Chlorella vulgaris BASED FUNCTIONAL FOOD DESIGN

by

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Chlorella vulgaris BASED FUNCTIONAL FOOD DESIGN

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ABSTRACT

Chlorella vulgaris BASED FUNCTIONAL FOOD DESIGN

Malnutrition affects people all around the world regardless of their economic income or geographic location. This problem is mainly caused by inadequate food intake and/or unhealthy eating habits. Today, the common diet of most people is high in simple carbohydrates, deficient in micronutrients and mostly depends on an animal protein which is responsible for occupying the majority of agricultural lands and usage of excess amounts of water. *Chlorella vulgaris* is an exceptional vegan-vegetarian food alternative by providing enough protein to compete with animal sources in addition to containing a broad range of vitamins and minerals like vegetables. From the environmental aspect, cultivation of *Chlorella vulgaris* aids by sequestering carbon dioxide without the need for fertile lands and uses 500% less water than meat production. In this study, preliminary nutritional analysis was done to assess the utilization of *Chlorella vulgaris* CCAP 211/11b as a functional food ingredient. For the experimental process, *Chlorella vulgaris* was grown under photoautotrophic and sterile conditions in two liter photobioreactors in sextuplicate batches. Harvested biomass of three batches were baked at 125°C for 15 and 35 min, separately. Nutritional profiles of both raw and baked biomasses were analyzed. Macronutrient compositions were determined by colorimetric methods and mass balance whereas vitamin contents were quantified by LC-MS/MS analysis. The results showed that baking at 125°C for 15 and 35 min had no statistically significant effect on the vitamin content of *Chlorella vulgaris* and the produced biomass has potential as a significant source of vegan-vegetarian protein and vitamin B12.

ÖZET

Chlorella vulgaris TABANLI FONKSİYONEL GIDA TASARIMI

Yetersiz beslenme ekonomik durum ve coğrafi konum fark etmeksizin dünyanın her yerinde insanları etkilemektedir. Bu problem hem gıda yetersizliği hem de kötü beslenme alışkanlıklarından kaynaklanmaktadır. Günümüzde, yaygın beslenme şekli yoğun miktarda basit karbonhidratlar içermekte, mikro besinler açısından zayıf kalmakta ve çoğunlukla hayvansal proteine dayanmaktır. Hayvansal protein üretimi tarım arazilerinin çoğunluğunu işgal etmekte ve aşırı su tüketimine sebep olmaktadır. *Chlorella vulgaris* hayvansal kaynaklarla yarışacak kadar protein ve sebzeler kadar vitamin ve mineral içeren, istisnai bir vegan-vejeteryan gıda alternatifidir. Çevre açısından bakıldığında *Chlorella vulgaris* yetiştirilmesi atmosferdeki karbondioksitin özümsenmesinin yanı sıra, verimli arazilere ihtiyaç duymamaktadır ve et üretimine göre %500 daha az su tüketmektedir. Bu çalışmada *Chlorella vulgaris* biyokütlesinin fonksiyonel gıda malzemesi olarak kullanılabilmesi için gereken besin değeri ön analizleri yapılmıştır. Deneysel süreçte, *Chlorella vulgaris* iki litrelik fotobiyoreaktörler içerisinde fotoototrofik ve steril koşullarla 6 paralel grup olarak yetiştirilmiştir. Hasatlanan biyokütlenin üç grubu 125°C'da ayrı miktarlarda 15 ve 35 dakika fırınlanmıştır. Ham ve fırınlanmış biyokütlelerin besin değerleri analiz edilmiştir. Makrobesin değerleri kolorimetrik metotlarla ve ağırlık dengesiyle belirlenip, vitamin değerleri LC-MS/MS ile analiz edilmiştir. Elde edilen veriler, *Chlorella vulgaris* biyokütlesinin 125°C'de 15 ve 35 dakika fırınlanmasının vitamin değerleri üzerinde istatistiksel olarak kayda değer bir etki yaratmadığını göstermiştir. Sonuç olarak, üretilen *Chlorella vulgaris* biyokütlesinin dikkate değer bir vegan-vejeteryan protein ve B12 vitamini kaynağı olduğu tespit edilmiştir.

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LIST OF SYMBOLS/ABBREVIATIONS

Symbol	Explanation	Unit
%	Percent	
λ	Lambda	
μ	Micro	
Ω	Ohm	
μL	Microliter	
μm	Micrometer	
Ca	Calcium	
$CuSO_4.5H2O$	Copper (II) Sulfate	
Fe	Iron	
HCl	Hydrochloric Acid	
I	Iodine	
Mg	Magnesium	
N_2	Nitrogen	
Na_2CO_3	Sodium Carbonate	
NaK Tartrate tetrahydrate	Potassium Sodium Tartrate Tetrahydrate	
NaOH	Sodium Hydroxide	
^oC	Degree Celsius	
Se	Selenium	
Zn	Zinc	
ω	Omega	

Abbreviation	Explanation
A	Retinol
ALA	α-Linolenic Acid
B1	Baked for 15 Min 1
B2	Baked for 15 Min 2
B3	Baked for 15 Min 3
BOD	Biochemical Oxygen Demand
C1	Control 1
C2	Control 2

C3	Control 3
CC	Cell Count
DHA	Docosahexaenoic Acid
DIW	Deionized Water
DMB	5,6-Dimethyl- Benzimidazole
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
DW	Dry Weight
EPA	Eicosanpentaenoic Acid
F1	Baked for 35 Min 1
F2	Baked for 35 Min 2
F3	Baked for 35 Min 3
FAO	Food and Agriculture Organization of United Nations
g	Gram
Gt	Giga Tonne
h	Hour
HDL	High Density Lipoprotein
kg	Kg
L	Liter
LA	Linoeic Acid
LC	Liquid Chromotography
LDL	Low Density Lipoprotein
M	Molar
MΩ	Megaohm
m^2	Meter Square
m^2	Meter Square
Max	Maximum
MBTH	3-Methyl-2-Benzothiazolione Hydrazine
MeOH	Methanol
mg	Milligram
Min	Minimum
min	Minute
mL	Milliliter
mM	Micromolar
MS	Mass Spectrometry

MUFA	Monounsaturated Fatty Acid
N	Normal
nm	Nanometer
OD	Optical Density
p	Probability
PUFA	Polyunsaturated Fatty Acid
R^2	R-Squared
RDV	Recommended Daily Value
ROS	Reactive Oxygen Species
rpm	Repetitions Per Minute
RT	Retention Time
s	Second
SFA	Saturated Fatty Acid
t	t-test Value
TAG	Triglyceride
TC/HDL	Total Cholesterol/High Density Lipoprotein
USDA	United States Department of Agriculture
Vitamin B1	Thiamine
Vitamin B12	Cobalamin
Vitamin B12m	Methylcobalamin
Vitamin B2	Riboflavin
Vitamin B3	Niacin
Vitamin B5	Pantothenic Acid
Vitamin B6	Pyridoxine
Vitamin B7	Biotin
Vitamin B9	Folate
Vitamin C	Ascorbic Acid
Vitamin E	Tocopherol
WHO	World Health Organization
ω-3 PUFA	Omega-3 Polyunsaturated Fatty Acid
ω-6 PUFA	Omega-6 Polyunsaturated Fatty Acid

1. INTRODUCTION

Earth's resources compensated the needs of humans in the era of hunter gatherers. Along with the increase in the human population, 7.5 billion needs to be fed now (United States Census Bureau, 2018). While the water from Fiji, matcha (powdered Japanese green tea leaves) from Japan is available on the shelves of markets, there is still widespread malnutrition around the world including developed countries. Undernourishment arises from the unbalanced distribution of food resources, overconsumption and lack of knowledge about nutrition where a wide range of food is available.

A healthy diet should involve a balanced mix of complex carbohydrates, unsaturated fats with smaller quantities of saturated fats and protein sources that provide a full range of essential amino acids. Conjointly, micronutrients are vital for the proper functioning of the body and required in diet. However, the common diet involves high amounts of simple carbohydrates, saturated and trans-fats, limited amounts of protein and is deficient in micronutrients. To crown it all, almost every food contains pesticides which is either used on vegetables and fruits or on the plants that is used for feeding animals.

Another contradictive aspect of the common diet is the environmentally unsustainable production of the highly consumed foods. For agriculture, large areas of fertile lands and forests are occupied by single species, environmentally persistent pesticides are used, and water resources are polluted and depleted in a non-sustainable way. Seventy five percent of the World's agricultural lands are used for animal raising (Foley et at., 2011) and 18% of the global greenhouse emissions are originated from the animal based food production (Cassidy et al., 2013).

Increasing knowledge about nutrition influenced the society and led to the emergence of "functional foods" (Gyenis et al., 2005). These foods provide higher amounts of nutrients in little volume compared to the classical ones. Concentrated amounts of nutrients offer to close the gap of the deficiency by aiding the common diet. Also, they have the potential to reduce the amount of agricultural products, especially livestock which is the main source of protein. Simply, a functional food can be made by fortifying food with vitamins, minerals or protein. However, it is possible to enrich foods with the natural components such as; matcha, egg yolk, cocoa, and algae as well.

In recent years, the potential of microalgae and cyanobacteria as food additive gained significance in the interest of achieving high nutritional content with a minimum amount. Both

microalgae and cyanobacteria are microscopic organisms that use sunlight as an energy source to convert carbon dioxide into biomass and oxygen. However, they are classified under different domains. Cyanobacteria belong to the Bacteria domain and microalgae belong to Eukarya (Figure 1.1). Despite this, both have edible species and serve to the same purpose by offering a broad range of vitamins, minerals along with proteins, lipids and certain amounts of carbohydrates.

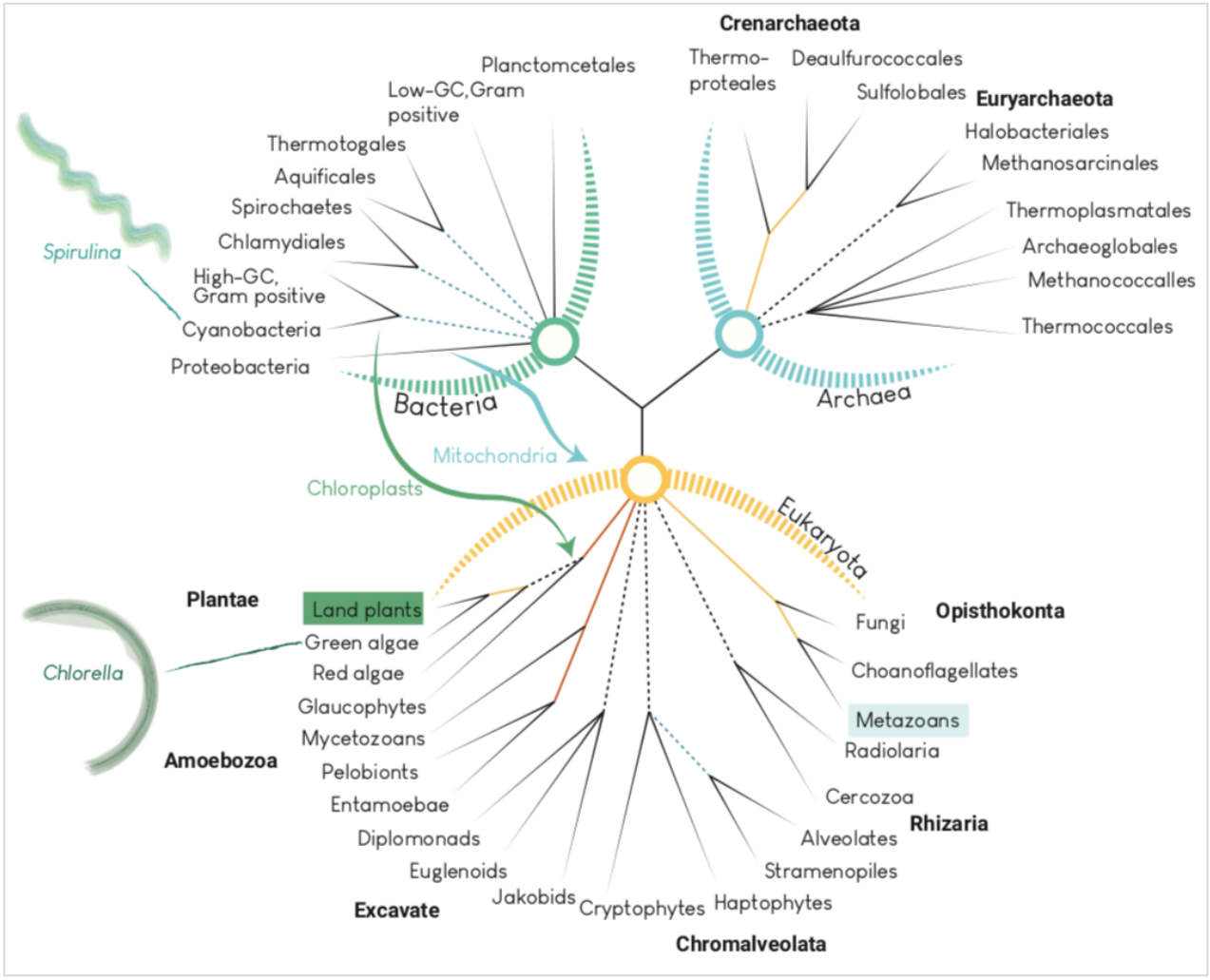

Figure 1.1. Phylogenic tree of cyanobacteria and microalgae (Delsuc et al., 2005).

History of the edible microscopic algae cells dates back to Aztecs. The people of Tenochtitlán harvested cyanobacteria from Lake Tezcoco and consumed in the form of dried cakes (Farrar, 1966). Later evidence of the edible cyanobacteria was found during a Belgian expedition to Sahara between 1964 and 1965. People of Chad harvested the cyanobacteria *Spirulina platensis* from Lake Chad and sold in the market as dried cakes (Leonard, 1966). Today, both microalgae and cyanobacteria are widely consumed around the world, especially in the Asian markets. The most well-known edible genera are microalgae *Chlorella* spp. and cyanobacteria *Spirulina* spp.

The main problem about consuming microalgae and cyanobacteria is the sharp seaweed taste which is commonly undesirable. Asian cuisine is already fused with the seaweeds which might be the reason for the high consumption of microalgae and cyanobacteria compared to other parts of the world. In the Western World, both are mostly consumed as supplements in the form of tablets. An effective fix for the taste is the fusion of microalgae or cyanobacteria with foods to benefit from their nutritional quality. Mixing with food is the best possible way to consume algae naturally without modulating its taste artificially. In order to achieve that, the nutritional value of the microalgae or cyanobacteria must be determined as preliminary work to create a healthy formulation for balanced nutrition.

In this study, the nutritional profile of *Chlorella vulgaris* was analyzed and the effects of baking on nutrients were explored for further applications in the food industry. Accordingly, the required amount of *Chlorella vulgaris* was determined for diverse nutritional benefits for various food applications.

2. LITERATURE REVIEW

2.1. Microalgae as Food; *Spirulina* vs. *Chlorella*

Microalgae have already been used in the food and beverages. Most popular ones are the *Spirulina* pastas, chocolates, protein bars and green juices (Lee and Marino, 2010). *Spirulina* is highly preferred considering its high protein and micronutrients content (Lee and Marino, 2010). *Spirulina* is also marketed as a vegan source of vitamin B12 which is a major problem for both vegetarians and vegans. Although there seems to be no compelling reason to use *Spirulina* considering its high protein value, current research show that vitamin B12 in commercially sold *Spirulina* is not bioavailable to humans (Helliwell et al., 2016; Heal et al., 2017; Watanabe, 2007).

Vitamin B12 is the term for "cobalamin" which is a cobalt centered, planar tetra-pyrrole ring called a corrin ring [(Helliwell et al., 2016) (Figure 2.1.)]. Only the cobalamins with 5,6-dimethyl-benzimidazole (DMB) located in their lower axial ligands are shown to be bioavailable to the humans [(Heal et al., 2017) (Figure 2.1.)]. *Spirulina* on the other hand, contains pseudocobalamin which has adenine located in its lower axial (Heal et al., 2017). When ingested, pseudocobalamin binds to same protein as cobalamin and occupy its place (Watanabe, 2007). It is suggested that, *Spirulina* extracts may interrupt metabolism of the humans but no such effect observed in the *Spirulina* supplemented rats (Watanabe, 2007).

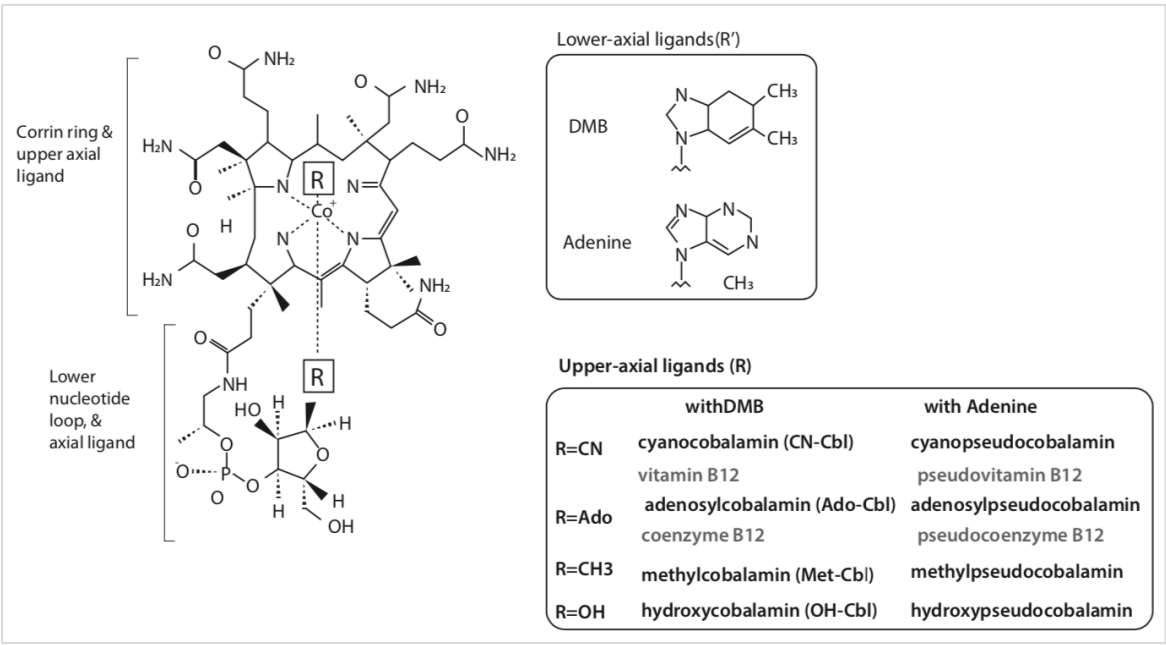

Figure 2.1. Chemical form of bioavailable cobalamin and pseudocobalamin (Helliwell et al., 2016).

Nevertheless, there is insufficient human research on the pseudocobalamin linked B12 deficiency to draw any firm conclusion on its health effects (Watanabe, 2007). However, protein occupancy puts forward the view that replacement of the pseudocobalamin with its already accessible bioavailable form might offer better functioning for the body. In light of this, eukaryotic green microalgae species, i.e. *Chlorella vulgaris*, was preferred instead. *Chlorella* vulgaris is a 2.5 billion years old freshwater microalgae with spherical eukaryotic cells that have a diameter between 2-10 μm (Safi et al., 2014). Compared to *Spirulina*, *Chlorella vulgaris* contains bioavailable form of the vitamin B12 to humans (Hiromi, Tomoyuki et al. 2002) and also offers high amounts of protein as well as wide range of vitamins, minerals and bioactive compounds (Wells et al., 2017).

2.2. Nutritional Value of *Chlorella vulgaris*

2.2.1. Carbohydrates

The very first problem of the common diet is high consumption of sugar. Sugar refers to the refined monosaccharides; i.e. glucose, fructose, galactose, extracted from sugar cane, beet, corn, etc. (FAO, 2019). Refined sugar can be present in various forms in foods (such as corn syrup, glucose syrup, fructose syrup, maltose, etc.) and most people consume sugar without realizing its presence in the juices, fizzy beverages and cereals (USDA, 2019). The problem about consuming refined sugar is, its long-term damaging effect on the carbohydrate metabolism (Harvard T.H. Chan School of Public Health, 2019). Over time, condition exacerbates and evolves to the type 2 diabetes which is expected to affect 366 million people by 2030 (Wild et al., 2004).

Experts from Harvard T.H. Chan School of Public Health reports that human body metabolizes carbohydrates by breaking them down into its monomers and releasing them to bloodstream. As a response, brain triggers the insulin secretion from pancreas. Insulin induces muscle cells and adipocytes to draw glucose from the bloodstream. Unlike other carbohydrates, when a food loaded with the refined sugar is ingested, blood sugar increases rapidly. As a response, insulin is secreted and blood sugar drops harshly below normal levels. Refined sugar induces dopamine secretion, thus creating a craving effect for more and repeating cycle of eating refined sugar loaded foods (Blum et al., 2014). Over time, the cells become more resistant to these spikes and become tolerant to the insulin. Type 2 diabetes is the condition when long term high blood sugar, insulin spikes cause cells to wear out, and consequently insulin production ends (Harvard T.H. Chan School of Public Health, 2019). Additionally, the brain requires stable blood sugar levels to operate and fluctuation of the blood sugar affect mood adversely (Sommerfield et al., 2004). Correspondingly, in order to achieve

a gradual increase and decrease in the blood sugar, slowly digested foods must be preferred (Harvard Medical School, 2019).

Carbohydrates are classified under three categories; sugars, complex carbohydrates and fiber (Harvard T.H. Chan School of Public Health, 2019). Sugars are not necessarily bad for the health. Fruits and milk contain naturally occurring sugars (WHO 2015) and both are packed with nutrients (WHO, 2001). In addition, fruits are high in fiber which slows the digestion to a healthy pace (Mann et al., 2007). Complex carbohydrates are starches found in the legumes (Crosby, 2019), grains, beans, starchy vegetables, pasta, bread and rice (British Nutrition Foundation, 2019). Complex carbohydrates are digested slowly and naturally present along with the fiber that slows digestion (Harvard T.H. Chan School of Public Health, 2019). Starches are not harmful unless they undergo refining process which accelerates the digestion and rapidly increase the blood sugar (Harvard T.H. Chan School of Public Health, 2019). A sub-branch, resistant starches are not digested in the small intestine and proceed directly to the large intestine (Birt et al., 2013). Therefore, blood sugar increases gradually (Weickert and Pfeiffer, 2008). Additionally, certain types of the resistant starches are prebiotics which are vital for the beneficial gut bacteria (Birt et al., 2013). Fiber is indigestible by the humans but crucial for the digestive health (Lattimer and Haub, 2010). Fiber lowers LDL by binding to the cholesterol, lowers the blood sugar levels by slowing digestion, provides fullness after eating and benefits the intestines by retaining water (Brown et al., 1999).

