KAUNAS UNIVERSITY OF TECHNOLOGY
FACULTY OF CHEMICAL TECHNOLOGY

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Optimization of extraction processes and development of biorefinery schemes for processing wild cyanobacteria into high-added value products

Master's Final Degree Project

Supervisor
Dr. Michail Syrpas

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Food Science and Safety (code 621E40001)

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KAUNAS, 2018

KAUNO TECHNOLOGIJOS UNIVERSITETAS
CHEMINĖS TECHNOLOGIJOS FAKULTETAS

Ekstrakcijos procesų optimizavimas ir biorafinavimo schemų sukūrimas perdirbant melsvabakteres į aukštos pridėtinės vertės produktus

Baigiamasis magistro projektas
Maisto mokslas ir sauga (kodas 621E40001)

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KAUNAS, 2018

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DECLARATION OF ACADEMIC INTEGRITY

5 June 2018
Kaunas

I confirm that the final project of mine, **Jolita Bukauskaitė**, on the topic „ **Optimization of extraction processes and development of biorefinery schemes for processing wild cyanobacteria into high-added value products** “ is written completely by myself; all the provided data and research results are correct and have been obtained honestly. None of the parts of this thesis have been plagiarised from any printed, Internet-based or otherwise recorded sources. All direct and indirect quotations from external resources are indicated in the list of references. No monetary funds (unless required by law) have been paid to anyone for any contribution to this project.

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Study field and area (study field group): Technological Sciences, Food Technology.

Keywords: Cyanobacteria; Supercritical CO_2 extraction; Phycobiliproteins; RSM optimization; Biorefinery.

Kaunas, 2018. 95 pages.

SUMMARY

Cyanobacteria are photosynthetic microorganisms widely distributed around the world. In water, cyanobacteria can live in large colonies and create water blooms. Over the last decades, the presence of wild cyanobacterial blooms has also been reported in the Curonian Lagoon. Recent reports indicate that priority should be given to the removal of wild cyanobacterial blooms from the Curonian Lagoon as a management measure. In this study, the utilization of wild cyanobacteria as a potential source of high added products is reported.

Lipophilic products from wild cyanobacteria was isolated from Curonian Lagoon by supercritical carbon dioxide extraction (SFE-CO_2) and the yield, under optimal conditions, was compared with conventional Soxhlet extraction. Under optimal conditions (42.5 MPa, 55 ºC and 120 min), SFE-CO_2 yielded 4.43 g/100 g DW of non-polar extract, showing 20% higher extraction efficiency at 3-fold lower extraction time as compared to the conventional Soxhlet extraction. The SFE-CO_2 extract under optimal conditions was also evaluated by determining fatty acids, tocopherols and *in vitro* antioxidant activity. Results showed that it was rich in α-linolenic acid (27% of total fatty acids) and α-tocopherol (293 μg/g extract).

Phycobiliproteins from wild cyanobacteria was isolated by several traditional extraction methods, of which the most effective was homogenization (one cycle, 30 min, 30 ºC), but after combining traditional methods with ultrasound assisted extraction (UAE) the freeze-thaw extraction with liquid nitrogen combined with UAE had the highest yield of phycobiliproteins (139.28 mg/g DW) under optimal conditions (one cycle of freeze-thaw and 99 % of amplitude, 9.39 min of UAE). The antioxidant activity of phycobiliproteins extracts was evaluated by *in vitro* methods.

Central composite design (CCD) and response surface methodology (RSM) were employed to optimize SFE-CO_2, phycobiliproteins extractions parameters (temperature, pressure, amplitude and time) to obtain high yield extracts.

Biorefinery was performed by removal of lipophilic fraction using SFE-CO_2 or solid liquid extraction (SLE) with hexane, and by removal of phycobiliproteins using homogenization combined with UAE, and then performing accelerated solvent extraction (ASE) to extract the remaining materials by polarity. The extracts yield after ASE from fresh wild cyanobacterial biomass was 5.9 and 2.8 times higher than extracts yield after ASE and after SFE with phycobiliproteins removal and extracts yield after ASE and after SLE with phycobiliproteins removal, respectively. The ASE and SLE extracts from fresh cyanobacterial biomass was also evaluated by determining fatty acids and all ASE extracts was evaluated by determining *in vitro* antioxidant activity. Results of fatty acids detection showed that the most abundant fatty acid was α-linolenic acid: 225.86 mg/g in ASE extract with hexane at 55 ºC (32 %), 260.85 mg/g in ASE extract with hexane at 70 ºC (34 %) and 430 mg/g in SLE extract (36 %). All these extracts showed higher yield of α-Linolenic acid comparing with SFE-CO_2 or Soxhlet extracts.

Thus, SFE-CO_2, phycobiliprotein extractions and ASE methods could be utilized to valorize wild cyanobacteria into high-added value products with various industrial applications.

Jolita Bukauskaitė. Ekstrakcijos procesų optimizavimas ir biorafinavimo schemų sukūrimas perdirbant melsvabakteres į aukštos pridėtinės vertės produktus. Magistro baigiamasis projektas / vadovas dr. Michail Syrpas; Kauno technologijos universitetas, Cheminės technologijos fakultetas.

Studijų kryptis ir sritis (studijų krypčių grupė): Technologijų mokslai, Maisto technologijos.

Reikšminiai žodžiai: Melsvabakterės, superkritinė CO_2 ekstrakcija, Fikobiliproteinai, RSM optimizacija, bioperdirbimas.

Kaunas, 2018. 95 puslapiai.

SANTRAUKA

Meslvabakterės yra fotosintetinantys mikroorganizmai plačiai paplitę visame pasaulyje. Vandenyje melsvabakterės gali gyventi, sudarydamos kolonijas, ir žydėti. Per pastaruosius dešimtmečius Kuršių mariose buvo pastebėtas laukinių melsvabakterių žydėjimas. Naujausios ataskaitos rodo, kad šį žydėjimą pasirinkta valdyti tiesiog pašalinant melsvabakteres iš telkinio. Šiame tyrime nagrinėjamas melsvabakterių, kaip galimo didelės pridėtinės vertės produktų šaltinio, panaudojimas.

Lipidų frakcija iš melsvabakterių, augusių Kuršių mariose, išgauta superkritinės anglies dvideginio ekstrakcijos (SFE-CO_2) metodu, o ekstrakto išeiga, gauta optimaliomis sąlygomis (42.5 MPa, 55 ºC and 120 min) buvo palyginta su tradicinio soksleto metodo metu gautu lipidų frakcijos kiekiu. Superkritinės ekstrakcijos metu gauto ekstrakto kiekis (4.43 g/100 g sausos medžiagos) buvo 20 proc. didesnis už ekstrakto kiekį išgautą soksleto metu, o metodo laikas buvo net tris kartus trumpesnis. SFE-CO_2 ekstraktas, gautas optimaliomis sąlygomis taip pat buvo įvertintas nustatant riebalų rūgščių ir tokoferolių kiekį bei antioksidantų aktyvumą. Tyrimai parodė, jog ekstrakte daugiausiai yra α-linoleno riebalų rūgšties (27 proc. bendro riebalų rūgščių kiekio) ir α-tokoferolio (293 μg/g ekstrakto).

Fikobiliproteinai iš laukinių melsvabakterių buvo išgauti keliais tradiciniais metodais, iš kurių efektyviausias buvo homogenizavimas (vienas ciklas, 30 min, 30 ºC), tačiau po tradicinių metodų taikymo kartu su ultragarso ekstrakcija (UAE) išryškėjo šaldymo-atšildymo ekstrakcijos, naudojant skystą azotą, kartu su ultragarsu pranašumas – optimaliomis sąlygomis (vienas ciklas šaldymo-atšildymo ekstrakcijos, 99 proc. amplitudė ir 9,39 minutės ultragarso) buvo gautas

didžiausias fikobiliproteinų kiekis (139.28 mg/g sausos medžiagos). Taip pat fikobiliproteinų ekstraktai buvo įvertinti antioksidantų nustatymo metodais.

Centrinio taško dizaino (CCD) ir atsako (RSM) metodikos buvo pasitelktos nustatant SFE-CO_2 ir fikobiliproteinų ekstrakcijų optimalias sąlygas.

Biorafinavimas buvo atliktas pašalinus iš laukinių melsvabakterių lipofilinę frakciją superkritinės arba paprastosios ekstrakcijos (SLE) metodais, pašalinus fikobiliproteinus homogenizatoriumi ir ultragarsu bei iš likusios biomasės atlikus pagreitintą skysčių ekstrakciją (ASE). Ekstraktų kiekis, po pagreitintos skysčių ekstrakcijos iš šviežios melsvadumblių biomasės, ir ekstraktų kiekiai, po SFE ir SLE bei fikobiliproteinų pašalinimo, skyrėsi atitinkamai 5,9 ir 2,8 kartus. Ekstraktai iš šviežios melsvadumblių biomasės po ASE ir SLE buvo įvertinti atlikus riebalų rūgščių nustatymą ir visuose ASE ekstraktuose buvo nustatytas antioksidantų aktyvumas. Riebalų rūgščių nustatymas parodė, kad daugiausiai buvo aptikta α-linoleno riebalų rūgšties: 225.86 mg/g ASE ekstrakto su heksanu 55 ºC temperatūroje (32 %), 260.85 mg/g ASE ekstrakte su heksanu 70 ºC temperatūroje (34 %) ir 430 mg/g SLE ekstrakte (36 %). Visuose šiuose ekstraktuose α-linoleno riebalų rūgšties buvo aptikta daugiau nei SFE-CO_2 ar soksleto ekstraktuose.

Taigi, tyrime pasirinkti metodai - superkritinė anglies dvideginio ekstrakcija, fikobiliproteinų ekstrakcijos ir pagreitinta skysčių ekstrakcija gali būti panaudotos aukštos pridėtinėsvertės produktų išgavimui iš melsvabakterių.

INTRODUCTION

Cyanobacteria are photosynthetic microorganisms that can be found in almost every terrestrial and aquatic habitat. In heavily eutrophicated water bodies, cyanobacteria can can create extensive surface water blooms. This kind of algal blooms has also been reported in the Curonian lagoon and the algal removal was chosen for their management. Cyanobacterial biomass is rich in high added value products – fatty acids, tocopherols, phycobiliproteins, several of which possess antioxidant activity. Cyanobacteria can be used as a feedstock, for biofuel production, cosmetics, as biofertilizers. Some cyanobacteria such as *Spirulina* can be used in medicine.

The aim of the research was determined due to the constant need for higher added value components and nutraceuticals and the interest in green extraction technologies utilizing algal feedstock. The utilization of a wild cyanobacterial bloom from Curonian Lagoon was used as a source for recovery of lipophilic and protein products. This cyanobacterial biomass coud be discussed as a source of higher added value compounds with food, biotechnological, pharmaceutical or agrochemical application. It is expected that such systematic approach may provide a promising platform in developing industrial scale clean production processes for converting wild cyanobacteria products into novel bioactive ingredients.

The main aim of this work – to optimize extraction processes and to develop biorefinery schemes for processing wild cyanobacteria, isolated from Curonian Lagoon, into high-added value products.

The following tasks were set to achieve this aim:

1. To determine chemical composition (nitrogen content, lipid content, mineral content and moisture) and *in vitro* antioxidant activity of wild cyanobacteria biomass;
2. To optimize isolation of lipophilic products from wild cyanobacteria by supercritical carbon dioxide extraction and to determine recovery of SFE-CO_2 comparing with Soxhlet extraction;
3. To evaluate SFE-CO_2 (obtained under optimal conditions) and soxhlet extracts by determination of their fatty acids profile, tocopherol content and *in vitro* antioxidant activity;
4. To evaluate the efficiency of conventional techniques and optimize ultrasound assisted technuqes for isolation of phycobiliproteins from wild cyanobacteria and determine the *in vitro* antioxidant activity of phycobiliprotein extracts under optimal conditions;
5. To develop consecutive multistep fractionation scheme using accelerated solvent extraction with different solvents of increasing polarity, different extraction temperatures. Select the most effective ASE conditions for isolation of polar extracts and valorize wild cyanobacteria biomass after lipophilic fraction and phycobiliproteins removal;

6. To evaluate the fatty acid profile of non-polar extracts obtained by solid-liquid extraction (Hexane) and ASE (Hexane) and conmpare their efficiency with SFE-CO2 extract under optimal conditions;
7. To determine *in vitro* antioxidant activity of ASE polar and non-polar extracts.
8. To evaluate nonpolar and polar fractions obtained after biorefinery by determination of preliminary phytochemical characterization of compounds by means of UPLC-TOF-MS.

1. LITERATURE REVIEW

1.1. Cyanobacteria

Cyanobacteria, also known as blue-green algae, represent a large group of photosynthetic bacteria, some of which are nitrogen fixing, that live in a wide variety of places freely or in symbiotic relationship with plants or fungi [1]. They can be found in unicellular or filamentous form and may also form colonies [2],[3].

Cyanobacteria can be found in lots of different places – oceans, fresh water, damp soil. Antartic rocks or almost every endolithic ecosystem [4]. Blue-green algae can also grow naturally in ponds and lakes where waters are calm and have less mixing. Aquatic cyanobacteria are known for their blooms (Figure 1) that can form in both freshwater and marine enviroments. These blooms can have the presence of blue-green paint or scum, depending on the species can be toxic and lead to the closure of recreational waters [5].

Figure 1. Cyanobacteria blooms [6]

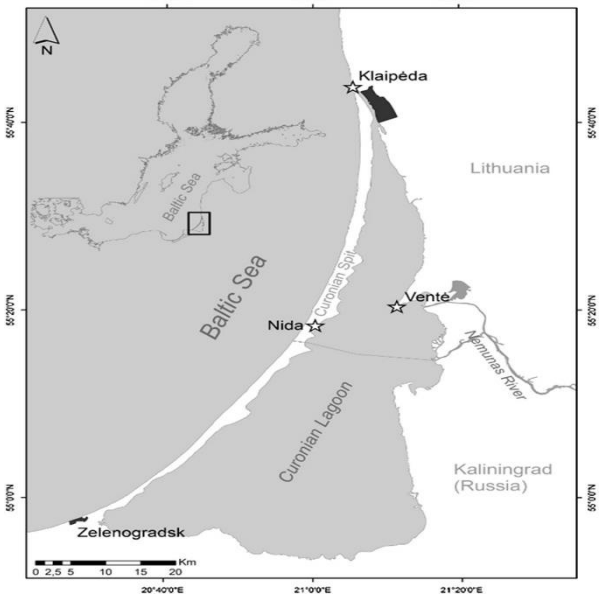


Figure 2. Curonian Lagoon in the map [7]

Cyanobacteria have been used since ancient times as source of food, because of their high content of bioactive and high-added value compounds [8],[9]. The high protein content of some blue-green algae species is one of the reasons to consider them as nontraditional source of proteins. Lipids and fatty acids from microalgae have gained particular interest because of the health benefits related to its usage. Batista et al. reported that *C. green* and *S. maxima* showed high protein (38 – 44 %.) and low fat (4 – 5 %.) content, *C. vulgaris* and *H. pluvialis* presented higher carotenoid content, higher

fat content, *D. vilkianum* and *I. galbana* showed high protein (38 – 40 %.) and fat (18 – 24 %.) content with PUFA‘s ω3, mainly EPA and DHA, and thats why they called microalgae the most promising food and functional food products sources [10]. Microalgae, including cyanobacteria, can be used as feedstock or for biofluel production [11],[12]. Various high-added compounds can be extracted from the cyanobacteria biomass – lipids, fatty acids, tocoferols, phycobiliproteins, carotenoids, antioxidants [8].

Besides their capacity to produce bioactive compounds, cyanobacteria attracted water management authorities attention due to their ability to form harmful algal blooms (HAB). This process can create dead zones in water, generate compounds that can negatively effect water quality and in extreme cases be lethal to humans or animals [13]. Toxic algal blooms can raise the health issues, but non-toxic blooms are also important, because they can effect environment and local economies. HAB in the Baltic sea created a lot environment and socioeconomic concerns [14],[15]. Massive algal blomms formation in this reagion is determined by several dominant species – *Aphanizomenon* sp., *Nodularia* sp. and *Dolichospermum* sp. [16]. HAB formation in the Curonian Lagoon (Figure 2) and the risk of exposure of concentrated cyanotoxins by the tourists, local comunities and animals has been confirmed [17]. The Curonian Lagoon and Curonian Spit, which separates Lagoon from the Baltic sea, listed as a UNESCO world heritage site [18].

Cyanobacteria ability to produce toxins and influence financial sectors of local economies in various countries have prioritized HAB management [13]. The removal of cyanobacteria has been suggested as an important management measure [16]. Interestingly, harvesting of *Aphanizomenon flos-aquae* blooms from Klamath Lake has already found commercial applications as food supplements [19].

1.2. Bioactive and high-added value compounds from cyanobacteria

1.2.1. Lipids

Lipids are hydrophobic or amphipathic molecules that may emerge by carbanion based condensations of thioesters and carbonation based condensations of isoprene units [20]. The main components of the microalgae lipid fraction are fatty acids, waxes, sterols, ketones, phospholipids, tocopherols and pigments as carotenoids [21].

1.2.1.1. Fatty acids

Microalgal fatty acids can be assorted in three groups: saturated (SFAs), mono-unsaturated and polyunsaturated (PUFAs). Polyunsaturated fats have more than one unsaturated carbon bond in

the molecule. PUFAs can lower risk of heart diseases and stroke because help to reduce bad cholesterol amount in blood. Commercially produced microalgal PUFAs are eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), oleic acid, linolenic acid and palmitic acid [21]. Lipid production in microalgae depends on the species and culture conditions such as nutients, light intensity periods, pH, temperature or association with other microorganisms [8].

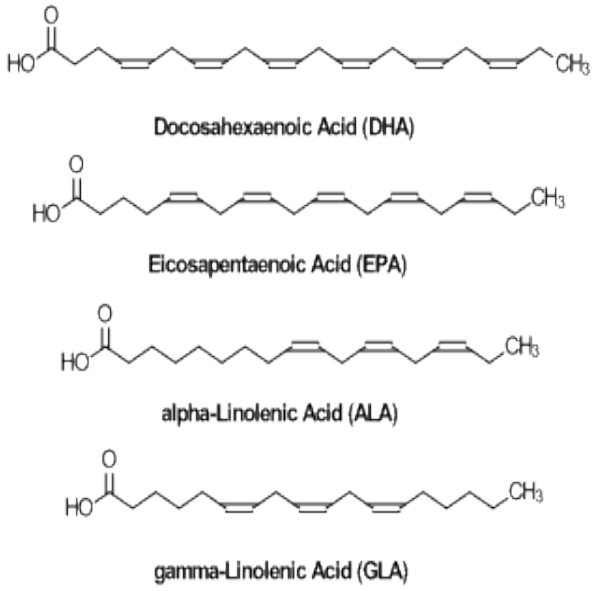


Figure 3. Chemical structure of polyunsaturated fatty acids [22].

Cyanobacteria are known to produce large amounts of α-linolenic (ALA) and γ-linolenic (GLA) acids (Figure 3) which have various applications. Wang et al. reported that the main fatty acids, extracted with supercritical carbon dioxide of *Spirulina platensis* were palmitic acid (35.3 %.), linoleic acid (20.6 %.) and linolenic acid (21.7 %.) [23]. Another research showed that *Arthrospira platensis* supplementation into rabbits diet increased GLA content of rabbit meat [24]. The γ-linolenic acid has an important role in human metabolism because of schizophrenis [25], dermatitis [26], sclerosis, diabetes [27], rheumatoid arthritis [28] treatments [29]. Guedes at al. study showed that Eustigmatophyceae members were the best producers of ALA, followed by Chlorophyceae, Prymnesiophyceae and Rhodophyceae [30]. Consumption of α-linolenic acid is associated with lower risk of cardio vascular diseases [31].

1.2.1.2. Tocopherols

Vitamin E is acomplex of lipid soluble antioxidants sythesized by photosynthetic organisms and found in nature as four tocopherols (α-, β-, γ-, δ-tocopherols) and four tocotrienols (α-, β-, γ-, δ-tocotrienols) [32]. Vitamin E is one of the most important compound of human diet and is well known for its antioxidant, anticancer [33], antihypertensive [34], neuroprotective, nephoprotective [35], anti-

inflammatory [36] activities activities and it is also used to increase stability and shef life of foods [37]. Due to their photoprotection and antioxidant properties tocopherols are also receiving attention in cosmetic and clinical dermatology [38].

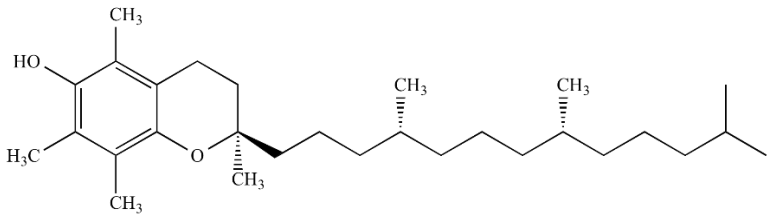


Figure 4. α-tocopherol [39].

Several studies have shown that alpha tocopherol (Figure 4) is the major tocopherol found in cyanobacteria. Wang et al. reported that under optimal conditions SFE extract of *Spirulina platensis* had a content 3.4 g/kg of α-tocopherol [23]. Another study showed that SFE *S. platensis* extracts have 12 time more tocopherols than initial material [40]. Alpha tocopherol is a vitamin and antioxidant that is important in protecting cells from oxidative stress, balancing normal coagulation, maintaining endothelial cell integrity and regulating immune function [32].

1.2.1.3. Carotenoids

Carotenoids are tetraterpenoids divided into two groups – carotenes and xanthophylls – and synthesized in plants, photosynthetic organisms and in some nonphotosynthetic bacteria, molds and yeasts. They are usually colored red, orange and yellow and are wide spread pigments found in nature. Carotenoids are important for plants protection against photooxidative damage [15].

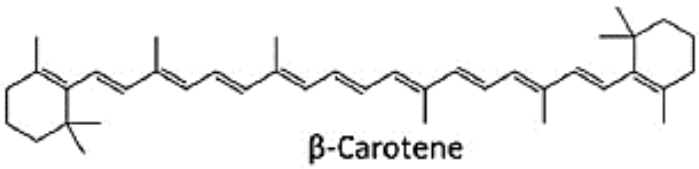


Figure 5. β-carotene [8]

Carotenoids are known as food colorants, feed or cosmetics additives, but research showed that they also have great health benefits. For example astaxanthin is strong antioxidant and has anti-imflammatory [41], anti-cancer, cardiovascular health effects [42], β-carotene (Figure 5) has antioxidant properties [43] and prevents night blindness and liver fibrosis [44], protects skin [15]. The two most recognized carotenoids are astaxanthin and β-carotene and they make-up almost half of carotenoid market. As antioxidants, carotenoids are sensitive to oxygen, light and heat, special care should be given in storage and handling [45].

