pretty comparable for all of the *Chlorella* powders ($p < 0.05$). There was only one peak visible in the chromatograms that eluted at CNCbl's retention time (Fig. 2A).

The figure is composed of three distinct figures:

2A: Presents an actual UHPLC chromatogram of comparison between *Spirulina* and *Chlorella* extracts, selected with the peaks of pseudo-vitamin B_{12} in spirulina and active vitamin B_{12} in *Chlorella*. The reference standard of cyanocobalamin is identified with a retention time (RT) of 3.4 minutes. 2B: Provides the MS/MS spectra for the pseudo-vitamin B_{12} peak from *Spirulina* and identifies the ions that are characteristic of this form.

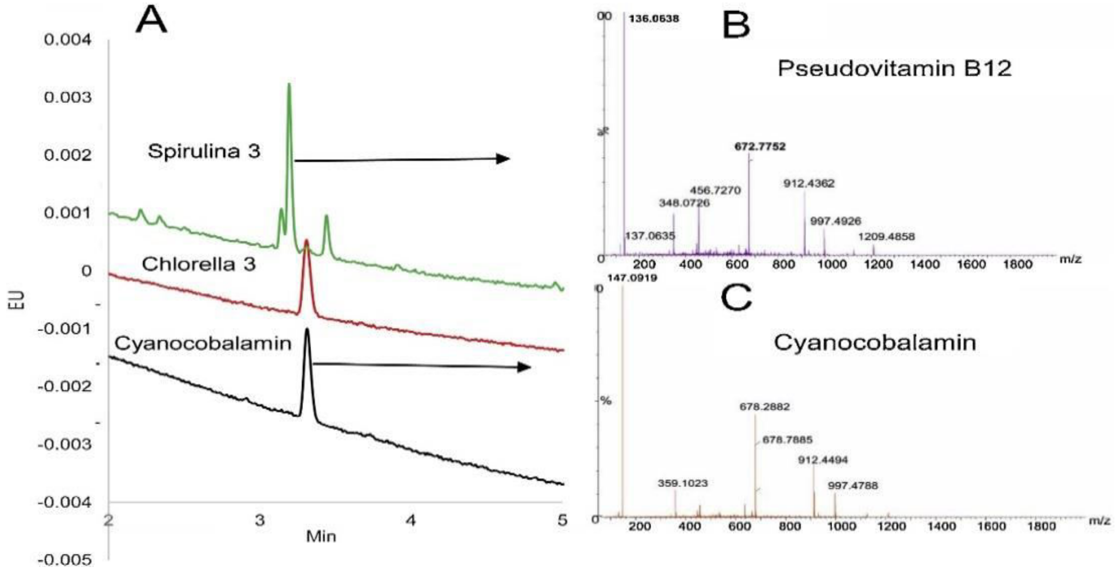

Figure 2A, B, C: UHPLC and MS/MS Identification of Vitamin B_{12} and Pseudo-Vitamin B_{12} in Microalgal Powders.

2C: Shows the MS/MS spectra of the vitamin B_{12} peak from *Chlorella*, which is a similar fragmentation pattern to cyanocobalamin proving that there is active B_{12} present.

The mass spectrum patterns between the peak and cyanocobalamin standard matched exactly. This occurred through their shared m/z 678.2882 peak and characteristic B12 vitamin patterns. The compound meets the specifications for cyanocobalamin due to its elution time plus unique mass spectral analysis (Fig. 2C). experiments reveal Spirulina has mostly pseudo-vitamin B12 as its main ingredient but this form does not offer meaningful nutrition to humans. The detection method reveals a peak from Chlorella that mirrors the cyanocobalamin standard which confirms that vitamin B12 exists in its active form. Additional testing needs to verify the precision of this discovery. *Spirulina* extract chromatograms displayed a primary peak eluting just prior to the CNCbl peak and a secondary, smaller peak with a retention time very close to that of the CNCbl standard (3.4 min) (Fig. 2A). The LC-MS/MS analysis proved that the peak that appeared at 3.3 min was in fact pseudo-vitamin B_{12}, which is similar to B_{12} but has adenine instead of 5,6-dimethylbenzimidazole (DMBI) as the lower ligand (m/z 136.0638). This peak made double-charged ions with a mass-to-charge ratio of 672.7762 [M+2H] 2+. When these ions broke apart, they made the fragment ions that are typical of pseudo-vitamin B_{12} (Fig. 2B). When the amount of "fake vitamin B_{12}" in the *Spirulina* powders was measured using the CNCbl calibration curve as well as assembled to the amount of "active vitamin B_{12}," the UHPLC results were more in line with the MBA results (Fig. 1). Before, it was found that most *Spirulina* tablets had the pseudo form (Chamlagain *et al.*, 2015; Edelmann *et al.*, 2019) and it made up about 83% of the entire content.