Knowing the amount of carbohydrate in foods does not provide sufficient information to draw any conclusion about their effect on the blood sugar. Many factors including processing and fiber content of the foods affect their increase rate of the blood sugar (Harvard T.H. Chan School of Public Health, 2019). Rather than just total the carbohydrate content, correct labels should include the food's carbohydrate type, fiber content and blood sugar increment rate.

Chlorella vulgaris contains roughly 8-20% carbohydrate of its dry weight (Table 2.1). Hundred g of *Chlorella vulgaris* meets approximately 7% of daily carbohydrate requirement advised by WHO (Table 2.1). However, depending on the growth conditions, content of carbohydrate can increase up to 55% (Chen et al., 2013). The dominant type of carbohydrate in *Chlorella vulgaris* is starch 46.5%, followed by monomeric and oligomeric sugars 26.4%, and structural polysaccharides 6.0% [(Figure 2.2) (Ortiz-Tena et al., 2016)].

Table 2.1. Carbohydrate content of *Chlorella vulgaris*.

Reference			RDV FAO & WHO (Mann et al., 2007)		100g *Chlorella vulgaris* %RDV*	
	Carbohydrate	Unit	Male*	Female*	Male*	Female*
***Chlorella vulgaris* (Tokusoglu et al., 2003)**	8.08	%DW	275-375g		2.94%	
***Chlorella vulgaris* (Batista et al., 2013)**	19.9	%DW			7.24%	
***Chlorella vulgaris* (Becker, 2007)**	12-17	%DW			5.27%	

*Male=65 kg, Female=55 kg, based on 2000 kcal/day diet.

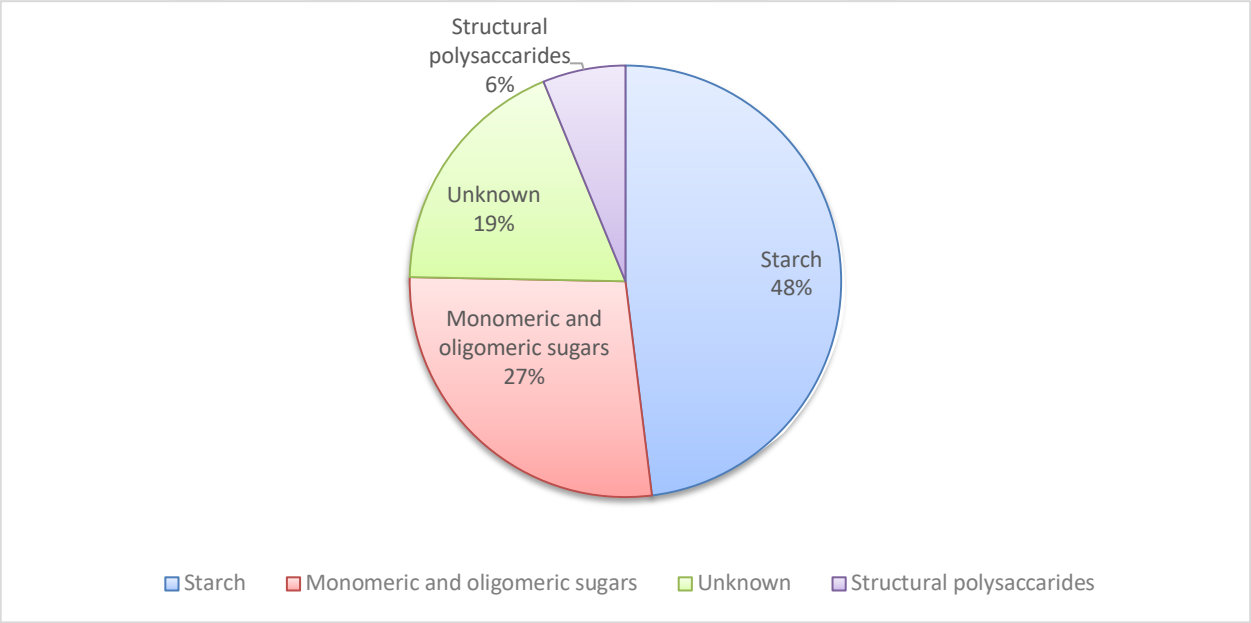

Figure 2.2. Types of carbohydrates in *Chlorella vulgaris* (Ortiz-Tena et al., 2016).

As for the blood sugar, it is shown that *Chlorella* has remedial effects on diabetes (Yamaguchi, 1996) and increases insulin sensitivity in the streptozotocin induced diabetic mice (Jong-Yuh et al., 2005) which exhibits a relatable example of its effect on the humans. The glycemic effect of *Chlorella vulgaris* is unknown, however its fiber content of 5.8-15.6% (Table 2.2) falls between 5% (low) and 20% (high) recommended by FDA (FDA, 2019).

Table 2.2. Fiber content of *Chlorella vulgaris.*

Reference			RDV FDA (FDA, 2019)		100g *Chlorella vulgaris* %RDV	
	Fiber	Unit	Male*	Female*	Male*	Female*
***Chlorella vulgaris* (Maruyama et al., 1997)**	5.8	%DW	25g		23.20%	
***Chlorella vulgaris* (Panahi et al., 2012)**	15.6	g/100g			62.40%	

*Male=65 kg, Female=55 kg, based on 2000 kcal/day diet.

Overall, carbohydrate content of *Chlorella vulgaris* is low regarding the daily carbohydrate requirements advised by the FDA (FDA, 2019) and its fiber content will aid by slowing the increment of blood sugar. As mentioned earlier, the dominant carbohydrate in *Chlorella vulgaris* is starch but there is no information available in literature whether it is resistant starch or not. As a precaution, biomass cultivated with low carbohydrate and high protein should be preferred to avoid rapid blood sugar increase caused by the starch as mentioned earlier.

2.2.2. Fatty Acids

Not so long ago, the fatty foods were banished from nutrition because of the presumption that consuming foods with saturated fats leads to gain weight, elevated cholesterol and cardiovascular diseases. This approach bloomed the idea that elimination of the animal fats by replacing them with hydrogenated vegetable oils. Later, it was also understood that hydrogenated vegetable oils contain trans-fats which possess greater danger to the heart and raise cholesterol (WHO and FAO, 2010).

In recent period, scientists understand the fat metabolism better. Roughly there are three key elements that regulate cholesterol; high density lipoprotein (HDL), low density lipoprotein (LDL) (Arnold and Kwiterovich Jr, 2003) and triglycerides (TAGs) (Singh and Singh, 2016). Since water cannot dissolve lipids, cholesterol travels through the body bounded to former named proteins. LDL works by transporting fatty acids from the liver to blood and tissues (Arnold and Kwiterovich Jr, 2003). HDL works by transporting the excess cholesterol to the liver which can be dismantled or used (Arnold and Kwiterovich Jr, 2003). TAGs are simply blood fats that used for transporting excess carbohydrates (Singh and Singh, 2016). High levels of TAGs, LDL combined with low levels of HDL possess high risk for cardiovascular diseases and inflammation [(Figure 2.3) (WHO, 2010; FAO, 2010; Singh and Singh, 2016)].



Figure 2.3. Illustration of cholesterol mechanism.

There are three types of nutritional fats; unsaturated, saturated and trans-fats (WHO and FAO, 2010). A healthy diet includes unsaturated fats along with limited amounts of saturated fats while excluding trans-fats (WHO and FAO, 2010). Trans-fats are excluded because they reduce good HDL levels resulting in increased risk of coronary heart disease, sudden cardiac death, metabolic syndrome components and diabetes (WHO and FAO, 2010). Consumption of trans-fats are limited to 1% of daily energy requirement (2 g for 2000 kcal diet) by WHO and FAO (WHO and FAO, 2010). On the other hand, saturated fats are harmful only if consumed in high amounts (WHO and FAO, 2010). Overconsumption of saturated fatty acids increase LDL, TC/HDL (Total cholesterol/High density lipoprotein) ratio (depending on type) and risk of diabetes (WHO and FAO, 2010). Therefore, saturated fatty acid (SFA) consumption is limited to 10% of daily energy requirement (WHO and FAO, 2010).

People are being exposed to high saturated and trans-fats due to excessive amounts of dairy, pastry, red meat consumption and fast food intake. Eliminating fats completely from the diet is harmful because consumption fatty acids are mandatory for healthy functioning of the human body (WHO and FAO, 2010). Healthy fatty acids are used as components of the cell and hormone structures, solubilize the vitamins A, D, E, and K, used for energy storage, and make up 60% of the brain (WHO and FAO, 2010). Monounsaturated fatty acids (MUFAs) decrease LDL and TC/HDL ratio (WHO and FAO, 2010). Polyunsaturated fatty acids (PUFAs) decrease the risk of cardiovascular diseases when consumed in place of saturated fats and lower the risk of diabetes (WHO and FAO, 2010). Best sources of the healthy fats are nuts and seeds, fatty fish, microalgae, olive oil, sesame oil, and avocados (The National Heart Foundation of Australia, 2019).

Among other microalgae species, *Chlorella vulgaris* is a well-studied, safe-to-consume algae (Lee and Marino, 2010). Some species of microalgae are not proven to be edible as whole biomass, therefore fatty acids are extracted to be capsulated and taken as supplements (Lee and Marino, 2010). Disadvantages of this approach are high costs of extractions at large scale, residual toxins in the case of chemical extraction and lower bioavailability due to separation (Cuellar-Bermudez et al., 2014). In contrast, *Chlorella vulgaris* is edible without the need of any processing (Lee and Marino, 2010). Predominantly, unsaturated fats make the 77% of *Chlorella vulgaris* lipids followed by 23% with

saturated fats (Figure 2.4). Since the foods high in unsaturated fats and low in saturated fats are favored due to health promoting effects, *Chlorella vulgaris* is a healthy and vegetarian-vegan alternative with respect to its lipid content. The lipid content and lipid profile of *Chlorella vulgaris* vary depending on the strains and the cultivation methods thus, the strain and the cultivation methods should be selected accordingly (Table 2.3, Table 2.4, Table 2.5, Table 2.6, Table 2.7, Table 2.8 and Table 2.9).

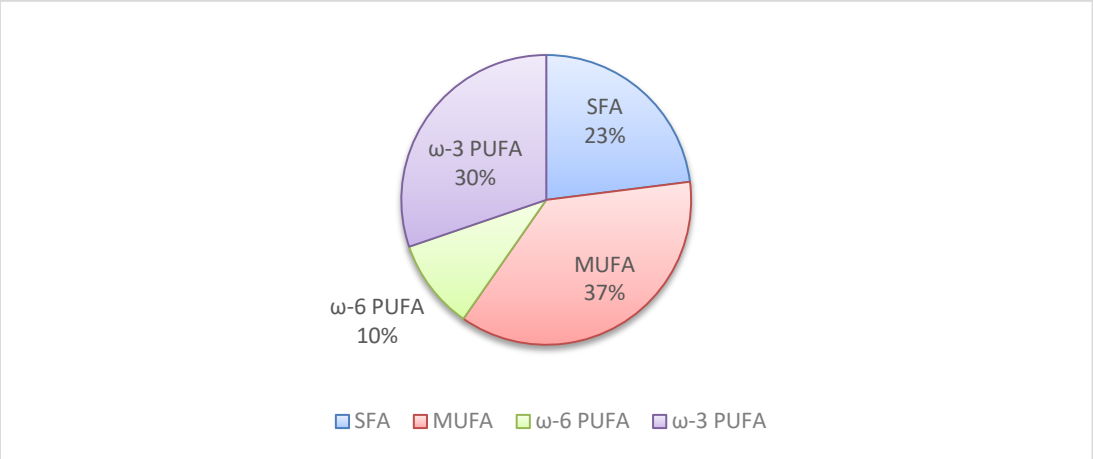

Figure 2.4. Types of lipids in *Chlorella vulgaris* (Tokusoglu et al., 2003).

Table 2.3. Lipid content of *Chlorella vulgaris* in various studies and daily recommended values by WHO (WHO and FAO, 2010).

Reference			**RDV WHO**		**100g *Chlorella vulgaris* %RDV**	
	Lipid	**Unit**	**Male***	**Female***	**Male***	**Female***
***Chlorella vulgaris* (Tokusoglu et al., 2003)**	13.32	%DW	44-78g		30.27%	
***Chlorella vulgaris* (Batista et al., 2013)**	5.1	%DW			11.59%	
***Chlorella vulgaris* (Becker, 2007)**	14-22	%DW			40.91%	

*Male=65 kg, Female=55 kg, 2000 kcal/day diet.

Table 2.4. SFA content of *Chlorella vulgaris* in various studies and daily recommended values by WHO (WHO and FAO, 2010).

Reference			**RDV WHO**		**100g *Chlorella vulgaris* %RDV**	
	SFA	**Unit**	**Male***	**Female***	**Male***	**Female***
***Chlorella vulgaris* (Tokusoglu et al., 2003)**	22.22	% of Total lipids	< 22g		13.45%	
***Chlorella vulgaris* (Batista et al., 2013)**	1254	mg/100g			5.70%	

*Male=65 kg, Female=55 kg, 2000 kcal/day diet.

Table 2.5. MUFA content of *Chlorella vulgaris* in various studies and daily recommended values by WHO (WHO and FAO, 2010).

Reference			**RDV WHO**		**100g *Chlorella vulgaris* %RDV**	
	MUFA	**Unit**	**Male***	**Female***	**Male***	**Female***
***Chlorella vulgaris* (Tokusoglu et al., 2003)**	35.44	% of Total lipid	33.44g		14.30%	
***Chlorella vulgaris* (Batista et al., 2013)**	836	mg/100g			2.53%	

*Male=65 kg, Female=55 kg, 2000 kcal/day diet.

Table 2.6. ω-6 PUFA content of *Chlorella vulgaris* in various studies and daily recommended values by WHO (WHO and FAO, 2010).

Reference			**RDV WHO**		**100g *Chlorella vulgaris* %RDV**	
	ω-6 PUFA	**Unit**	**Male***	**Female***	**Male***	**Female***
***Chlorella vulgaris* (Tokusoglu et al., 2003)**	9.73	% of Total lipid	6-20g		21.60%	
***Chlorella vulgaris* (Batista et al., 2013)**	428	mg/100g			16.18%	

*Male=65 kg, Female=55 kg, 2000 kcal/day diet.

Table 2.7. ω-3 PUFA content of *Chlorella vulgaris* in various studies and daily recommended values by WHO (WHO and FAO, 2010).

Reference			**RDV WHO**		**100g *Chlorella vulgaris* %RDV**	
	ω-3 PUFA	**Unit**	**Male***	**Female***	**Male***	**Female***
***Chlorella vulgaris* (Tokusoglu et al., 2003)**	29.21	% of Total lipid	1-4g		97.27%	
***Chlorella vulgaris* (Batista et al., 2013)**	971	mg/100g			10.70%	

*Male=65 kg, Female=55 kg, 2000 kcal/day diet.

Table 2.8. Fatty acid profile of *Chlorella vulgaris* (Tokusoglu et al., 2003).

Fatty acid profile of *Chlorella vulgaris* (% of total lipid)					
Fatty Acid	**Amount**	**Unit**	**Fatty Acid**	**Amount**	**Unit**
C14:0	0.38	% of total lipid	**C18:2 ω-6**	9.73	% of total lipid
C16:0	15.41	% of total lipid	**C18:3 ω-3**	1.93	% of total lipid
C16:1 ω-7	1.17	% of total lipid	**C20:0**	0.19	% of total lipid
C18:0	6.24	% of total lipid	**C20:5 ω-3**	3.23	% of total lipid
C18:1 ω-9	33.14	% of total lipid	**C22:5 ω-3**	3.11	% of total lipid
C18:1 ω-7	1.13	% of total lipid	**C22:6 ω-3**	20.94	% of total lipid

Table 2.9. Fatty acid profile of *Chlorella vulgaris* (Batista et al., 2013).

Fatty acid profile of *Chlorella vulgaris*					
Fatty Acid	**Amount**	**Unit**	**Fatty Acid**	**Amount**	**Unit**
C14:0	124	mg/100g	**C20:5 ω-3 (EPA)**	19	mg/100g
C16:0	1016	mg/100g	**C22:6 ω-3 (DHA)**	16	mg/100g
C18:0	25	mg/100g	**Other PUFA ω-3**	111	mg/100g
Other SFA	88	mg/100g	**Σ PUFA ω-3**	971	mg/100g
Σ SFA	1254	mg/100g	**C18:2 ω-6**	292	mg/100g
C16:1	78	mg/100g	**C18:3 ω-6 (GLA)**	112	mg/100g
C18:1	449	mg/100g	**C20:4 ω-6**	-	mg/100g
Other MUFA	110	mg/100g	**C22:5 ω-6**	4	mg/100g
Σ MUFA	836	mg/100g	**Other PUFA ω-6**	20	mg/100g
C16:4 ω-3	165	mg/100g	**Σ PUFA ω-6**	428	mg/100g
C18:3 ω-3 (ALA)	661	mg/100g	**EPA+DHA**	35	mg/100g
C18:4 ω-3	-	mg/100g			

In addition to the previously explained benefits, sufficient consumption of the unsaturated fatty acids is particularly important since the human body cannot synthesize certain types of them de novo (Oregon State University, 2019). These are alpha-linolenic acid (ALA) and linoleic acid (LA) which are referred as the essential fatty acids (Oregon State University, 2019). Despite common knowledge, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are not essential fatty acids, as the human body can synthesize them from ALA and LA by alternative desaturation and chain elongation [(Figure 2.5.) (Oregon State University, 2019)]. Alpha-linolenic acid, an ω-3 fatty acid, is the precursor for biosynthesis of EPA and DHA (Oregon State University, 2019) and linoleic acid, an ω-6 fatty acid, is the precursor for biosynthesis of arachidonic acid (AA) (Oregon State University, 2019). Yet, it is advised to consume the EPA and DHA due to need of long processes for conversion their production by body is limited (Oregon State University, 2019).

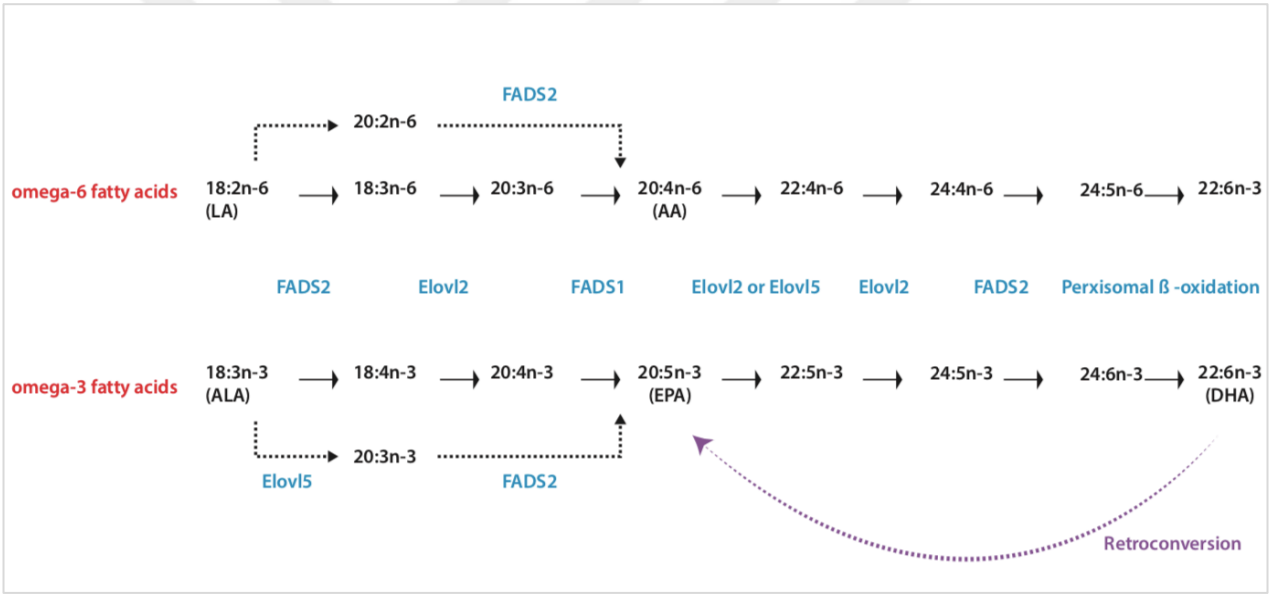

Figure 2.5. Conversion of EPA and DHA from ALA and LA (Oregon State University, 2019).

DHA is a crucial fatty acid for brain and retina (Imhoff-Kunsch et al., 2011). Benefits of EPA are less understood relative to DHA, however, several studies show that consuming combination of EPA and DHA has a synergistic effect albeit consuming solely deactivates some of the benefits (Cottin et al., 2011). Intake of EPA and DHA combination is shown to improve insulin sensitivity, protect blood pressure and lower the TAG levels (Jong-Yuh and Mei-Fen 2005; Kris-Etherton et al., 2009; Cottin et al., 2011). Richest sources of EPA and DHA are oily fish (The National Heart Foundation of Australia, 2019). If consumption of fish is limited, ω-3 fish oil supplements are favored. It is advised to consume fish 2-3 portions a week or take fish oil supplements to meet the daily recommended EPA+DHA combined value of 250 mg to 2 g by WHO (WHO and FAO, 2010).

The main drawback of eating fish and taking fish oil supplements is presence of high levels of heavy metals; such as arsenic, cadmium, mercury and lead (Canli and Atli, 2003; Jaishankar et al., 2014). Heavy metals tend to accumulate in human body and cause DNA damage, induce oxidative stress, and damage to heart and brain (Jaishankar et al., 2014). Besides, farmed fish contain residual antibiotics that are used in aquaculture (Romero et al., 2012). Consequently, fish oil supplements are required to be purified of toxic compounds prior to consumption.