Wang et al. reported that under optimal conditions (48 °C, 20 MPa, 4 h) SFE extracts of *S. platensis* contained 77.8 g/kg of β-carotene [23]. Another research with SFE extracts of *Nannochloropsis gadita* showed that yield of oil and carotenoids varied between 110.1 and 152.9 g/kg of oil in dry substrate and between 393.0 and 773.7 mg/kg of carotenoids in dry substrate respectively [46]. These studies indicate that the yield of bioactive components depends on chosen method, conditions and material. Most studies have shown that beta carotenoids are most commonly found in cyanobacteria and they are important as antioxidant vitamins and phytochemicals, also they are good blue light filters, enriched in lipophilic compartments and this makes them photoprotectants [15].

1.2.2. Phycobiliproteins

The algae proteins are in a diverse range of forms and cellular locations. Phycobiliproteins are a kind of algal proteins which exhibit great bioactive potential [21]. Phycobiliproteins are water soluble, deep colored proteins that are produced by cyanobacteria and rhodophyta. They are part of photosynthesis, because phycobiliproteins capture light energy and pases to chlorophylls. Phycobiliproteins like the blue pigment phycocyanin, red pigment phycoerythrin and light blue pigment allophycocyanin differ in their special properties. These cyanobacterial proteins are strongly fluorescent markers, they have antioxidant properties, also can reduse oxidative stress and neutralize the reactive oxygen species [47]. Phycobiliproteins can have a „c“ or „r“ letter before name and that depends on which microalgae, cyanobacteria or rhodophyta, produces them. Thus, c-phycocyanin is produced by cyanobacteria and r-phycocyanin produced by rhodophyta.

Phycoerythrin (PE) is a red coloured phycobiliprotein which is found in cyanobacterial or red algae chloroplasts. Phycoerythrin has some physical properties that make it suitable in molecular biology and clinical research. PE can be used as a reagent in fluorescence microscopy, diagnostics or as a label for biological molecules [8].

Phycocyanin (Figure 6) is a blue coloured phycobiliprotein. c-Phycocyanin is the major protein in *Spirulina* sp. and constitutes up to 20 % of its dry weight. Phycocyanin is a pigment commonly used in cosmetics and food. The use of this phycobiliprotein in food and other applications is limited because of its sensitivity to heat [8]. Chaiklahan et al. study about phycocyanin extracted from *Spirulina* sp. stability showed that the critical temperature for phycocyanin stability is 47 °C and that sodium chloride, glucose and sucrose demonstrated potential for protection [48].

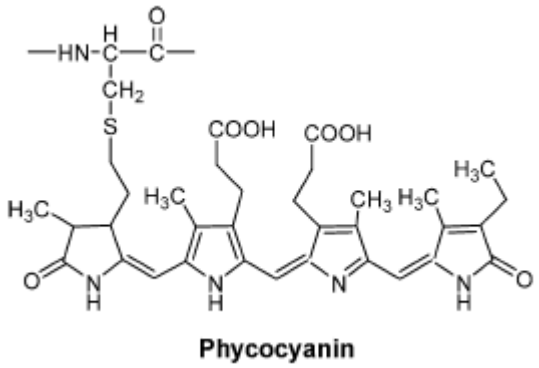


Figure 6. C-phycocyanin [49].

Allophycocyanin (APC) is an intensely bright blue phycobiliprotein isolated from red algae. APC and CPC have the major absorption (λmax) in the visible region of 650–655 nm and 610–620 nm, respectively, with emission light at 660 nm and 637 nm respectively [50].

1.2.3. Antioxidants

Reactive oxygen species (ROS) are natural byproducts of living organism. Exaggerated amounts of ROS may cause cell stucture damage contributing to various diseases, such as atherosclerosis, diabetes, chronic inflammation, cancer, stroke, ageing processes. Antioxidants may protect organisms from damages caused by ROS [51]. Antioxidants are considered as nutraceuticals with potential health benefits [52].

There are a lot of methods to measure antioxidant capacity from various bioresourses [53]. TPC, $ABTS^{\cdot+}$ and DPPH assys have been comonly used for microalgae [54],[55]. These methods are more closely related with antioxidant activity of substituents extracted from cyanobacteria or other matrices, but bioactive compounds can have limited solubility, as they could be chemically bound to the matrix. Thus, total antioxidant capacity of biomass may be underestimated [56]. For these reasons „QUENCHER“ was proposed to estimate the antioxidant activity of unextracted biomaterials [57].

The antioxidant capacity in biological systems and foods is affected by many factors, such as antioxidants partitioning properties between aqueous and lipid phases, the physical state of oxidizable substrate and the oxidation conditions [58].

TPC method counts on the transfer of electrons from phenolic compounds and formation of blue color complexes that can be detected spectophotometrically at 750-765 nm [59]. The total phenol content assay is simple, convenient and reproducible. However, there is a disagreement on what is actually detected in total antioxidant capacity assays – only phenols or phenols with reducing agents [51]. DPPH radical is an organic nitrogen radical that has purple color. The radical scavenging

capacity is determined by measuring absorbance at 517 nm [60]. The method is simple and rapid, but requires diligence because DPPH can only be desolved in organic solvent, it is sensitive to light and oxygen [51],[52]. ABTS is a peroxidase substrate, which, after oxidation by peroxyl or other radicals, forms cation $ABTS^{\cdot+}$, which is deep green and can be determined by spectrophotometer at 600-750 nm [61]. The antioxidant capacity is determined by the ability of compounds to reduse the intensity of the radical color and is expressed relative to Trolox. $ABTS^{\cdot+}$ is soluble in both organic and aqueous media [51].

1.3. Conventional and high-pressure processes extraction of bioactives

Choice of extraction method is very significant for maximal extract yield and to reduce loss of bioactive compounds and usage of solvents and energy. Also the choice is important for environment safety too. All extractions have pluses and minuses that are related to time, solvents, the complexity and cost of equipment use.

1.3.1. Soxhlet

Soxhlet extraction was developed in 1879 and has been the most widely used standard extraction technique for over a century. The sample is packed in filter paper or patron and placed in extractor that is slowly filled with condensed solvent from a distilation flask (Figure 7). When the solvent reaches overflow level, a siphon aspirates liquid from extractor and unleashes it with extracted analytes back into the distillation flask. The operation is repeated until extraction is finish [62].

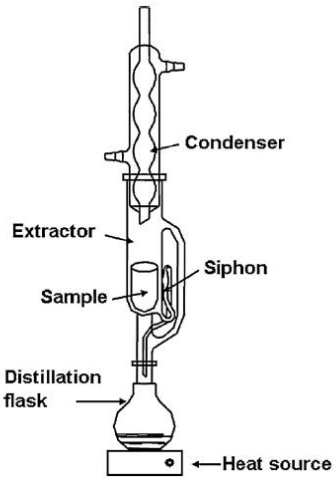


Figure 7. Conventional Soxhlet extractor [62].

Conventional Soxhlet extraction has some advantages. The sample has repeatedly contact with fresh solvent, which facilitates displacement of the extraction equilibrium. Also, no filtration is

required and several extraxtions can be done in parallel which is facilitated by the low cost of equipment. In addition, Soxhlet extraction is a very simple methodology that requires little training [62].

Soxhlet extraction also has disadventages. This technique requires a lot of time and energy and it also polluting the environment with hazardous solvents [63]. Extraction is carried out at the solvent boiling point, which can result in thermal decomposition of thermolabile components. Also, The conventional Soxhlet extraction is limited by solvent and it is difficult to automate [62].

1.3.2. Solid-liquid extraction

This method is applied to the extraction of bioactive components from the material. Extraction is based on penetration of the solvent into the material cells and dissolution of the components. The mechanism is simple and can be divided in 5 steps. When the cell contacts the solvent, the solvent penetrates through the cell membrane into the cytoplasm and interacts with soluble components and forms a solvent-component complex. Because of the concentration gradient, this complex diffuses across the cell membrane and stays in the solvent [12].

By choosing different solvents, different compounds such as oils or proteins can be extracted. The extraction efficiency can be improved by adjusting the temperature and using the shaker. Selecting the appropriate temperature for extraction changes the properties of the solvent, which can accelerate the extraction. Also, using a shaker during extraction, the material and solvent contact area increase and the extraction accelerates [64].

Solvent extraction has some advantages, it is cheap and easy to scale up, and disadvantages like it takes long time, cots large amount of solvents, which is not environmental friendly, require further treatment – evaporation to concentrate the extract.

1.3.3. Supercritical fluid extraction

Supercritical fluid extraction (SFE) is a sustainable extraction technology, it uses eco-friendly solvents and require lower energy input while reducing extraction time [63]. This technology finds application in natural products used as ingredients, food suplements or active compounds extraction [20]. Supercritical fluid extraction is based on the solvating properties of a supercritical fluid (SF), which can be received by applying temperature and pressure above critical point of a compound [65]. When fluid is forsed by pressure an temperature above its critical point, it becomes a supercritical fluid and under these comditions the fluid properties is placed between gas and liquid properties – SF density is similar to a liquid and its viscosity is similar to a gas, so its diffusivity is

between the two stages (Figure 8). Due to their low viscosity and high diffusivity, supercritical fluids have better transport properties than liquids, they can easily go through materials and can give faster extraction yields. SF density can be modified by changing its temperature and pressure [66].

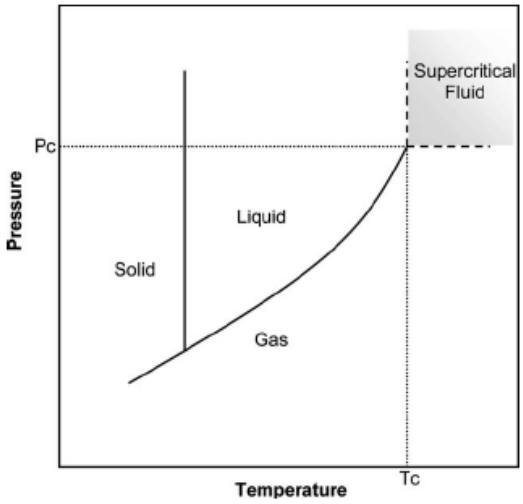


Figure 8. Typical phase diagram for a pure compound [66].

Supercritical fluid extraction can be changed by adding liquid modifiers (i.e ethanol) and it can help to obtain a desired polarity of CO_2 based fluids. The fluids properties can be manipulated by adding modifier or changing the molar ratio of it. Modifier selection can influence extraction efficiency and reduce the extraction time. There are three ways to introduce a liquid modifier into the SFE system, using premixed fluids from a cylinder, direct spiking and using second pump [67].

Extraction by supercritical fluid depends on intrinsic tunable characteristics of SF like temperature and pressure and extrinsic characteristics like the features of the sample matrix, interaction with targeted analytes and many environmental factors [65]. Supercritical CO2 and hexane have similar dissolving properties. This means, that CO_2 is very good solvent for non polar materials and leaves no residue in extracts. The low viscosity of CO_2 allows to penetrate into material more easily and decreases extraction time [68].

Supercritical fluid ectraction is an important alternative to traditional solvent extraction methods and has some advantages. When CO2 is used as a SF, this technique does not require organic solvents, which can beharmful for environment. SFE method exploits the unique properties of gases above their critical points and extracts soluble components from material. This technique uses carbon dioxide as a SF and can extract phenolics, flavonoids which could be used in food applications as it is nontoxic, readily available and easy to remove. Also it has been used to separate components like carotenoids and –linolenic acid. SFE extracts quality is higher than extractions with organic solvents, which can leave toxic residual solvent or induce thermal degradation of components in extract [69].

However, Supercritical fluid extraction also has some disadvantages. The major one is that SFE equipment is expensive. Also, there is limited knowledge of the behavior of using mixture with co-solvents. Moreover, when pure CO2 is used this technology has limited applications for isolation of polar compounds, because extracted metabolites are preferably nonpolar or moderately nonpolar. For these reasons, when compounds of interest have a more polar nature a polar co-solvent has to be used [20].

1.3.4. Pressurized liquid extraction

Pressurized liquid extraction (PLE) involves the application of technique that has been refined in literature as accelerated solvent extraction (ASE), pressurized fluid extraction (PFE) or pressurized hot-solvent extraction (PHSE). In any case, this extraction is based on the use of solvents to carry out extractions at high temperatures and pressures (below their critical points), that solvent could hold its liquid state during the whole extraction process [24][25]. PLE process can be defined in these steps: desorption – analytes are removed from the biomass; diffusion – analytes are diffused through the pores of the biomass; analytes are transferred into the bulk solvent and then collected. There are dynamic and static systems of PLE. The dynamic set up consist of a continuus pumping of solvent through the extraction cell and the static set up consists of extraction cycle(s). To optimize the pressurized liquid extraction process, it is important to examine parameters such as pressure, temperature, time, solvents, extraction mode, matrix composition. [20].

Pressurized liquid extraction has some advantages. One of them is the wide variety of solvents that can used, both polar and nonpolar. Extraction can be used for extracting compound from solid or semi-solid samples. Another advantage is the reduction in time.

Also PLE has disadvantages. For example, extraction can be exhaustive and it results in a nonselective extraction of compounds. Moreover, the equipment is expensive. However, these problems can be solved by using adsorbents in the extraction cell and high cost can be balanced by reduction in the solvent volume, time and waste [20].

1.3.5. Ultrasound assisted extraction

Ultrasound technology can be used to extract bioactive compounds such as antioxidants, oil and pigments from natural products, also for protein or oil extraction [20].

Ultrasounds are sonic waves that mechanicaly deformate solid, liquid or gaseous samples. Ultrasound is characterised by sound power (W), sound intensity (W/m2) and sound energy density (W/m3). Depending on the intensity, UAE can be separated into two main groups: low intensity and

high intensity. Low intensity ultrasound – high frequency, low power – is involved in nondestructive analysis, for quality assessment. This analytical technique is applied to provide the physicochemical properties of food – ripeness, firmness, sugar content. High intensity ultrasound – low frequency, high power – can alter food properties physically or chemically. Also it is used to improve efficiency of sample preparation [72].

The effects caused by ultrasound are attributed to the cavitation phenomena. The bubbles created by the ultrasound wave are able to grow during the rarefaction phases and decrease during compression phases. When bubble reaches critical size it collapses and release energy. When bubbles collapse on the surface of solid, the high pressure and temperature release shock waves directed toward the solid surface. This phenomenon is responsible for cell wall destruction and releasing of the cellular content into the surrounding phase [20].

The advantages of UAE include the inproved mass transfer, cell disruption, penetration and increased yields of bioactive compounds [73].

1.3.6. Freeze-thaw extraction

Freeze-thaw extraction is carried out for the recovery of water-soluble compounds such as phycobiliproteins. The extraction process is simple and fast and based on water crystals formation in the sample cells. During the extraction, the sample is poured into water and subsequently is freezed using a freezer or liquid nitrogen. Water forms crystals that damage the sample cells and break them down During the thawing phase the soluble components from the cell enter the water/solvent mixture. Depending on the sample, sample to water ratio or the equipment used for freezing, it may be necessary to repeat freezing-thawing to complete the extraction [74].

1.3.7. Homogenization

This extraction is based on mechanical destruction of the sample cells using a homogenizer. The main extraction factors are temperature, rotor speed, time, and selected solvent. By adjusting these factors, extraction can be accelerated and improved. The homogenizer extraction mechanism is simple - the components of mechanically destructed cells enter the solvent. The homogenizer can be used for protein extraction or sample preparation. The homogenizer extraction advantages are that equipment is cheap and it is easy to use [75].

1.4. Biorefinery concept

Microalgal biomass has become an important alternative source for renewable fuels. The growing population and increased demand for energy have become the causes of renewable fuel research. Certain approaches have been suggested for extraction of valuable compounds from microalgal biomass, which could expand the market for micronutrient products and open up new opportunities for biofuels and high-value compounds from microalgae. The full use of microalgae biomass is shown in figure 9 [76].

Cyanobacteria are rich in secondary metabolites, which can find potential applications as biofuels, pharmaceuticals, biocides, biofertilizerrs, chelators, suplements and cosmetics [77].

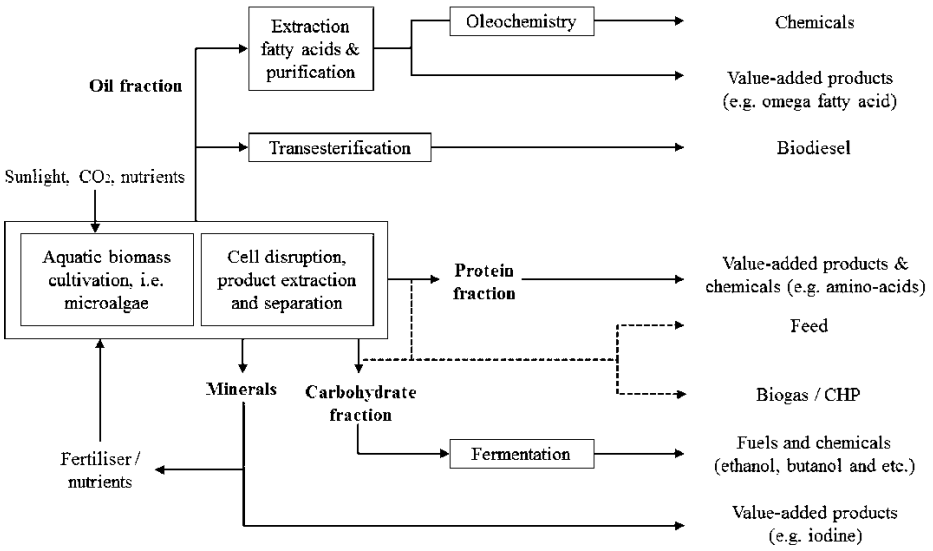


Figure 9. The applications of aquatic biomass cultivation processing system [78].

Cyanobacteria produces toxic bioactive metabolites which have inhibitory properties against macrophytes, microbes, and are toxic to vertebrates and invertebrates. Therefore, they could be developed into herbicides, secticides and algaecides [77]. Berry et al. studied the insecticidal and herbicidal properties of cyanobacteria. It was found that about 53% isolates from cyanobacteria inhibit the growth of sympathetic photoautotrophs. As well as a study showed that about 26 % isolates extracted from cyanobacteria inhibit the development of mosquito larvae [79].

Biofertilizers are more environmentally friendly than chemical fertilizers and can be a good alternative. Cyanobacterial species that are used as biofertilizers must be able to fix nitrogen , increase biomass accumulation, also they should not produce cyanotoxins, because of their negative effect on plant growth [77]. Hussain et al. research was focused on screening of wild and rhizospheric

cyanobacteria for *in vitro* phytohormones production and growth stimulation in wheat. Results showed that the maximum increase in gain weight was 43 %, demonstrated in wheat plants with *Chroococcidiopsis* sp. under natural conditions. Other cyanobacterial species also had positive effect on wheat growth, for example *Phormidium* sp. enhanced root groth by 24 % and *Anabaena* sp. was able to release 153 % more cytokinins that *Synechocystis* sp [80].

Cyanobacteria gained particular interest because of their capability to fix nitrogen. They may not produce as many lipids as other algae, but they can be manipulated to generate biofuels [77]. Cyanobacterial secondary products can also be useful for biofuel production due to their capability use carbon dioxide to produce alcohols, alkanes, alkenes, terpenes, esters, which can reduce the carbon dioxide emissions during intermediate processing [81].

Siderophores reduce copper toxicity in cyanobacteria – Synechococcus produce copper chelators and allow cyanobacteria to control the toxicity and availability of copper in the external environment [82].

Cyanobacterial secondary metabolites the pharmacological activities research has been focused on their medicinal benefits as anti inflammatory, antimicrobial, antitumor, antiparasitic properties. Research has proven that cyanobacterial toxins contain compounds to target aids, cancer nad other diseases, however that requires more research and clinical experimentations [77].

The mycosporine-like amino acids (MAAs) and scytonemin possess the necessary UV-absorption qualities to be appealing for cosmetic applications. They have preventative effects on photoaging and skin cancer [77]. Schmid et al. reported that cream which contains 0.005% MAAs from red algae encapsulated in liposomes can minimize the lipid peroxidation by 37%. After four weeks of topical application, the skin‘s smoothness was improved by 12% and firmness – by 10% [83]. Cyanobacteria *Nostoc punctiforme* can block approximately 90% of UV radiation and shares similar properties with MAAS [84],[85].

Cyanobacteria gained popularity for human consumption due to their unsaturated fatty acids and high protein content. Cyanobacteria are commonly used for food and feed. For example, *Athrospira* sp. and *Spirulin*a sp. are used as food supplement due to their high protein content [77]. Nagaka et al. conducted a study of 13 cyanobacterial species, that identified protein, carbohydrate and pigment content in cyanobacteria. The Bradford method was used to obtain the protein content. The results showed that *Spirulina subsalsa* (71%) had the highest protein content, and *Lyngbya semiplena* (19%) had the lowest [86]. Phycocyanin is one of cyanobacteria major proteins and it can be used as food colourant. Also phycocyanin has antiviral, antifungal functions. As cyanobacterial biomass finds application as food or feed supplements, it is very important to check cyanobacterial biomass for cyanotoxins [87].

2. MATERIALS AND METHODS

2.1. Algal biomass

Wild cyanobacteria were collected from within the sampling area off Nida, Lithuania, on October 06, 2016 when the Curonian Lagoon was experiencing a strong bloom of cyanobacteria. Plankton net (mesh diameter 20 μm) was used for sampling. All biomass samples were frozen (-20 °C) immediately after collection. Frozen samples were transferred to the laboratory and were subsequently freeze dried (-50 °C and 0.5 mbar) in a Freeze-Drying Plant Sublimator 4x5x6 (Zirbus Technology, Bad Grund/Harz, Germany). Dried samples were kept in the freezer (-18 °C) in hermetically sealed opaque glass vials flushed with nitrogen prior to any analysis.

Samples for phytoplankton community investigation were taken from the water surface (0.1 m) using 50 mL plastic containers, fixed with acid Lugol's solution and kept in the dark at +4 ^{o}C prior to microscopical analysis. Microscopic examination of the collected samples indicated that filamentous cyanobacterium *Aphanizomenon flos-aquae* embedded into typical fascicle-like colonies exceeded 96 % of the total phytoplankton biomass.