Most cyanobacteria, including *Spirulina*, produce and use pseudo vitamin B_{12} as a co-factor for a specialized form of methionine synthase (Edelmann *et al.*, 2012) that favours adenine over DMB as a weaker ligand often in the form of B_{12} (Helliwell *et al.*, 2011). Supplements made from *Spirulina* biomass are not a dependable source of bioactive B_{12} so that pseudo-vitamin B_{12} has a binding affinity to human intrinsic factor that is 500 times lower than that of B_{12} with DMB (Helliwell *et al.*, 2011). It is unclear why active B_{12} is present in *Spirulina* powders. Like *Chlorella* sp., *Spirulina* may also take up B_{12} from the growth medium (Bito *et al.*, 2016). However, (Watanabe *et al.*, 2013) illustrated that when *Spirulina platensis* was grown in a synthetic media with CNCbl, it did not acquire exogenous B_{12}. In contrast, the experimented LC-MS/MS of both types in the consumable cyanobacterium Nostoc flagelliforme suggests that *N. flagelliforme* may produce both pseudo vitamin B_{12} and active B_{12}. Spirulina's one-carbon metabolism uses only pseudo vitamin B_{12}, whereas most *Chlorella* species and plants do not require B_{12} as a coenzyme for METH at all. METE, or methionine synthase, is present in algae deficient B_{12} (Edelmann *et al.*, 2012), which functions without B_{12}. Algae can also produce both methionine synthase isoforms, though. These species employ METH if extracellular B_{12} is accessible; otherwise, they use METE (Helliwell *et al.*, 2011). According to the manufacturers, either *C. pyrenoidosa* or *C. vulgaris* was used to make the *Chlorella* sp. powders used in this investigation. B_{12} has been demonstrated to not be necessary for *C. pyrenoidosa* or *C. vulgaris* (Croft *et al.*, 2005; Kittaka-Katsura *et al.*, 2002). But they can gather or take in extracellular CNCbl, and they can even change excess CNCbl into B_{12} coenzymes (Bito *et al.*, 2016).

According to the review, the active B_{12} in the *Chlorella* powders used in this study most likely came from bacteria that produce B_{12} or from B_{12} that was added to the growth medium. In addition to that, one of the powders of *Chlorella* used in this research did not include any B_{12}. Some researcher also found that some *Chlorella* products had an abnormally low amount of B_{12} in their formulations (Bito *et al*., 2016). In place of a single microfibrillar layer, certain *Chlorella* genotypes form through hard triple laminar layer as the outermost part of the cell wall. Because of this, it might be harder for substances with a large molecular weight, like B_{12}, to enter the cells (Helliwell *et al*., 2011; Sañudo-Wilhelmy *et al*., 2014). In order to compare and contrast *Chlorella* as well as *Spirulina* powders as sources of B_{12}, it may be said that *Chlorella* sp. powders are typically superior (Bajaj and Singhal, 2020; Bishop and Zubeck, 2012). A daily meal of *Chlorella* sp. powder consisting of five grams and having either 0.25 micrograms per gram or two micrograms per gram of active B_{12} will supply at least fifty percent of the dietary referenced consumption of 2.4 micrograms of B_{12}.

Conclusion

The results of this study showed that both *Chlorella* sp. powders and *N. gaditana* powder contain active B_{12} in varying amounts. Only one of the four powders that were evaluated had an active B_{12} concentration that was high enough that one serving (5 g) would deliver more than the recommended daily allowance. On the other hand, every single *Spirulina* powder had extremely high levels of a substance called pseudo-vitamin B_{12}, but just a trace amount of the real vitamin B_{12}. The MBA overstated the B_{12} concentration because it was unable to distinguish between active and inactive forms of the vitamin. In general, the findings of this inquiry have brought our information on the B_{12} vitamin. The quantity of industrial microalgae is up to date and has shown the significance of extraction and quantification procedures in B_{12} vitamin testing.

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