In pursuit of finding an alternative, it was realized that fish also acquire EPA and DHA from external sources (Wells et al., 2017). One of such primary source is microalgae consumed by the fish (National Institutes of Health, 2018). Microalgae are abundant sources of healthy fats that some species are rich in EPA and DHA naturally, while others can synthesize increased amounts under various stress conditions (Adarme-Vega et al, 2012). Refraining from fish consumption offers safe intake of EPA and DHA without interference of heavy metals and advantageous for vegetarians and vegans, as well. The first drawback of consuming non-animal sources is the bioavailability and microalgae derived lipids have bioavailability ratios between 50-100% which is efficient to consume (Wells et al. 2017).

EPA and DHA content of *Chlorella vulgaris* varies between strains and growth conditions (Table 2.10). Depending on the EPA and DHA content, the base food can be enriched with complementary ω-3 rich seeds and nuts (flaxseeds, walnuts, etc.) besides *Chlorella vulgaris* to meet the daily requirements advised by WHO (WHO and FAO, 2010).

Table 2.10. EPA+DHA content of *Chlorella vulgaris* in various studies and daily recommended values by WHO (WHO and FAO, 2010).

Reference			**RDV WHO**		**100g *Chlorella vulgaris* %RDV**	
	EPA+DHA	**Unit**	**Male***	**Female***	**Male***	**Female***
***Chlorella vulgaris* (Tokusoglu et al., 2003)**	24.13	% of T. lipid	0.250-2 g		160.71%	
***Chlorella vulgaris* (Batista et al., 2013)**	35	mg/100g			1.75%	

*Male=65 kg, Female=55 kg, 2000 kcal/day diet.

2.2.3. Protein

Every piece of the human body comprises some kind of protein (Harvard T.H. Chan School of Public Health, 2019). Therefore, protein is a requisite constituent of a healthy diet. Structure of the proteins are made up by linking amino acids (Harvard T.H. Chan School of Public Health, 2019). Human body is able to synthesize amino acids except the essential eight (Table 2.11), that are required to be taken externally (Schaafsma, 2000). Consequently, wide range of amino acid profiles which involves essential amino acids are important as well as high protein content when choosing foods (Schaafsma, 2000). The essential amino acids tend to be present in the animal sources as full spectrum, however a balanced diet plan offers enough intake of protein from the plant sources, likewise (National Research Council, 1989).

Animal based protein is advantageous due to high rates of protein availability (Gavelle et al., 2017) and presence of full spectrum of the amino acids (Hoffman and Falvo, 2004). Most common animal-based protein sources are red meat, dairy, fish, seafood and poultry. Drawback of consuming red meat and dairy is their high saturated fat content (Wood et al., 2008). In comparison, fish and poultry have lower amounts of saturated fat (American Heart Association, 2019). Plus, fish are rich sources of unsaturated fatty acids and significant micronutrients such as selenium and phosphorus (WHO, 2001).

Table 2.11. Essential amino acids (Schaafsma, 2000).

Essential Amino Acids
Phenylalanine
Threonine
Tryptophan
Methionine
Isoleucine
Leucine
Lysine
Histidine

Plant based diet is advantageous because the plants are packed with vitamins, minerals, fiber and contain lower amounts of saturated fat (Harvard Medical School, 2019). Bioavailability of the plant protein is slightly lower than the animal protein and it is hard to obtain the essential amino acids from

a single plant source (Hoffman and Falvo, 2004). Yet, it is possible to obtain required amounts of protein and the essential amino acids by combining multiple plant sources like vegans and vegetarians (de Gavelle et al., 2017). Plant based diets offer more healthy gut microbiota despite animal based diet which increases the number of inflammatory bowel disease triggering microorganisms (David et al., 2014). In addition, prolonged fiber intake offered by plant based diet preserves *Prevotella* genus that is source of inter-individual gut microbiota variation which decreases in the case of animal protein consumption (David et al., 2014).

In light of these, *Chlorella vulgaris* possess great potential for covering the protein and amino acid deficit of a plant-based diet. Protein amount of *Chlorella vulgaris* varies between 38-58% of its dry weight which is enough to compete with animal sources (Table 2.12). Distinctively, amino acid profile of *Chlorella vulgaris* is comparable with that of a whole egg which contains all essential amino acids required for the humans (Table 2.13 and Table 2.14).

Table 2.12. Protein content of *Chlorella vulgaris* and daily recommended values by WHO (WHO, 2007).

Reference			**RDV WHO**		**100g *Chlorella vulgaris* %RDV**	
	Protein	**Unit**	**Male***	**Female***	**Male***	**Female***
***Chlorella vulgaris* (Tokusoglu et al., 2003)**	47.82	%DW	43g	36g	111.21%	132.83%
***Chlorella vulgaris* (Batista et al., 2013)**	38	%DW			88.37%	105.56%
***Chlorella vulgaris* (Becker, 2007)**	51-58	%DW			126.74%	151.39%

*Male=65 kg, Female=55 kg, 2000 kcal/day diet.

Table 2.13. Amino acid profile of *Chlorella vulgaris* and comparison with a whole egg.

(Becker, 2007)			
Amino acid	***Chlorella vulgaris***	**Egg**	**Unit**
Isoleucine	3.8	6.6	g/100g protein
Leucine	8.8	8.8	g/100g protein
Valine	5.5	7.2	g/100g protein
Lysine	8.4	5.3	g/100g protein
Phenylalanine	5.0	5.8	g/100g protein
Tyrosine	3.4	4.2	g/100g protein
Methionine	2.2	3.2	g/100g protein
Cysteine	1.4	2.3	g/100g protein
Tryptophan	2.1	1.7	g/100g protein
Threonine	4.8	5.0	g/100g protein
Alanine	7.9	-	g/100g protein
Arginine	6.4	6.2	g/100g protein
Asparagine	9.0	11.0	g/100g protein
Glutamine	11.6	12.6	g/100g protein
Glycine	5.8	4.2	g/100g protein
Histidine	2.0	2.4	g/100g protein
Proline	4.8	4.2	g/100g protein
Serine	4.1	6.9	g/100g protein

Table 2.14. Recommended daily values of essential amino acids by WHO and amino acid profile of *Chlorella vulgaris* (WHO, 2007).

(Becker, 2007)			**RDV WHO**		**100g *Chlorella vulgaris* %RDV**	
Amino acid	***Chlorella vulgaris***	**Unit**	**Male***	**Female***	**Male***	**Female***
Histidine	2	g/100g protein	650 mg	550 mg	308%	364%
Isoleucine	3.8	g/100g protein	1300 mg	1100 mg	292%	345%
Leucine	8.8	g/100g protein	2535 mg	2145 mg	347%	410%
Lysine	8.4	g/100g protein	1950 mg	1650 mg	431%	509%
Methionine	2.2	g/100g protein	650 mg	550 mg	338%	400%
Phenylalanine	5	g/100g protein	1625 mg	1375 mg	308%	364%
Threonine	4.8	g/100g protein	975 mg	825 mg	492%	582%
Tryptophan	2.1	g/100g protein	260 mg	220 mg	808%	955%
Valine	5.5	g/100g protein	1690 mg	1430 mg	325%	385%

*Male=65 kg, Female=55 kg, 2000 kcal/day diet.

Another compelling reason to consume *Chlorella vulgaris* as the protein source is, its low saturated fat content (Table 2.4) and high protein to carbohydrate ratio (Table 2.15). Excluding the high carbohydrate cultivated biomass, *Chlorella vulgaris* has a protein to carbohydrate ratio higher than 2.7 which is an indication of quality of safe foods for maintaining healthy blood lipid levels [(Bahadoran et al., 2013) (Table 2.15)]. Ultimately, *Chlorella vulgaris* is a significant and ideal vegetarian-vegan alternative to the animal protein with its adequate amino acid profile.

Table 2.15. Protein to carbohydrate ratio of *Chlorella vulgaris.*

Reference	Protein to Carbohydrate Ratio
Chlorella vulgaris **(Tokusoglu et al., 2003)**	5.9
Chlorella vulgaris **(Batista et al., 2013)**	1.9
Chlorella vulgaris **(Becker, 2007)**	3.8

2.2.4. Vitamins and Minerals

Micronutrients such as vitamins and minerals are required for healthy development and functioning of the human body. Through a well-balanced diet, vitamins and minerals can be acquired in adequate amounts. Compared to the macronutrients, vitamins and minerals are required in far less amounts, and as such they are called micronutrients. Yet, micronutrients play vital roles and need to be consumed at right amounts (Table 2.16, Table 2.17), neither insufficient nor in excess (WHO, 2001). Animal and plant-based foods cover different types of micronutrients, therefore both required be consumed or supplemented (Watanabe, 2014; Yabuta et al. 2014). While animal based diet is rich in vitamin A, D, B12, zinc and iron, plant based diet is rich in vitamin C, E, folate and magnesium (Watanabe, 2014; Yabuta et al., 2014).

Table 2.16. Significance, effects of deficiency and overdoses of the vitamins (WHO, 2001).

Vitamin	Significance	Deficiency	Overdose
B1 (Thiamin)	Co-enzyme functions in metabolism of carbohydrates and branched-chain amino acids	Beri-beri, polyneuritis, and Wernicke-Korsakoff syndrome	No toxicity
B2 (Riboflavin)	Co-enzyme functions in numerous oxidation and reduction reactions	Growth, cheilosis, angular stomatitis, and dermatitis	No toxicity
B3 (Niacin)	Co-substrate/co-enzyme for hydrogen transfer with numerous dehydrogenases	Pellagra with diarrhea, dermatitis, and dementia	Hepatotoxicity, dermatologic problems Max: 35mg/day
B5 (Pantothenic acid)	Constituent of co-enzyme A and phosphopantetheine involved in fatty acid metabolism	Fatigue, sleep disturbances, impaired coordination, and nausea	No toxicity
B6 (Pyridoxine)	Co-enzyme functions in metabolism of amino acids, glycogen, and sphingoid bases	Naso-lateral seborrhea, glossitis, and peripheral neuropathy (epileptiform convulsions in infants)	Neurotoxicity Max: 100mg/day
B7 (Biotin)	Co-enzyme functions in bicarbonate-dependent carboxylations	Fatigue, depression, nausea, dermatitis, and muscular pains	No toxicity
B9 (Folic acid, folate)	Blood cell formation, co-enzyme in one-carbon transfer mechanism	Cardiovascular disease, colorectal cancer, foetal neural tube defects during pregnancy,	Masking diagnosis of pernicious anemia Max: 1000µg/day
B12 (Cobalamin)	Co-enzyme in methylation such as myelin basic protein, co-enzyme in metabolism of propionate, amino acids	Pernicious anemia, atrophic gastritis, subacute combined degeneration of the spinal cord and peripheral nerves, ataxia, paralysis, death	Absorption is limited to 1,2-2µg per meal No toxicity but high intakes should be avoided
C (Ascorbic acid)	Antioxidant, electron donor for enzymes, participate in collagen hydroxylation, iron absorption promotion,	Scurvy, anemia, follicular hyperkeratosis, petechial hemorrhages, swollen-bleeding gums, joint pain	Diarrhea, gastrointestinal disturbances, hyperoxaluria, red cell hemolysis Max: 1g/day
A (Retinol)	Functioning of the visual system, growth and development, maintenance of epithelial cellular integrity, immune system function, reproduction	Irreversible blindness, poor reproductive health, increased risk of anemia, slowed growth and development	Liver damage, bone abnormalities, joint pain, alopecia, headaches, vomiting, skin desquamation Recommended safe intake: 500µg for females, 600µg for males daily
D	Maintaining blood levels of Ca and P, mineralization of bone, muscle contraction, nerve conduction, cellular function, modulating transcription of cell cycle proteins, bone resorption, immuno-modulating, secosteroid	Declining of bone mass	Hypercalciuria, hypercalcemia Recommended safe intake: 5µg/day
E (Tocopherols)	Antioxidant, protection of PUFAs, cell membranes, prevent lipid peroxidation,	Cell membrane damage, cardiac myopathies, skeletal myopathies, neuropathies, liver necrosis, muscle and neurological problems	Low toxicity Pro-oxidant damage at >1000mg/day feeding supplements
K	Ca binding, coagulation	Bleeding	Natural vitamin K has no toxicity Neonatal hemolysis, liver damage

*Max values represent amounts for adults.

Table 2.17. Significance, effects of deficiency and overdoses of the minerals (WHO, 2001).

Mineral	Significance	Deficiency	Overdose
Ca (Calcium)	Skeleton rigidity, neuromuscular and cellular functions, intracellular signaling	Controversial	Milk-alkali syndrome Max: 3g/day
I (Iodine)	Synthesis of thyroid hormones	Goitre, hypothyroidism, impaired mental function	Iodine induced hyperthyroidism Recommended safe intake: 2μg/kg/day
Fe (Iron)	Oxygen carrier, transport medium for electrons within cells, integrated part of enzyme systems	Iron deficiency anemia, impaired oxidative metabolism, impaired defense functions of body	Regulated by the body
Mg (Magnesium)	Co-factor in energy metabolism, RNA and DNA synthesis, maintenance of the electrical potential of nervous tissues and cell membranes	Neurologic defect, neuromuscular defect, anorexia, nausea, muscular weakness, lethargy, weight loss, hyperirritability, hyperexcitability, muscular spasm	Nausea, hypotension, diarrhea Max: 350mg
Se (Selenium)	Antioxidant, maintenance of defenses against infection, modulation of growth and development	Muscular weakness, myalgia, development of congestive heart failure, Keshan disease, Kashin-Beck disease, susceptibility to infections	Hair loss, structural changes in keratinization of hair and nails, development of icteroid skin, gastrointestinal disturbances, nail dystrophy Max: 400μg/day
Zn (Zinc)	Component of enzymes participating in synthesis and degradation of carbohydrates, lipids, proteins, nucleic acids and micronutrients; cell, organ integrity; essential role in polynucleotide transcription and genetic expression; central role in immune system	Growth retardation, delayed sexual and bone maturation, skin lesions, diarrhea, alopecia, impaired appetite, increased susceptibility to infections, behavioral changes, impaired taste and wound healing	Nausea, vomiting, diarrhea, fever, lethargy Max: 45mg/day for males

*Max values represent amounts for adults.

A plant based diet involving vegetables, fruits, whole-grains, legumes, nuts and seeds has satisfactory amounts of micronutrients to meet the daily requirements except the vitamin B12 (Watanabe et al., 2014). Cobalamin (vitamin B12) is present in plants sources in trace amounts, therefore, only animal sources are able to provide the vitamin B12 to humans (Watanabe et al., 2014). Recommended intake of vitamin B12 is 2,4 μg for 65 kg male, 55 kg female and 2000 kcal/day diet (WHO, 2001). As shown in Table 2.18, animal based foods are able to compensate daily

recommended vitamin B12 need even in small amounts. Since vegans do not consume any animal products, they are obligated to take vitamin B12 externally. Although vegetarians able to acquire the vitamin B12 from dairy and eggs, the vitamin B12 in eggs have low absorption rate, i.e. <9%, (Watanabe, 2007). Milk is a significant source of the vitamin B12 but thermal processes decrease the B12 content by 30% at 2-5 min of boiling and 59% at 30 min of boiling (Watanabe, 2007). In the case of insufficient dairy intake or thermal processing, experiencing deficiency is inevitable.

Table 2.18. Vitamin B12 contents of the various foods (USDA, 2015).

Food	**Vitamin B12 amount (µg) per 100 g**
Mollusks, cooked	98.9
Beef liver, cooked	96.0
Salmon, smoked	18.1
Chicken giblets, cooked	13.3
Mozarella cheese	2.3
Grass-fed beef, raw	1.97
Turkey, cooked	1.8
Egg, whole, cooked	1.1
Sheep milk	0.7
Cow milk (reduced fat)	0.5
Salted butter	0.2

As mentioned earlier, *Chlorella vulgaris* contains bioavailable form of vitamin B12 in contrast to plants and cyanobacteria. Vitamin B12 content in *Chlorella vulgaris* varies depending on cultivation process which allows microalgae to acquire the vitamin B12 (Hiromi Kittaka-Katsura, 2002; Maruyama et al., 1997). Depending on the content, daily adequate intake of vitamin B12 can be supplied by *Chlorella vulgaris* [(Watanabe et al. 2014) (Table 2.19 and Table 2.20)]. Equally important, *Chlorella* consumption doubled the amount of B12 in the blood serum of vegans (Watanabe, 2007) which proves that *Chlorella vulgaris* is a reliable source vitamin B12.

Table 2.19. Vitamin content of *Chlorella vulgaris* and daily recommended values by WHO (WHO, 2001).

Micronutrients	**(Maruyama et al., 1997)**		**RDV WHO**		**100g *Chlorella vulgaris* %RDV**	
Vitamins	***Chlorella vulgaris***	**Unit**	**Male***	**Female***	**Male***	**Female***
A	-	-	600µg	600µg	-	-
B1	24	µg/g DW	1.2mg	1.1mg	200%	218%
B2	60	µg/g DW	1.3mg	1.1mg	462%	545%
B3	-	-	16mg	14mg	-	-
B5	-	-	5mg	5mg	-	-
B6	10	µg/g DW	1.3mg	1.3mg	77%	77%
B7	-	-	30µg	30µg	-	-
B9	-	-	400µg	400µg	-	-
B12	0.001**	µg/g DW	2.4µg	2.4µg	4%	4%
C	1000	µg/g DW	45mg	45mg	222%	222%
D	-	-	5µg	5µg	-	-
E	200	µg/g DW	10mg	7.5mg	200%	267%
K	-	-	65µg	55µg	-	-
β-carotene	-	-	3592.8µg	2994µg	-	-

*Male=65 kg, Female=55 kg, 2000 kcal/day diet.
**2-6 µg/g DW if B12 added to medium.

Table 2.20. Vitamin content of *Chlorella vulgaris* and daily recommended values by WHO (WHO, 2001).

Micronutrients	**(Panahi et al., 2012)**		**RDV WHO**		**100g *Chlorella vulgaris* %RDV**	
Vitamins	***Chlorella vulgaris***	**Unit**	**Male***	**Female***	**Male***	**Female***
A	-	-	600µg	600µg	-	-
B1	1.5	mg/100g	1.2mg	1.1mg	125%	136%
B2	4.8	mg/100g	1.3mg	1.1mg	369%	436%
B3	23.8	mg/100g	16mg	14mg	149%	170%
B5	1.3	mg/100g	5mg	5mg	26%	26%
B6	1.7	mg/100g	1.3mg	1.3mg	131%	131%
B7	191.6	µg/100g	30µg	30µg	639%	639%
B9	26.9	µg/100g	400µg	400µg	7%	7%
B12	125.9	µg/100g	2.4µg	2.4µg	5246%	5246%
C	15.6	mg/100g	45mg	45mg	35%	35%
D	-	-	5µg	5µg	-	-
E	-	-	10mg	7.5mg	-	-
K	-	-	65µg	55µg	-	-
β-carotene	180.8	mg/100g	3592.8µg	2994µg	5032%	6039%

*Male=65 kg, Female=55 kg, 2000 kcal/day diet.

Second issue with plant-based diet is low absorption of plant-sourced iron (WHO, 2001). Non-heme is the form of iron present in plant foods (WHO, 2001). Phytates (grains, seeds, nuts, vegetables, roots, fruits), iron-binding polyphenols (tea, coffee, cocoa), galloyl groups (green leafy vegetables, herbs, spices) in plant foods and soy proteins inhibit iron absorption (WHO, 2001). Therefore, non-heme iron has an absorption rate between 2-10% whereas heme iron in animal foods has 20-30% absorption rate (WHO, 2001). Enhancing iron absorption is possible with intake of vitamin C, citric acid, cysteine containing peptides, and fermented foods (WHO, 2001). Calcium is another inhibitory factor for iron absorption which eliminates the dairy as iron source (WHO, 2001). Hence, vegetarians, vegans and women need to pay extra attention to their iron intake. Women lose 0.56 mg iron per day during 28 days of menstruation cycle (WHO, 2011). Insufficient iron intake causes iron deficiency anemia, impaired oxidative metabolism and impaired defense functions of body (WHO, 2001). Meanwhile, iron is the most common deficient nutrient in the world and iron deficiency anemia affects 600-700 million people worldwide (WHO, 2001).

Chlorella vulgaris is a good non-animal source of iron with the content of 166.3 – 259.1 mg per 100 g (Table 2.21, Table 2.22). In comparison, lentils have 6.51 mg iron per 100 g which is regarded as one of the best vegetarian/vegan sources of iron (USDA, 2019). However, further research is needed to determine absorption efficiency of iron in *Chlorella vulgaris* regarding presence of phytates, iron-binding polyphenols, galloyl groups and effect of calcium.

Table 2.21. Mineral content of *Chlorella vulgaris* and daily recommended values by WHO (WHO, 2001).

Micronutrients	**(Tokusoglu et al., 2003)**		**RDV WHO**		**100g *Chlorella vulgaris* %RDV**	
Minerals	***Chlorella vulgaris***	**Unit**	**Male***	**Female***	**Male***	**Female***
Ca	593.7	mg/100g DW	1000mg	1000mg	59%	59%
Mg	344.3	mg/100g DW	260mg	220mg	132%	157%
Se	0.07	mg/100g DW	34µg	26µg	206%	269%
Zn	1.19	mg/100g DW	7mg	4.9mg	17%	24%
Fe	259.1	mg/100g DW	14mg	29mg	1851%	893%
I	-	mg/100g DW	130µg	110µg	-	-
Na	1346.4	mg/100g DW	< 2g /day		67%	67%

*Male=65 kg, Female=55 kg, 2000 kcal/day diet.

Table 2.22. Mineral content of *Chlorella vulgaris* and daily recommended values by WHO (WHO, 2001).

Micronutrients	**(Batista et al., 2013)**		**RDV WHO**		**100g *Chlorella vulgaris* %RDV**	
Minerals	***Chlorella vulgaris***	**Unit**	**Male***	**Female***	**Male***	**Female***
Ca	4.73	%DW	1000mg	1000mg	473%	473%
Mg	1.46	mg/kg	260mg	220mg	0%	0%
Se	-	-	34µg	26µg	-	-
Zn	17.5	mg/kg	7mg	4.9mg	25%	36%
Fe	166.3	mg/kg	14mg	29mg	119%	57%
I	-	-	130µg	110µg	-	-
Na	0.98	mg/kg	< 2g /day		0%	0%

*Male=65 kg, Female=55 kg, 2000 kcal/day diet.