2.2. Chemicals

2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) ($ABTS^{\bullet+}$, Sigma-Aldrich, Steinheim, Germany), 2,2-diphenyl-1-picrylhydrazyl hydrate ($DPPH^{\bullet}$, free radical, 95%), 3,4,5-trihydroxybenzoic acid (gallic acid, 99%, Sigma-Aldrich, Steinheim, Germany), 2-(3-hydroxy-6-oxo-xanthen-9-yl)benzoic acid (Fluorescein (FL), Fluka Analytical, Bornem, Belgium), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, 97%, Sigma-Aldrich, Steinheim, Germany), Folin & Ciocalteu's phenol reagent ((2M), Fluka Analytical, Bornem, Belgium), NaCl, KCl, KH_2PO_4, $K_2S_2O_8$ (Lach-Ner, Brno, Czech Republic), Na_2HPO_4 (Merck KGaA, Darmstadt, Germany), Na_2CO_3 (Sigma-Aldrich), H_2SO_4, NaOH, H_3PO_4, (Sigma-Aldrich), HCl (35-38%, Chempur, Piekary Slaskie, Poland), acetonitrile, methanol, dichlormetane, hexane (HPLC grade, Sigma-Aldrich Chemie, Steinheim, Germany), boron trifluoride (24% methanol solution, Acros organics, Geel, Belgium), tridecanoic acid (Sigma-Aldrich Chemie, Germany), microcrystalline cellulose (20 μm, Sigma-Aldrich, St. Louis, MO, USA), catalytic tablet (K_2SO_4, $CuSO_4$, Sigma-Aldrich), ASE filters (Glass Fiber_(X)_Cellulose, Dionex Corporation, Sunnyvale, CA,USA), diatomaceous earth (100 % SiO_2, Dionex Corporation, Sunnyvale, CA, USA), cotton-wool (Bella-cotton, Poland), ethanol (96.3%, food grade, Stumbras, Kaunas, Lithuania), nitrogen liquid (AGA SIA, Riga, Latvia), carbon dioxide

gases and nitrogen gases (99.9%, Gaschema, Jonava region, Lithuania). All solvents used for extraction and chomatographic analysis were of analytical and HPLC-grade respectively.

2.3. Determination of the selected chemical composition indices

2.3.1. Moisture content

To the heated, dry, constant weight glass with cap and rod, 3 ± 0.001 g of cyanobacteria biomass were added and dried in the oven at 100-105°C for 3 hours, afterwards placed in a desiccator for 25 minutes and weighted on the analytical balances. The heating-weighting procedure afterwards was repeated two times per day (at the same time in mornings and evenings) until variation between two weighting results was less than 0.005 g. Experiments were performed in duplicate. Moisture content was calculated using the formula below (g/100g).

$$x = \frac{(m_1 - m_2) * 100}{m_1 - m}; g/100g \qquad (1)$$

m – glass with cap and rod weight g; m_1 – glass weight with sample before drying g; m_2 – glass weight with sample after drying, g.

2.3.2. Mineral content

3 ± 0.001 g of cyanobacteria biomass was added to dry, constant weight crucible, heated on electric hotplate and kept in muffle for ~16 hours at 600-650°C, afterwards placed in a desiccator for 25 minutes and weighted on the analytical balances. The heating-weighting procedure was repeated until variation between two weighting results was less than 0.005 g. Experiments were performed in duplicate. Ash (mineral) content, expressed as a percentage, is calculated by the following formula:

$$x = \frac{(m_2 - m) * 100}{m_1 - m}; g/100g \qquad (2)$$

m – crucible weight, g; m_1 – crucible weight with sample, g; m_2 – crucible weight with burned sample, g.

2.3.3. Protein content by Kjeldahl method

To a Kjeldahl flask, 1 ± 0.001 g of cyanobacteria biomass, 20 ml of conc. H_2SO_4 and catalyst tablet, containing 3.5 g K_2SO_4 and 0.4 g $CuSO_4$, were added, and mineralized until solution in the flask became transparent (heating intensity 60%, time – 90 min). The solution was distillated with automatic steam distillation system under the following conditions: 3 s NaOH and 3 s H_3BO_4 filing parameters, distillation time 300 min, steam intensity 80%. Distillate was collected in flask, followed

with the addition of Tashiro indicator and titration with 0.01 N HCl until the colour change from light green to grey-violet. Experiments were performed in triplicate. Control sample was prepared and analysed following the above described conditions. The nitrogen content, expressed as a percentage, was calculated using the following formula:

$$x = \frac{0.0014*A}{m} * 100; g/100g \qquad (3)$$

A – 0.1N HCl amount, used for distillate titration, ml; m – sample weight, g; 0.0014 – nitrogen amount equivalent 1 ml 0.1 N HCl. Protein material amount is calculated by multiplying the amount of nitrogen from the conversion factor 6.25.

2.3.4. Spectrophotometric estimation of phycobiliproteins

UV-Vis absorbance was recorded in a model spectrophotometer. The equations of Bennett and Bogorad and the extinction coeffiecients from Bryant et al. were used to calculate the amount of C-PC (equation 4), C-PE (equation 5) and C-APC (equation 6) in the samples.

$$C - PC(mg\ ml^{-1}) = \frac{(OD_{624} - 0.7OD_{650})}{7.38} \qquad (4)$$

$$C - APC(mg\ ml^{-1}) = \frac{(OD_{650} - 0.19OD_{624})}{5.65} \qquad (5)$$

$$C - PE(mg\ ml^{-1}) = \frac{(OD_{540} - 2.8(C-PC) - 1.34(C-APC))}{12.7} \qquad (6)$$

C-PC – phycocyanin, C-APC – allophycocyanin, C-PE – phycoerythrin

2.4. Conventional extraction techniques

2.4.1. Soxhlet extraction

Soxhlet extraction was performed in an automated Soxhlet extractor EZ100H (Behr Labor-Technik, Düsseldorf, Germany), using 5 ± 0.001 g of wild cyanobacterial biomass, loaded into a Whatman™ cellulose Soxhlet extraction thimbles (single 1 mm wall) between two layers of defatted cotton wool and inserted into an inner tube of the Behr Labor Technik™ glass Soxhlet extraction chamber. Non-polar fraction was isolated using hexane, applying the rate of extraction of 1 cycle per 5 min and total extraction time of 360 min (6 hours). After extraction, organic solvent was evaporated in a Büchi V–850 Rotavapor R–210 (Flawil, Switzerland). Extraction yield was determined gravimetrically (± 0.001 g) and expressed as g/100 g DW. Dry extract was kept under the nitrogen

flow for 15 min to remove organic solvent residues and stored at -18 °C prior to the analysis. The solid residue was collected, dried (50 °C) in and kept in a dry, well-ventilated place prior to the *in vitro* antioxidant activity assessment. Extraction experiments were performed in triplicate.

2.4.2. Solid-liquid extraction

Solid-liquid extraction (SLE) for oil extraction was performed in a thermostatically controlled shaker from 10 ± 0.001 g of cyanobacteria biomass and 50 mL of hexane (*solid: liquid* ratio *1:5*) at the following conditions: temperature 50°C time 180 min, 950 rpm, followed by the rapid cooling and centrifugation (9000 rpm, 10 min) and filtration and the whole cycle was repeated second time with a fresh hexane added on the same biomass. Organic solvents from the optically clear supernatants were evaporated in a Büchi V–850 Rotavapor R–210 (Flawil, Switzerland). Dry extracts were kept under the nitrogen flow for 15 min to remove organic solvent residues and stored at -18°C prior to the analysis. SLE-He extracts yield was determined gravimetrically (± 0.001 g) and expressed as g/100 g DW. The solid residues were collected, dried (50°C) in and kept in a dry, well-ventilated place prior to the analysis.

SLE for protein extraction was performed in a thermostatically controlled shaker from 0.08 ± 0.001 g of cyanobacteria biomass and 50 mL of water (*solid: liquid* ratio *1:625*) at the following conditions to find the highest yield: temperature 20°C time 60, 120, 180, 240 and 300 min, 950 rpm, and four cycles adding fresh distilled water, followed by centrifugation (9000 rpm, 10 min) and filtration. Experiments were performed in triplicates. Phycobiliproteins content was calculated using equations described in section 2.3.4 of the materials and methods.

2.5. High-pressure extraction techniques

2.5.1. Supercritical CO_2 extraction

2.5.1.1. Experimental design

Response surface methodology (RSM) and central composite design (CCD) were utilized for the experimental design setup of SFE-CO_2. Three independent variables and their variation levels were chosen, as follows: pressure (10-50 MPa), temperature (30-70 °C) and dynamic extraction time (30-120 min). The number of experiments was defined, based on the equation 1:

$$N = 2^f + 2f + c \qquad (7)$$

Where: f - the number of factors; c – the number of centre points.

Complete design consisting of 20 experimental runs with 8 factorial points, 6 axial and 6 centre points was established using the software Design-Expert trial version 8.0.7.1 (Stat–Ease Inc.,

Minneapolis, MN). As a response factor (RF), the total yield of lipophilic extract was selected. The multiple regression equation was used to fit the second-order polynomial equation, expressing the yield of SFE-CO_2 extract as a function of independent variables (Equation 2):

$$Y = \beta 0 + \sum_{i=1}^{3} \beta iXi + \sum_{i=1}^{3} \beta iiXi^2 + \sum_{i=1}^{3} \sum_{j>1}^{3} \beta ijXiXj \quad (8)$$

Where: Y – the predicted response (SFE-CO_2 extract yield, g/100 g DW); β_0 – a constant; β_i, β_{ii} and β_{ij} – the coefficients for linearity; X_i and X_j – independent variables.

All extraction experiments were performed in duplicate and in random order. Results were analysed using Design-Expert trial version 8.0.7.1 software. Statistical significance of the model and each variable was determined using the Student test (*p*-value) at 5% probability level ($p<0.05$). The adequacy of the model was determined by evaluating the '*lack of fit*' coefficient and the Fisher test value (*F*-value) obtained from analysis of variance.

2.5.1.2. Extraction procedure

SFE-CO_2 was performed in a Helix extractor (Applied Separation, Allentown, PA). Each extraction was carried out from 5 ± 0.001 g of freeze-dried wild cyanobacterial biomass, placed in a 50 mL cylindrical vessel (320 mm length, 14 mm inner diameter) between two layers of defatted cotton wool. The temperature of the extraction vessel was controlled by a surrounding heating jacket. The flow rate of CO_2 in the system (v) was controlled manually by the micro-metering valve (back-pressure regulator) and kept at 2-3 standard litres per min (SL/min) during all experiments. The volume of CO_2 consumed was measured by a ball float rotameter and a digital mass flow meter in SL/min at standard state: pressure (P) = 100 kPa, temperature (T) = 20 °C, density (ρ) = 0.0018 g/mL. The conditions for each extraction experiment were set at a range of variables: pressure (10-50 MPa), temperature (30-70 °C) and dynamic extraction time (30-120 min). A static time of 10 min was included in to the total extraction time in all extractions. Extraction yield was determined gravimetrically (± 0.001 g) and expressed as g/100 g DW. All extracts were collected in the glass bottles and stored at -20°C. The solid residues after SFE-CO_2 was collected and kept in a dry, well-ventilated place prior to the *in vitro* antioxidant activity assessment.

2.5.2. Pressurised liquid extraction

Pressurised liquid extraction was performed in ASE-350 (Thermo Scientific Dionex, Sunnyvale, CA, USA) apparatus following modified procedure of Kraujalis et al. (2013) [**Error! eference source not found.**] from 10 ± 0.001 g of cyanobacteria biomass, 3 ± 0.001 g of cyanobacteria biomass after solid-liquid extraction with hexane, 3 ± 0.001 g of cyanobacteria biomass after SFE-CO_2 extraction was mixed respectively with 10, 2 and 2 g diatomaceous earth and placed

to 5 and 66 ml stainless-steel extraction cells, with two cellulose filters in the both ends to avoid particle release to the system. Cyanobacteria biomass was extracted with hexane (55°C), acetone (55 and 70°C), ethanol (55 and 70°C) and water (55 and 140°C). Cyanobacteria biomasses after solid-liquid extraction and SFE-CO_2 extraction were extracted with acetone (70°C), ethanol (70°C) and water (140°C). All extractions were done in the same period of time - 5min x 3 cycles. The system pressure (103 bar or 10.3 MPa), pre-heating time (5 min), cell flush volume (100%) and purge time (120 s) with nitrogen to collect the extracts in the vials was kept constant for all PLE experiments. Organic solvents were evaporated with rotary evaporator (BUCHI Labotechnic, Switzerland). H_2O extracts were additionally freeze-dried (-50ºC, 0.5 mbar) to remove residual water. The yields of extracts were determined gravimetrically (± 0.001 g) and expressed as g/100g DW, extract were kept in brown glass bottles in the freezer prior to the analysis. The solid residues were collected, dried (50°C) in and kept in a dry, well-ventilated place prior to the analysis. All extractions were performed in duplicates.

2.6. Other extraction techniques

2.6.1. Freeze-thaw extraction

Freeze-thaw extraction was performed in centrifugal tubes at the following conditions to find the highest yield: different cyanobacteria biomass and water ratio – 1:100, 1:200, 1:400, 1:625, and four cycles adding fresh distilled water, followed by centrifugation (9000 rpm, 20 min) and filtration. Freezing time was 5 min and thawing time was 15 min. Water with proteins was measured with spectrophotometer. Experiments were performed in triplicates. Phycobiliproteins content was calculated based on the equations described in section 2.3.4 of the materials and methods.

2.6.2. Homogenization

Extraction with homogenizer was performed in centrifugal tube placed in glass of water using homogenizer (IKA labortechnik) from 0.08 ± 0.001 g of cyanobacteria biomass and 50 mL of water (*solid: liquid* ratio *1:625*) at the following conditions to find the highest yield: temperature 20, 30, 40 and 50°C, time 5, 15, 30, 45 and 60 min, 950 rpm, and five cycles adding fresh distilled water, followed by centrifugation (9000 rpm, 20 min) and filtration. Experiments were performed in triplicates. Phycobiliproteins content was calculated based on the equations described in section 2.3.4 of the materials and methods.

2.6.3. Ultrasound assisted extraction

UAE extraction was performed in centrifugal tube placed in glass of water using handheld ultrasonic homogenizer (UP200Ht, Germany) from 0.08 ± 0.001 g of cyanobacteria biomass and 50 mL of water (*solid: liquid* ratio *1:625*) at the following conditions to find the highest yield: time 1-10 min, amplitude 20-100 proc., followed by centrifugation (9000 rpm, 20 min) and filtration. Experiments were performed in triplicates. After necessary dilutions phycobiliproteins content was calculated based on the equations described in section 2.3.4 of the materials and methods.

Response surface methodology (RSM) utilized for the experimental design setup of UAE. Two independent variables and their variation levels were chosen, as follows: time (1-10 min) and amplitude (20-100 proc.). The response factor (RF) was the yields of PC, APC, PE and total. The number of experiments was defined, based on the equation 18:

$$N = 2^f + 2f + c \qquad (9)$$

Where: f - the number of factors ; c – the number of centre points.

Complete design consisted of 13 experimental runs with 4 factorial points, 4 axial and 5 centre points was established using the software Design-Expert trial version 8.0.7.1 (Stat–Ease Inc., Minneapolis, MN).. The multiple regression equation was used to fit the second-order polynomial equation, expressing the yields of UAE extracts as a function of independent variables (Equation 19):

$$Y = \beta 0 + \sum_{i=1}^{3} \beta iXi + \sum_{i=1}^{3} \beta iiXi^2 + \sum_{i=1}^{3} \sum_{j>1}^{3} \beta ijXiXj \qquad (10)$$

Where: Y – the predicted response (yield, g/100 g DW); β_0 – a constant; β_i, β_{ii} and β_{ij} – the coefficients for linearity; X_i and X_j – independent variables.

All extraction experiments were performed in duplicate and in random order. Results were analysed using Design-Expert trial version 8.0.7.1 software. Statistical significance of the model and each variable was determined using the Student test (*p*-value) at 5% probability level ($p<0.05$). The adequacy of the model was determined by evaluating the '*lack of fit*' coefficient and the Fisher test value (*F*-value) obtained from analysis of variance.

2.6.4. Homogenization and UAE, SLE and UAE, Freeze-thaw and UAE

Extractions were performed combining two techniques – homogenization optimal conditions (one cycle, 30 min and 30 °C) with UAE, SLE optimal conditions (one cycle and 60 min) with UAE and freeze-thaw optimal conditions (one cycle) with UAE. Extractions were performed from 0.08 ± 0.001 g of cyanobacteria biomass and 50 mL of water (*solid: liquid* ratio *1:625*) at the following

conditions: optimal conditions of homogenizer, SLE or freeze-thaw, UAE time 2-10 min, amplitude 50-100 proc., followed by centrifugation (9000 rpm, 20 min) and filtration. Experiments were performed in triplicates. After necessary dilutions phycobiliprotein content was calculated based on the equations described in section 2.3.4 of the materials and methods.

Response surface methodology (RSM) utilized for the experimental design setup of UAE. Two independent variables and their variation levels were chosen, as follows: time (2-10 min) and amplitude (50-100 proc.). The response factor (RF) was the yield of PC, APC, PE and total. The number of experiments was defined, based on the equation 23:

$$N = 2^f + 2f + c \quad (11)$$

Where: f - the number of factors ; c – the number of centre points.

Complete design consisted of 13 experimental runs with 4 factorial points, 4 axial and 5 centre points was established using the software Design-Expert trial version 8.0.7.1 (Stat–Ease Inc., Minneapolis, MN).. The multiple regression equation was used to fit the second-order polynomial equation, expressing the yield of element as a function of independent variables (Equation 24):

$$Y = \beta 0 + \sum_{i=1}^{3} \beta iXi + \sum_{i=1}^{3} \beta iiXi^2 + \sum_{i=1}^{3} \sum_{j>1}^{3} \beta ijXiXj \quad (12)$$

Where: Y – the predicted response (yield, g/100 g DW); β_0 – a constant; β_i, β_{ii} and β_{ij} – the coefficients for linearity; X_i and X_j – independent variables.

All extraction experiments were performed in duplicate and in random order. Results were analysed using Design-Expert trial version 8.0.7.1 software. Statistical significance of the model and each variable was determined using the Student test (*p*-value) at 5% probability level ($p<0.05$). The adequacy of the model was determined by evaluating the '*lack of fit*' coefficient and the Fisher test value (*F*-value) obtained from analysis of variance.

2.7. Phytohemical characterization of extracts

2.7.1. Non-volatile compound analysis by UPLC/ESI-QTOF-MS

Phytochemical composition of aqueous extracts after Soxhlet, SFE-CO_2 extract at optimal conditions, SLE extracts (mechanical shaking hexane extracts), PLE extracts (hexane, acetone, ethanol, water extracts), were screened on an Acquity UPLC system (Waters, Milford, USA) equipped with a Bruker maXis UHR-TOF mass spectrometer (Bruker Daltonics, Bremen, Germant), binary solvent delivery system, an auto sampler with a 10 µL sample loop, column manager,

photodiode array (PDA) detector and an Acquity BEH C18 column (1.7 m. 50 x 2.1 mm, i.d.), as previously described by Kraujalyte and Venskutonis (2013) [88] with following modifications. The mobile phase initially consisted of eluent A (0.4 v/v formic acid in ultra-pure water), followed by an increase from 0% to 100% of eluent B (acetonitrile) over 9 min. During the following 2 min, the amount of eluent B was maintained at 100 for 1 min, followed by the re-introduced initial conditions over 1 min and the equilibration time of 1 min. Separation of compounds was performed at 25°C; the column was equilibrated for 1 min before each run; the flow rate 0.4 mL/min; extract concentration 1 mg/ml; injection volume 1 μL. The effluent (monitored at 254 nm) from the PDA detector was introduced directly into the UHR-TOF mass spectrometer equipped with an ESI source. MS data was recorded in ESI negative ionization mode. The capillary voltage was maintained at +4000 V with the end plate offset at -500 V. Nitrogen was used as the drying and nebulizing gas at a flow rate of 10.0 L/min and a pressure of 2.0 bar. For the instrument control and data acquisition, the Compass 1.3 (HYStar 3.2 SR2) software was used. Preliminary peak identification was carried out by comparing accurate masses of compounds with hose reported in literature sources and free chemical databases (Metlin, Chempspider).

2.8. Fatty acid composition analysis by gas chromatography

FAMEs were prepared by using boron trifluoride as a catalyst. Briefly, for triglycerides esterification and free fatty acids saponification, 0.5 ± 0.001 g of extract and 4 mL of methanolic NaOH (0.5 M) was poured into 50 mL round-bottomed flask and refluxed for 5-10 min until disappearance of the fatty phase. After esterification, 5 mL of 24% boron trifluoride/methanol complex was added, mixture was boiled for 2 min., cooled to room temperature, diluted with 5 mL n-hexane followed with the addition of NaCl, well-shaken and left still until layers separated. For analysis, 100 μL of hexane phase was diluted with 900 μL of pure GC-grade hexane, containing tridecanoic acid as internal standard (final concentration 0.1 mg/mL) for compound quantification. The samples were analysed by means of gas chromatography with flame ionization (GC-FID) on an HRGC 5300 (Mega Series, Carlo Erba, Milan, Italy) equipped with a flame ionization detector and 100 m length 0.25 mm (id), 0.20 μm film thickness fused silica capillary column SP™–2560 (Supelco, Bellefonte, PA, USA). Analysis parameters were as follows: injection temperature 220 °C; detector's temperature 240 °C; split ratio 100:1; oven temperature was programmed from 80 °C to 240 °C at 4 °C/min and held isothermal for 5 min; carrier gas, helium at a flow rate of 20 cm^3/s; injection volume – 1μL. A standard FAME mixture of 37 fatty acids (C8-C24) was used for compound identification. In the ASE extracts, a standard of tridecanoic acid, which was added to the

sample before saponification, was used to determine the fatty acids content by mg/g of the extract. The experiments were performed in triplicate.

2.9. Determination of tocopherols

Tocopherol content in extracts (10 mg/mL) was analysed by high pressure liquid chromatography (HPLC), based on the method of Panfil et al. with slight modifications [89]. Perkin Elmer Series 200 HPLC system was equipped with a normal phase column (particle size 5 μm, 250 mm × 4.6 mm), applying isocratic elution with hexane: isopropanol (98:2, v/v). Injection volume was 20 μL and flow rate 1.6 mL/min. Tocopherols were detected using fluorescence detector at 290 nm excitation and 330 nm emission and identified by comparing the retention time of peaks to those of pure standard solutions. The calibration curves (peak area versus injected amount) were used to determine the quantity of tocopherols. Analyses were performed in triplicate.

2.10. *In vitro* antioxidant capacity assessment

For $DPPH^{\cdot}$ and $ABTS^{\bullet+}$ scavenging capacities and total phenolic content (TPC) determination, extracts obtained under optimized extraction conditions were dissolved in acetone-methanol mixture (1:9, v/v) and further diluted with methanol to a final concentration of 100-1000 μg/mL. Antioxidant capacity of freeze-died cyanobacterial biomass and solid residues after Soxhlet and optimized SFE-CO_2 was evaluated using QUENCHER procedure [57]. The samples for these measurements were prepared and analysed following the protocol of Kitrytė et al. [90]. Briefly, solid samples were mixed with microcrystalline cellulose at a concentration of 500 μg/mg. Prior to the analysis, a series of "solid dilutions" of stock mixture with microcrystalline cellulose were performed to a final concentration of samples 10-100 μg/mg.

Absorbance was measured on a Spectronic Genesys 8 spectrophotometer (Thermo Spectronic, Rochester, NY). Radical scavenging capacity was expressed as Trolox equivalent antioxidant capacity (TEAC) in mg TE/g extract or dry biomass weight (DW), TPC as gallic acid equivalents in mg GAE/g extract or DW), calculated by means of dose-response curves for Trolox and gallic acid, respectively. All the experiments were performed in pentaplicate.

2.10.1. Total phenolic content (TPC) by Folin-Ciocalteu's assay

TPC of extracts was determined by the modified procedure of Singleton et al [91]. Briefly, 300 μL of extract (200 μg/mL) or MeOH (blank) were mixed with 1500 μL of Folin-Ciocalteu's reagent (1:9, v/v) and 1200 μL of Na_2CO_3 solution (75 g/L), left in dark for 2 hours and absorbance

was measured at 760 nm. For solids (QUENCHER), 10 mg of sample (20 μg/mg) or cellulose (blank) were mixed with 150 μL of distilled H_2O, 750 μL of Folin-Ciocalteu's reagent (1:9, v/v), and 600 μL of Na_2CO_3 solution, vortexed for 15 s, shaken at 250 rpm for 2 hours in the dark, centrifuged (1960 x g, 5 min) and the absorbance of optically clear supernatant was measured at 760 nm.

2.10.2. The $ABTS^{\bullet+}$ scavenging assay

The $ABTS^{\bullet+}$ scavenging assay was carried out by the modified procedure of Re et al. [92]. Briefly, phosphate buffered saline (PBS) solution (75 mmol/L; pH 7.4) was prepared by dissolving 8.18 g NaCl, 0.27 g KH_2PO_4, 1.42 g Na_2HPO_4 and 0.15 g KCl in 1 L of ultra-pure water. The $ABTS^{\bullet+}$ solution was prepared by mixing 50 mL of ABTS (2 mmol/L PBS) with 200 μL $K_2S_2O_8$ (70 mmol/L) and allowing the mixture to stand in the dark at room temperature for 15-16 h before use. For analysis, 3000 μL of working $ABTS^{\bullet+}$ solution (AU 0.700±0.010 at 734 nm) was mixed with 50 μL of extract (1000 μg/mL) or MeOH (blank), left in dark for 2 hours and the absorbance was measured at 734 nm. For solids, 10 mg of sample (10 μg/mg) or cellulose (blank) were mixed with 25 μL of MeOH and 1500 μL of working $ABTS^{\bullet+}$ solution, vortexed for 15 s, shaken at 250 rpm for 2 hours in the dark, centrifuged (1960 x g, 5 min) and the absorbance of optically clear supernatant was measured at 734 nm.

2.10.3. The $DPPH^{\cdot}$ scavenging assay

The $DPPH^{\cdot}$ assay was carried out by the modified procedure of Brand-Williams et al. [93]. Briefly, to a 2000 μL of a ~89.7 μmol/L (final absorption adjusted to 0.800±0.010 AU at 517 nm) $DPPH^{\cdot}$ methanolic solution 1000 μL of extract (100 μg/mL) or MeOH (blank) were added, the mixtures were left in dark and absorbance was measured after 2 hours at 517 nm. For solids, 10 mg of sample (100 μg/mg) or cellulose (the blank) were transferred to a centrifugation tube, mixed with 500 μL of MeOH and 1000 μL of $DPPH^{\bullet}$ methanolic solution (AU 0.800 ± 0.010 at 517 nm), vortexed for 15 s, shaken at 250 rpm for 2 hours in the dark, centrifuged (1960 x g, 5 min) and the absorbance of optically clear supernatant was measured at 517 nm.

2.11. Statistical analysis

Mean values and standard deviations were calculated using MS Excel 2016. GraphPad Prism 6.01 software (2012) was used for statistical data analysis, applying unpaired t-test ($p < 0.05$) or one-way analysis of the variance (ANOVA) and the Tukey's posthoc test to compare the means that showed significant variation ($p < 0.05$) and conducting bivariate correlation analysis to calculate Pearson correlation coefficients.

3. RESULTS AND DISCUSSION

3.1. Recovery of lipophilic products from wild cyanobacteria isolated from the Curonian Lagoon by means of supercritical carbon dioxide extraction

(Submitted to Algal Research Journal by M. Syrpas, J. Bukauskaitė, R. Paškauskas, L. Bašinskienė, P. R. Venskutonis)

3.1.1. Chemical composition of wild cyanobacteria biomass

At the first step of this study, the chemical composition of cyanobacterial biomass was determined by conventional methods. By Soxhlet, Kjeldahl, mineral content and moisture testing methods were determined fat, protein, minerals and water contents and yields expressed as a percentage.

Table 1. Chemical composition values (%) of wild cyanobacterial biomass

Soxhlet (Hexane) extraction yield, %	Nitrogen content, Kjeldahl, %	Mineral content, %	Water content, %
3.68 ± 0.14	67.35 ± 4.25	5.01 ± 0.15	6.29 ± 0.01

Values are reported as mean value ± standard deviation.

According to the results (Table 1), the largest part of the biomass of wild cyanobacteria is nitrogen content (67.35 %), and the smallest part is fats (3.68 %). Results are in agreement with previous studies. Batista et al. reported research of comparison of microalgal biomass and used same methods (Soxhlet, Kjeldahl, ash and water content) to determine chemical compositions of six microalgae species. One of microalgal species was *Spirulina maxima* and the results showed that it had 3.6 % fats, 44.9 % proteins, 30.9 % of total ash content, 11.3 % water [10]. Chemical composition of *Spirulina* sp. biomass was analyzed by the same manner and results were similar – 8.3 % of moisture, 10.2 % of ash content, 5.4 % of lipids and 41.9 % of crude proteins [94]. Safi et al. determined that *H. pluvialis* had 51.7 % total proteins, *P. cruentum* had 57.3 % total proteins and *A. platensis* had 53.5 % total protein content using *Lowry* method [95]. The lipophilic content obtained from wild cyanobacterial biomass is similar to lipophilic content from *Spirulina* sp., however, the protein content is higher comparing to *Spirulina* sp. or other results done by *Lowry* method.

3.1.2. Optimization of supercritical carbon dioxide extraction

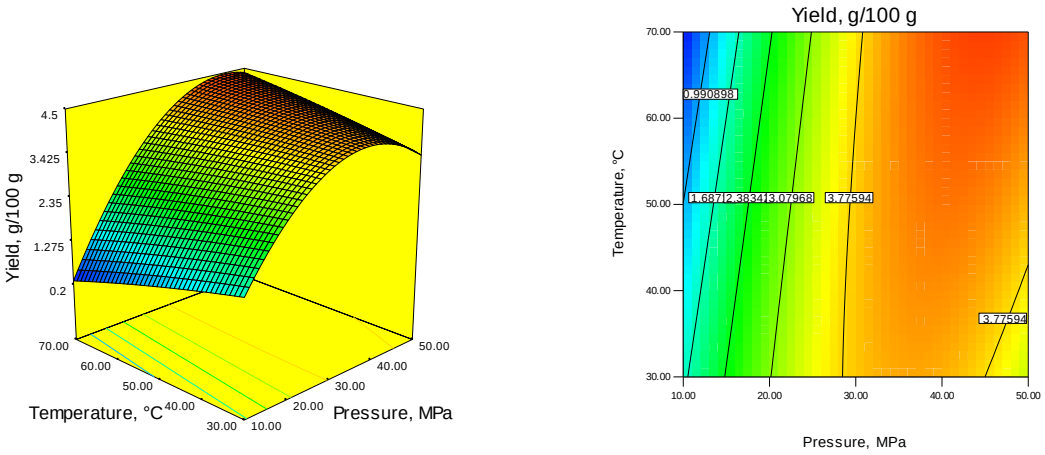
The recovery of lipophilic products from wild cyanobacteria was performed by SFE-CO_2 and Soxhlet extraction and the obtained results were compared. CCD and RSM were used to optimize the effect of the most important independent variables on the total SFE-CO_2 yield, selected as the response factor. The complete CCD matrix along with the predicted and experimental values of obtained SFE-CO_2 yields is presented in Table 2. According to the results, the lipophilic extract yield varied from 0.12 to 4.76 g/100 g of wild cyanobacterial biomass under different tested SFE-CO_2 conditions. The yield of lipophilic fraction after 360 min Soxhlet extraction was 3.68 g/100g and comparing both techniques SFE-CO2 extraction achieved efficiency which was from 3 to 129 % under experimental conditions.

3D and 2D response surface plots showing the effect of extraction time, temperature, pressure and their interactions on obtained SFE-CO_2 extract yields are presented in Figure 10. Figure 10 A illustrates the effect of temperature and pressure at a constant extraction time of 75 min. Results show that increased temperature has negative effect in low pressure levels but it was not significant at 30-50 MPa pressure ranges. Increased pressure has positive effect on SFE-CO_2 yield, reaching maximum values when temperature was >50 and pressure in the range of 30-50 MPA. Based on the results in Table 2, the lowest yields (0.12-0.44 g/100g) were obtained at the lowest pressure (10 MPa) and the highest temperature (70 °C), while the decrease of temperature (30 °C) resulted higher yields (1.21-2.04 g/100 g). This adverse effect of temperature could be explained by the physicochemical properties of compressed CO_2. The increase of temperature at lower operating pressure levels (e.g. at 10 MPa) remarkably decreases CO_2 density (e.g. from 772 kg/m^3 at 30 °C to 226 kg/m^3 at 70 °C) and thus induces weaker diffusivity and solvating power. On the other hand, at high pressure levels the so-called "enhanced solubility effect" could be observed, when the increasing vapour pressure of solute outweighs the decreasing solvating power of CO_2. Figure 10 B shows that increasing pressure over time at a constant temperature of 50 °C, yield increases. In this case extraction pressure has a very strong effect comparing with time which has weak linear effect, resulting higher yield values at >30 MPa pressure and >75 minutes extraction time. Figure 10 C shows the effects of time and temperature on extraction yield at a constant pressure of 30 MPa. The results indicate positive linear effect of extraction time towards total SFE-CO_2 extract yield with maximum value at the longest time (120 min). The effect of increasing temperature for the longer extraction experiments was negative, but for extractions until 60 minutes effect was negligible.

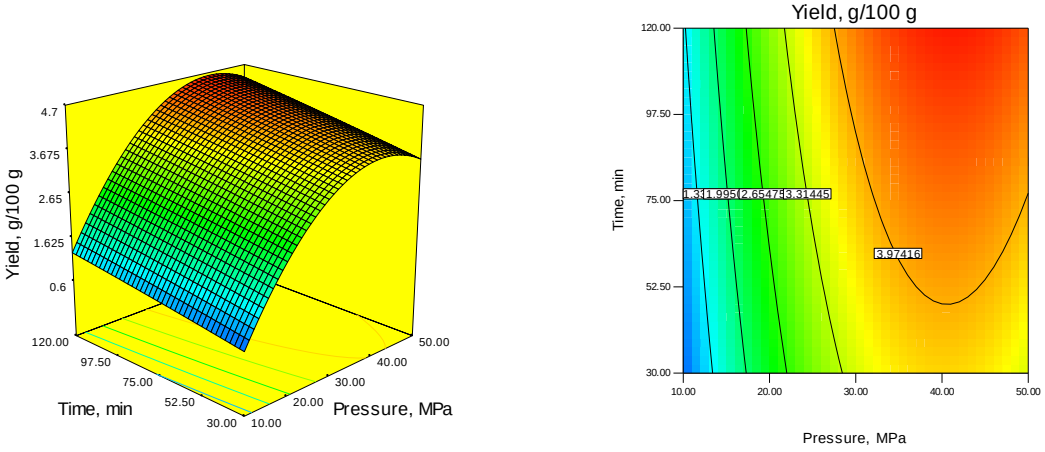
Table 2. Central composite design matrix for SFE-CO_2 optimisation for wild cyanobacterial biomass and values of observed responses: experimental (actual) and predicted SFE-CO_2 extract yields (g/100 g DW) and SFE-CO_2 efficiency in comparison to Soxhlet extraction (%)

Run No.	SFE-CO_2 parameters			SFE-CO_2 extract yield[1], g/100 g DW		SFE-CO_2 efficiency[2], %	
	P, MPa	T, °C	τ, min	Experimental	Predicted	Experimental	Predicted
1	30	50	75	3.73 ± 0.11^{f}	3.70	101 ± 3^{f}	101
2	50	50	75	3.94 ± 0.12^{fg}	4.00	107 ± 3^{fg}	109
3	30	50	120	4.19 ± 0.03^{fg}	4.19	114 ± 1^{fg}	114
4	30	50	75	3.86 ± 0.12^{f}	3.83	105 ± 3^{f}	104
5	10	30	30	1.21 ± 0.08^{c}	1.19	33 ± 2^{c}	32
6	30	50	75	3.97 ± 0.16^{fg}	4.00	108 ± 4^{fg}	109
7	50	30	30	2.95 ± 0.23^{e}	2.86	80 ± 6^{e}	78
8	10	70	120	0.44 ± 0.02^{ab}	0.50	12 ± 1^{ab}	14
9	30	50	75	3.85 ± 0.08^{f}	3.83	105 ± 2^{f}	104
10	30	50	30	3.31 ± 0.03^{e}	3.43	90 ± 1^{e}	93
11	10	50	75	0.89 ± 0.03^{bc}	0.99	24 ± 1^{bc}	27
12	50	30	120	3.89 ± 0.07^{f}	3.92	106 ± 2^{f}	107
13	50	70	120	4.76 ± 0.24^{h}	4.75	129 ± 7^{h}	129
14	30	30	75	3.70 ± 0.15^{f}	3.65	101 ± 4^{f}	99
15	30	50	75	3.89 ± 0.13^{f}	3.83	106 ± 4^{f}	104
16	50	70	30	3.95 ± 0.15^{fg}	4.00	107 ± 4^{fg}	109
17	30	50	75	3.91 ± 0.20^{fg}	3.83	106 ± 5^{fg}	104
18	10	30	120	2.04 ± 0.06^{d}	1.96	55 ± 2^{d}	53
19	10	70	30	0.12 ± 0.01^{a}	0.05	3 ± 0^{a}	1
20	30	70	75	3.73 ± 0.01^{f}	3.70	101 ± 0^{f}	101
Optimal conditions							
	42.5	55	120	4.43 ± 0.25^{gh}	4.68	120 ± 7^{gh}	127

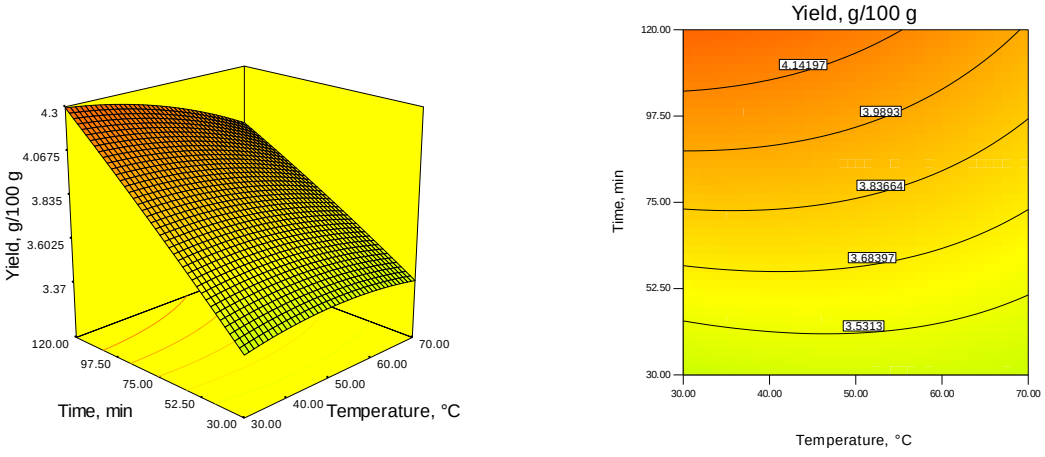
SFE-CO_2: supercritical carbon dioxide extraction; P: extraction pressure; T: extraction temperature. τ: extraction time; [1]: SFE-CO_2 extract yields were expressed as g/100 g DW of biomass sample prior SFE-CO_2. [2]: compared to Soxhlet extraction and calculated as [$Yield_{SFE\text{-}CO2}$/$Yield_{Soxhlet}$] *100. Experimental values are reported as mean value ± standard deviation of two replicates. In all cases relative standard deviation did not exceed 10%. Different superscript letters within the same column indicate significant differences (one-way ANOVA and Tukey's test, $p < 0.05$).



A: Effect of temperature and pressure at constant time of 75 min



B: Effect of time and pressure at constant temperature of 50 °C



C: Effect of time and temperature at constant pressure of 30 MPa

Figure 10. Response surface 3D and 2D plots showing the effects of independent variables (temperature, pressure and time) on SFE-CO_2 extract yields (g/100 g DW) from wild cyanobacterial biomass (**A**: effect of temperature and pressure at constant time of 75 min; **B:** effect of time and pressure at constant temperature of 50 °C: **C**: effect of time and temperature at constant pressure of 30 MPa)

Analysis of variance (ANOVA), presented in Table A.1. (Appendices), was used to identify statistically significant effects of the tested variables on the experimental response factor using the Student test (*p*-value) at 5% probability level ($p<0.05$). Based on the obtained experimental values, a quadratic regression model was built. The adequacy of the model was determined by evaluating the '*lack of fit*' coefficient and the Fisher test value (*F*-value) obtained from analysis of variance. Analysis of this quadratic regression model showed that the model itself was significant according to the Student test ($p < 0.0001$, F=330.07), whereas the "*lack of fit*" was not significant ($p > 0.05$) relative to the pure error, with a *p*-value of 0.1450. The obtained results showed that all three model variables (P, T, τ), PT interaction and second-order term P^2 were significant ($p < 0.05$) on the total SFE-CO_2 extract yield. The factor with the largest effect was pressure P with *F* value of 1814.93, followed by extraction time τ (F = 118.55) and temperature T (F = 5.18). Interaction between the pressure and temperature (PT) had significant effects on SFE-CO_2 yield in a linear manner ($p < 0.05$, F=215.66), indicating synergistic effect between these factors, while the effects of the other factor interactions were not significant in this model ($p > 0.05$). Similarly, second-order term of pressure (P^2) was the fifth significant factor ($p < 0.0001$) with determined *F* value of 417.52. The main discussed effects of three evaluated SFE-CO_2 parameters and various interactions thereof are summarized in the Pareto chart in Figure A.1. It could be seen that approximately 95% of the observed responses in this model derives from the effects of P (~45%), P^2 (~23%), PT interactions (~15%) and τ (~11%), while contribution of other factors is significantly smaller.

The close fit between experimental and predicted values within the investigated experimental range complemented the information, provided by the statistical indicators (Figure A.2.). Second order polynomial regression model, describing relationship between dependent and independent variables (P, T, τ), is summarized in equation 13:

$$Yield_{SFECO_2} = 3.83 + 1.48 * P - 0.079 * T + 0.38 * \tau + 0.57 * PT + 0.075 * P\tau - 0.08 * T\tau - 1.35 * P^2 - 0.053 * T^2 - 0.018 * \tau^2 \quad (13)$$

Based on higest SFE-CO_2 yields, 42.5 MPa pressure, 55 °C temperature and 120 min parmeters were chosen as optimal conditions. Under these conditions, 4.43 g/100 g DW of lipophilic fraction from wild cyanobacterial biomass was isolated, showing 20% higher extraction efficiency at 3-fold lower extraction time as compared to the conventional Soxhlet extraction (120 min *versus* 360 min).

In this study, the significant influence of extraction pressure on the yield was highlighted. This is in agreement with previous studies summarized in review of Crampon et al. where it is generally concluded that the higher the applied pressure the higher is the extraction efficiency and/or

faster the extraction kinetics is achieved [96]. Moreover, the retrograde behaviour of temperature on the extraction yield was also observed. Several authors have reported increased extraction efficiency with the addition of co-solvent(s) [96]. However due to different polarities, the addition thereof leads to a less selective extraction process [67] and current legislative safety regulations may not allow the use of co-solvent(s) at industrial scale [97]. Interestingly, Pyo and Shin reported that microcystins, the toxins that are present in several cyanobacterial blooms, are sparingly soluble in pure supercritical CO_2 [98]. However, these toxins could be extracted with very high recovery rates from cyanobacterial samples with the addition of methanol, acetic acid or acetonitrile as a co-solvent [98],[99]. Therefore, it could be assumed that SFE may offer an additional advantage over conventional extraction techniques in utilizing wild cyanobacteria for multipurpose applications.

3.1.3. Fatty acid profile of non-polar extracts from wild cyanobacteria

In a next step, the fatty acid profile of the extracts from wild cyanobacterial biomass was assessed by means of GC-FID. As reported in Table 3, six saturated, four monounsaturated and three polyunsaturated fatty acids were identified in these extracts, comprising on average 24%, 15% and 42% of the total GC peak area, respectively.

The most abundant fatty acid was polyunsaturated α-linolenic acid (ω-3) which average of amounting reached 30% in the triglycerides from two different extraction techniques. Second most abundant fatty acid was saturated palmitic acid with ~15% of the total peak area, followed by polyunsaturated linoleic (~11%), monounsaturated oleic (~8%), saturated myristic (~6%) and monounsaturated elaidic (~4%) and palmitoleic (~2%) acids (Table 3).

The discussed fatty acid profile is in close agreement with the ones reported for *Aphanizomenon* cultures from Scandinavian coastal waters [16], cyanobacterial biomass isolated from Portuguese freshwater habitats [100] and *Aphanizomenon* cultures related with harmful cyanobacterial blooms in the Baltic Sea [101]. According to the classification of Los and Mironov, the fatty acid profile of the wild cyanobacteria used in this study relates to cyanobacteria of group 4 which is described by the amount of α-linolenic, palmitic, palmitoleic, oleic and linoleic acids [102]. Besides the potential use of the lipophilic fraction from filamentous cyanobacteria for biodiesel production [103], specific fatty acids like α-linolenic acid can find applications in the food, biotechnological, nutraceutical and pharmaceutical industries [104].