Nowadays, there is a widespread usage of laboratory-made supplements to compensate daily required vitamin and mineral intake. However, recent studies have shown that, lab-made synthetic vitamins are beneficial only in the case of deficiency and do not prevent diseases (Mitka, 2014). Supplementing a diet which comprise solely of animal products/plant foods with vitamins is shown to be not effective against developing vitamin deficiencies (Mitka, 2014). In light of this, preferring natural ingredients for functional foods is a far-sighted decision. Therefore, *Chlorella vulgaris* is an outstanding supplement candidate for people preferring either (or both) animal and plant-based diets can benefit considering its significant vitamin and mineral content including the bioavailable vitamin B12.

2.3. *Chlorella vulgaris* as a Functional Food Ingredient; Serving Size, Effect of Cooking on Nutritional Value and Digestibility

2.3.1. Serving Size of *Chlorella vulgaris*

As explained previously, the inconvenience of eating pure microalgae is its sharp seaweed taste. Although some people comfortable with consuming it, majority of people experience difficulty. Therefore, a functional food with *Chlorella vulgaris* was aimed to be designed in this study. Beyond the taste, there are limitations about micronutrients that need to be considered. While some micronutrients do not possess toxicity at excess amounts, majority of vitamins and minerals are

dangerous at high concentrations (Table 2.16, Table 2.17). In light of this, serving size of the chosen microalgae must be calculated according to former limitations that advised by WHO in order to prevent possible health effects. Since vitamin B1, B2, B5, B7 and K have no toxicity (WHO, 2001), measures should include remaining vitamins and minerals.

Similar to other cell components, vitamin and mineral concentrations in *Chlorella vulgaris* differ depending on the cultivation methods and strains (Maruyama et al., 1997; Panahi et al., 2012). Therefore, amount of *Chlorella vulgaris* that can be allowed to add in intended functional food changes accordingly (Table 2.23, Table 2.24, Table 2.25, Table. 2.26). Considering the maximum limits, allowed amounts of *Chlorella vulgaris* for consumption in the following articles are:

- Biomass of *Chlorella vulgaris* 1 (Table 2.23): Limiting factor is vitamin C and allowed weight is 1 kg.
- Biomass of *Chlorella vulgaris* 2 (Table 2.24): Limiting factor is β-carotene and allowed weight is 1.99 g for males, 1.66 g for females.
- Biomass of *Chlorella vulgaris* 3 (Table 2.25): Limiting factor is Mg and allowed weight is 101.66 g.
- Biomass of *Chlorella vulgaris* 4 (Table. 2.26): Limiting factor is Ca and allowed weight is 63.4 g.

Table 2.23. Allowed weight of *Chlorella vulgaris* considering maximum limits of vitamins advised by WHO (WHO, 2001).

Micronutrients	**(Maruyama et al., 1997)**		**Max value WHO**		**Allowed amount of *Chlorella vulgaris***	
Vitamins	***Chlorella vulgaris***	**Unit**	**Male***	**Female***	**Male***	**Female***
A	-	-	600µg/day	500µg/day	-	
B1	24	µg/g DW	No toxicity		No restriction	
B2	60	µg/g DW	No toxicity		No restriction	
B3	-	-	35 mg/day		No restriction	
B5	-	-	No toxicity		No restriction	
B6	10	µg/g DW	100mg/day		1 kg	
B7	-	-	No toxicity		No restriction	
B9	-	-	1000µg/day		-	
B12	0.001*	µg/g DW	1.2-2µg/meal		2 kg	
C	1000	µg/g DW	1g/day		1 kg	
D	-	-	5µg/day		-	
E	200	µg/g DW	1000mg/day		5 kg	
K	-	-	No toxicity		-	
β-carotene	-	-	3592.8µg	2994µg	-	

*Male=65 kg, Female=55 kg, 2000 kcal/day diet.

Table 2.24. Allowed weight of *Chlorella vulgaris* considering maximum limits of vitamins advised by WHO (WHO, 2001).

Micronutrients	**(Panahi et al., 2012)**		**Max value WHO**		**Allowed amount of *Chlorella vulgaris***	
Vitamins	***Chlorella vulgaris***	**Unit**	**Male***	**Female***	**Male***	**Female***
A	-	-	600µg/day	500µg/day	-	
B1	1.5	mg/100g	No toxicity		No restriction	
B2	4.8	mg/100g	No toxicity		No restriction	
B3	23.8	mg/100g	35 mg/day		147.06g	
B5	1.3	mg/100g	No toxicity		No restriction	
B6	1.7	mg/100g	100mg/day		5882.35g	
B7	191.6	µg/100g	No toxicity		No restriction	
B9	26.9	µg/100g	1000µg/day		3717.47g	
B12	125.9	µg/100g	1.2-2µg/meal		1.59g	
C	15.6	mg/100g	1g/day		6410.26g	
D	-	-	5µg/day		-	
E	-	-	1000mg/day		-	
K	-	-	No toxicity		-	
β-carotene	180.8	mg/100g	3592.8µg	2994µg	1.99g	1.66g

*Male=65 kg, Female=55 kg, 2000 kcal/day diet.

Table 2.25. Allowed weight of *Chlorella vulgaris* considering maximum limits of minerals advised by WHO (WHO, 2001).

Micronutrients	**(Tokusoglu et al., 2003)**		**Max value WHO**		**Allowed amount of *Chlorella vulgaris***	
Minerals	***Chlorella vulgaris***	**Unit**	**Male***	**Female***	**Male***	**Female***
Ca	593.7	mg/100g DW	3g/day		505.31g	
Mg	344.3	mg/100g DW	350mg		**101.66g**	
Se	0.07	mg/100g DW	400µg/day		571.43g	
Zn	1.19	mg/100g DW	45mg/day	-	3781.51g	-
Fe	259.1	mg/100g DW	Regulated by the body		-	
I	-	mg/100g DW	30µg/kg/day		-	-
Na	1346.4	mg/100g DW	< 2g /day		148.53g	

*Male=65 kg, Female=55 kg, 2000 kcal/day diet.

Table 2.26. Allowed weight of *Chlorella vulgaris* considering maximum limits of minerals advised by WHO (WHO, 2001).

Micronutrients	**(Batista et al., 2013)**		**Max value WHO**		**Allowed amount of *Chlorella vulgaris***	
Minerals	***Chlorella vulgaris***	**Unit**	**Male***	**Female***	**Male***	**Female***
Ca	4.73	%DW	3g/day		**63.4g**	
Mg	1.46	mg/kg	350mg		239726.0g	
Se	-	-	400µg/day		571.4g	
Zn	17.5	mg/kg	45mg/day	-	2571.4g	-
Fe	166.3	mg/kg	Regulated by the body		-	
I	-	-	30µg/kg/day		-	-
Na	0.98	mg/kg	< 2g /day		2040816.3g	

*Male=65 kg, Female=55 kg, 2000 kcal/day diet.

Carotenoids tend to present abundantly in microalgae (Lee and Marino, 2010) which is a major restriction for consumption. Concentration of β-carotene in *Chlorella vulgaris* differs from 7 µg to 12000 µg per 1 g dry weight which can be used from 250 mg to 427.7 g depending on the biomass (Safi et al., 2014). In short, nutritional value of the microalgae must be determined strictly prior to addition to foods and intake of nutrients in other meals/foods should be taken into consideration.

2.3.2. Effect of Cooking on Nutritional Value of *Chlorella vulgaris*

Cooking has profound effects on nutritional value of the foods. While accessibility of some vitamins increases with the nutrient releasing process of cooking (Lee et al., 2018), some vitamins tend to degrade due to thermal sensitivity (Rickman et al., 2007). Thermal effects on nutritional value varies between foods which becomes favorable in some cases. Water soluble vitamins, especially thiamine (vitamin B1) and vitamin C, known as being susceptible to thermal degradation (Lee et al., 2018). However, duration and method of cooking induces thermal degradation at different rates. For instance, decrease of vitamin C content varies between 7-55% and 11-66% for thiamine respectively, based on the cooking method (Rickman et al., 2007). Rest of B vitamins are reported to be more stable against cooking neglecting the decrease from leaching caused by cooking conditions (Rickman et al., 2007). On the other hand, a study of Lee et al. shown that cooking various vegetables had enhancing effect on fat soluble vitamins, including β-carotene and α-tocopherol (Lee et al., 2018). This effect is explained by the release of fat soluble vitamins by disintegration of cell walls during cooking (Lee et al., 2018). Similarly, cooking enhanced accessibility of β-carotene in broccoli, chard, mallow and spinach whereas decreased in carrot, crown daisy, perilla leaf and zucchini (Lee et al., 2018) which emphasizes the requirement of individual analysis for each vitamin.

In the pursuit of designing functional foods with *Chlorella vulgaris*, stability of vitamins against thermal effects is important for calculation of dietary intake of vitamins. Equally important, conservation of vitamins during cooking process offers far more options and recipes than raw consumption. And yet, none of the studies show the effect of cooking on vitamins of *Chlorella vulgaris.* Hence, effect of cooking on the values of B vitamins and α-tocopherol in *Chlorella vulgaris* were quantified in this study.

2.3.3. Digestibility of *Chlorella vulgaris*

Digestibility of microalgae is a controversial topic. Cellulosic cell wall of microalgae (roughly 10% of the weight) makes it harder to digest (Becker, 2007). Cellulose is categorized under insoluble fibers which do not form gels and become slightly fermented (Lattimer and Haub, 2010). Gastrointestinal track of humans lack the enzyme that digests cellulose (Dhingra et al., 2012), therefore, breaking the cell walls of microalgae is thought to increase their digestibility. On the other hand, processing releases nutrients and makes them more susceptible to oxidation and degradation (Rickman et al., 2007). A study on rats has shown that processing makes protein slightly more accessible and concluded that *Chlorella vulgaris* is an adequate protein source without cell wall

disruption (Komaki et al., 1998). In addition, two-thirds of the fiber in most fiber-rich foods is insoluble-fiber, as well (Lattimer and Haub, 2010). Thus, cell walls of *Chlorella vulgaris* were chosen to be not disrupted in this study.

2.3.4. Safety of *Chlorella vulgaris* Consumption and Side Effects

Chlorella vulgaris is one of the four types of microalgae that is permitted by EU to be consumed as food (Probst et al., 2015). Depending on the cultivation, *Chlorella vulgaris* harvest may contain toxic compounds, heavy metals and pesticides (Spiegel et al., 2013). Regarding toxicity, *Chlorella vulgaris* itself has no toxic effect when grown as axenic but presence of other species such as *Aphanizomenon flos-aqua* in harvest is shown to be toxic when consumed (Spiegel et al., 2013). Considering heavy metals, *Chlorella vulgaris* is able to absorb heavy metals from their surroundings, as a result if any heavy metals are present in cultivation medium, they might end up in human diet (Spiegel et al., 2013). In light of these, microalgae should be grown axenically and culture medium should be tested for the presence of heavy metals, pesticides, and any other potentially toxic chemical.

Apart from external pollution, *Chlorella vulgaris* also has allergen properties. A study by Tiberg et al. has shown that *Chlorella vulgaris* extracts have IgE binding which is an antibody used in allergenic response of the body (Tiberg et al., 1990). Also, another study by Tiberng et al. on children has shown that individuals who possessed allergic skin response to 5 mg/mL *Chlorella vulgaris* extract also possessed allergic skin response to tree, grass, weed pollen and animal dander (Tiberg et al., 1995). The allergenic response to *Chlorella vulgaris* is highly correlated with mold allergy (Tiberg et al., 1995). In addition, *Chlorella vulgaris* is found to be weak a allergen according to conjunctival provocation tests which concluded in study as *Chlorella vulgaris* weak allergenic response compared to tree, grass, weed pollen, animal dander, molds, mites and food or 5 mg/mL is an low amount for triggering strong allergic response (Tiberg et al., 1995). Another study by Bernstein and Safferman has shown that *Chlorella vulgaris* extracts induced histamine release in individuals who have skin sensitivity to house dust (Bernstein and Safferman, 1973; Fleurence and Levine, 2018). According to a report published in 2007, an 11-year old boy was diagnosed with acute tubulointerstitial and hospitalized for the evaluation of glucosuria, proteinuria and leukocyturia who had been using ten 200 mg *Chlorella* tablets per day (Yim et al., 2007). The renal function of the boy enhanced after corticosteroid therapy and disuse of *Chlorella* tablets (Yim et al., 2007). According to skin tests, it was found that the boy was also allergic to *Dermatophagoides pteronyssinus* (house dust mite), *Dermatophagoides farina* (another house dust mite), mugwort, cat hair and feather mixture (Yim et al., 2007). Supporting the previous study, allergic reaction to *Chlorella* coexisted with other allergies.

According to another report by Yavasoglu et al, a 49 year old healthy female was diagnosed with thrombocytopenia after taking 1,080 mg *Chlorella* tablets per day (Yavasoglu et al., 2018). Platelet count of the patient was recovered after two weeks after quitting the use of *Chlorella* tablets (Yavasoglu et al., 2018). Overall, people who consider using *Chlorella* should do a consumption test run with small doses to observe possible side effects with *Chlorella* which produced and approved as edible and people who have allergies should also be tested for *Chlorella vulgaris* allergy prior to consumption.

2.4. Antioxidants

Accompanying macro and micronutrients, there are other bioactive molecules that improve well-being in various pathways which are not typically listed under nutritional requirements. Antioxidants are the most well-known bioactive molecules that act by neutralizing reactive oxygen species (ROS) and as a result cellular material and DNA is protected (Birben et al., 2012). Microalgae possess great potential of containing antioxidants due to need of neutralizing ROS generated during photosynthesis (Goiris et al., 2015). Ascorbic acid (vitamin C) and tocopherol (vitamin E) are the most abundant compounds in microalgae with antioxidant abilities (Safi et al. 2014). Considered as the most potent antioxidant, astaxanthin is a relatively-newly discovered, powerful antioxidant that is present in certain fish, seafood and microalgae [(Figure 2.6) (Ambati et al., 2014)].

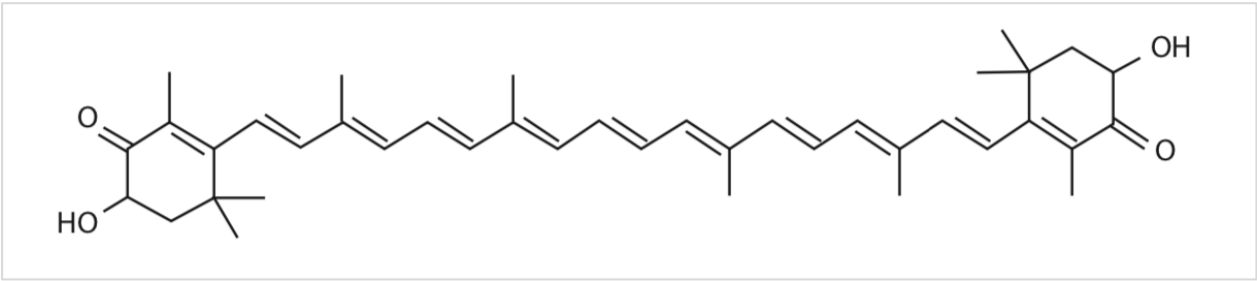

Figure 2.6. Chemical structure of astaxanthin (Inoue et al., 2017).

Astaxanthin belongs to carotenoid family along with well-known β-carotene and lutein (Ambati et al., 2014). Claimed benefits of astaxanthin are cardiovascular, skin, ocular, neurological, gastro-, heap- protective effects, reduced oxidative stress and anti-inflammation, anti-diabetic, and anti-cancer activity (Yuan et al., 2011). Astaxanthin's unique features are its high potency, ability to cross blood brain barrier unlike other known antioxidants and not possessing toxic characteristics at high doses (Grimmig et al., 2017). However, a key study has shown that supplementation of healthy mice with 20 mg/kg body weight astaxanthin for 30 days resulted in decreased levels of glutathione which

is a natural antioxidant of the body (Otton et al., 2010). On the other hand, astaxanthin is present in wild salmonids up to 26-38 mg/kg which is already consumed by humans (Ambati et al., 2014). Supposing moderate amounts of salmon consumption, two portions a week had no glutathione decreasing effect (García-Rodríguez et al., 2012). By these means, the former negative effect might be caused by consuming isolated form of astaxanthin rather than in whole food like in the case of ineffectiveness of vitamins. Meanwhile, along with the common trend of consuming antioxidants, antioxidative-stress is a disregarded substantial issue. Opposed to oxidative stress, antioxidative stress disrupts the signaling process of cells (Poljsak and Milisav, 2012). Reactive oxygen species are vital for cells to activate adaptive responses (Schulz et al., 2007). It is proven that, antioxidative stress restricts mitochondrial hormesis which is naturally triggered by ROS (Schulz et al., 2007). In addition, surplus of antioxidants is able to increase cancer risk by aiding survival of dysfunctional cells which would normally undergo destruction processes (Poljsak and Milisav, 2012). ROS are beneficial in balanced concentrations for fighting against microorganisms and unwanted, damaged, precancerous, or cancerous cells (Poljsak and Milisav, 2012). It is suggested that allowed antioxidant capacity is controlled via homeostatic mechanisms by cells (Poljsak and Milisav, 2012). Apparently, that capacity is not exceeded by consumption of antioxidant containing whole foods regarding their effect on glutathione levels (Lotito and Frei, 2006; García-Rodríguez et al., 2012). Although isolated astaxanthin is considered as safe to taken as supplement, its interference with human body's natural antioxidant mechanism is unknown. Therefore, it is required to be proven to not produce that such effects prior to consumption. In light of this, addition of astaxanthin from *Haematococcus pluvialis* to biomass of *Chlorella vulgaris* was excluded from this study as originally considered for the thesis proposal.

2.5. Environmental Value of Microalgae as a Food

Global warming is a heated discussion that is majorly turning around the increased greenhouse gas (GHG) emissions. Agriculture contributes significant amounts of methane, nitrous oxides, and carbon dioxide to the atmosphere. While transportation and industry can face the belt-tightening policies, agriculture is the last to cut production back due to its vital importance. Currently, large areas are used for food production even by destructing huge forest areas like Amazons which is one of the major carbon sequestering lands (Reuters, 2019). Besides, agriculture is occupying lands with single species where many different species naturally inhabit. This leads to loss of biodiversity which consequently affects various environmental systems and nutrient cycles (National Research Council, 1992).

At first glance, agriculture seems harmless by cultivating plants which are carbon capturing entities. However, agricultural practices went beyond converting available areas to deforestation (Reuters, 2019). Forests are capable of carbon sequestering far better than farms (FAO, 2019). By deforestation for farming, a carbon dioxide sink will be replaced with a lower capacity one. As a result, present amount of carbon dioxide will be increase in the first place. Plus, additional carbon dioxide is generated during the process of farming and machinery use (FAO, 2019). Even though current carbon dioxide emissions were stopped, prolonged amount of time will be required for the recovery of atmosphere (Solomon et al., 2008). In the pursuit of accelerated recovery, carbon capture is necessary. Microalgae are outstanding agricultural products with respect to high carbon capture with no requirement of fertile area and pesticides (Sayre, 2010). To begin with, microalgae and cyanobacteria are responsible for fixing 50 Gt of carbon per year which is equivalent of approximately 50% of the carbon dioxide sequestration on Earth (Basu and Mackey, 2018). Cultivation of microalgae is advantageous over plant cultivation because microalgae offer faster carbon dioxide capture due to rapid growth rates relative to plants. On top of all, microalgae can be cultivated even in desert climates. For instance, Israel hosts large microalgae cultivating areas which are placed on non-fertile soils (Israel21C, 2019). In fact, areas like Arizona (USA) (Treece, 2011) and Israel (Israel21C, 2019) possess a greater chance to grow microalgae considering all-year sunshine receival. By means of these advantages, microalgae possess a solution for production of nutritious food without occupying fertile or forest areas while recycling carbon dioxide in a more efficient way.

Another environmentally important aspect is the improper usage of water for agriculture and livestock. Livestock is the main protein source across the world (FAO, 2019). For the purpose of placing meat on the market counters, slaughtering is required. When the water usage for feed crops, drinking water of animals and the water needed for slaughterhouse are taken into account all together, 15 tones of water is used for production of only one kg of red meat (Mekonnen and Hoekstra, 2011) whereas production of one kg of microalgae consumes 2.6-6.8 tones of water (Martins et al., 2018). In addition, slaughterhouses generate high amounts of polluted wastewater. Common municipal wastewater has BOD values ranging between 100-300 (FAO, 2019) whereas slaughterhouse wastewater has BOD values ranging between 610-4635 (Bustillo-Lecompte et al., 2016) which indicates a highly polluted water that cannot be discharged in nature without treatment. Although most slaughterhouses have wastewater treatment units, effluent is not free from pharmaceuticals (Bustillo-Lecompte and Mehrvar, 2017) and pesticides (USDA, 1988) which are present in animals at concentrated levels due to bioaccumulation and biomagnification. In comparison, microalgae cultivation does not require pharmaceuticals or pesticides and effluent is safe to discharge to soil (Sayre, 2010). The 75% of agricultural lands are occupied for livestock raising (Foley et at., 2011).

Cassidy et al. suggested that if current agricultural lands used directly for human food rather than animal feed and biofuel feedstock, harvest could feed additional 4 billion people which makes nearly half population of the World (Cassidy et al., 2013; United States Census Bureau, 2018). Obviously, abandoning meat production completely is not plausible in today's world. However, microalgae based protein may aid in reducing the amount of meat produced while decreasing the burden on natural sources such as water and forest lands occupied for agriculture.

3. PURPOSE

Nutritional value of the microalgae is crucial for the determination of its serving size. Designing microalgae based functional foods take shape according to the allowed amount for consumption. Serving size of microalgae will define the functional food's ability to meet daily nutrient requirements and limitations for safe consumption. The aim of this study was to design a functional food based on microalgae *Chlorella vulgaris* and to determine the possibility of being a vegetarian-vegan vitamin B12 source. As reviewed in previous section, macronutrient and mineral content of *Chlorella vulgaris* is well studied. However, its vitamin content is either sourced from commercial companies or measured by biological methods. Additionally, the thermal stability of its nutrients is unknown which is a significant gap of knowledge considering the variety of the potential functional foods. Subsequently, the central themes of this study were:

- Quantifying macronutrient and vitamin content of *Chlorella vulgaris* to compare with daily nutrient requirements stated by WHO.
- Quantifying vitamin content of *Chlorella vulgaris* with analytical methods, LC-MS/MS analysis.
- Determining the thermal stability of water-soluble vitamins with LC-MS/MS analysis following baking the biomass at 125°C for 15 and 35 min.
- Quantifying bioavailable vitamin B12 content in *Chlorella vulgaris*.
- Estimating the serving size of *Chlorella vulgaris* according to the results.