Table 3. Fatty acid content of non-polar (Soxhlet and SFE-CO_2) extracts, obtained from wild cyanobacterial biomass after Soxhlet and optimized SFE-CO_2 (42.5 MPa, 55 °C, 120 min)

Fatty acid	Profile, % of the total GC-FID peak area	
	Soxhlet extract	SFE-CO_2 extract
C12:0	0.75±0.01[a]	0.76±0.00[a]
C14:0	7.23±0.07[b]	5.51±0.16[a]
C14:1	0.63±0.02[b]	0.47±0.02[a]
C16:0	15.01±0.12[a]	15.53±0.24[b]
C16:1	2.16±0.01[b]	1.70±0.08[a]
C18:0	1.25±0.01[a]	1.89±0.02[b]
C18:1n9t	1.76±0.12[a]	6.41±0.20[b]
C18:1n9c	8.49±0.06[b]	6.46±0.12[a]
C18:2n6c	8.14±0.00[a]	13.04±0.11[b]
C20:0	-[nd]	0.13±0.00
C18:3n3	33.81±0.24[b]	27.07±0.1[a]
C22:0	-[nd]	0.10±0.01
C20:3n3	0.64±0.01[b]	0.44±0.01[a]
SFAs	24.24	23.92
MUFAs	13.04	17.04
PUFAs	42.59	40.55

SFE-CO_2: supercritical carbon dioxide extraction. C12:0 – Lauric acid; C14:0 – Myristic acid; C14:1 – Myristoleic acid; C16:0 – Palmitic acid; C16:1 – Palmitoleic acid; C18:0 – Stearic acid; C18:1n9t – Elaidic acid; C18:1n9c – Oleic acid; C18:2n6c – Linoleic acid; C20:0 – Arachdic acid; C18:3n3 - α-Linolenic acid; C22:0 – Behenic; C20:3n3 – cis-11,14,17-Eicosatrienoic acid; results expressed as % of biomass sample. Values are reported as mean value ± standard deviation. In all cases relative standard deviation did not exceed 10%. Different superscript letters within the same line indicate significant differences (unpaired t-test, $p < 0.05$)

3.1.4. Tocopherol content in lipophilic extracts of wild cyanobacteria

The tocopherol content of the studied extracts of wild cyanobacteria was analysed by means of normal phase HPLC. As reported in Table 4, cyanobacterial extracts were characterized mainly by the presence of α-tocopherol; β-and γ-isomers were present in remarkably (10-fold) smaller amounts, whereas δ-tocopherol was not detected at all. This is in agreement with the previous studies: for example, among 12 cultured cyanobacteria the average α-tocopherol content was approximately 125 and 58 μg/g dry matter for the stationary and late logarithmic growth phase respectively [105]. Mendiola et al in samples from *Arthrospira platensis* extracted with SFE-CO_2 (36.1 MPa and 55 °C) reported 5.15 mg Vitamin E/g extract with a yield of 0.6%, which equals ~31 μg/g of DW [40]. Hernandez et al. reported that the highest content of α-tocopherol (5.01 μg/g) in *Arthrospira platensis* was obtained under 60 °C, 15 MPa and 53.33% of ethanol as a co-solvent in supercritical fluid

extraction [63]. Nevertheless, an important factor that must be always evaluated is the chemical composition of the specific feedstock. It is known that at higher pressures a competitive extraction between tocopherol and other diluting materials (glycerides, free fatty acids, pigments) can occur resulting in lower tocopherol concentrations [40].

Table 4. Tocopherol content in Soxhlet and SFE-CO_2 (42.5 MPa, 55 °C, 120 min) extracts, obtained from wild cyanobacterial biomass

Tocopherol	Amount of tocopherols, μg/g			
	in extract		in DW[1]	
	Soxhlet	SFE-CO_2	Soxhlet	SFE-CO_2
α-Tocopherol	326.68 ± 8.34^{d}	293.73 ± 9.55^{c}	12.02 ± 0.31^{c}	13.01 ± 0.42^{d}
β-Tocopherol	17.94 ± 0.92^{a}	38.00 ± 2.55^{b}	0.66 ± 0.03^{a}	1.68 ± 0.11^{b}
γ-Tocopherol	11.04 ± 0.69^{a}	18.88 ± 0.55^{a}	0.41 ± 0.03^{a}	0.84 ± 0.02^{a}
Total	355.65	350.61	13.08	15.53

SFE-CO_2: supercritical carbon dioxide extraction; [1]: g/100 g DW of crude (unextracted) biomass; -[nd]: not detected. Data expressed as mean value ± standard deviation of three replicates. Different superscript letters within the individual block of data (Amount of tocopherols μg/g in extract or Amount of tocopherols μg/g in DW) indicate significant differences (one-way ANOVA and Tukey's test, $p < 0.05$).

3.1.5. *In vitro* antioxidant activity assessment of lipophilic extracts and solid fractions

As reported in Table 5, TPC, $TEAC_{ABTS}$ and $TEAC_{DPPH}$ values of lipophilic extracts were 60.88 mg GAE/g, 65.19 mg TE/g and 52.29 mg TE/g, respectively. When recalculated for one gram of crude (unextraced) biomass, the recovery of antioxidant equivalents by Soxhlet/SFE-CO_2 in TPC, $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ assays were 2.06/2.93 mg GAE, 2.33/2.97 mg TE and 1.81/2.45 mg TE, respectively. Consequently, the antioxidant capacity of SFE-CO_2 extract as well as the recovery of antioxidants was by 27-42% higher as compared to the Soxhlet extraction. Using QUENCHER procedure, the highest TPC value (23.49 mg GAE/g DW) was obtained for the wild cyanobacterial biomass prior extractions; after removing the lipophilic fraction by means of SFE-CO_2 or Soxhlet extraction it reduced on average by 32%. It is rather unlikely that the observed reduction in TPC content could occur due to the phenolic compound removal with non-polar solvents, such as supercritical CO_2 or hexane. However, the reactivity of the Folin –Ciocalteu's reagent is not limited solely to phenolics and many other compounds of varying classes are known to reduce this reagent too; thus, this assay generally measures overall reducing capacity of the sample. No significant changes in $TEAC_{ABTS}$ and $TEAC_{DPPH}$ values for cyanobacterial biomass was observed prior and after non-polar fraction removal. This indicates that a considerable part of compounds with *in vitro* antiradical capacity remains in the solid residues after SFE-CO_2 or Soxhlet extraction, which could

be further treated to obtain antioxidant-rich fractions of higher polarity with potential multipurpose applications.

Table 5. Total phenolic content (TPC), DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ scavenging capacity of Soxhlet and SFE-CO_2 (42.5 MPa, 55 °C, 120 min) extracts, crude material and solid residues, obtained from wild cyanobacterial biomass after extraction

***In vitro* antioxidant capacity**	**Extracts**		**Crude biomass**	**Solid residues after extraction**	
	Soxhlet	SFE-CO_2		After Soxhlet	After SFE-CO_2
TPC, mg GAE/g:					
mg /g extract*	55.68±3.95^{a}	66.09±0.79^{b}	-^{na}	-^{na}	-^{na}
mg /g DW[1**]	2.06±0.15^{a}	2.93±0.03^{a}	23.49±1.51^{c}	16.13±1.26^{b}	15.97±1.20^{b}
$TEAC_{ABTS}$, mg TE/g:					
mg /g extract*	63.30±2.76^{a}	67.08±3.06^{a}	-^{na}	-^{na}	-^{na}
mg /g DW[1**]	2.33±0.10^{a}	2.97±0.14^{a}	57.14±5.06^{b}	57.18±4.31^{b}	56.89±3.92^{b}
$TEAC_{DPPH}$, mg TE/g:					
mg /g extract*	49.18±3.50^{a}	55.40±2.36^{b}	-^{na}	-^{na}	-^{na}
mg /g DW[1**]	1.81±0.13^{a}	2.45±0.10^{b}	5.04±0.18^{c}	5.38±0.13^{d}	4.88±0.15^{c}

[1]: g/100 g DW, mg GAE or TE/g DW of crude (unextracted) biomass; -^{na} : not applicable; SFE-CO_2: supercritical carbon dioxide extraction. Data expressed as mean value ± standard deviation of five replicates. *: Different superscript letters within the same line for individual *in vitro* antioxidant activity assessment assays indicate significant differences (unpaired t-test, $p < 0.05$). **: Different superscript letters within the same line for individual *in vitro* antioxidant activity assessment assays indicate significant differences (one-way ANOVA and Tukey's test, $p < 0.05$).

In addition, calculated Pearson correlation coefficients between different antioxidant activity indices indicate the presence of a strong positive correlation (0.9279-0.9859, $p<0.05$) between TPC and DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ scavenging assays (Table 6). To the best of our knowledge, this is the first report characterizing *in vitro* antioxidant potential of both extracts and solid fractions from wild cyanobacteria.

Table 6. Analysis of correlation between different antioxidant activity indices of non-polar (SFE-CO_2) extracts and solid residues, crude material and solid residues, obtained from wild cyanobacteria biomass after Soxhlet and optimized SFE-CO_2 (42.5 MPa, 55 ºC, 120 min)

Antioxidant activity indices[1]	**TPC**	**ABTS$^{\bullet+}$**	**DPPH$^{\bullet}$**
TPC	1	0.9453*	0.9279*
ABTS$^{\bullet+}$		1	0.9859**
DPPH$^{\bullet}$			1

SFE-CO_2: supercritical carbon dioxide extraction. [1] expressed as mg GAE/g DW or mg TE/g DW; *: correlation is significant at the p<0.05 level (two-tailed). Pearson correlation coefficients were calculated using GraphPad Prism 6.01 software (2012).

It should be noted that generally studies regarding antioxidant potential of cyanobacteria are focused on the antioxidant properties and activity of pigments and most notably on phycocyanin [106] or, in some other cases, polysaccharides [107]. In a study with selected cyanobacterial and microalgal strains from the Coimbra Collection of Algae, the antioxidant capacity of the cyanobacterial strains was in the range of 22-39 mg TE/100 g of fresh biomass in the ABTS assay, whereas the reported IC_{50} $DPPH^{\bullet}$ scavenging values varied from 289 to 1034 mg/mL [108]. Singh et al. reported that the TPC of cell-free extracts of twenty terrestrial cyanobacteria was 23-290 mg of GAE/g of fresh weight with IC_{50} values of 1-4 mg/mL and 0.2-1 mg/mL in the $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ assays, respectively [109]. However, direct comparison of these values is rather complex, since most of these studies focus on specific strains grown under optimal conditions.

3.2. Phycobiliproteins extraction from wild cyanobacteria

3.2.1. Optimization of three traditional protein extractions

Three different methods have been selected for the recovery of phycobiliproteins, freeze-thaw, homogenization, solid-liquid extraction, due to their ability to break the cells. For each method, optimal conditions for obtaining highest phycobiliproteins yield were determined. Later, all three methods under optimal conditions were combined with ultrasound assisted extraction, with the expectation that a higher yield of phycobiliproteins would be obtained. The process of experiment is demonstrated in Figure 11. CCD and RSM were used to optimize combined methods on phycobiliproteins yields. For all phycobiliproteins extractions water was used as a solvent.

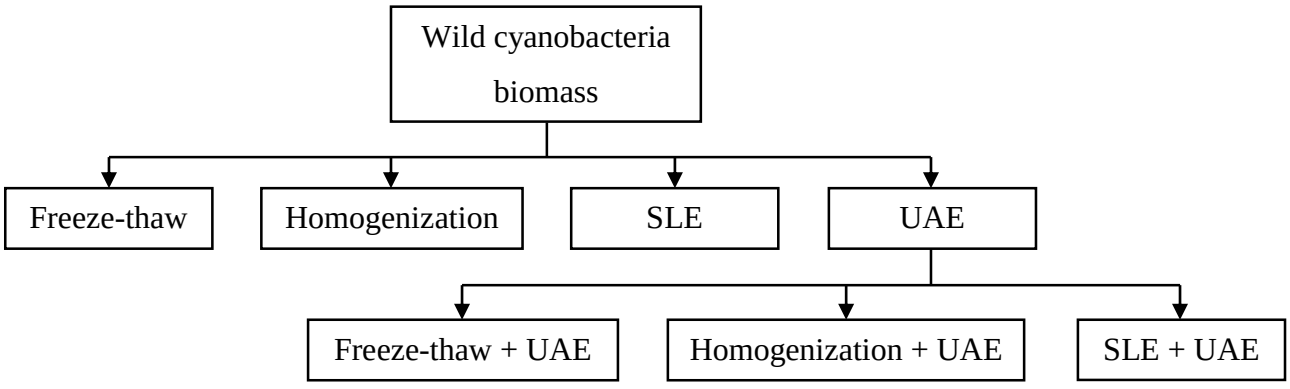


Figure 11. Experiment of phycobiliproteins extraction process

SLE – solid-liquid extraction; UAE – ultrasound assisted extraction.

3.2.1.1. Freeze-thaw extraction

Liquid nitrogen was used for freeze-thaw extraction. Due to water crystals after freezing process the cells were break and the phycobiliproteins were released. The experiment was done by cycles to detect phycobiliproteins content left in cyanobacterial biomass. After each cycle, the water with phycobiliproteins was poured in another vessel and prepared for measurement, and fresh water was added to the remaining biomass. One cycle takes 20 minutes, 5 minutes for freezing and 15 minutes for thawing.

Table 7. PC and APC yields (mg/g DW) after three cycles of freeze-thaw extraction from wild cyanobacteria biomass

Proteins	**Number of cycles, yield mg/g DW**		
	I	**II**	**III**
PC	26.64 ± 0.91	4.29 ± 0.31	2.05 ± 0.38
APC	17.25 ± 0.36	2.60 ± 0.28	1.85 ± 0.08

PC – phycocyanin, APC – allophycocyanin. Values are reported as mean value ± standard deviation.

Phycocyanin and allophycocyanin contents were determined during the study, and phycoerythrin content was too small to determ. Based on the obtained results (Table 7), phycocyanin and allophycocyanin contents were highest in the first cycle, respectively 26.64 and 17.25 mg/g DW, and in the other cycles yields decreases. In the second cycle, the yield of phycocyanin is lower by 6.2 times compared to the yield in the first cycle and the yield of allophycocyanin is lower by 6.6 times compared to the yield in the first cycle, and in the third cycle yields are lower respectively by 13 and 9.3 times compared to the yields in the first cycle. Based on phycobiliproteins yields one cycle of freeze-thaw extraction was chosen as optimal.

3.2.1.2. Homogenization

The homogenizer mechanically damages the cyanobacterial cells and releases phycobiliproteins. In order to obtain the highest yield of phycobiliproteins, it was decided to optimize the influence of three parameters – experiment cycles, time and temperature. The phycocyanin and allophycocyanin contents were determined in the experiment, and phycoerythrin content was too low.

Three cycles were chosen to evaluate the changes of phycobiliproteins yields. After each cycle, the water with phycobiliproteins was poured in another vessel and prepared for measurement, and fresh water was added to the remaining biomass.

Table 8. PC and APC yields (mg/g DW) after three cycles of homogenisation extraction from wild cyanobacteria biomass

Proteins	Number of cycles, yield mg/g DW		
	I	II	III
PC	29.01 ± 0.18	3.90 ± 0.05	1.97 ± 0.05
APC	20.32 ± 0.81	4.98 ± 0.30	3.44 ± 0.18

PC – phycocyanin, APC – allophycocyanin. Values are reported as mean value ± standard deviation.

Based on the obtained results (Table 8), phycocyanin and allophycocyanin contents were highest in the first cycle, respectively 29.01 and 20.32 mg/g DW, and in the other cycles yields decreases. In the second cycle, the yield of phycocyanin is lower by 7.4 times compared to the yield in the first cycle and the yield of allophycocyanin is lower by 4 times compared to the yield in the first cycle, and in the third cycle yields are lower respectively by 14.7 and 5.9 times compared to the yields in the first cycle. Based on phycobiliproteins yields one cycle of freeze-thaw extraction was chosen as optimal.

Phycobiliproteins yields changes were determined at selected time intervals. According to results (Table 9), phycocyanin and allophycocyanin contents were highest after 60 minutes respectively 32.27 and 22.2 mg/g DW, and lowest after 5 minutes respectively 27.29 and 18.88 mg/g DW. Phycocyanin and allophycocyanin contents increased 1.2 times comparing yields after 5 and 60 minutes. Based on phycobiliproteins yields 30 minutes of homogenization was chosen as optimal.

Table 9. PC and APC yields (mg/g DW) after different periods of time of homogenisation extraction from wild cyanobacteria biomass

Proteins	Time min, yield mg/g DW				
	5	15	30	45	60
PC	27.29 ± 0.50	28.37 ± 0.24	30.72 ± 0.03	31.80 ± 0.83	32.27 ± 0.25
APC	18.88 ± 0.50	19.96 ± 0.37	21.24 ± 0.28	21.98 ± 0.70	22.20 ± 0.42

PC – phycocyanin, APC – allophycocyanin. Values are reported as mean value ± standard deviation.

The effect of temperature on the phycobiliproteins yields was checked every 10 °C, starting 20 °C and ending with 50 °C. Phycocyanin yield was increasing all the time and the highest yield was 36.45 mg/g DW at 50 °C. Allophycocyanin yield was increasing until 30 °C and from 30 °C started to decrease due to denaturation. The highest yield of allophycocyanin was 20.82 mg/g DW at 30 °C (Table 10). 30 °C temperature was chosen as optimal because until that both yields of phycobiliproteins were increasing. Based on phycobiliproteins yields one cycle, 30 minutes and 30 °C was chosen as optimal conditions.

Table 10. PC and APC yields (mg/g DW) after applying different temperature of homogenisation extraction from wild cyanobacteria biomass

Proteins	Temperature °C, yield mg/g DW			
	20	30	40	50
PC	29.42 ± 0.29	32.05 ± 0.03	32.08 ± 0.72	36.45 ± 0.28
APC	19.84 ± 0.22	20.82 ± 0.39	20.28 ± 0.51	11.86 ± 0.03

PC – phycocyanin, APC – allophycocyanin. Values are reported as mean value ± standard deviation.

3.2.1.3. Solid-liquid extraction

During the solid-liquid extraction, the phycobiliproteins are transferred to the water due to the concentration gradient. In order to obtain the highest yield of phycobiliproteins, it was decided to optimize the influence of two parameters – experiment cycles and time. Three cycles were chosen to evaluate the changes of phycobiliproteins yields. After each cycle, the water with phycobiliproteins was poured in another vessel and prepared for measurement, and fresh water was added to the remaining biomass.

Table 11. PC and APC yields (mg/g DW) after three cycles of solid-liquid extraction from wild cyanobacteria biomass

Proteins	Number of cycles, yield mg/g DW		
	I	II	III
PC	28.87 ± 0.09	2.33 ± 0.03	2.02 ± 0.02
APC	18.59 ± 0.20	1.86 ± 0.07	1.18 ± 0.06

PC – phycocyanin, APC – allophycocyanin. Values are reported as mean value ± standard deviation.

Based on the obtained results (Table 11), phycocyanin and allophycocyanin contents were highest in the first cycle, respectively 28.87 and 18.59 mg/g DW, and in the other cycles yields decreases. In the second cycle, the yield of phycocyanin is lower by 12.4 times compared to the yield in the first cycle and the yield of allophycocyanin is lower by 10 times compared to the yield in the first cycle, and in the third cycle yields are lower respectively by 14.3 and 15.8 times compared to the yields in the first cycle. Based on phycobiliproteins yields one cycle of solid-liquid extraction was chosen as optimal.

Table 12. PC and APC yields (mg/g DW) after different periods of time of solid-liquid extraction from wild cyanobacteria biomass

Proteins	Time min, yield mg/g DW			
	60	120	180	240
PC	28.93 ± 0.04	31.89 ± 0.39	33.01 ± 0.23	33.41 ± 0.26
APC	18.43 ± 0.01	20.38 ± 0.10	20.68 ± 0.33	20.63 ± 0.27

PC – phycocyanin, APC – allophycocyanin. Values are reported as mean value ± standard deviation.

Phycobiliproteins yields changes were determined at selected time intervals. According to the results (Table 12), phycocyanin content was highest after 240 minutes (33.41 mg/g DW), and lowest after 60 minutes (28.93 mg/g DW), allophycocyanin content was highest after 180 minutes (20.68 mg/g DW) and after 240 minutes yield was almost the same, and the lowest content of allophycocyanin was after 60 minutes (18.43 mg/g DW). Phycocyanin and allophycocyanin contents increased 1.2 times comparing yields after 5 and 60 minutes. 60 minutes were chosen as optimal for the extraction time difference in comparison with other methods. Based on phycobiliproteins yields one cycle, 60 minutes were chosen as optimal conditions.

Comparing all three methods, freeze-thaw, homogenization and solid-liquid extraction, was determined that after one cycle of experiment the highest total phycobiliproteins yield was after homogenization (29,01 mg/g DW or 46.4 μg/ml) and the lowest after freeze-thaw extraction (26.64 mg/g DW or 42.6 μg/ml). Comparing homogenization with solid-liquid extraction was determined that after optimal time, 30 minutes for homogenization and 60 minutes for SLE, total phycobiliproteins yield was respectively 30.72 and 28.93 mg/g DW (49 and 46 μg/ml), so according to the time, homogenization was superior than solid-liquid extraction Based on the results, homogenization was the best method to extract phycobiliproteins comparing with freeze-thaw and SLE.

These results are in agreement with previous studies, however, high influence for the yield has microalgal species, chosen methods and solvents. Abalde et al. reported about three methods to extract phycocyanin from cyanobacterium *Synechococcus* sp. and results showed that the best method was freeze-thaw in freezer (13.42 μg/ml), followed by freeze-thaw in liquid nitrogen (9.41 μg/ml) and sonication (7.44 μg/ml) when water was a solvent [110]. The other research showed that *Spirulina platensis* had more phycocyanin and proved that with study, when biomass was freezed, homogenized and left in water for 24 h and the yield of phycocyanin reached 13.73 mg/g DW [111]. Ilter et al. did the same experiment and results was 11.22 mg/g of phycocyanin from *S. platensis* [112]. Moraes et al. showed that one cycle of freezing and thawing and leaving biomass for 24 h (yield was 18 mg/g DW of phycocyanin from *S. platensis*) had not any difference comparing with the same method after leaving biomass for 48 h (yield was 17 mg/g DW) [113]. Moreover, Tavanandi et al. used three methods, maceration, homogenization, freeze thawing, to extract phycocyanin from *A. platensis* and the phosphate buffer was chosen as a solvent. Results showed that phycocyanin yields were higher using phosphate buffer as a solvent (55.91, 52.11, 73.73 mg/g DW, respectively) [114]. However, direct comparison of these values is rather complex, since most of these studies focus on specific strains grown under optimal conditions.

3.2.2. Optimization of ultrasound assisted extraction (UAE) parameters

The effectiveness of ultrasound assisted extraction depends on amplitude and time. CCD and RSM were used to optimize the effect of two factors, amplitude and time, on the phycocyanin, allophycocyanin, phycoerythrin and total phycobiliproteins yields as the response factors. The complete matrix with actual and predicted values of phycobiliproteins yields is presented in Table 13. According to the results, total phycobiliproteins yield varied from 51.70 to 129.41 mg/g DW of starting wild cyanobacteria biomass under different tested UAE conditions.

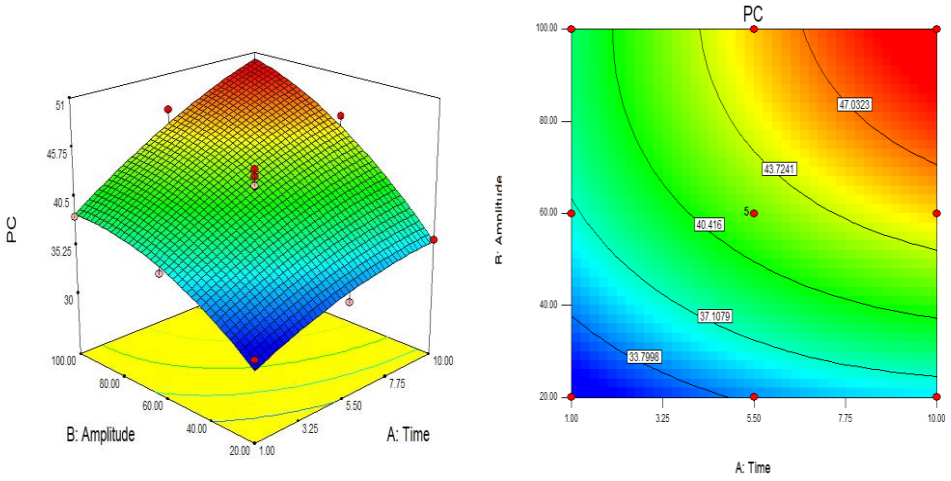
Table 13. Central composite design matrix for UAE optimisation for wild cyanobacterial biomass and values of observed responses: PC, APC, PE actual yields (mg/g DW) and predicted (P) yields of each phycobiliprotein (mg/g DW)

No.	UAE parameters		Phycobiliprotein yield, mg/g DW							
	A, %	τ, min	PC	PC P	APC	APC P	PE	PE P	Total yield	Total yield P
1	60	5.5	42.78 ± 0.46	42.06	52.11 ± 1.26	53.20	11.57 ± 0.78	13.06	106.46 ± 1.59	108.32
2	20	5.5	33.05 ± 0.91	34.18	28.34 ± 0.35	33.21	3.75 ± 0.20	4.65	65.14 ± 1.47	72.04
3	60	5.5	41.13 ± 0.08	42.06	52.51 ± 1.17	53.20	14.40 ± 0.41	13.06	108.05 ± 1.50	108.32
4	100	10	49.10 ± 1.31	50.34	61.90 ± 0.18	67.24	18.08 ± 0.17	19.34	129.08 ± 1.30	136.93
5	100	5.5	47.10 ± 0.81	45.55	65.42 ± 3.83	59.51	16.15 ± 0.52	15.41	128.68 ± 5.16	120.47
6	60	5.5	40.64 ± 1.66	42.06	50.77 ± 0.59	53.20	11.67 ± 0.13	13.06	103.07 ± 2.12	108.32
7	60	10	46.41 ± 0.37	45.26	64.87 ± 0.34	59.58	18.14 ± 0.84	16.43	129.41 ± 1.55	121.27
8	60	5.5	41.79 ± 0.48	42.06	52.93 ± 0.70	53.20	13.03 ± 0.85	13.06	107.75 ± 1.06	108.32
9	20	1	31.52 ± 0.44	30.49	20.76 ± 3.45	15.94	-0.58 ± 0.45	-1.92	51.70 ± 3.40	44.51
10	60	5.5	43.53 ± 0.52	42.06	56.67 ± 0.88	53.20	14.76 ± 0.31	13.06	114.96 ± 1.71	108.32
11	60	1	36.06 ± 0.19	36.79	30.32 ± 0.67	34.58	4.06 ± 0.48	5.92	70.44 ± 1.34	77.29
12	100	1	38.38 ± 0.37	38.69	38.96 ± 1.78	39.53	8.25 ± 0.17	7.72	85.58 ± 2.32	85.93
13	20	10	38.28 ± 1.80	35.78	38.28 ± 1.31	38.23	7.02 ± 0.04	7.47	81.18 ± 0.46	81.48
Optimal conditions (minimum time)										
	100	8.72	48.29 ± 0.17	49.16	65.03 ± 0.36	66.29	17.80 ± 0.40	18.61	131.12 ± 0.88	134.06

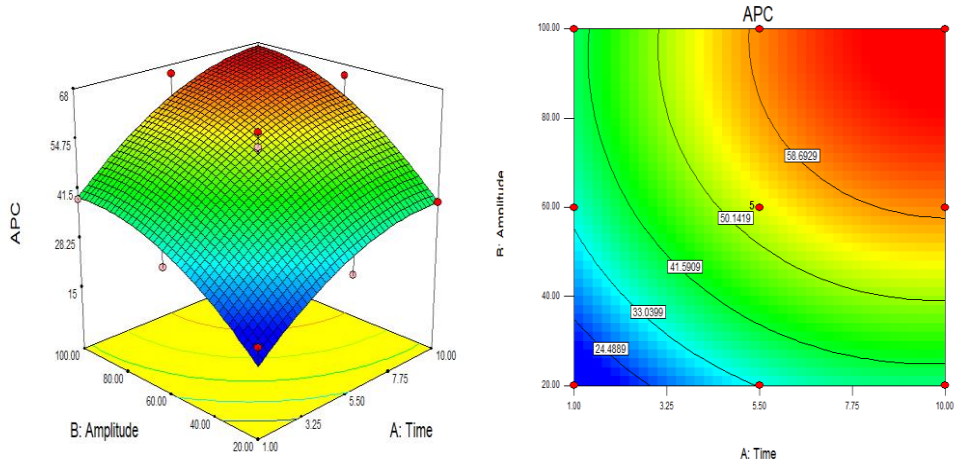
UAE – ultrasound assisted extraction; A – extraction amplitude (%); τ - extraction time (min); PC – phycocyanin; APC – allophycocyanin; PE – phycoerythrin; P – predicted value; extract yields were expressed as mg/g DW of biomass sample. Values are reported as mean value ± standard deviation. In all cases relative standard deviation did not exceed 10%.

3D and 2D response surface plots showing the extraction time and amplitude effect on phycobiliproteins yields (Figure 12). Figure 12 A shows the effect of time and amplitude for phycocyanin yield. Results shows that increasing both parameters, time and amplitude, the phycocyanin yield increases, both maximum time (10 min) and amplitude (100%) gave the maximum yield of phycocyanin (49.10 mg/g DW). Figure 12 B shows the effect of time and amplitude for

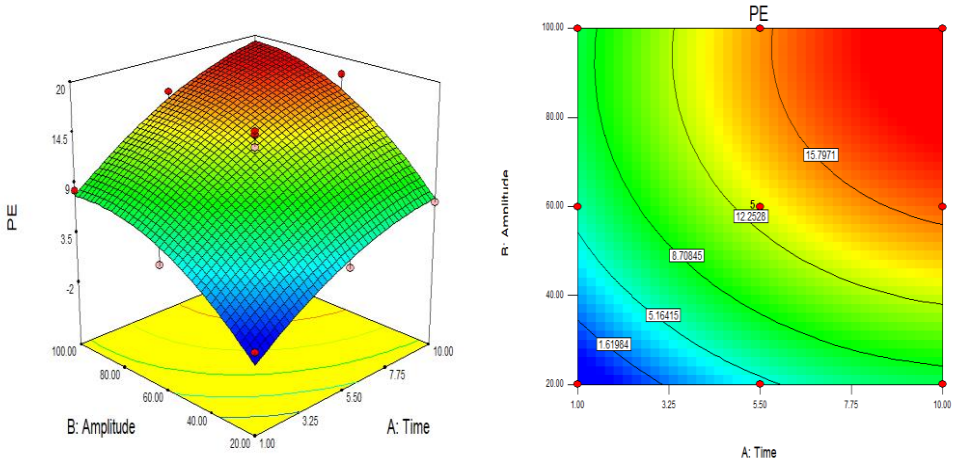
allophycocyanin yield. Results shows that increasing both parameters, time and amplitude, the allophycocyanin yield increases to maximum (65.42 mg/g DW) until amplitude reaches 100 % and time 5.5 minutes, with the longer extraction time at the same amplitude, yield of allophycocyanin starting to decrease. Such a change can be caused by allophycocyanin sensitivity to temperature. Figure 12 C shows the effect of time and amplitude for phycoerythrin yield. Results shows that increasing both amplitude and time increases the yield of phycoerythrin. The maximum yield is reached applying 60 % amplitude and time for 10 minutes, which shows that phycoerythin sensitive for temperature as allophycocyanin. Figure 12 D shows the effect of time and amplitude for total phycobiliproteins yield. Results shows that increasing both parameters, time and amplitude, the total yield increases. As given in the Table 13, the yield of phycobiliproteins depends on time, because for each level of amplitude there is three levels of time and in each amplitude level yield of phycobiliproteins is the lowest at 1 minute of extraction and the highest at 10 minutes.



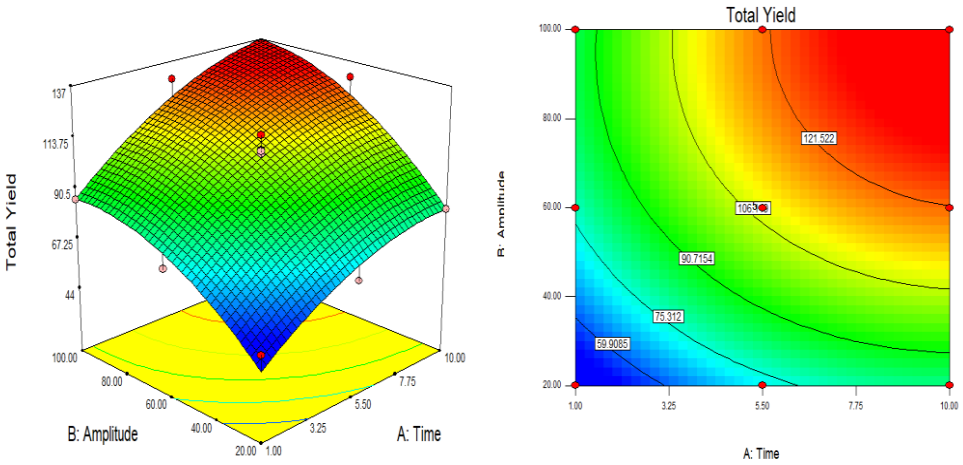
A: Effect of time and amplitude for phycocyanin yield (mg/g DW)



B: Effect of time and amplitude for allophycocyanin yield (mg/g DW)



C: Effect of time and amplitude for phycoerythrin yield (mg/g DW)



D: Effect of time and amplitude for total phycobiliproteins yield (mg/g DW)

Figure 12. Response surface 3D and 2D plots showing the effects of independent variables (time, min and amplitude, %) on ultrasound assisted extraction extract yields (mg/g DW) from wild cyanobacterial biomass (**A:** Effect of time and amplitude for phycocyanin yield (mg/g DW); **B:** Effect of time and amplitude for allophycocyanin yield (mg/g DW); **C:** Effect of time and amplitude for phycoerythrin yield (mg/g DW); **D:** Effect of time and amplitude for total phycobiliproteins yield (mg/g DW)).

The optimal conditions to obtain the highest UAE extraction yield were 100 % amplitude and 8.72 minutes. Under these conditions were extracted 48.29 mg/g DW of phycocyanin, 65.03 mg/g DW of allophycocyanin, 17.80 mg/g DW of phycoerythrin and total yield of phycobiliproteins was 131.12 mg/g DW.

Analysis (Table A.2.) of phycocyanin yield quadratic regression model showed that the model itself was significant according to the Student test ($p < 0.0001$, F=34.01), whereas the "*lack of fit*" was not significant ($p > 0.05$) relative to the pure error, with a p-value of 0.2626. The obtained results showed that A τ interaction and second-order term τ^2 were not significant ($p > 0.05$) on the

phycocyanin yield from UAE extract. The factor with the largest effect was amplitude A with *F* value of 98.06, followed by extraction time τ (*F* = 54.38). Interaction between the time and amplitude (A τ) had not significant effects on phycocyanin yield in a linear manner ($p > 0.05$, *F*=5.12).

The adequacy of the model can be also deduced from the determination coefficient (R^2 = 0.9605), which indicates good fit of the model to the experimental data. Moreover, this design could be used for modelling design space as the adjusted R-squared value of 0.9322 (a measure of the amount of variation about the mean) and predicted R-squared of 0.7397 are in reasonable agreement. The coefficient of variation (CV) of the model was 3.47%, meaning that model can be considered as reasonably reproducible. The close fit between experimental and predicted values within the investigated experimental range complemented the information, provided by the statistical indicators (Figure A.3.). Second order polynomial regression model, describing relationship between dependent and independent variables (P, T, τ), is summarized in equation 25:

$$Yield_{PC} = 24.77 + 0.98 * \tau + 0.26 * A + 8.84 * \tau A - 0.05 * \tau^2 - 1.37 * A^2 \qquad (25)$$

Analysis (Table A.3.) of allophycocyanin yield quadratic regression model showed that the model itself was significant according to the Student test ($p = 0.0006$, *F*=18.71), whereas the "*lack of fit*" was significant ($p < 0.05$) relative to the pure error, with a *p*-value of 0.0219. The obtained results showed that A τ interaction and second-order term τ^2 were not significant ($p > 0.05$) on the allophycocyanin yield from UAE extract. The factor with the largest effect was amplitude A with *F* value of 5.13, followed by extraction time τ (*F* = 4.11). Interaction between the time and amplitude (A τ) had not significant effects on allophycocyanin yield in a linear manner ($p > 0.05$, *F*=0.29).

The adequacy of the model can be also deduced from the determination coefficient (R^2 = 0.9304), which indicates good fit of the model to the experimental data. R-squared value was 0.8806 and predicted R-squared was 0.3601, due to significant "*lack of fit*". The coefficient of variation (CV) of the model was 10.64 %. The close fit between experimental and predicted values within the investigated experimental range complemented the information, provided by the statistical indicators (Figure A.4.). Second order polynomial regression model, describing relationship between dependent and independent variables (P, T, τ), is summarized in equation 14:

$$Yield_{APC} = -3.87 + 5.65 * \tau + 0.8 * A + 7.53 * \tau A - 0.30 * \tau^2 - 4.28 * A^2 \qquad (14)$$

Analysis (Table A.4.) of phycoerythrin yield quadratic regression model showed that the model itself was significant according to the Student test ($p = 0.0002$, *F*=27.01), whereas the "*lack of fit*" was not significant ($p > 0.05$) relative to the pure error, with a *p*-value of 0.2943. The obtained results showed that A τ interaction and second-order term τ^2 were not significant ($p > 0.05$) on the

phycoerythrin yield from UAE extract. The factor with the largest effect was amplitude A with *F* value of 8.63, followed by extraction time τ (*F* = 3.33). Interaction between the time and amplitude (A τ) had not significant effects on phycoerythrin yield in a linear manner ($p > 0.05$, *F*=0.42).

The adequacy of the model can be also deduced from the determination coefficient (R^2 = 0.9507), which indicates good fit of the model to the experimental data. Moreover, this design could be used for modelling design space as the adjusted R-squared value of 0.9155 (a measure of the amount of variation about the mean) and predicted R-squared of 0.6841 are in reasonable agreement. The coefficient of variation (CV) of the model was 15.86%, meaning that model can be considered as reasonably reproducible. The close fit between experimental and predicted values within the investigated experimental range complemented the information, provided by the statistical indicators (Figure A.5.). Second order polynomial regression model, describing relationship between dependent and independent variables (P, T, τ), is summarized in equation 15:

$$Yield_{PE} = -10.03 + 2.00 * \tau + 0.34 * A + 3.09 * \tau A - 0.09 * \tau^2 - 1.89 * A^2 \qquad (15)$$

Analysis (Table A.5.) of total phycobiliproteins yield quadratic regression model showed that the model itself was significant according to the Student test ($p = 0.0002$, *F*=25.09), whereas the "*lack of fit*" was not significant ($p > 0.05$) relative to the pure error, with a *p*-value of 0.0571. The obtained results showed that A τ interaction and second-order term τ^2 were not significant ($p > 0.05$) on the total yield from UAE extract. The factor with the largest effect was amplitude A with *F* value of 6.75, followed by extraction time τ (*F* = 3.79). Interaction between the time and amplitude (A τ) had not significant effects on total yield in a linear manner ($p > 0.05$, *F*=0.82).

The adequacy of the model can be also deduced from the determination coefficient (R^2 = 0.9471), which indicates good fit of the model to the experimental data. Moreover, this design could be used for modelling design space as the adjusted R-squared value of 0.9094 (a measure of the amount of variation about the mean) and predicted R-squared of 0.5460 are in reasonable agreement. The coefficient of variation (CV) of the model was 17.62%, meaning that model can be considered as reasonably reproducible. The close fit between experimental and predicted values within the investigated experimental range complemented the information, provided by the statistical indicators (Figure A.6.). Second order polynomial regression model, describing relationship between dependent and independent variables (P, T, τ), is summarized in equation 16:

$$Yield_{TOTAL} = 10.88 + 8.63 * \tau + 1.40 * A + 0.02 * \tau A - 0.45 * \tau^2 - 7.54 * A^2 \qquad (16)$$

3.2.3. Optimization of freeze-thaw and UAE extractions parameters

The effectiveness of freeze-thaw combined with ultrasound assisted extraction depends on amplitude and time. CCD and RSM were used to optimize the effect of two factors, amplitude and time, on the phycocyanin, allophycocyanin, phycoerythrin and total phycobiliproteins yields as the response factors. The complete matrix with actual and predicted values of phycobiliproteins yields is presented in Table 14. According to the results, total phycobiliproteins yield varied from 86.50 to 139.04 mg/g DW of starting wild cyanobacteria biomass under different tested UAE conditions.

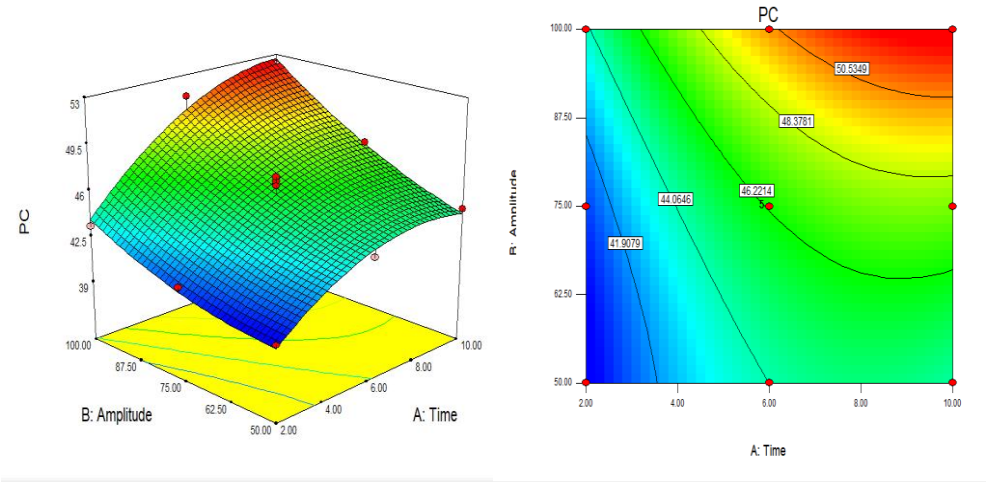
Table 14. Central composite design matrix for combined freeze-thaw and UAE extractions optimisation for wild cyanobacterial biomass and values of observed responses: PC, APC, PE actual yields (mg/g DW) and predicted (P) yields of each phycobiliprotein (mg/g DW)

No.	Freeze-thaw+UAE parameters		Phycobiliprotein yield, mg/g DW							
	A, %	τ, min	PC	PC P	APC	APC P	PE	PE P	Total yield	Total yield P
1	50	6	43.47 ± 0.53	44.07	50.21 ± 0.44	52.15	12.51 ± 0.35	13.82	106.18 ± 1.33	110.03
2	100	2	43.31 ± 0.95	43.87	48.67 ± 0.96	47.30	12.19 ± 0.41	12.60	104.16 ± 0.41	103.77
3	50	2	39.99 ± 0.56	39.75	38.41 ± 1.01	37.42	8.09 ± 0.07	7.22	86.50 ± 1.64	84.39
4	75	6	46.79 ± 0.65	46.30	61.90 ± 0.85	61.29	17.11 ± 0.36	17.50	125.80 ± 1.85	125.10
5	75	6	46.42 ± 0.18	46.30	63.00 ± 0.33	61.29	17.74 ± 0.23	17.50	127.16 ± 0.38	125.10
6	50	10	44.68 ± 0.04	44.32	57.82 ± 0.13	56.88	15.57 ± 0.20	15.13	118.07 ± 0.37	116.33
7	75	2	41.25 ± 0.88	40.92	44.29 ±1.65	46.65	10.59 ± 0.67	11.06	96.13 ± 3.20	98.62
8	100	10	52.25 ± 1.70	52.69	67.74 ± 3.59	66.43	19.05 ± 0.67	19.91	139.04 ± 1.22	139.03
9	75	10	47.70 ± 1.36	47.62	63.69 ± 0.96	65.94	19.08 ± 0.34	18.67	130.47 ± 2.65	132.22
10	75	6	45.34 ± 0.55	46.30	61.11 ± 0.01	61.29	16.85 ± 0.05	17.50	123.30 ± 0.51	125.10
11	75	6	47.09 ± 0.27	46.30	62.98 ± 0.50	61.29	18.33 ± 0.22	17.50	128.40 ± 0.02	125.10
12	75	6	45.48 ± 0.28	46.30	62.08 ± 0.51	61.29	17.53 ± 0.18	17.50	125.09 ± 0.97	125.10
13	100	6	51.32 ± 0.09	50.31	59.19 ± 0.17	61.87	20.16 ± 0.22	18.90	130.68 ± 0.14	131.08
Optimal conditions										
	99	9.39	53.78 ± 0.47	52.25	66.17 ± 0.73	66.59	19.33 ± 0.21	20.08	139.28 ± 0.88	138.92

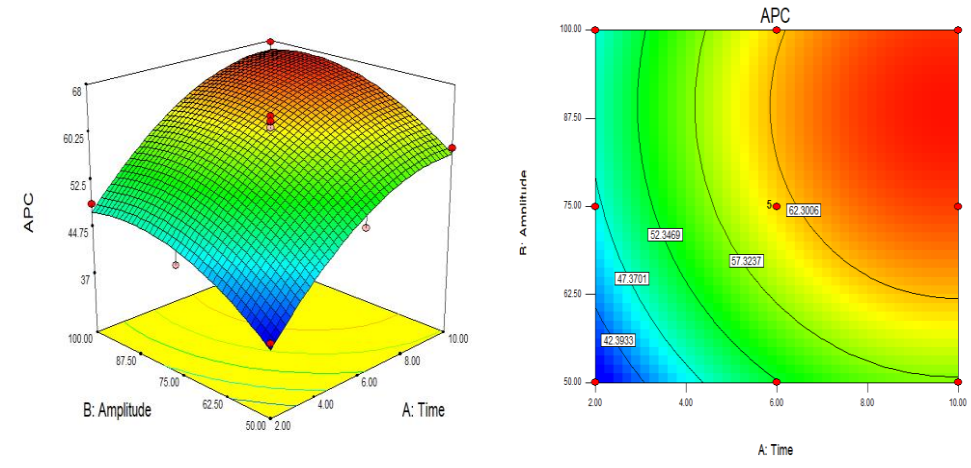
UAE – ultrasound assisted extraction; A – extraction amplitude (%); τ - extraction time (min); PC – phycocyanin; APC – allophycocyanin; PE – phycoerythrin; P – predicted value; extract yields were expressed as mg/g DW of biomass sample. Values are reported as mean value ± standard deviation. In all cases relative standard deviation did not exceed 10%.