The outcomes of the study determined the nutritional value of the *Chlorella vulgaris* that was grown in given conditions, serving size according to that value, possibility of the addition of biomass in baking recipes and confirmed the presence of vegetarian-vegan vitamin B12 in the bioavailable form. Finally, culture growth data provided a basis for scale-up production.

4. MATERIALS AND METHODS

This chapter describes methods, tools, and reagents used for cultivation of *Chlorella vulgaris* and quantification of carbohydrate, protein, lipid and vitamin values of the biomass. Primarily, the characterization of the used strain, culture medium, cultivation conditions in photobioreactor (PBR) were given in detail. Following, colorimetric methods used for the determination of carbohydrate and protein content were described. Lastly, the extraction method of vitamins and analysis with LC-MS/MS were presented.

4.1. Cultivation and Harvest

Strain CCAP 211/11B of *Chlorella vulgaris* (Figure 4.1) was used as the model organism throughout this study, and maintained at 25ºC under 1200 lux intensity, 12:12 h dark:light cycle in a growth chamber (Nüve GC401, Turkey). Seed cultures of *Chlorella vulgaris* were cultivated in Modified Bold's 3N (MB3N) culture medium (Table 4.1) and passaged every 10 days.

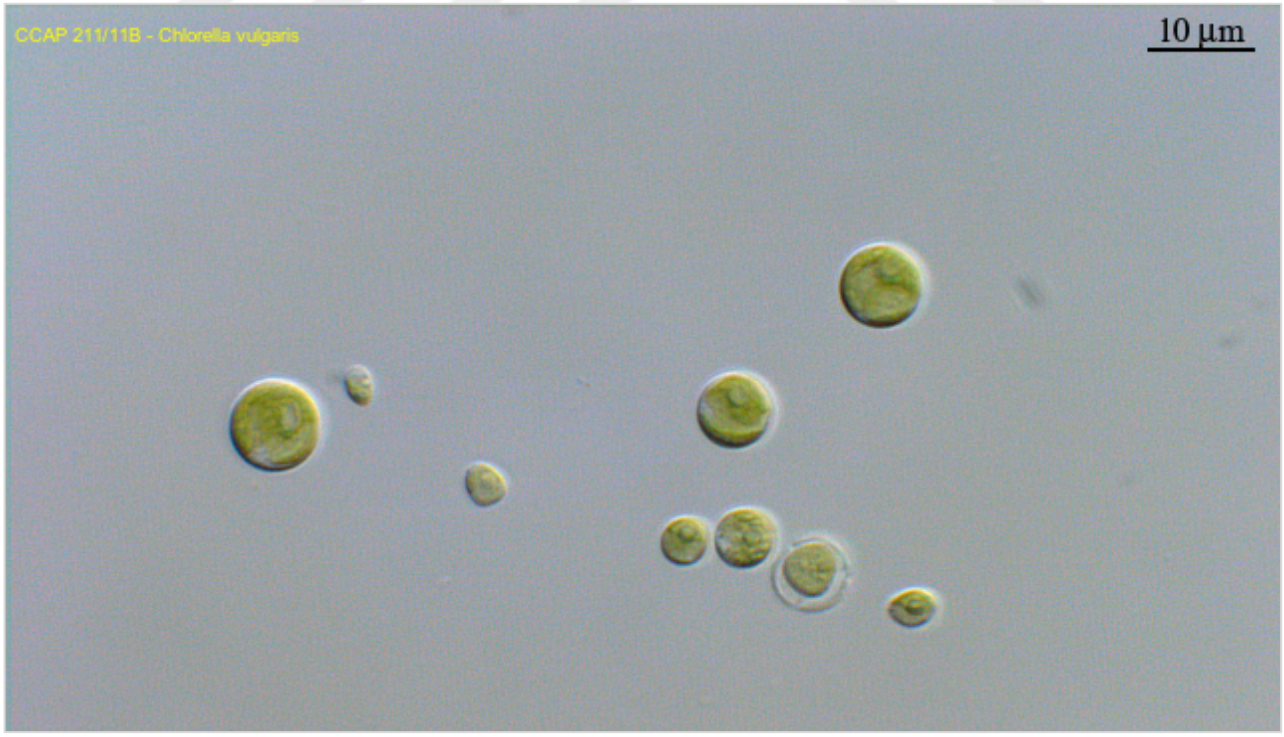

Figure 4.1. Microscope image of *Chlorella vulgaris* CCAP 211/11b (image credit: CCAP, 2018).

For experimental biomass production, three custom-made batch type reactors were used in a single batch. A Specifically, reactors consisted of a 2 L Erlenmeyer flask, air diffuser stone with 0.5

mm pore size, a silicone cap with one air inlet and two exhaust outlets made of borosilicate glass pipes (Figure 4.2). Both inlet and outlets were closed with sterile air filters with 0.22 µm pore size to ensure axenic growth. Each reactor was cleansed and autoclaved prior to usage.

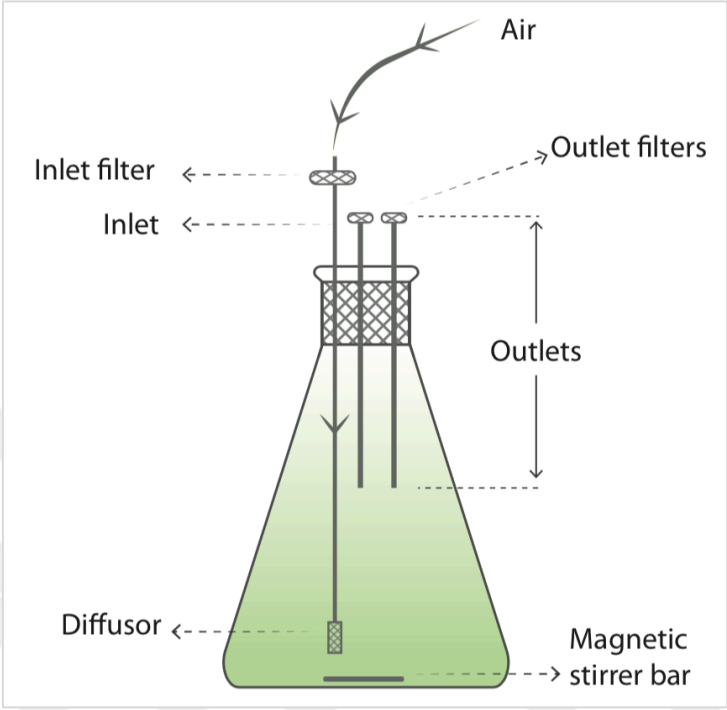

Figure 4.2. Scheme of custom-made batch type reactor.

Chlorella vulgaris CCAP 211/11B was cultivated by inoculating with $5x10^6$ cells/ml concentration from seed cultures harvested at mid-exponential growth phase. A working volume of 1.6 L with modified Bold's 3N (MB3N) medium (Table 4.1) was kept constant per each 2 L Erlenmeyer batch type reactor. Reactors were placed in front of two white LED spotlights with the light intensity of 29600 lux (400µmol photons/s·m^2) with a light:dark cycle of 14:10 h (Yuvraj et al., 2016; Guccione et al., 2014; Lohman et al., 2015). The temperature remained between 24-28 ºC and pH fluctuated in the range of 6.5-11 (Guccione et al., 2014). Air flow was kept at 0.5 L/min per each reactor by using filtered dry air. No additional carbon dioxide was supplemented. Each reactor was continuously stirred with a magnetic stirrer (appx. 200 rpm). After 8 days of cultivation, biomass was harvested by centrifugation (Boeco M240, Germany) at 4500 rpm 25 ºC for 10 min and freeze-dried (Hypercool H4055, Korea) for 36 hours. Freeze-dried biomass was stored in -20 ºC for further processing.

Table 4.1. Ingredients of MB3N algal growth culture medium (UTEX, 2019).

Order	Component	Stock solution	Quantity used	Final conc.
	Nutrients			
1	$NaNO_3$	10g/400mL dH_2O	30mL/L	8.82mM
2	$CaCl_2$.2H2O	1g/400mL dH_2O	10mL/L	0.17mM
3	$MgSO_4$.7H2O	3g/400mL dH_2O	10mL/L	0.3mM
4	K_2HPO_4	3g/400mL dH_2O	10mL/L	0.43mM
5	KH_2PO_4	7g/400mL dH_2O	10mL/L	1.29mM
6	NaCl	1g/400mL dH_2O	10mL/L	0.43mM
7	**P-IV Metal Solution**	-	6mL/L	-
8	**Soilwater: GR+ Medium**	-	40mL/L	-
	Vitamins			
9	Vitamin B12	-	1mL/L	-
10	Biotin vitamin solution	-	1mL/L	-
11	Thiamine vitamin solution	-	1mL/L	-

Optical density (λ=680 nm), pH and temperature data were gathered every day using spectrophotometer (Hach DR3900, US) and pH-temperature probe (SI Analytics Handylab, UK). Cell count data gathered with a hemocytometer (Neubauer improved, Germany) every other day. Decreased amount of medium volume was restored by adding fresh media.

4.2. Baking

Baking was done following the parameters of Gouveia et al. applied on *Chlorella vulgaris* cookies (Gouveia et al., 2007) using an oven (Daihan ON-105, Korea). Batches were separated to two groups as control (raw) and baked. For the carbohydrate, lipid and protein contents 15 minutes of baking was done. For vitamins, baking was done with two different durations, 15 and 35 minutes, in order to observe degradation rate of vitamins.

4.3. Analysis

4.3.1. Carbohydrate Analysis

Carbohydrate extraction was performed via two-step sulfuric acid hydrolysis and soluble carbohydrate determination by spectrophotometry (Van Wychen and Laurens, 2017). The method was preferred owing to its ability to respond to different aldehyde functional groups when different variants of carbohydrates are present.

Primarily, glucose standard stock solution for the calibration curve was prepared using 25 mg glucose with ≥ 99.5% purity (Sigma G8270, Germany) and 100 mL of deionized water (DIW). For correction, a separate glucose stock solution was prepared for calibration verification standard as well. Prior to spectrophotometric measurement and sample hydrolysis, following analysis reagents were prepared: 0.5 M 200 mL NaOH, 3 mg/mL MBTH (3-methyl-2-benzothiazolione hydrazine) solution, 1 mg/mL DTT (dithiothreitol) solution, 0.25 M 200 mL HCl (hydrochloric acid) and a ferric solution (200 mg 0.5% ferric ammonium sulfate dodecahydrate, 200 mg 0.5% sulfamic acid and 0.25 M 40 mL HCl) based on instructions as provided elsewhere (Van Wychen and Laurens, 2017).

Secondly, sample hydrolysis was performed. Samples were homogenized with porcelain mortart and pestle. For each sample, 25 ± 2.5mg freeze-dried microalgae were placed in 10 mL glass tubes, weighed and dried overnight in an oven at 40°C. Oven-dried samples were cooled in the desiccator for 24 h and re-weighed. At the end, moisture contents of algae were calculated taking the difference between pre- and post-dry weights. Moisture contents of the samples were checked to be under 10%.

Afterwards, 250 μL of 72% (w/w) sulfuric acid solution was added to each tube and placed in a water bath at 30 ± 3°C for 1 hour. Following, samples were removed from the water bath and 7 mL of DIW was added. Glass tubes were sealed and autoclaved for 1 hour at 121°C. Samples allowed to cool in an autoclave for 15 min and 1 h at room temperature. At the final step, hydrolyzed samples were filtered with 0.22 μm filters and proceeded to concentration analysis. In order to start spectrophotometric measurements, glucose standards for calibration curve were prepared using glucose standard stock solution (0.25 mg/mL) with the following concentrations (Table 4.2).

Table 4.2. Glucose standards' concentrations for calibration curve.

Conc. of stock (mg/mL)	Dilution stock + water (µL)	Final concentration (mg/mL)
0.25	0 + 500	0
0.25	20 + 480	0.010
0.25	30 + 470	0.015
0.25	50 + 450	0.025
0.25	75 + 425	0.0375
0.25	100 + 400	0.050

Sample dilutions of 1:10 with 50 µL hydrolysate, 1:20 with 25 µL were done with a total volume of 0.5 mL. Calibration verification standard was prepared from its glucose stock solution (0.25 mg/mL) using 75 µL and 425 µL DIW. MBTH working solution comprising 3 mg/mL of MBTH solution, 1 mg/mL DTT solution was prepared freshly with a volume of 500 µL for each sample. Following, 500 µL of 0.5 M NaOH added to each tube (including standards and samples) and vortexed. 500 µL of MBTH working solution was added to each tube and vortexed. Right after, tubes were placed in a pre-heated oven at 80 ± 3°C for 15 ± 1 min. NaOH, MBTH additions and placing in oven performed sequentially at a quick pace. At the end of the 15 min, the oven was turned off and 1 mL of ferric solution was added. Following the reaction, 2.5 mL DIW was added and vortexed. Subsequently, absorbances at 620 nm were measured for each sample hydrolysate and standard with the spectrophotometer (Hach DR3900) to determine final concentrations. Finally, carbohydrate contents of the biomasses were calculated by using the absorbance values of the extracts and the correlation equation was obtained by the calibration curve as shown in Figure 4.3:

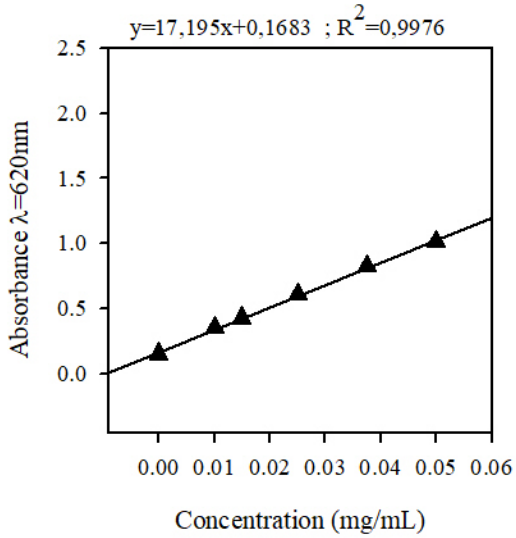

Figure 4.3. Glucose calibration curve.

4.3.2. Protein Analysis

Protein extraction was performed via alkaline extraction and quantification was done by Lowry assay (Safi et al., 2013). This procedure only quantified the hydro-soluble proteins. Due to overestimating results obtained from trichloroacetic acid (TCA) extraction, this method was chosen.

Firstly, standard concentrations were prepared using bovine serum albumin (BSA) (Sigma P536) with concentrations from 0 to 3 mg/mL and a final volume of 200 µL (Table 4.3). Lowry reagent C was prepared by mixing 50 mL Lowry reagent A [2% (w/v) Na_2CO_3 in 0.1N NaOH)] and Lowry reagent B [(0.5% (w/v) $CuSO_4$.5H2O in 1% (w/v) NaK tartrate tetrahydrate]. As for extraction, a few drops of 2N NaOH was added to 50mL DIW and pH is fixed to 12 using a pH meter (SI Analytics Handylab).

Table 4.3. Bovine serum albumin standards' concentrations for calibration curve.

Conc. of stock (mg/mL)	Dilution stock + water (µL)	Final concentration (mg/mL)
5	0 + 200	0
5	20 + 180	0.5
5	40 + 160	1
5	60 + 140	1.5
5	80 + 120	2
5	100 + 100	2.5
5	120 + 80	3

At the second step, 5mg of freeze-dried algae of each sample was weighed in screw-capped microcentrifuge tubes. 250 µL from alkaline NaOH solution was added to each tube and vortexed carefully for all solids to remain in solution. Tubes were incubated in 40°C water bath for 1 h and vortexed every 10 min. Following incubation, tubes were centrifuged at 5000xg for 10 min. 200 µL of supernatants were placed in clean microcentrifuge tubes, 1 mL of Lowry reagent C was added to each sample and standard tubes, vortexed and left to incubation for 10 min. After incubation, 100 µL of 1 N Folin-Ciocalteu's phenol reagent was added to each tube, vortexed and left to incubation for 30 min. Absorbances were read at 750 nm and respective protein concentrations were determined. Lastly, hydro soluble protein content calculations were done by using absorbance values of extracts and equation obtained from the BSA calibration curve as shown in Figure 4.4:

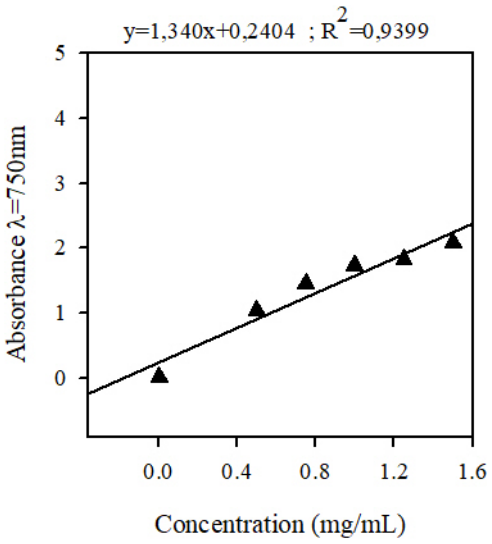

Figure 4.4. BSA calibration curve.

4.2.3. Lipid Analysis

Lipid extraction was performed by mechanical cell disruption and chemical extraction following a protocol by Breuer et al. (Breuer et al., 2013). Primarily, 300 mg of 0.1 mm and 100 mg of 0.5 mm glass beads were weighed, placed in the screw-capped bead beating tubes and autoclaved. Following, 50±0.9 mg of microalgae was weighed and placed in bead beating tubes. 1 mL of 4:5 chloroform:methanol mixture (v/v) was added to each tube. Bead beating (Bertin Minilys, France) was done for 8 times at 2500 rpm for 1 min with 2 min intervals of cooling on ice. Microalgae and beads were transferred to glass vials and bead beating tubes were washed 3 times with 1 mL of 4:5 chloroform:methanol mixture (v/v). At the end, the total volume in glass vials was kept at 4 mL total. Vials were vortexed for 5 sec and sonicated for 10 min. 2.5 mL water containing 50 mM Tris and 1 M NaCl with pH 7 were added to the vials. Following, vials were vortexed for 5 sec, sonicated for 10 min and centrifuged at 1200g for 5 min. The bottom phase (chloroform) was transferred to a new vial using a Pasteur pipette without interfering the other phase. Extraction was repeated by adding 1 mL of chloroform to previous vials, vortexed for 5 sec, sonicated 10 min and centrifuged at 1200g for 5 min. Bottom phase was collected using Pasteur pipette. As the last step, chloroform was evaporated under N_2 gas and pre-weighed vials weighed again to quantify total lipid content amount.

4.3.4. Vitamin Analysis

Vitamin extractions were performed via mechanical disruption and chemical extraction. Protocols of Lock et al. were modified for both water-soluble and fat-soluble vitamin extractions (Lock, 2013; Lock and Noestheden, 2014).

For the water-soluble vitamin extractions, 300 mg of 0.1 mm and 100 mg of 0.5 mm glass beads were weighed, placed in the screw-capped bead beating tubes and autoclaved. 250 ± 0.9 mg of microalgae were weighed, placed in bead beating tubes. One mL DIW was added to each tube. Bead beating was done for 8 times at 2500 rpm for 1 min with 2 min intervals of cooling on ice. Microalgae and beads were transferred to amber glass vials to protect from light driven oxidation and bead beating tubes were washed 3 times with 1 mL DIW. Four mL of acetonitrile and 80 µL formic acid were added to each amber vial and vortexed for 1 min. Roller mixing was done for 10 min in a dark room. Centrifugation was done at 4500 rpm for 15 min and the supernatant was filtered. A total of 900 µL of filtrate, and 100 µL of internal standard (2500 µg/L cyanocobalamin) were added to chromatography vials and analyzed in a triple-quadrupole liquid chromatography-mass spectrometer (LC-MS/MS) system (AB SciEx 4500, USA).

For the fat-soluble vitamin extractions, 300 mg of 0.1 mm and 100 mg of 0.5 mm glass beads were weighed, placed in screw-capped bead beating tubes and autoclaved. 250 ± 0.9 mg of microalgae were weighed and placed in bead beating tubes. 1 mL of methanol was added to each tube. Bead beating was done for 8 times at 2500 rpm for 1 min with 2 min intervals of cooling on ice. Microalgae and beads were transferred to amber glass vials to protect from light driven oxidation and bead beating tubes were washed 2 times with 1 mL methanol. 15 mL of hexane was added to vials and vortexed for 30 sec. Centrifugation was done at 3000 rpm for 5 min. 10 mL from top layer (hexane) was taken, placed in new amber glass vials. Glass vials containing 10 mL hexane were placed under N_2 gas for evaporation. Following, 2mL of methanol was added to vials to reconstitute, vortexed 10 sec and filtered. Lastly, 1/100 dilution was done with methanol. A total of 900 µL of dilute, 100 µL of internal standard (2500 µg/L cyanocobalamin) was added to chromatography vials and analyzed in LC-MS/MS.

For LC-MS/MS analysis, firstly multiple reaction monitoring (MRM) of vitamin standards were quantified (Appendix A). Method was optimized according to vitamins and retention times were determined (Appendix A). Methods listed in Table 4.4 and Table 4.5 were used for both analyzing

water-soluble and fat-soluble vitamins. As an internal standard, cyanocobalamin (synthetic vitamin B12) was used.

Table 4.4. Scheduled LC-MS/MS method for water- and fat-soluble vitamins.

Oven temperature	40°C
Aqueous (pumpA)	MeOH w. 0.1% FA
Organic (pumpB)	DIW w. 0.1 Formic Acid
Pump maximum pressure	1240 bar
Pump minimum pressure	0 bar
Equilibration Time	2 min

Table 4.5. Pump timetable of the used LC-MS/MS method.

Pump Timetable			
Pump time	**Pump flow (mL/min)**	**Pump fraction A%**	**Pump fraction B%**
00:00:01	0.60	12	88
00:01:30	0.60	12	88
00:02:00	0.60	75	25
00:04:00	0.60	75	25
00:04:30	0.60	100	0
00:14:00	0.60	100	0

Vitamin standard concentrations for calibration curves were prepared according to detection range of LC-MS/MS (Figure 4.5-14). The vitamin contents of control, baked, and final groups were calculated using areas obtained from LC-MS/MS analysis (Appendix E) and equations obtained from calibration curves of vitamin standards (Figure 4.5-14).

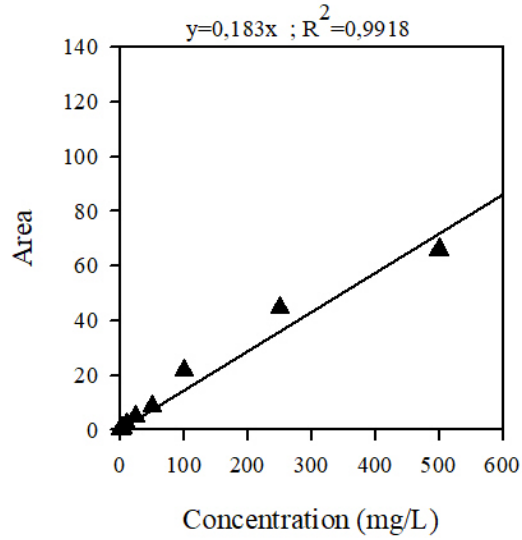

Figure 4.5. Calibration curve of vitamin B1.

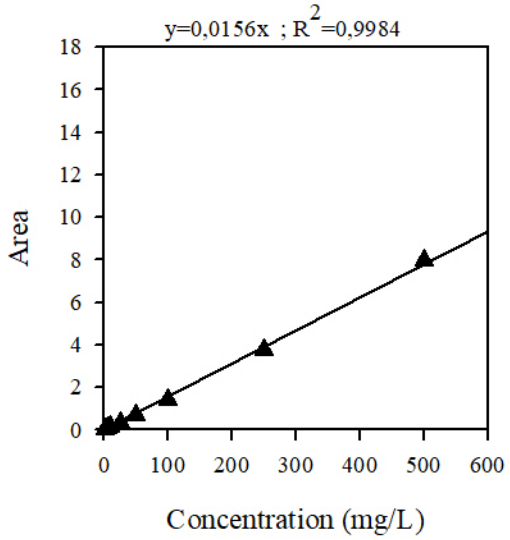

Figure 4.6. Calibration curve of vitamin B2.

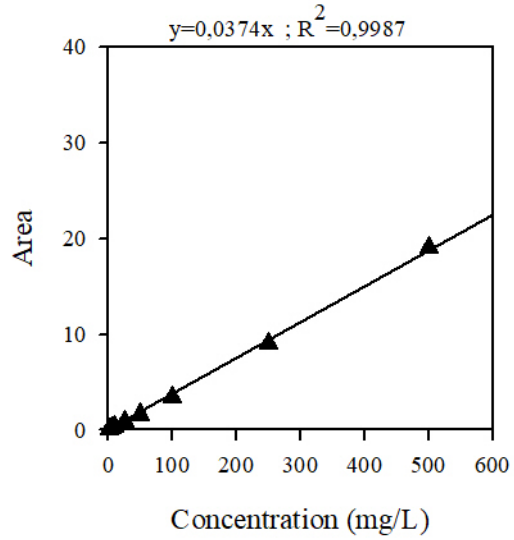

Figure 4.7. Calibration curve of vitamin B3.

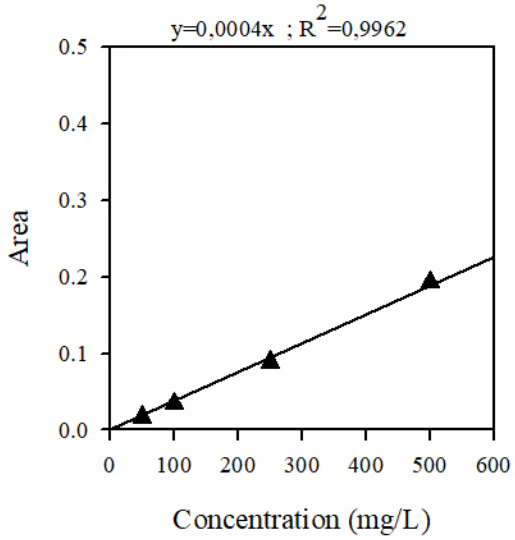

Figure 4.8. Calibration curve of vitamin B5.

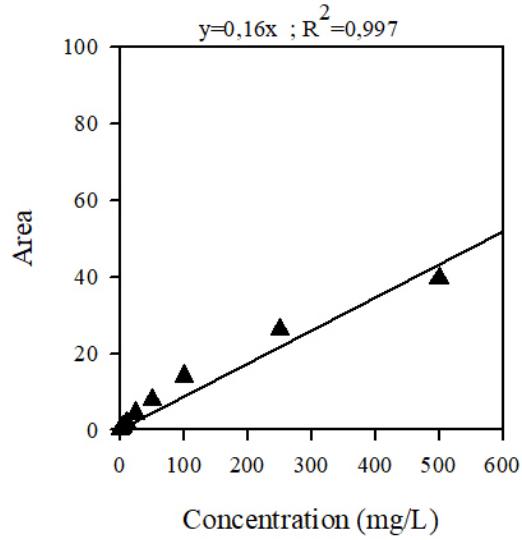

Figure 4.9. Calibration curve of vitamin B6.

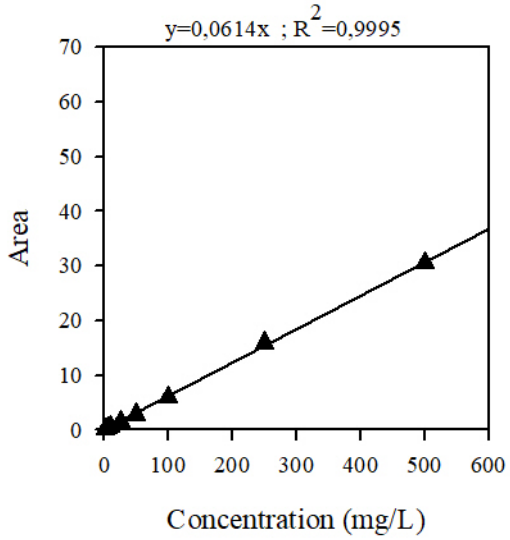

Figure 4.10. Calibration curve of vitamin B7.

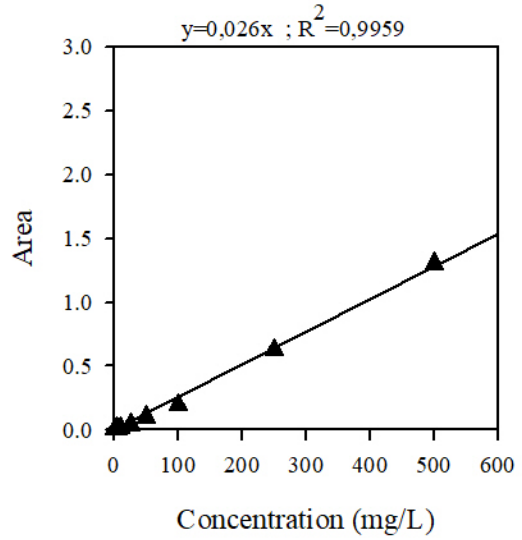

Figure 4.11. Calibration curve of vitamin B9.

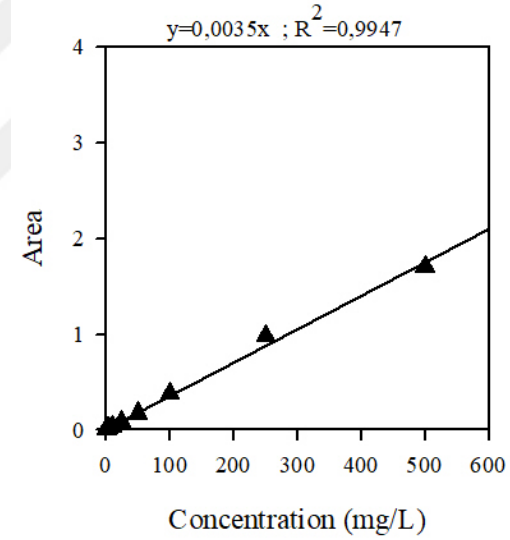

Figure 4.12. Calibration curve of vitamin B12m (methylcobalamin).

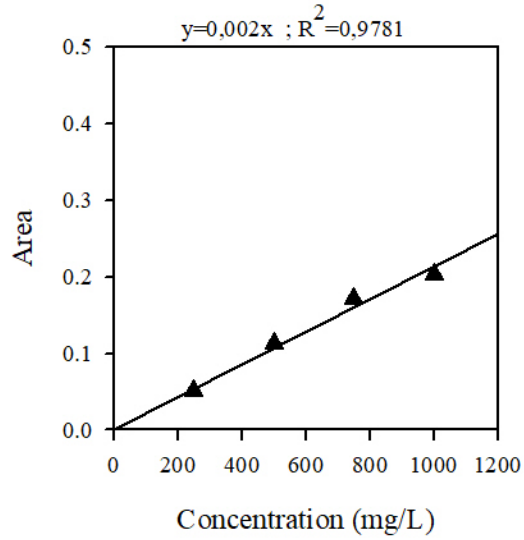

Figure 4.13. Calibration curve of vitamin C.

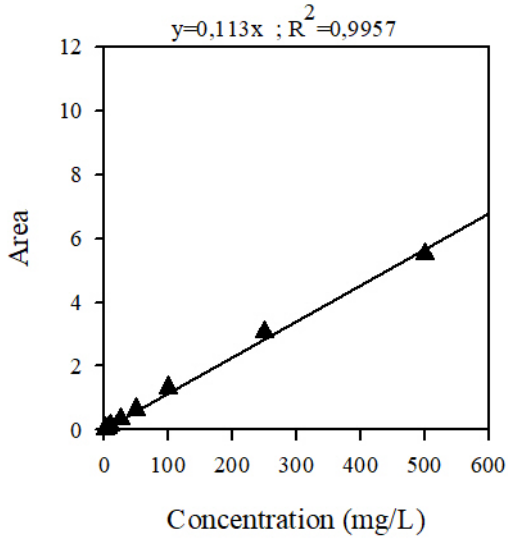

Figure 4.14. Calibration curve of vitamin E.

Lastly, 2.5µg/L B1, 25µg/L B2, 25µg/L B3, 500µg/L B5, 2.5µg/L B6, 10µg/L B7, 250µg/L B9, 50µg/L B12m, 250µg/L B12m 500µg/L C and 50µg/L E were used in an additional run to visualize vitamin peaks (Figure 4.15).

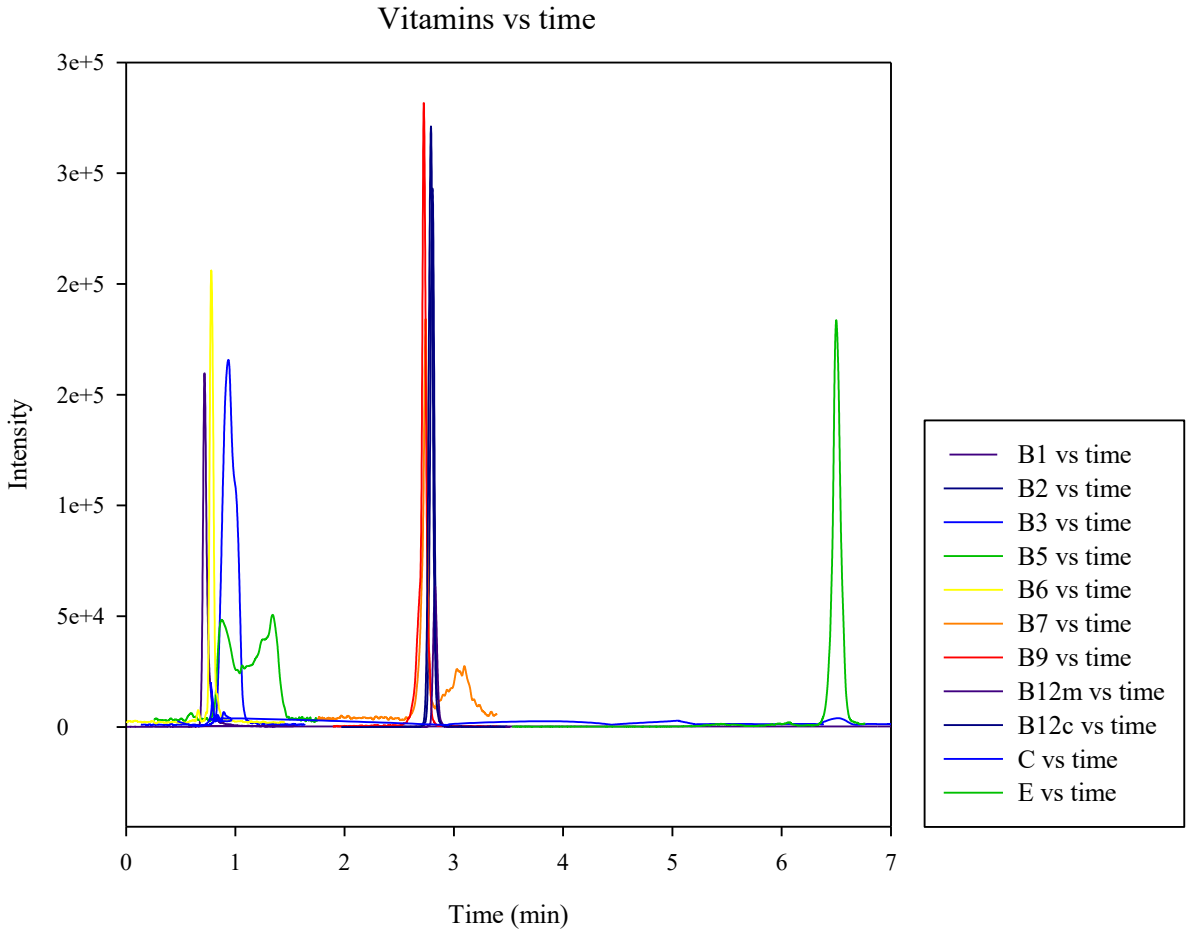

Figure 4.15. Visual demonstration of vitamin peaks observed in LC-MS/MS analysis.

4.3.5. Method for Application of the t-test

In order to determine significance of the obtained results, two-tailed, paired t-test was applied to results with probability of 0.05. Microsoft Excel version 16.26 was used for calculation of t values (Microsoft, Redmond, WA, USA) . Tests were applied for the carbohydrate, lipid and protein results of the biomass before and after baking at 125°C for 15 minutes. For the vitamins, t-test was applied for the results of raw biomass coupled with results of baking at 125°C for 35 and results of baking at 125°C for 15 minutes.

5. RESULTS AND DISCUSSION

In this chapter, results of the growth of *Chlorella vulgaris* strain CCAP 211/11b in batch type photobioreactor, macronutrient and vitamin analyses were presented. Efficiency of the used batch type photobioreactor was discussed and the results of carbohydrate, lipid and vitamin results were evaluated according to daily recommended values of WHO.

5.1. Biomass Growth Results

The type of photobioreactor used to grow microalgae directly affects productivity. In this study, a batch type PBR was used to grow *Chlorella vulgaris* (Figure 4.2, Figure 5.1). Tops of the Erlenmeyer flasks were enclosed with parafilm to prevent leakage and contamination. A total of 6 batches operated at different times with same growth conditions but inoculated with separate cultures of CCAP 211/11b *Chlorella vulgaris* for biological replication. Three batches were marked as control and three batches were separated to be baked at 125°C for 15 min following the baking parameters of Gouveia et al. (Gouveia et al., 2007). Concentrations of the sextuplicate batches are given in Table 5.1. Due to evaporation 4.8 L of three flasks' starting volume is reduced to 3.9 L.



Figure 5.1. Pictures of the batch type photobioreactors utilized during the course of the study.

Overall, water consumption per 1 gram of produced *Chlorella vulgaris* was:

$$Water\ consumption = \frac{1.6L}{2.4381g}$$

$$Water\ consumption = 0.66\ L/g$$

Microalgae usually grow following five phases; lag or acclimatization phase, log or exponential growth phase, declining growth phase, stationary phase and decline/death phase (Blair et al., 2014). In this study, all six batches were harvested at the early stationary phase. Despite inoculation of all six batches with $5x10^6$ cells/mL of seed cultures, minor differences observed on the growth graphs (Figure 5.2-19). Details of the growth data are provided in Appendix B, Appendix C and Appendix D. Despite these minor differences, final weights were compatible with each other as shown in Table 5.1.

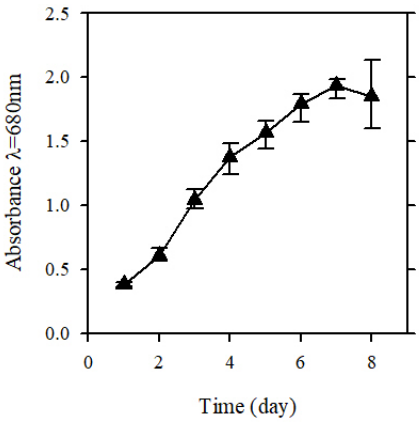

Figure 5.2. Optical density of batch C1.

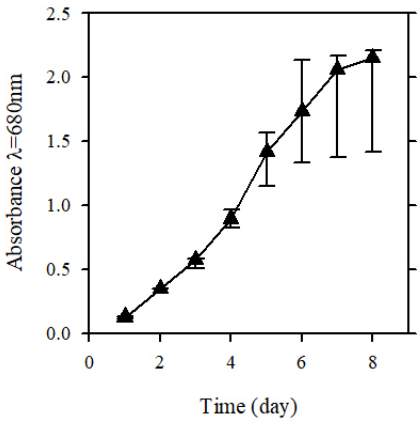

Figure 5.3. Optical density of batch C2.

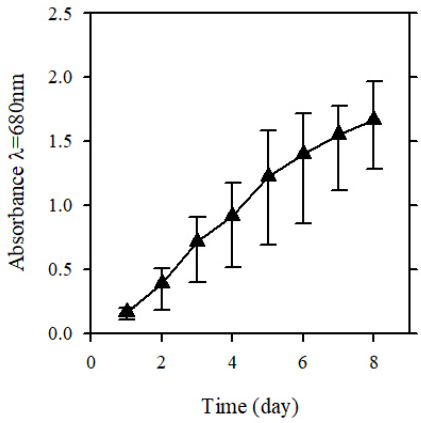

Figure 5.4. Optical density of batch C3.

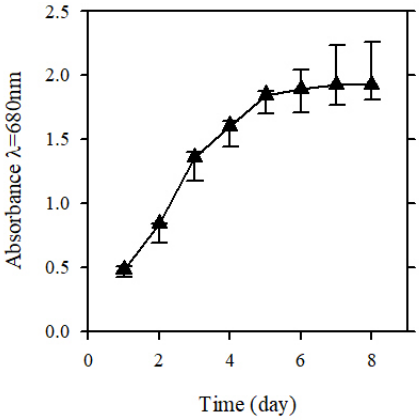

Figure 5.5. Optical density of batch B1.

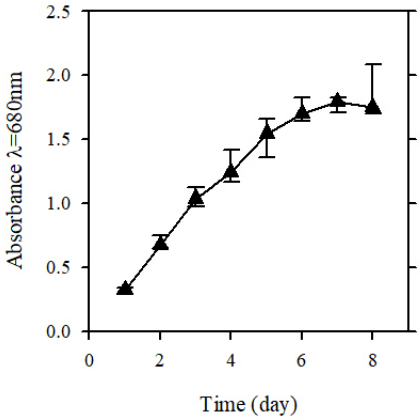

Figure 5.6. Optical density of batch B2.

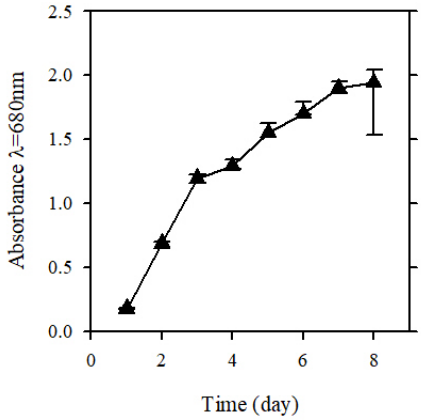

Figure 5.7. Optical density of batch B3.

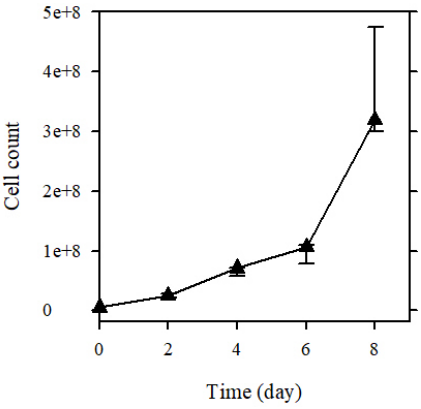

Figure 5.8. Cell count of batch C1.

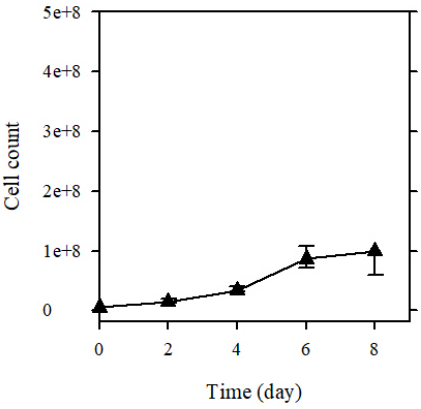

Figure 5.9. Cell count of batch C2.

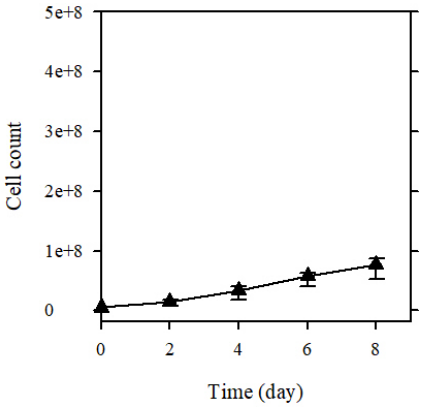

Figure 5.10. Cell count of batch C3.