3D and 2D response surface plots showing the extraction time and amplitude effect on phycobiliproteins yields (Figure 13). Figure 13 A shows the effect of time and amplitude for phycocyanin yield. Results shows that increasing both parameters, time and amplitude, the phycocyanin yield increases, both maximum time (10 min) and amplitude (100%) gave the maximum yield of phycocyanin (52.25 mg/g DW). Figure 13 B shows the effect of time and amplitude for

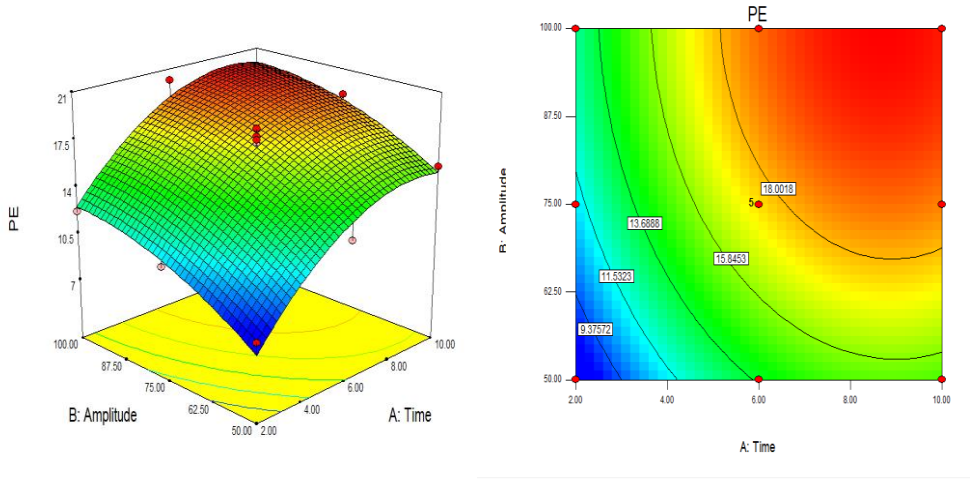
allophycocyanin yield. Results shows that increasing both parameters, time and amplitude, the allophycocyanin yield increases to maximum (67.74 mg/g DW) when amplitude reaches 100 % and time 10 minutes. Figure 13 C shows the effect of time and amplitude for phycoerythrin yield. Results shows that increasing both amplitude and time increases the yield of phycoerythrin. The maximum yield is reached applying 100 % amplitude and time for 6 minutes, at the longer extraction time with the same amplitude yield of phycoerythrin decreasing that can be caused by phycoerythrin sensitivity for high temperature. Figure 13 D shows the effect of time and amplitude for total phycobiliproteins yield. Results shows that increasing both parameters, time and amplitude, the total yield increases. As given in the Table 14, the yields of phycobiliproteins depends on time, because for each level of amplitude there is three levels of time and in each amplitude level yields of phycobiliproteins is the lowest at 2 minute of extraction and the highest at 10 minutes.



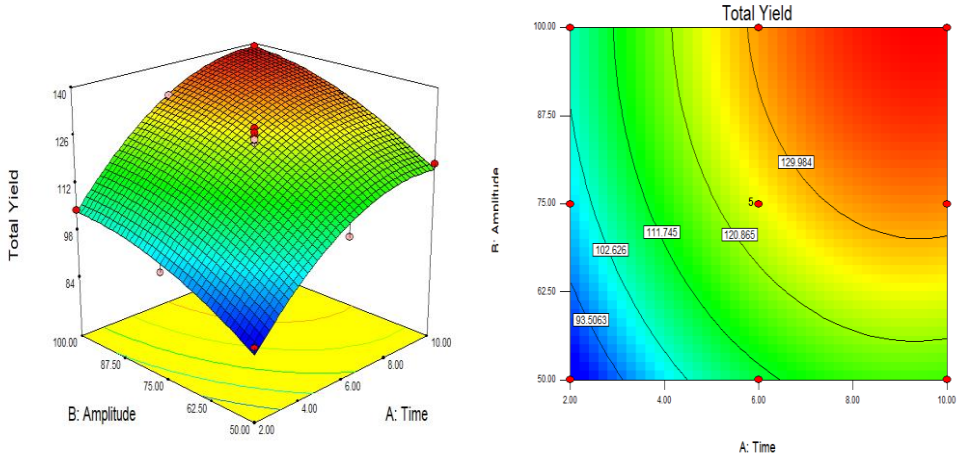
A: Effect of time and amplitude for phycocyanin yield (mg/g DW)



B: Effect of time and amplitude for allophycocyanin yield (mg/g DW)



C: Effect of time and amplitude for phycoerythrin yield (mg/g DW)



D: Effect of time and amplitude for total phycobiliproteins yield (mg/g DW)

Figure 13. Response surface 3D and 2D plots showing the effects of independent variables (time, min and amplitude, %) on combined freeze-thaw and ultrasound assisted extractions extract yields (mg/g DW) from wild cyanobacterial biomass (**A:** Effect of time and amplitude for phycocyanin yield (mg/g DW); **B:** Effect of time and amplitude for allophycocyanin yield (mg/g DW); **C:** Effect of time and amplitude for phycoerythrin yield (mg/g DW); **D:** Effect of time and amplitude for total phycobiliproteins yield (mg/g DW)).

The optimal conditions to obtain the highest UAE extraction yield were 99 % amplitude and 9.39 minutes. Under these conditions were extracted 53.78 mg/g DW of phycocyanin, 66.17 mg/g DW of allophycocyanin, 19.33 mg/g DW of phycoerythrin and total yield of phycobiliproteins was 139.28 mg/g DW.

Analysis (Table A.6.) of phycocyanin yield quadratic regression model showed that the model itself was significant according to the Student test ($p < 0.0001$, F=42.54), whereas the "*lack of fit*" was not significant ($p < 0.05$) relative to the pure error, with a p-value of 0.4143. The obtained results showed that A τ interaction and second-order term τ^2 were significant ($p < 0.05$) on the

phycocyanin yield from freeze-thaw with UAE extract. The factor with the largest effect was amplitude A with *F* value of 3.27, followed by extraction time τ (*F* = 17.15). Interaction between the time and amplitude (A τ) had significant effects on phycocyanin yield in a linear manner ($p < 0.05$, *F*=6.79).

The adequacy of the model can be also deduced from the determination coefficient (R^2 = 0.9681), which indicates good fit of the model to the experimental data. Moreover, this design could be used for modelling design space as the adjusted R-squared value of 0.9454 (a measure of the amount of variation about the mean) and predicted R-squared of 0.8269 are in reasonable agreement. The coefficient of variation (CV) of the model was 1.78%, meaning that model can be considered as reasonably reproducible. The close fit between experimental and predicted values within the investigated experimental range complemented the information, provided by the statistical indicators (Figure A.7.). Second order polynomial regression model, describing relationship between dependent and independent variables (P, T, τ), is summarized in equation 17:

$$Yield_{PC} = 40.11 + 1.56 * \tau - 0.15 * A + 0.01 * \tau A - 0.13 * \tau^2 + 1.42 * A^2 \qquad (17)$$

Analysis (Table A.7.) of allophycocyanin yield quadratic regression model showed that the model itself was significant according to the Student test ($p < 0.0001$, *F*=37.02), whereas the "*lack of fit*" was significant ($p < 0.05$) relative to the pure error, with a *p*-value of 0.0103. The obtained results showed that A τ interaction and second-order term τ^2 were not significant ($p > 0.05$) on the allophycocyanin yield from freeze-thaw with UAE extract. The factor with the largest effect was amplitude A with *F* value of 10.51, followed by extraction time τ (*F* = 14.31). Interaction between the time and amplitude (A τ) had not significant effects on allophycocyanin yield in a linear manner ($p > 0.05$).

The adequacy of the model can be also deduced from the determination coefficient (R^2 = 0.9636), which indicates good fit of the model to the experimental data. Moreover, this design could be used for modelling design space as the adjusted R-squared value of 0.9375 (a measure of the amount of variation about the mean) and predicted R-squared of 0.7648 are in reasonable agreement. The coefficient of variation (CV) of the model was 3.85 %, meaning that model can be considered as reasonably reproducible. The close fit between experimental and predicted values within the investigated experimental range complemented the information, provided by the statistical indicators (Figure A.8.). Second order polynomial regression model, describing relationship between dependent and independent variables (P, T, τ), is summarized in equation 18:

$$Yield_{APC} = -17.95 + 6.22 * \tau + 1.23 * A - 8.42 * \tau A - 0.31 * \tau^2 - 6.86 * A^2 \qquad (18)$$

Analysis (Table A.8.) of phycoerythrin yield quadratic regression model showed that the model itself was significant according to the Student test (p = 0.0001, F=32.47), whereas the "*lack of fit*" was not significant (p > 0.05) relative to the pure error, with a p-value of 0.0648. The obtained results showed that A τ interaction and second-order term τ^2 were not significant (p < 0.05) on the phycoerythrin yield from freeze-thaw with UAE extract. The factor with the largest effect was amplitude A with F value of 3.68, followed by extraction time τ (F = 19.57). Interaction between the time and amplitude (A τ) had not significant effects on phycoerythrin yield in a linear manner (p > 0.05, F=0.094).

The adequacy of the model can be also deduced from the determination coefficient (R^2 = 0.9587), which indicates good fit of the model to the experimental data. Moreover, this design could be used for modelling design space as the adjusted R-squared value of 0.9291 (a measure of the amount of variation about the mean) and predicted R-squared of 0.6486 are in reasonable agreement. The coefficient of variation (CV) of the model was 6.30 %, meaning that model can be considered as reasonably reproducible. The close fit between experimental and predicted values within the investigated experimental range complemented the information, provided by the statistical indicators (Figure A.9.). Second order polynomial regression model, describing relationship between dependent and independent variables (P, T, τ), is summarized in equation 19:

$$Yield_{PE} = -12.76 + 3.05 * \tau + 0.39 * A - 1.52 * \tau A - 0.17 * \tau^2 - 1.83 * A^2 \qquad (19)$$

Analysis (Table A.9.) of total phycobiliproteins yield quadratic regression model showed that the model itself was significant according to the Student test (p < 0.0001, F=78.24), whereas the "*lack of fit*" was not significant (p > 0.05) relative to the pure error, with a p-value of 0.1514. The obtained results showed that A τ interaction and second-order term τ^2 were not significant (p < 0.05) on the total yield from freeze-thaw with UAE extract. The factor with the largest effect was amplitude A with F value of 7.86, followed by extraction time τ (F = 35.64). Interaction between the time and amplitude (A τ) had not significant effects on total yield in a linear manner (p > 0.05, F=0.38).

The adequacy of the model can be also deduced from the determination coefficient (R^2 = 0.9824), which indicates good fit of the model to the experimental data. Moreover, this design could be used for modelling design space as the adjusted R-squared value of 0.9699 (a measure of the amount of variation about the mean) and predicted R-squared of 0.8976 are in reasonable agreement. The coefficient of variation (CV) of the model was 2.27 %, meaning that model can be considered as reasonably reproducible. The close fit between experimental and predicted values within the investigated experimental range complemented the information, provided by the statistical indicators

(Figure A.10.). Second order polynomial regression model, describing relationship between dependent and independent variables (P, T, τ), is summarized in equation 20:

$$Yield_{TOTAL} = 9.40 + 10.83 * \tau + 1.46 * A + 8.28 * \tau A - 0.60 * \tau^2 - 7.27 * A^2 \quad (20)$$

3.2.4. Optimization of homogenizer and UAE extractions parameters

The effectiveness of homogenization combined with ultrasound assisted extraction depends on amplitude and time. CCD and RSM were used to optimize the effect of two factors, amplitude and time, on the phycocyanin, allophycocyanin, phycoerythrin and total phycobiliproteins yields as the response factors. The complete matrix with actual and predicted values of phycobiliproteins yields is presented in Table 15. According to the results, total phycobiliproteins yield varied from 83.11 to 137.42 mg/g DW of starting wild cyanobacteria biomass under different tested UAE conditions.

Table 15. Central composite design matrix for combined homogenizer and UAE extractions optimisation for wild cyanobacterial biomass and values of observed responses: PC, APC, PE actual yields (mg/g DW) and predicted (P) yields of each phycobiliprotein (mg/g DW)

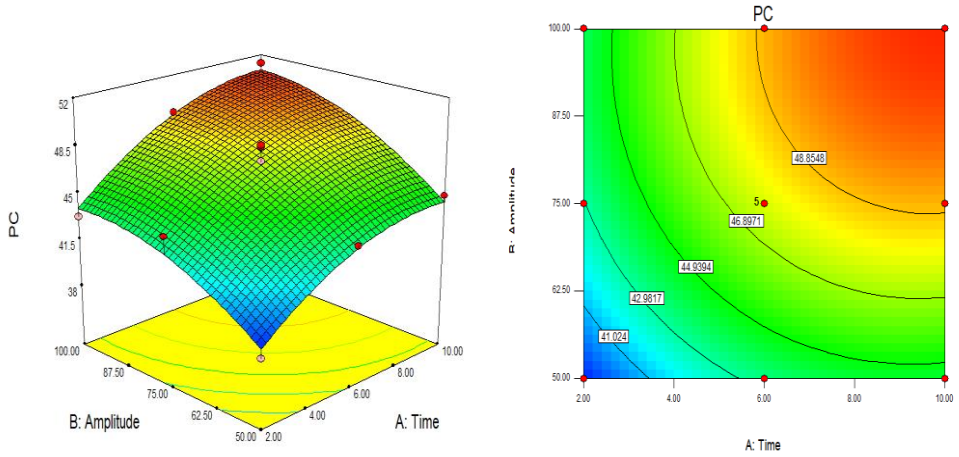
No.	Homogenizer +UAE parameters		Phycobiliprotein yield, mg/g DW							
	A, %	τ, min	PC	PC P	APC	APC P	PE	PE P	Total yield	Total yield P
1	50	10	44.86 ± 0.29	44.38	58.93 ± 1.51	59.35	15.41 ± 0.59	15.41	119.20 ± 1.21	119.14
2	75	10	48.06 ± 0.29	49.03	68.78 ± 0.07	67.29	18.61 ± 0.22	19.07	135.45 ± 0.59	135.38
3	50	6	43.55 ± 0.23	43.37	56.23 ± 0.63	55.17	14.81 ± 0.50	13.79	114.58 ± 0.90	112.33
4	75	2	44.21 ± 0.05	42.92	53.77 ± 0.30	51.83	14.11 ± 0.27	12.52	112.08 ± 0.52	107.27
5	100	10	51.31 ± 1.16	50.81	63.94 ± 0.72	65.02	20.62 ± 0.41	20.17	135.88 ± 0.85	136.00
6	75	6	47.43 ± 0.84	47.63	65.63 ± 0.08	65.91	17.61 ± 0.05	17.85	130.67 ± 0.87	131.38
7	75	6	45.97 ± 0.42	47.63	62.39 ± 1.48	65.91	15.76 ± 0.05	17.85	124.13 ± 1.94	131.38
8	100	2	43.28 ± 0.87	43.91	53.85 ± 0.01	55.15	13.86 ± 0.19	14.43	110.99 ± 0.69	113.49
9	75	6	48.58 ± 0.15	47.63	66.78 ± 0.54	65.91	18.64 ± 0.28	17.85	133.99 ± 0.97	131.38
10	75	6	47.40 ± 0.42	47.63	65.49 ± 1.48	65.91	17.85 ± 0.56	17.85	130.74 ± 2.46	131.38
11	50	2	38.41 ± 0.41	39.07	37.65 ± 0.47	38.30	7.05 ± 0.30	8.06	83.11 ± 1.18	85.43
12	100	6	49.15 ± 0.52	49.01	68.80 ± 0.88	66.43	19.48 ± 0.01	19.36	137.42 ± 1.41	134.80
13	75	6	48.44 ± 0.24	47.63	65.81 ± 0.37	65.91	18.25 ± 0.50	17.85	132.50 ± 1.11	131.38
Optimal conditions										
	94	8.75	48.79 ± 0.15	50.45	67.27 ± 0.53	68.16	21.15 ± 0.25	20.89	137.21 ± 0.81	138.89

UAE – ultrasound assisted extraction; A – extraction amplitude (%); τ - extraction time (min); PC – phycocyanin; APC – allophycocyanin; PE – phycoerythrin; P – predicted value; extract yields were expressed as mg/g DW of biomass sample. Values are reported as mean value ± standard deviation. In all cases relative standard deviation did not exceed 10%.

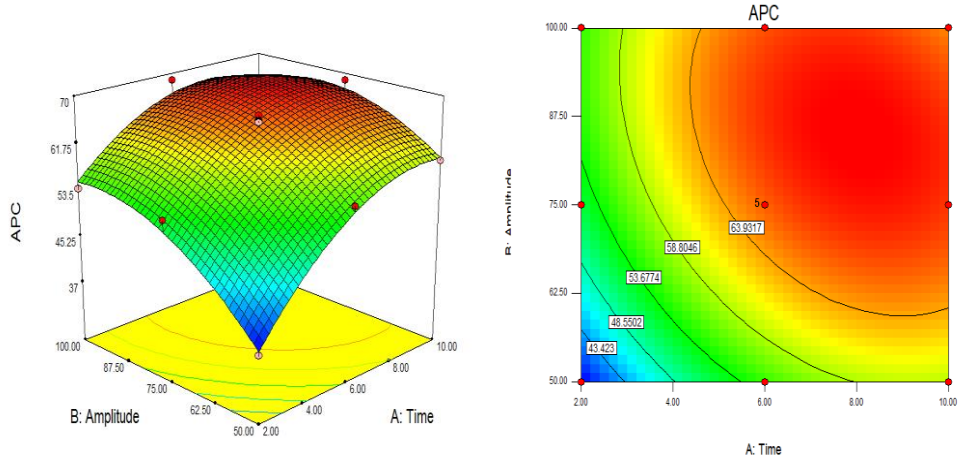
3D and 2D response surface plots showing the extraction time and amplitude effect on phycobiliproteins yields (Figure 14). Figure 14 A shows the effect of time and amplitude for phycocyanin yield. Results shows that increasing both parameters, time and amplitude, the phycocyanin yield increases, both maximum time (10 min) and amplitude (100%) gave the maximum yield of phycocyanin (51.31 mg/g DW). Figure 14 B shows the effect of time and amplitude for allophycocyanin yield. Results shows that increasing both parameters, time and amplitude, the allophycocyanin yield increases to maximum (68.80 mg/g DW) until amplitude reaches 100 % and time 6 minutes, with the longer extraction time at the same amplitude, yield of allophycocyanin starting to decrease. Such a change can be caused by allophycocyanin sensitivity to temperature. Figure 14 C shows the effect of time and amplitude for phycoerythrin yield. Results shows that increasing both amplitude and time increases the yield of phycoerythrin. Figure 14 D shows the effect of time and amplitude for total phycobiliproteins yield. Results shows that increasing both parameters, time and amplitude, the total yield increases. As given in the Table 15, the yield of phycobiliproteins depends on time, because for each level of amplitude there is three levels of time and in each amplitude level yield of phycobiliproteins is the lowest at 1 minute of extraction and the highest at 10 minutes.

The optimal conditions to obtain the highest UAE extraction yield were 94 % amplitude and 8.75 minutes. Under these conditions were extracted 48.79 mg/g DW of phycocyanin, 67.27 mg/g DW of allophycocyanin, 21.15 mg/g DW of phycoerythrin and total yield of phycobiliproteins was 137.21 mg/g DW.

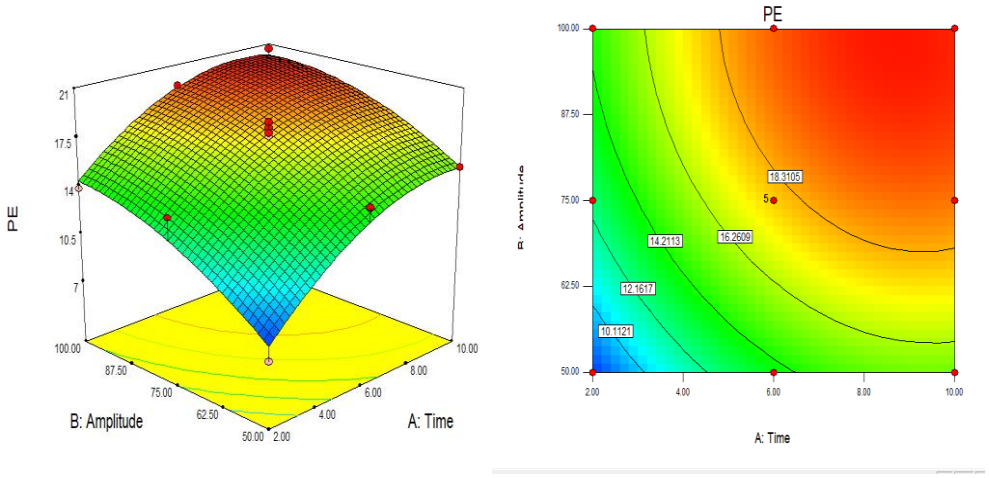
Analysis (Table A.10.) of phycocyanin yield quadratic regression model showed that the model itself was significant according to the Student test ($p = 0.0004$, F=21.10), whereas the "*lack of fit*" was not significant ($p > 0.05$) relative to the pure error, with a p-value of 0.4138. The obtained results showed that A τ was not significant ($p > 0.05$), and τ^2 was significant ($p < 0.05$) on the phycocyanin yield from homogenization with UAE extract. The factor with the largest effect was amplitude A with F value of 4.77, followed by extraction time τ (F = 6.32). Interaction between the time and amplitude (A τ) had not significant effects on phycocyanin yield in a linear manner ($p >$ 0.05, F=0.53).