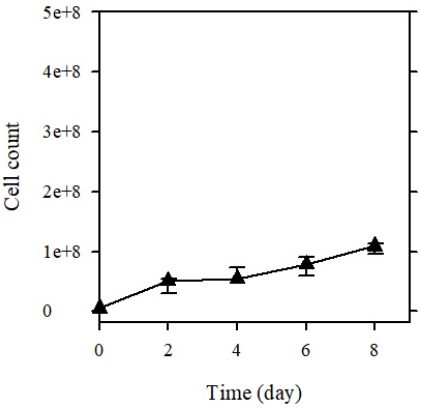

Figure 5.11. Cell count of batch B1.

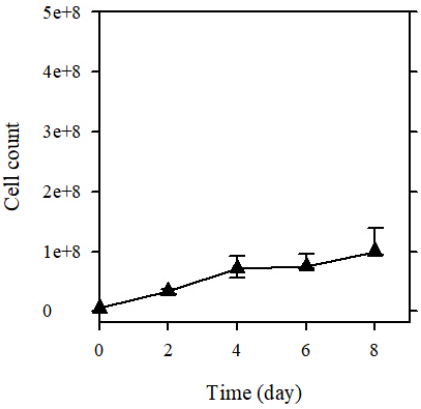

Figure 5.12. Cell count of batch B2.

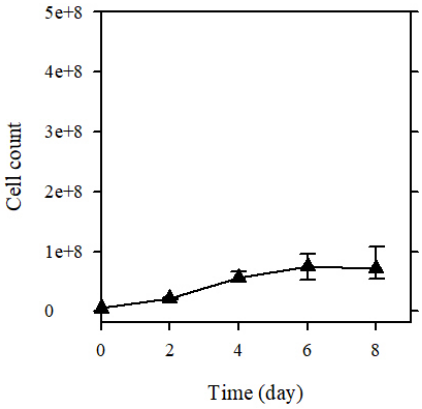

Figure 5.13. Cell count of batch B3.

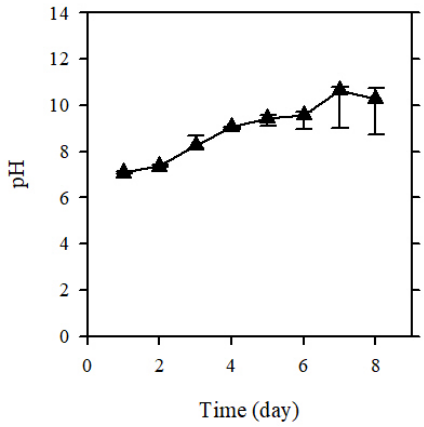

Figure 5.14. pH of batch C1.

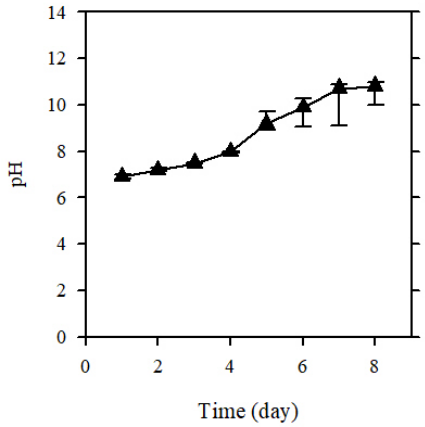

Figure 5.15. pH of batch C2.

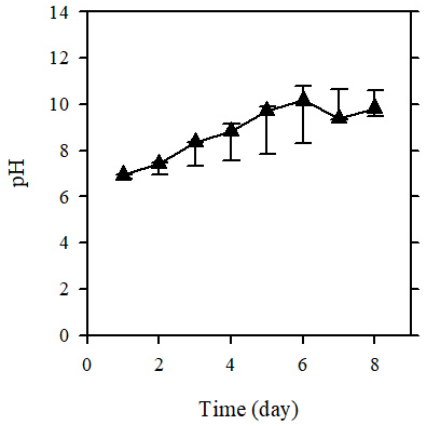

Figure 5.16. pH of batch C3.

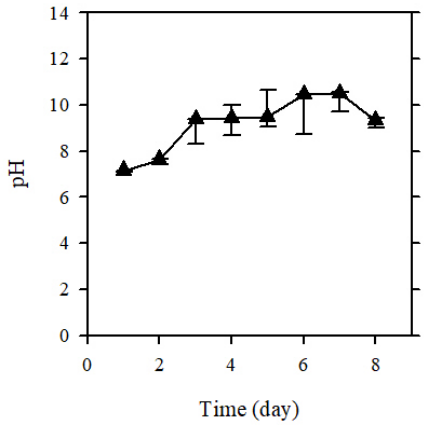

Figure 5.17. pH of batch B1.

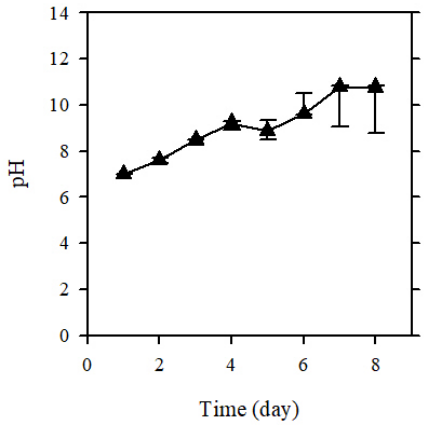

Figure 5.18. pH of batch B2.

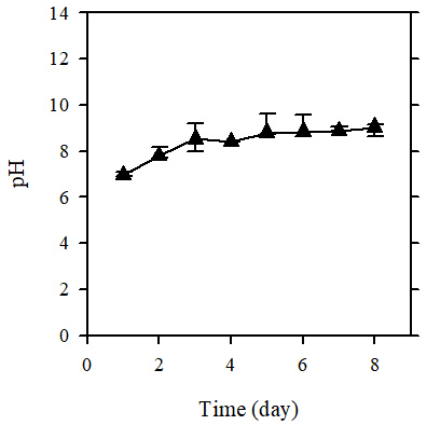

Figure 5.19. pH of batch B3.

Table 5.1. Biomass concentrations and dry weight measurements of six batches.

Batch	Freeze dried weight (g)	Final volume of medium (L)	Concentration (g/L)
C1	2.5488	3.9	0.654
C2	2.2631	3.9	0.580
C3	2.6544	3.9	0.681
B1	2.5657	3.9	0.658
B2	2.1543	3.9	0.552
B3	2.5624	3.9	0.657
Average	2.4581	3.9	0.630

5.2. Carbohydrate, Lipid and Protein Content

The primary criteria for the decision of utilizing the produced *Chlorella vulgaris* biomass is its carbohydrate, lipid and protein composition. Since meeting daily requirements for protein is harder when feeding on a plant based diet, high protein composition offers to compensate for the deficit. On the other hand, plant based diet provides adequate amounts of carbohydrates, especially fiber. Taken together, high protein and low carbohydrate composition in the biomass is preferred.

Carbohydrate content of the control (raw) and the baked (125°C for 15 minutes) groups were calculated using the glucose calibration curve equation and absorbance values. The volumes of the extracts were 11.75 mL and 1:25 dilution was done. The results of total carbohydrate calculation of the control and baked groups were presented in Table 5.2.

Table 5.2. Total carbohydrate contents of control (raw) and baked *Chlorella vulgaris.*

	Algae weight (mg)	**Abs. at λ=620nm**	**Calculated conc. (mg/mL)**	**Glucose (mg)**	**Carbohydrate %**	**Average**
C1	25.0	0.582	0.601	7.067	28%	27%
C2	23.9	0.512	0.500	5.872	25%	
C3	26.5	0.611	0.644	7.563	29%	
B1	25.0	0.575	0.591	6.948	28%	26%
B2	25.0	0.540	0.540	6.350	25%	
B3	23.2	0.488	0.465	5.462	24%	

As shown in Table 5.3, the carbohydrate content of the 100 g raw biomass (control) was low according to daily requirements advised by WHO. As discussed in the literature review section, a slight difference between values in Table 2.1 and Table 5.3 was related to different growth conditions. Plus, t-test ($t=0.47$, $p=0.05$) result has shown that baking at 125°C for 15 min had no significant difference on carbohydrate content. Overall, 27 g carbohydrate in 100 g of *Chlorella vulgaris*, i.e. %28, was satisfactory by the means of targeting a biomass with low carbohydrate and high protein.

Table 5.3. Carbohydrate content of control (raw) and baked *Chlorella vulgaris* and daily recommended values by WHO (Mann et al., 2007).

Batch			**RDV (WHO,FAO)**		**100g *Chlorella vulgaris* %RDV***	
	Carbohydrate	**Unit**	**Male***	**Female***	**Male***	**Female***
Control	27	%DW	275-375g		9.82%	
Baked	26	%DW			9.46%	

*Male=65 kg, Female=55 kg, 2000 kcal/day diet.

Protein content of the control (raw) and the baked (125°C for 15 minutes) groups were calculated using the BSA calibration curve equation and absorbance values. It should be noted that, only water-soluble proteins in *Chlorella vulgaris* were assayed in this study and the total protein content was calculated using the proportion rate of the hydro-soluble to total protein given by Safi et al. (Safi et al., 2013). The volume of the extracts was 1.3 mL and no dilution was done. Calculated hyro-soluble and total protein contents are presented in Table 5.4.

Table 5.4. Protein contents of control (raw) and baked *Chlorella vulgaris.*

	m (mg)	**Abs. at λ=750nm**	**Conc. (mg/mL)**	**Prot. (mg)**	**Water soluble protein %**	**Average water soluble protein**	**Calculated total protein %**	**Average total protein**
C1	5.1	1.474	0.920	1.197	23%	26%	55%	61%
C2	5.5	1.971	1.291	1.679	31%		71%	
C3	5.0	1.543	0.972	1.263	25%		59%	
B1	5.6	1.405	0.869	1.130	20%	24%	47%	56%
B2	5.1	1.697	1.087	1.413	28%		64%	
B3	5.1	1.493	0.935	1.215	24%		55%	

The protein contents as presented in Table 5.4 were consistent with studies in Table 2.12 and suggested that the protein content of *Chlorella vulgaris* was similar to animal sources. As comparison, 100 g of grass-fed beef contains 19.4 g of protein, whereas 100 g of raw *Chlorella vulgaris* contains 61 g of protein (Table 5.5). Therefore, it is a good candidate for supporting the diet with its protein value.

According to t-test results, the difference between baked and control protein values were found to be significant (t=0.04, p=0.05). However, the protein value of the baked group (56g per 100g) was only slightly lower than the control group (61g per 100g). Therefore, baked biomass did not lose its high protein quality.

Table 5.5. Protein content of control (raw) and baked *Chlorella vulgaris* and daily recommended values by WHO (WHO, 2007).

Batch			**RDV (WHO,FAO)**		**100g *Chlorella vulgaris* %RDV**	
	Protein	**Unit**	**Male***	**Female***	**Male***	**Female***
Control	61	%DW	43g	36g	141.86%	169.44%
Baked	56	%DW			130.23%	155.56%

*Male=65 kg, Female=55 kg, 2000 kcal/day diet.

Lipid content of the *Chlorella vulgaris* batches resulted in relatively low according to earlier studies presented in Table 2.3. Consequently, as seen in Table 5.6, this particular strain of *C. vulgaris* biomass was not recommended as a "complete source" for fatty acids. In order to determine its unsaturated fatty acid value, further GC analysis should be done for the determination of the fatty acid profile.

Table 5.6. Lipid content of control (raw) and baked *Chlorella vulgaris* and daily recommended values by WHO (WHO and FAO, 2010).

Batch			**RDV (WHO,FAO)**		**100g *Chlorella vulgaris* %RDV**	
	Lipid	**Unit**	**Male***	**Female***	**Male***	**Female***
Control	7%	%DW	44-78g		15.90%	
Baked	9%	%DW			20.45%	

*Male=65 kg, Female=55 kg, 2000 kcal/day diet.

The difference between lipid content results (Table 5.7) among the control group was possibly result of the physical inequality among harvest that was shown in Figure 5.20. Although all batches had same growth conditions and inoculated with $5x10^6$ cells/ml, this difference in formation observed

after lyophilization process. Samples C2, C3, B1 and B3 were clumped whereas C1 and B2 were in the form of powder. Therefore, extractability of the nutrients had slight differences.

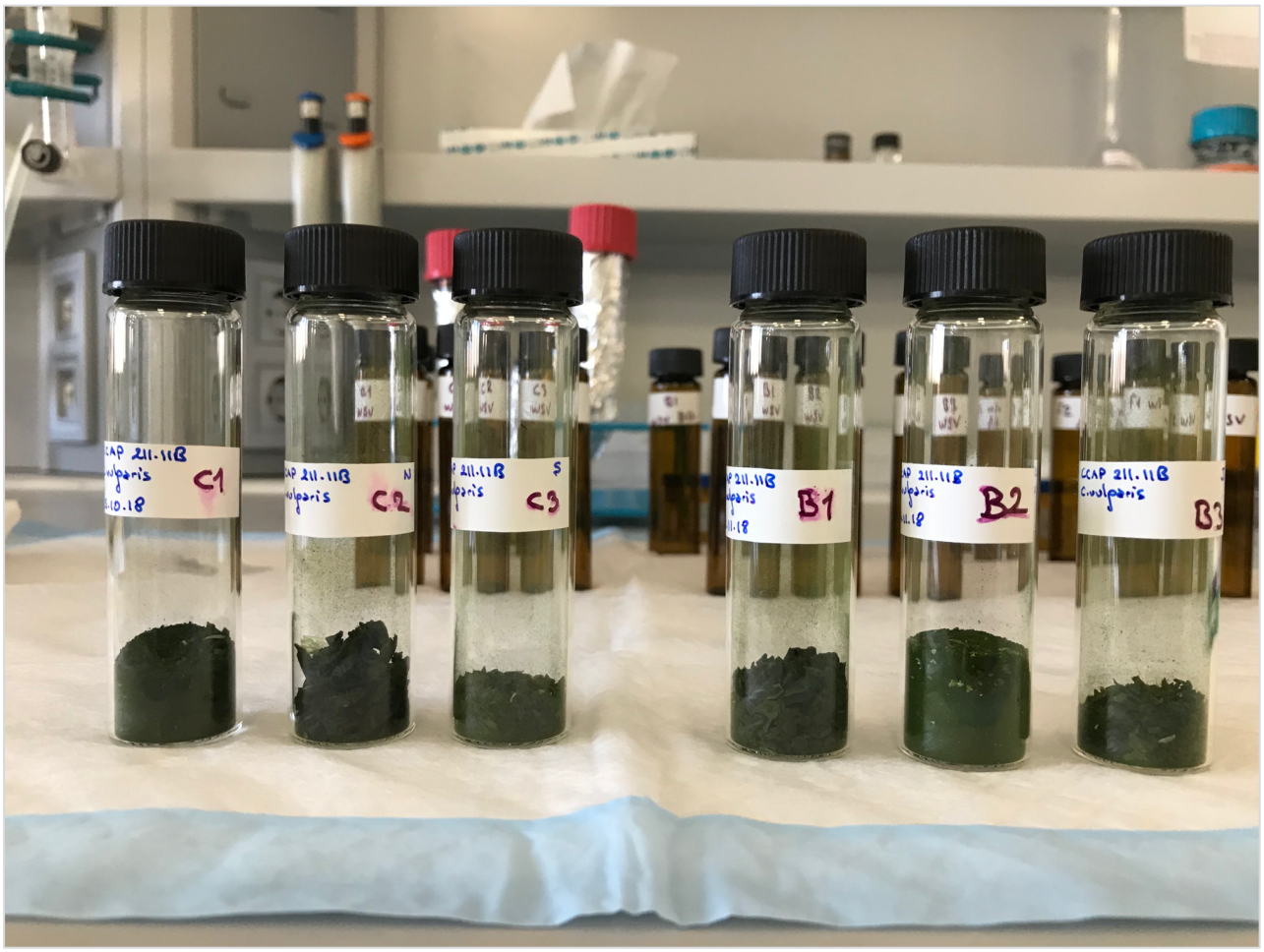

Figure 5.20. Physical differences between harvested biomass of sextuplicate batches.

As for the thermal effects, baking at 125°C for 15 min had no significant difference on lipid ($t=0.22$, $p=0.05$) content according to the t-test (Table 5.7). Overall, these results suggest that *Chlorella vulgaris* can be added to short-duration baking recipes considering the carbohydrate, lipid and protein contents.

Table 5.7. Lipid contents of control (raw) and baked *Chlorella vulgaris.*

	Algae weight (mg)	Vial weight (g)	Final weight (g)	Total lipid (mg)	Lipid %	Average lipid %
C1	50.4	2.8547	2.8560	1.3	8%	7%
		2.8069	2.8095	2.6		
C2	50.7	2.7555	2.7579	2.4	8%	
		2.7900	2.7917	1.7		
C3	50.3	2.7802	2.7814	1.2	6%	
		2.8853	2.8870	1.7		
B1	50.5	2.7220	2.7241	2.1	8%	9%
		2.7437	2.7454	1.7		
B2	50.5	2.7809	2.7850	4.1	11%	
		2.7389	2.7402	1.3		
B3	50.6	2.8146	2.8173	2.7	9%	
		2.8017	2.8037	2.0		

5.3. Vitamin Analysis with LC-MS/MS and Vitamin Content of *Chlorella vulgaris*

The aim of the vitamin analysis was obtaining analytical results and observation of the thermal stability of vitamins through the baking process at 125°C. In this analysis, an additional baking step was added to observe the degradation rate of the water-soluble vitamins which are more sensitive to thermal effects compared to the fat-soluble vitamins as discussed earlier. In order to assess the thermal degradation rate, 250 mg biomass of B1, B2 and B3 were baked for only 15 min at 125°C, and a separate 250 mg aliquots of B1, B2 and B3 were baked for 35 min at 125°C.

Vitamin composition results of the raw biomass in Table 5.8 were relatively low regarding the vitamins B1, B2, B3, B6, B7, C, and E, whereas, high for vitamin B9. Vitamin B5 values remained same and B12 value was higher than those of reported before (Maruyama et al., 1997) and lower than reported by another group [(Panahi et al., 2012) (Table 2.19, Table 2.20)]. Apparently, vitamin B12 was the biggest contributor by the means of daily nutritional requirements (Table 5.8). Regarding the daily advised amount of vitamin B12 by WHO, 2.4 µg, 5.57 g of the produced *Chlorella vulgaris* would be enough to provide daily bioavailable vitamin B12 intake.

Table 5.8. Analyzed vitamin content of *Chlorella vulgaris* and daily recommended values by WHO (WHO, 2001).

Macronutrients	**Control**		**RDV (WHO,FAO)**		**100g *Chlorella vulgaris* %RDV**	
Vitamins	***Chlorella vulgaris***	**Unit**	**Male***	**Female***	**Male***	**Female***
B1	75.623	µg/100g	1.2mg	1.1mg	6%	7%
B2	1.839	µg/100g	1.3mg	1.1mg	0%	0%
B3	237.549	µg/100g	16mg	14mg	1%	2%
B5	1928.888	µg/100g	5mg	5mg	39%	39%
B6	0.000	µg/100g	1.3mg	1.3mg	0%	0%
B7	29.192	µg/100g	30µg	30µg	97%	97%
B9	66.490	µg/100g	400µg	400µg	17%	17%
B12	43.095	µg/100g	2.4µg	2.4µg	1796%	1796%
C	4786.299	µg/100g	45mg	45mg	11%	11%
E	701.681	µg/100g	10mg	7.5mg	70%	70%

*Male=65 kg, Female=55 kg, 2000 kcal/day diet.

As discussed before, serving size should be determined according to the upper limits of nutrients considering the negative health effects caused by their overdose (Table 2.16, Table 2.17). For each nutrient, allowed amounts for consumption were calculated according to the upper limits stated by WHO and given in Table 5.9. The limiting nutrient is the one which allows the lowest amount to consume. The evaluation with current data has shown that the limiting nutrient was vitamin B12 (Table 5.9) and the upper limit for consumption of the produced biomass would be a total of 13.8 g for three meals (4.6 g for each meal). According to the report of WHO, upper limit of vitamin B12 is stated as 1.2-2 µg due to limited absorption of the human body rather than toxic effects (WHO, 2001). Also it was mentioned that absorption of the vitamin B12 in foods vary between 9-60% depending on the reference and foods (WHO, 2001). Despite the absence of reported side effects of taking 1000 µg of vitamin B12, it is advised to be avoided except the individuals with malabsorption (WHO, 2001). On the other hand, the foods with high vitamin B12 content such as mollusks, beef liver and salmon (Table 2.18) are consumed commonly which exceed the 2 µg limit of the vitamin B12 intake. For instance, serving size of the salmon is 113 g as recommended by USDA and contains 20.5 µg of vitamin B12 (USDA, 2016). Therefore, serving size of the *Chlorella vulgaris* can be regulated according to the safely consumed foods with high vitamin B12 contents and the second limiting factor. In the case of the biomass produced in this study, the second limiting nutrient was vitamin C

with 208.9 g, however, the other undetermined micronutrient contents should also be assayed before drawing any conclusions about the serving size.

Table 5.9. Upper limits for vitamin intake and maximum serving sizes of the raw biomass (WHO, 2001).

Micronutrients	**Control**		**Max value (WHO, FAO)**		**Allowed amount of *Chlorella vulgaris***	
Vitamins	***Chlorella vulgaris***	**Unit**	**Male***	**Female***	**Male***	**Female***
A	-	µg/100g	600µg/ day	500µg/ day	-	
B1	75.62	µg/100g	No toxicity		-	
B12	43.10	µg/100g	1.2-2µg/meal		**4.6g**	
B2	1.84	µg/100g	No toxicity		-	
B3	237.55	µg/100g	35 mg/day		12794.7g	
B5	1928.89	µg/100g	No toxicity		-	
B6	0.00	µg/100g	100mg/day		-	
B7	29.19	µg/100g	No toxicity		-	
B9	66.49	µg/100g	1000µg/day		1504.0g	
C	4786.30	µg/100g	1g/day		208.9g	
D	-	µg/100g	5µg/day		-	
E	701.68	µg/100g	1000mg/day		1426.5g	
K	-	µg/100g	No toxicity		-	
β-carotene	-	µg/100g	3592.8µg	2994µg	-	

*Male=65 kg, Female=55 kg, 2000 kcal/day diet.

The most important finding was, baking at 125°C for 15 or 35 min had no significant effect on the vitamin composition of *Chlorella vulgaris* except the vitamin B3 (Table 5.10) according to the t-test results (Table 5.11). The increment of the vitamin B3 content can be explained by the release of nutrients by the baking which causes the further disintegration of the cell walls (Lee et al., 2018). Although thermal stability of the carbohydrate, lipid and protein content is relatively possible, durability of the water-soluble vitamins were unexpected. Consequently, *Chlorella vulgaris* cookies baked by Gouveia et al. would have retained their nutritional value (Gouveia et al., 2007).

Table 5.10. Analyzed vitamin contents of the control (raw) and baked *Chlorella vulgaris.*

	Control (µg/100g)	Baked 15 min (µg/100g)	Baked 35 min (µg/100g)
B1	75.62	136.03	250.11
B2	1.84	11.36	26.25
B3	237.55*	1943.11*	2110.37*
B5	1928.89	1548.45	1438.75
B6	0.00	0.00	0.00
B7	29.19	20.80	20.30
B9	66.49	51.59	61.16
B12m	43.10	33.25	39.99
C	4786.30	3759.37	1710.27
E	701.68	489.85	-

*$P < 0.05$, t=0,04 (raw-baked 15), t=0,02 (raw-baked 35).

Table 5.11. t-test values.

Vitamin B1			**Vitamin B2**			**Vitamin B3**		
Control 1	0.46	**Baked 1**	**Control 1**	0.17	**Baked 1**	**Control 1**	0.04	**Baked 1**
Control 1	0.13	**Final 1**	**Control 1**	0.27	**Final 1**	**Control 1**	0.02	**Final 1**
Vitamin B5			**Vitamin B7**			**Vitamin B9**		
Control 1	0.28	**Baked 1**	**Control 1**	0.19	**Baked 1**	**Control 1**	0.62	**Baked 1**
Control 1	0.27	**Final 1**	**Control 1**	0.14	**Final 1**	**Control 1**	0.91	**Final 1**
Vitamin B12m			**Vitamin C**			**Vitamin E**		
Control 1	0.34	**Baked 1**	**Control 1**	0.24	**Baked 1**	**Control 1**	0.68	**Baked 1**
Control 1	0.78	**Final 1**	**Control 1**	0.28	**Final 1**	**Control 1**	0.16	**Final 1**

Considering the 13.8 g serving size, the produced biomass of *Chlorella vulgaris* was able to compensate one fifth of the daily protein requirements which is equal to the protein in a bowl of a Greek yoghurt (100g) (USDA, 2019) and provides all the daily vitamin B12 need, additionally (Table 5.12). Plus, durability of the vitamins during the baking process allows *Chlorella vulgaris* to be added to short-duration baking recipes with retaining its vitamin-rich status.

Table 5.12. Nutritional profile of the determined serving size 13.8g and recommended daily values by WHO and FAO (WHO, 2001; Mann et al., 2007; WHO 2007; WHO, 2010; FAO, 2010).

			RDV (WHO,FAO)		13.8g Chlorella vulgaris RDV%	
Nutrient	**Amount per 100g**	**Unit**	**Male***	**Female***	**Male***	**Female***
Carbohydrate	27	g	275-375g		0.1%	
Lipid	7	g	44-78g		1.6%	
Protein	61	g	43g	36g	19.6%	23.4%
B1	75.62	µg	1.2mg	1.1mg	0.9%	0.9%
B2	1.84	µg	1.3mg	1.1mg	0.0%	0.0%
B3	237.55	µg	16mg	14mg	0.2%	0.2%
B5	1928.89	µg	5mg	5mg	5.3%	5.3%
B6	0	µg	1.3mg	1.3mg	0.0%	0.0%
B7	29.19	µg	30µg	30µg	13.4%	13.4%
B9	66.49	µg	400µg	400µg	2.3%	2.3%
B12m	43.10	µg	2.4µg	2.4µg	247.8%	247.8%
C	4786.30	µg	45mg	45mg	1.5%	1.5%
E	701.68	µg	10mg	7.5mg	1.0%	1.3%

*Male=65 kg, Female=55 kg, 2000 kcal/day diet.

6. CONCLUSIONS AND RECOMMENDATIONS

In conclusion, the data that has been collected and analyzed in this study has shown that, usage of *Chlorella vulgaris* as a functional food ingredient has attention-worthy environmental and nutritional advantages. Microalgae cultivation contributes to environmental conservation by sequestering CO_2 without usage of fertile lands and pesticides. Currently, red meat is the primary source of protein which occupies majority of agricultural lands and consumes excess water. Microalgae production is much more water efficient (over 500% per kg) compared to meat production. Depending on the cultivation conditions, microalgae are nutritious functional food ingredients that offer to support both animal and plant based diets.

Specifically, this study has shown that, *Chlorella vulgaris* strain CCAP 211/11b had high protein and bioavailable vitamin B12 content with relatively lower amounts of vitamin B1, B2, B3, B6, B7, C, E. This nutritional profile was more suitable for supporting plant based diets. Serving size of this biomass was as low as 13.8 g which is enough to provide daily vitamin B12 needs by consuming 4.6 g for three meals. 13.8 g biomass contains satisfactory 8.4 g of protein which is equivalent of one and a half large eggs approximately (USDA, 2019). In addition, water consumption of the biomass production was 0.66 L/g which makes one liter per one gram protein whereas one gram of meat protein production requires 75 liters of water (USDA, 2019; Mekonnen and Hoekstra, 2011).

The second major finding was baking at 125°C for either 15 or 35 min was found to cause no significant degrading effect on the nutritional value of the *Chlorella vulgaris* which gives the flexibility to add biomass into cooked recipes. However, the full nutritional content is required to be further analyzed prior to consumption to determine limitations for preventing negative health effects. Further research can be conducted to explore:

- Whole nutritional profile with analytical tools since this study comprised of analysis of water-soluble vitamins.
- Glycemic index of *Chlorella vulgaris* since there is no information in academic literature.
- Degradation temperatures and durations of *Chlorella vulgaris* vitamins for additional cooking methods, such as microwave, etc.

Conclusively, the findings in this study suggested that *Chlorella vulgaris* is a significant potential source for vegan-vegetarian protein and vitamin B12 with high opportunity to be added to several baking recipes.

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APPENDIX A: MRM TABLE and RETENTION TIMES OF VITAMIN STANDARDS

Vitamin	Q1 (Da)	Q3 (Da)	RT (min)	DP (volts)	EP (Volts)	CE (Volts)	CXP (Volts)
B1 1	265.100	122.000	0.64	6.000	10.000	23.000	6.000
B1 2	265.100	80.900	0.64	6.000	10.000	43.000	6.000
B2 1	376.900	243.000	2.73	111.000	10.000	33.000	8.000
B2 2	376.900	172.000	2.73	111.000	10.000	51.000	10.000
B3 1	123.832	79.900	0.88	61.000	10.000	29.000	6.000
B3 2	123.832	77.900	0.88	61.000	10.000	31.000	8.000
B5 1	220.100	98.000	1.00	61.000	10.000	20.000	8.000
B5 2	220.100	90.000	1.00	61.000	10.000	20.000	6.000
B6 1	170.000	134.000	0.70	16.000	10.000	19.000	8.000
B6 2	170.000	152.000	0.70	16.000	10.000	19.000	6.000
B7 1	245.100	226.900	2.64	51.000	10.000	21.000	10.000
B7 2	245.100	98.900	2.64	51.000	10.000	35.000	10.000
B9 1	442.200	294.900	2.65	71.000	10.000	23.000	12.000
B9 2	442.200	176.000	2.65	71.000	10.000	57.000	12.000
B12m 1	673.000	665.000	2.77	50.000	10.000	25.000	4.000
B12m 2	673.000	971.500	2.77	50.000	10.000	38.000	14.000
B12c 1	678.400	147.000	2.72	91.000	10.000	71.000	12.000
B12c 2	678.400	358.900	2.72	91.000	10.000	33.000	16.000
C 1	172.900	112.500	0.80	-65.000	10.000	-14.000	-9.000
C 2	172.900	142.800	0.80	-65.000	10.000	-14.000	-7.000
E 1	431.400	165.100	6.01	120.000	9.000	40.000	15.000
E 2	431.400	137.100	6.01	120.000	9.000	68.000	19.000

APPENDIX B: OPTICAL DENSITY DATA of 6 *Chlorella vulgaris* BATCHES

Optical Density C1								
Days	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Flask 1	0.400	0.667	1.126	1.488	1.608	1.847	1.985	1.605
Flask 2	0.367	0.595	0.977	1.246	1.445	1.654	1.977	2.137
Flask 3	0.373	0.567	1.018	1.392	1.656	1.871	1.833	1.818
OD average	0.380	0.610	1.040	1.375	1.570	1.791	1.932	1.853
Std. dev.	0.018	0.052	0.077	0.122	0.111	0.119	0.086	0.268

Optical Density C2								
Days	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Flask 1	0.116	0.351	0.582	0.963	1.572	2.136	2.172	2.215
Flask 2	0.127	0.345	0.508	0.821	1.150	1.333	1.376	1.415
Flask 3	0.131	0.350	0.575	0.894	1.420	1.735	2.057	2.151
OD average	0.125	0.349	0.555	0.893	1.381	1.735	1.868	1.927
Std. dev.	0.008	0.003	0.041	0.071	0.214	0.402	0.430	0.445

Optical Density C3								
Days	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Flask 1	0.202	0.51	0.907	1.176	1.584	1.719	1.771	1.753
Flask 2	0.181	0.481	0.839	1.061	1.399	1.623	1.778	1.966
Flask 3	0.104	0.18	0.402	0.515	0.691	0.859	1.114	1.281
OD average	0.162	0.390	0.716	0.917	1.225	1.400	1.554	1.667
Std. dev.	0.052	0.183	0.274	0.353	0.471	0.471	0.381	0.351

Optical Density B1								
Days	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Flask 1	0.507	0.842	1.357	1.605	1.847	2.043	2.239	2.262
Flask 2	0.422	0.693	1.176	1.440	1.702	1.713	1.771	1.807
Flask 3	0.482	0.845	1.402	1.640	1.880	1.896	1.926	1.927
OD average	0.470	0.793	1.312	1.562	1.810	1.884	1.979	1.999
Std. dev.	0.044	0.087	0.120	0.107	0.095	0.165	0.238	0.236

Optical Density B2								
Days	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Flask 1	0.339	0.650	1.036	1.243	1.546	1.646	1.710	1.699
Flask 2	0.291	0.674	0.972	1.164	1.362	1.698	1.827	2.088
Flask 3	0.321	0.746	1.126	1.415	1.660	1.831	1.795	1.753
OD average	0.317	0.690	1.045	1.274	1.523	1.725	1.777	1.847
Std. dev.	0.024	0.050	0.077	0.128	0.150	0.095	0.060	0.211

Optical Density B3								
Days	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Flask 1	0.177	0.703	1.228	1.34	1.623	1.797	1.954	2.041
Flask 2	0.181	0.693	1.157	1.269	1.52	1.692	1.901	1.942
Flask 3	0.176	0.65	1.194	1.295	1.55	1.703	1.861	1.536
OD average	0.178	0.682	1.193	1.301	1.564	1.731	1.905	1.840
Std. dev.	0.003	0.028	0.036	0.036	0.053	0.058	0.047	0.268

APPENDIX C: CELL COUNT DATA of 6 *Chlorella vulgaris* BATCHES

Cell count C1					
Days	**0**	**2**	**4**	**6**	**8**
Flask 1	5.00E+06	2.86E+07	7.19E+07	1.09E+08	3.00E+08
Flask 2	5.00E+06	2.51E+07	5.69E+07	7.88E+07	4.75E+08
Flask 3	5.00E+06	2.14E+07	7.06E+07	1.06E+08	3.19E+08
CC average	5.00E+06	2.50E+07	6.65E+07	9.79E+07	3.65E+08
Std. dev.	0.00E+00	3.63E+06	8.32E+06	1.66E+07	9.61E+07

Cell count C2					
Days	**0**	**2**	**4**	**6**	**8**
Flask 1	5.00E+06	1.25E+07	4.13E+07	1.08E+08	9.88E+07
Flask 2	5.00E+06	1.38E+07	3.31E+07	7.12E+07	6.00E+07
Flask 3	5.00E+06	2.00E+07	3.13E+07	8.62E+07	9.88E+07
CC average	5.00E+06	1.54E+07	3.52E+07	8.83E+07	8.59E+07
Std. dev.	0.00E+00	4.02E+06	5.32E+06	1.83E+07	2.24E+07

Cell count C3					
Days	**0**	**2**	**4**	**6**	**8**
Flask 1	5.00E+06	1.44E+07	4.00E+07	6.38E+07	8.76E+07
Flask 2	5.00E+06	1.88E+07	3.31E+07	5.76E+07	7.62E+07
Flask 3	5.00E+06	6.88E+06	1.81E+07	4.12E+07	5.26E+07
CC average	5.00E+06	1.33E+07	3.04E+07	5.42E+07	7.21E+07
Std. dev.	0.00E+00	6.01E+06	1.12E+07	1.17E+07	1.79E+07

Cell count B1					
Days	**0**	**2**	**4**	**6**	**8**
Flask 1	5.00E+06	5.00E+07	5.44E+07	5.88E+07	1.14E+08
Flask 2	5.00E+06	3.00E+07	5.44E+07	7.88E+07	9.63E+07
Flask 3	5.00E+06	5.44E+07	7.28E+07	9.12E+07	1.09E+08
CC average	5.00E+06	4.48E+07	6.05E+07	7.63E+07	1.06E+08
Std. dev.	0.00E+00	1.30E+07	1.06E+07	1.63E+07	9.01E+06

Cell count B2					
Days	**0**	**2**	**4**	**6**	**8**
Flask 1	5.00E+06	3.38E+07	7.13E+07	7.50E+07	1.00E+08
Flask 2	5.00E+06	2.88E+07	5.56E+07	7.13E+07	1.39E+08
Flask 3	5.00E+06	3.75E+07	9.25E+07	9.50E+07	9.38E+07
CC average	5.00E+06	3.33E+07	7.31E+07	8.04E+07	1.11E+08
Std. dev.	0.00E+00	4.39E+06	1.85E+07	1.28E+07	2.44E+07

Cell count B3					
Days	**0**	**2**	**4**	**6**	**8**
Flask 1	5.00E+06	2.00E+07	6.63E+07	9.62E+07	1.08E+08
Flask 2	5.00E+06	2.13E+07	4.88E+07	5.26E+07	7.12E+07
Flask 3	5.00E+06	2.38E+07	5.63E+07	7.50E+07	5.50E+07
CC average	5.00E+06	2.17E+07	5.71E+07	7.46E+07	7.79E+07
Std. dev.	0.00E+00	1.91E+06	8.78E+06	2.18E+07	2.69E+07

APPENDIX D: pH DATA of 6 *Chlorella vulgaris* BATCHES

pH C1								
Days	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Flask 1	7.138	7.407	8.703	8.978	9.434	8.955	10.636	10.266
Flask 2	7.076	7.328	8.106	9.061	9.596	9.586	9.007	8.757
Flask 3	7.069	7.355	8.281	9.082	9.107	9.716	10.781	10.763
pH average	7.094	7.363	8.363	9.040	9.379	9.419	10.141	9.929
Std. dev.	0.038	0.040	0.307	0.055	0.249	0.407	0.985	1.045

pH C2								
Days	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Flask 1	6.80	7.13	7.49	7.99	8.93	9.06	10.90	10.81
Flask 2	7.00	7.26	7.51	7.92	9.70	10.26	10.71	9.99
Flask 3	6.90	7.18	7.48	7.98	9.20	9.90	9.10	10.99
pH average	6.90	7.19	7.49	7.96	9.28	9.74	10.24	10.59
Std. dev.	0.10	0.06	0.02	0.04	0.39	0.62	0.99	0.53

pH C3								
Days	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Flask 1	6.94	7.46	8.36	9.14	9.73	10.78	10.67	10.62
Flask 2	6.93	7.41	8.34	8.81	9.90	10.16	9.33	9.83
Flask 3	6.78	6.94	7.32	7.59	7.86	8.30	9.38	9.50
pH average	6.88	7.27	8.01	8.51	9.16	9.75	9.79	9.98
Std. dev.	0.09	0.28	0.59	0.82	1.13	1.29	0.76	0.58

pH B1								
Days	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Flask 1	7.158	7.646	9.403	9.445	9.490	8.714	9.699	9.338
Flask 2	7.091	7.428	8.330	8.700	9.068	10.483	10.489	9.016
Flask 3	7.167	7.631	9.387	10.010	10.631	10.449	10.576	9.417
pH average	7.139	7.568	9.040	9.385	9.730	9.882	10.255	9.257
Std. dev.	0.042	0.122	0.615	0.657	0.809	1.012	0.483	0.212

pH B2								
Days	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Flask 1	7.005	7.479	8.511	9.221	8.517	9.625	10.787	10.761
Flask 2	6.995	7.612	8.286	9.286	8.863	9.550	9.056	8.788
Flask 3	7.002	7.710	8.496	8.900	9.339	10.517	10.818	10.819
pH average	7.001	7.600	8.431	9.136	8.906	9.897	10.220	10.123
Std. dev.	0.005	0.116	0.126	0.207	0.413	0.538	1.008	1.156

pH B3								
Days	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Flask 1	7.115	8.194	7.974	8.421	9.611	9.559	8.851	9.034
Flask 2	6.897	7.805	8.524	8.391	8.805	8.842	9.049	8.633
Flask 3	6.953	7.705	9.204	8.426	8.717	8.649	8.875	9.168
pH average	6.988	7.901	8.567	8.413	9.044	9.017	8.925	8.945
Std. dev.	0.113	0.258	0.616	0.019	0.493	0.479	0.108	0.278

APPENDIX E: CALIBRATION DATA FOR VITAMINS

B1 Area	INT B12c Area	Vitamin/B12c	Vitamin Concentration (µg/L)
8.81E+04	9.96E+05	0.09	1
3.62E+05	9.58E+05	0.38	2.5
6.97E+05	1.03E+06	0.68	5
1.85E+06	9.79E+05	1.89	10
4.92E+06	1.04E+06	4.75	25
8.86E+06	1.04E+06	8.51	50
2.26E+07	1.04E+06	21.73	100
4.51E+07	1.01E+06	44.48	250
6.56E+07	9.95E+05	65.91	500

B2 Area	INT B12c Area	Vitamin/B12c	Vitamin Concentration (µg/L)
1.51E+04	9.96E+05	0.02	1
3.14E+04	9.58E+05	0.03	2.5
7.04E+04	1.03E+06	0.07	5
1.31E+05	9.79E+05	0.13	10
3.53E+05	1.04E+06	0.34	25
7.03E+05	1.04E+06	0.67	50
1.44E+06	1.04E+06	1.38	100
3.78E+06	1.01E+06	3.73	250
7.90E+06	9.95E+05	7.94	500

B3 Area	INT B12c Area	Vitamin/B12c	Vitamin Concentration (µg/L)
2.92E+04	9.96E+05	0.03	1
7.28E+04	9.58E+05	0.08	2.5
1.44E+05	1.03E+06	0.14	5
2.98E+05	9.79E+05	0.30	10
8.15E+05	1.04E+06	0.79	25
1.66E+06	1.04E+06	1.59	50
3.51E+06	1.04E+06	3.38	100
9.13E+06	1.01E+06	9.01	250
1.89E+07	9.95E+05	18.98	500

B5 Area	INT B12c Area	Vitamin/B12c	Vitamin Concentration (µg/L)
1.71E+04	1.04E+06	0.02	50
3.55E+04	1.04E+06	0.03	100
8.93E+04	1.01E+06	0.09	250
1.92E+05	9.95E+05	0.19	500

B6 Area	INT B12c Area	Vitamin/B12c	Vitamin Concentration (µg/L)
1.55E+05	9.96E+05	0.16	1
3.99E+05	9.58E+05	0.42	2.5
8.02E+05	1.03E+06	0.78	5
1.73E+06	9.79E+05	1.76	10
4.43E+06	1.04E+06	4.28	25
8.16E+06	1.04E+06	7.83	50
1.45E+07	1.04E+06	13.95	100
2.64E+07	1.01E+06	26.07	250
3.94E+07	9.95E+05	39.59	500

B7 Area	INT B12c Area	Vitamin/B12c	Vitamin Concentration (µg/L)
5.79E+04	9.96E+05	0.06	1
1.33E+05	9.58E+05	0.14	2.5
2.65E+05	1.03E+06	0.26	5
5.37E+05	9.79E+05	0.55	10
1.44E+06	1.04E+06	1.39	25
2.91E+06	1.04E+06	2.79	50
6.18E+06	1.04E+06	5.94	100
1.61E+07	1.01E+06	15.87	250
3.04E+07	9.95E+05	30.52	500

B9 Area	INT B12c Area	Vitamin/B12c	Vitamin Concentration (µg/L)
1.59E+03	9.96E+05	0.002	1
2.74E+03	9.58E+05	0.003	2.5
7.43E+03	1.03E+06	0.007	5
1.47E+04	9.79E+05	0.015	10
3.91E+04	1.04E+06	0.038	25
1.01E+05	1.04E+06	0.097	50
2.03E+05	1.04E+06	0.195	100
6.31E+05	1.01E+06	0.622	250
1.30E+06	9.95E+05	1.302	500

B12m Area	INT B12c Area	Vitamin/B12c	Vitamin Concentration (µg/L)
3.18E+03	9.96E+05	0.003	1
8.63E+03	9.58E+05	0.009	2.5
1.47E+04	1.03E+06	0.014	5
2.91E+04	9.79E+05	0.030	10
8.36E+04	1.04E+06	0.081	25
1.78E+05	1.04E+06	0.171	50
3.90E+05	1.04E+06	0.375	100
9.95E+05	1.01E+06	0.982	250
1.69E+06	9.95E+05	1.701	500

C Area	INT B12c Area	Vitamin/B12c	Vitamin Concentration (µg/L)
5.08E+04	1.01E+06	0.05	250
1.12E+05	9.95E+05	0.11	500
1.60E+05	9.35E+05	0.17	750
1.75E+05	8.64E+05	0.20	1000

E Area	INT B12c Area	Vitamin/B12c	Vitamin Concentration (µg/L)
1.22E+04	9.96E+05	0.01	1
3.17E+04	9.58E+05	0.03	2.5
6.42E+04	1.03E+06	0.06	5
1.34E+05	9.79E+05	0.14	10
3.47E+05	1.04E+06	0.34	25
6.62E+05	1.04E+06	0.64	50
1.37E+06	1.04E+06	1.32	100
3.10E+06	1.01E+06	3.06	250
5.47E+06	9.95E+05	5.49	500