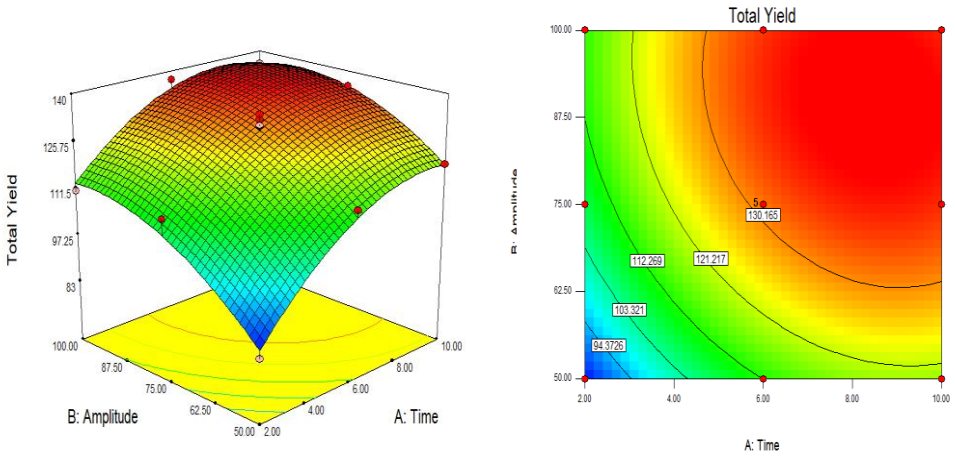
A: Effect of time and amplitude for phycocyanin yield (mg/g DW)



B: Effect of time and amplitude for allophycocyanin yield (mg/g DW);



C: Effect of time and amplitude for phycoerythrin yield (mg/g DW)



D: Effect of time and amplitude for total phycobiliproteins yield (mg/g DW)

Figure 14. Response surface 3D and 2D plots showing the effects of independent variables (time, min and amplitude, %) on combined homogenasation and ultrasound assisted extractions extract yields (mg/g DW) from wild cyanobacterial biomass (**A:** Effect of time and amplitude for phycocyanin yield (mg/g DW); **B:** Effect of time and amplitude for allophycocyanin yield (mg/g DW); **C:** Effect of time and amplitude for phycoerythrin yield (mg/g DW); **D:** Effect of time and amplitude for total phycobiliproteins yield (mg/g DW)).

The adequacy of the model can be also deduced from the determination coefficient (R^2 = 0.9378), which indicates good fit of the model to the experimental data. Moreover, this design could be used for modelling design space as the adjusted R-squared value of 0.8933 (a measure of the amount of variation about the mean) and predicted R-squared of 0.6545 are in reasonable agreement. The coefficient of variation (CV) of the model was 2.36 %, meaning that model can be considered as reasonably reproducible. The close fit between experimental and predicted values within the investigated experimental range complemented the information, provided by the statistical indicators (Figure A.11.). Second order polynomial regression model, describing relationship between dependent and independent variables (P, T, τ), is summarized in equation 21:

$$Yield_{PC} = 19.76 + 1.70 * \tau + 0.43 * A + 3.97 * \tau A - 0.10 * \tau^2 - 2.29 * A^2 \qquad (21)$$

Analysis (Table A.11.) of allophycocyanin yield quadratic regression model showed that the model itself was significant according to the Student test ($p < 0.0001$, F=41.48), whereas the "*lack of fit*" was not significant ($p > 0.05$) relative to the pure error, with a p-value of 0.2263. The obtained results showed that A τ interaction and second-order term τ^2 were significant ($p < 0.05$) on the allophycocyanin yield from homogenization with UAE extract. The factor with the largest effect was amplitude A with F value of 17.08, followed by extraction time τ (F = 26.40). Interaction between the time and amplitude (A τ) had significant effects on allophycocyanin yield in a linear manner ($p <$ 0.05).

The adequacy of the model can be also deduced from the determination coefficient (R^2 = 0.9674), which indicates good fit of the model to the experimental data. Moreover, this design could be used for modelling design space as the adjusted R-squared value of 0.9440 (a measure of the amount of variation about the mean) and predicted R-squared of 0.8375 are in reasonable agreement. The coefficient of variation (CV) of the model was 3.39 %, meaning that model can be considered as reasonably reproducible. The close fit between experimental and predicted values within the investigated experimental range complemented the information, provided by the statistical indicators (Figure A.12.). Second order polynomial regression model, describing relationship between dependent and independent variables (P, T, τ), is summarized in equation 22:

$$Yield_{APC} = -35.40 + 8.79 * \tau + 1.62 * A - 0.03 * \tau A - 0.40 * \tau^2 - 8.17 * A^2 \qquad (22)$$

Analysis (Table A.12.) of phycoerythrin yield quadratic regression model showed that the model itself was significant according to the Student test (p = 0.0007, F=18.19), whereas the "*lack of fit*" was not significant (p > 0.05) relative to the pure error, with a p-value of 0.3416. The obtained results showed that A τ was not significant (p > 0.05) and τ^2 were significant (p < 0.05) on the phycoerythrin yield from homogenization with UAE extract. The factor with the largest effect was amplitude A with F value of 2.98, followed by extraction time τ (F = 7.76). Interaction between the time and amplitude (A τ) had not significant effects on phycoerythrin yield in a linear manner (p > 0.05).

The adequacy of the model can be also deduced from the determination coefficient (R^2 = 0.9285), which indicates good fit of the model to the experimental data. Moreover, this design could be used for modelling design space as the adjusted R-squared value of 0.8775 (a measure of the amount of variation about the mean) and predicted R-squared of 0.6077 are in reasonable agreement. The coefficient of variation (CV) of the model was 7.52 %, meaning that model can be considered as reasonably reproducible. The close fit between experimental and predicted values within the investigated experimental range complemented the information, provided by the statistical indicators (Figure A.13.). Second order polynomial regression model, describing relationship between dependent and independent variables (P, T, τ), is summarized in equation 23:

$$Yield_{PE} = -13.31 + 2.66 * \tau + 0.44 * A - 4.02 * \tau A - 0.13 * \tau^2 - 2.04 * A^2 \qquad (23)$$

Analysis (Table A.13.) of total phycobiliproteins yield quadratic regression model showed that the model itself was significant according to the Student test (p < 0.0001, F=34.78), whereas the "*lack of fit*" was not significant (p > 0.05) relative to the pure error, with a p-value of 0.4148. The obtained results showed that A τ was not significant (p > 0.05) and τ^2 were significant (p < 0.05) on

the total yield from homogenization with UAE extract. The factor with the largest effect was amplitude A with *F* value of 10.90, followed by extraction time τ (*F* = 18.03). Interaction between the time and amplitude (A τ) had not significant effects on total yield in a linear manner ($p > 0.05$).

The adequacy of the model can be also deduced from the determination coefficient (R^2 = 0.9613), which indicates good fit of the model to the experimental data. Moreover, this design could be used for modelling design space as the adjusted R-squared value of 0.9337 (a measure of the amount of variation about the mean) and predicted R-squared of 0.8246 are in reasonable agreement. The coefficient of variation (CV) of the model was 3.19 %, meaning that model can be considered as reasonably reproducible. The close fit between experimental and predicted values within the investigated experimental range complemented the information, provided by the statistical indicators (Figure A.14.). Second order polynomial regression model, describing relationship between dependent and independent variables (P, T, τ), is summarized in equation 24:

$$Yield_{TOTAL} = -28.95 + 13.15 * \tau + 2.49 * A - 0.03 * \tau A - 0.63 * \tau^2 - 0.01 * A^2 \qquad (24)$$

3.2.5. Optimization of solid-liquid and UAE extractions parameters

The effectiveness of solid-liquid extraction combined with ultrasound assisted extraction depends on amplitude and time. CCD and RSM were used to optimize the effect of two factors, amplitude and time, on the phycocyanin, allophycocyanin, phycoerythrin and total phycobiliproteins yields as the response factors. The complete matrix with actual and predicted values of phycobiliproteins yields is presented in Table 16. According to the results, total phycobiliproteins yield varied from 79.99 to 132.26 mg/g DW of starting wild cyanobacteria biomass under different tested UAE conditions.

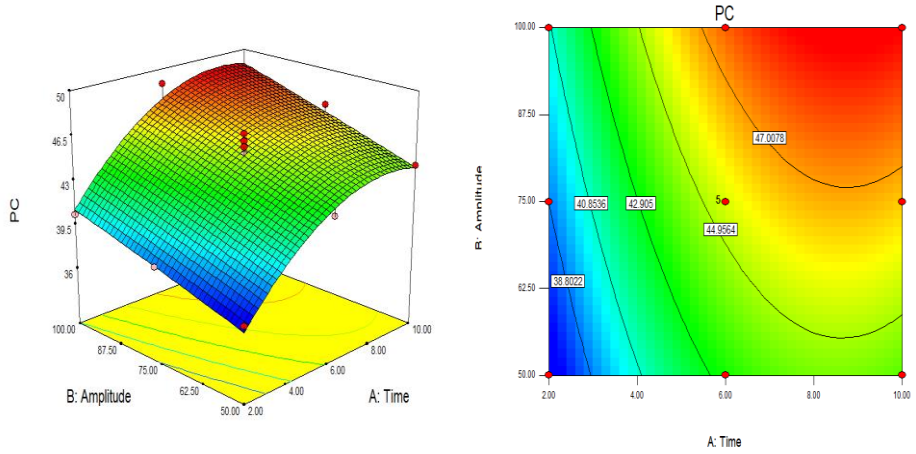
3D and 2D response surface plots showing the extraction time and amplitude effect on phycobiliproteins yields (Figure 15). Figure 15 A shows the effect of time and amplitude for phycocyanin yield. Results shows that increasing both parameters, time and amplitude, the phycocyanin yield increases, both maximum time (6 min) and amplitude (100%) gave the maximum yield of phycocyanin (48.78 mg/g DW), with the longer extraction time at the same amplitude, yield of phycocyanin starting to decrease, that may show phycocyanin sensitivity to temperature. Figure 15 B shows the effect of time and amplitude for allophycocyanin yield. Results shows that increasing both parameters, time and amplitude, the allophycocyanin yield increases to maximum (64.60 mg/g DW) until amplitude reaches 100 % and time 10 minutes. Figure 15 C shows the effect of time and amplitude for phycoerythrin yield. Results shows that increasing both amplitude and time increases the yield of phycoerythrin. The maximum yield (19.85 mg/g DW) is reached applying 100 %

amplitude and time for 10 minutes, which shows that phycoerythin sensitive for temperature as allophycocyanin. Figure 15 D shows the effect of time and amplitude for total phycobiliproteins yield. Results shows that increasing both parameters, time and amplitude, the total yield increases. As given in the Table 16, the yield of phycobiliproteins depends on time, because for each level of amplitude there is three levels of time and in each amplitude level yield of phycobiliproteins is the lowest at 1 minute of extraction and the highest at 10 minutes.

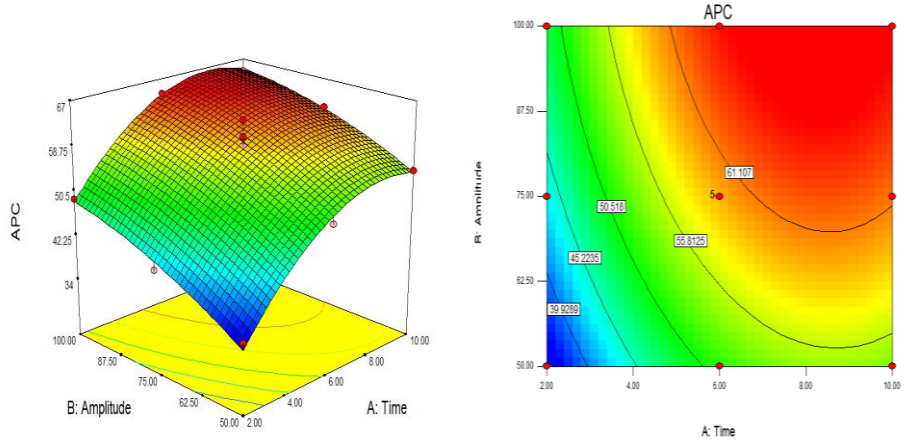
Table 16. Central composite design matrix for combined solid-liquid and UAE extractions optimisation for wild cyanobacterial biomass and values of observed responses: PC, APC, PE actual yields (mg/g DW) and predicted (P) yields of each phycobiliprotein (mg/g DW)

No.	SLE+UAE parameters		Phycobiliprotein yield, mg/g DW							
	A, %	τ, min	PC	PC P	APC	APC P	PE	PE P	Total yield	Total yield P
1	100	10	48.09 ± 0.53	48.84	64.60 ± 0.44	65.11	19.56 ± 0.20	19.85	132.26 ± 1.18	133.79
2	75	2	38.80 ± 0.08	38.82	41.87 ± 0.27	43.46	9.69 ± 0.03	9.80	90.36 ± 0.37	92.08
3	50	2	37.21 ± 0.34	36.75	35.79 ± 0.23	34.63	6.99 ± 0.13	6.79	79.99 ± 0.01	78.17
4	75	10	47.13 ± 0.94	46.54	61.67 ± 0.52	61.39	17.43 ± 0.37	17.16	126.23 ± 0.05	125.09
5	75	6	45.07 ± 0.05	45.50	58.73 ± 0.30	59.57	15.76 ± 0.17	15.96	119.56 ± 0.08	121.03
6	75	6	43.34 ± 0.28	45.50	55.99 ± 0.93	59.57	14.95 ± 0.11	15.96	114.29 ± 0.76	121.03
7	100	2	40.31 ± 0.52	40.74	49.12 ± 0.88	48.70	12.36 ± 0.24	12.46	101.79 ± 1.63	101.90
8	75	6	46.11 ± 0.76	45.50	60.49 ± 1.25	59.57	15.49 ± 0.30	15.96	122.09 ± 1.71	121.03
9	75	6	46.70 ± 0.56	45.50	63.51 ± 0.43	59.57	17.26 ± 0.36	15.96	127.46 ± 0.50	121.03
10	50	10	44.23 ± 0.10	44.09	54.34 ± 0.60	54.11	14.12 ± 0.18	14.10	112.68 ± 0.68	112.29
11	75	6	45.69 ± 0.19	45.50	60.45 ± 0.67	59.57	16.18 ± 0.33	15.96	122.32 ± 1.19	121.03
12	50	6	42.63 ± 0.43	43.24	50.13 ± 1.04	51.52	12.70 ± 0.46	12.92	105.46 ± 1.93	107.68
13	100	6	48.78 ± 1.32	47.61	64.14 ± 0.62	64.05	19.02 ± 0.14	18.63	131.94 ± 0.85	130.29
Optimal conditions										
	99	8.85	46.24 ± 0.35	48.93	60.57 ± 0.54	66.13	24.58 ± 0.21	19.85	131.39 ± 0.44	134.91

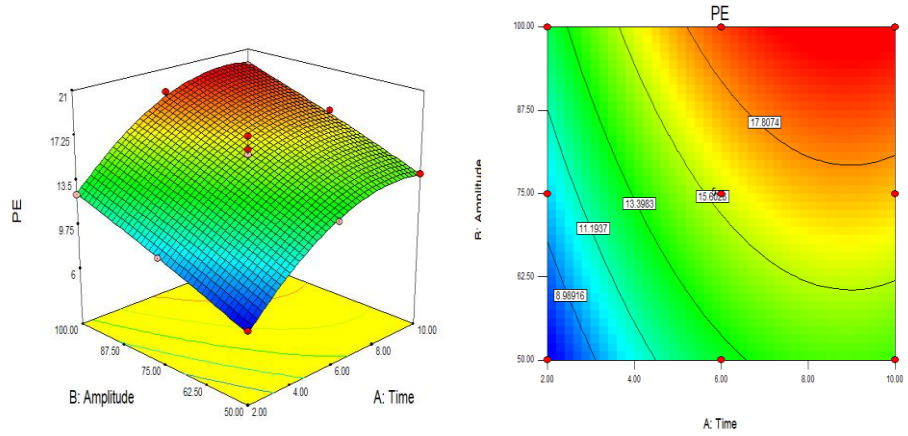
UAE – ultrasound assisted extraction; A – extraction amplitude (%); τ - extraction time (min); PC – phycocyanin; APC – allophycocyanin; PE – phycoerythrin; P – predicted value; extract yields were expressed as mg/g DW of biomass sample. Values are reported as mean value ± standard deviation. In all cases relative standard deviation did not exceed 10%.



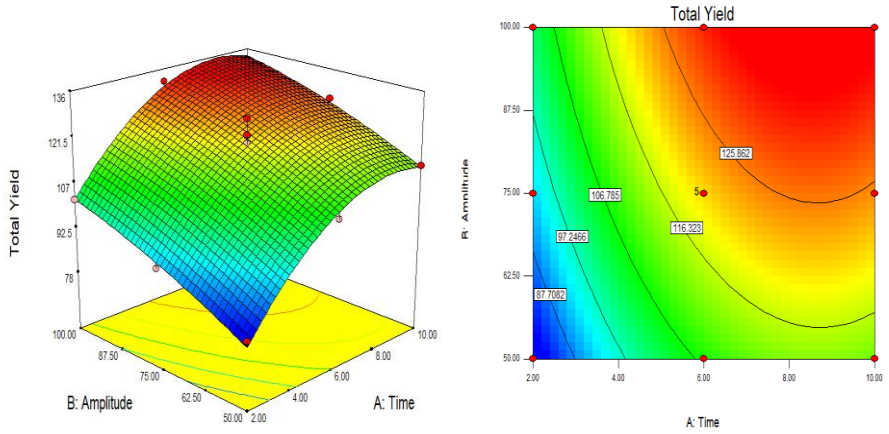
A: Effect of time and amplitude for phycocyanin yield (mg/g DW)



B: Effect of time and amplitude for allophycocyanin yield (mg/g DW)



C: Effect of time and amplitude for phycoerythrin yield (mg/g DW)



D: Effect of time and amplitude for total phycobiliproteins yield (mg/g DW)

Figure 15. Response surface 3D and 2D plots showing the effects of independent variables (time, min and amplitude, %) on combined solid-liquid and ultrasound assisted extractions extract yields (mg/g DW) from wild cyanobacterial biomass (**A:** Effect of time and amplitude for phycocyanin yield (mg/g DW); **B:** Effect of time and amplitude for allophycocyanin yield (mg/g DW); **C:** Effect of time and amplitude for phycoerythrin yield (mg/g DW); **D:** Effect of time and amplitude for total phycobiliproteins yield (mg/g DW)).

The optimal conditions to obtain the highest UAE extraction yield were 99 % amplitude and 8.85 minutes. Under these conditions were extracted 46.24 mg/g DW of phycocyanin, 60.57 mg/g DW of allophycocyanin, 24.58 mg/g DW of phycoerythrin and total yield of phycobiliproteins was 131.39 mg/g DW.

Analysis (Table A.14.) of phycocyanin yield quadratic regression model showed that the model itself was significant according to the Student test (p = 0.0005, F=20.74), whereas the "*lack of fit*" was not significant (p > 0.05) relative to the pure error, with a p-value of 0.6328. The obtained results showed that A τ was not significant (p > 0.05) and τ^2 were significant (p < 0.05) on the phycocyanin yield from SLE with UAE extract. The factor with the largest effect was amplitude A with F value of 0.011, followed by extraction time τ (F = 15.78). Interaction between the time and amplitude (A τ) had not significant effects on phycocyanin yield in a linear manner (p > 0.05).

The adequacy of the model can be also deduced from the determination coefficient (R^2 = 0.9368), which indicates good fit of the model to the experimental data. Moreover, this design could be used for modelling design space as the adjusted R-squared value of 0.8916 (a measure of the amount of variation about the mean) and predicted R-squared of 0.7408 are in reasonable agreement. The coefficient of variation (CV) of the model was 2.67 %, meaning that model can be considered as reasonably reproducible. The close fit between experimental and predicted values within the investigated experimental range complemented the information, provided by the statistical indicators

(Figure A.15.). Second order polynomial regression model, describing relationship between dependent and independent variables (P, T, τ), is summarized in equation 25:

$$Yield_{PC} = 26.99 + 2.94 * \tau + 0.09 * A + 1.91 * \tau A - 0.18 * \tau^2 - 1.21 * A^2 \qquad (25)$$

Analysis (Table A.15.) of allophycocyanin yield quadratic regression model showed that the model itself was significant according to the Student test ($p < 0.0001$, F=35.16), whereas the "*lack of fit*" was not significant ($p > 0.05$) relative to the pure error, with a p-value of 0.8282. The obtained results showed that A τ was not significant ($p > 0.05$) and τ^2 were significant ($p < 0.05$) on the allophycocyanin yield from SLE with UAE extract. The factor with the largest effect was amplitude A with F value of 1.67, followed by extraction time τ (F = 26.75). Interaction between the time and amplitude (A τ) had not significant effects on allophycocyanin yield in a linear manner ($p > 0.05$).

The adequacy of the model can be also deduced from the determination coefficient (R^2 = 0.9617), which indicates good fit of the model to the experimental data. Moreover, this design could be used for modelling design space as the adjusted R-squared value of 0.9344 (a measure of the amount of variation about the mean) and predicted R-squared of 0.8922 are in reasonable agreement. The coefficient of variation (CV) of the model was 4.14 %, meaning that model can be considered as reasonably reproducible. The close fit between experimental and predicted values within the investigated experimental range complemented the information, provided by the statistical indicators (Figure A.16.). Second order polynomial regression model, describing relationship between dependent and independent variables (P, T, τ), is summarized in equation 26:

$$Yield_{APC} = -28.95 + 13.15 * \tau + 2.49 * A - 0.03 * \tau A - 0.63 * \tau^2 - 0.01 * A^2 \qquad (26)$$

Analysis (Table A.16.) of phycoerythrin yield quadratic regression model showed that the model itself was significant according to the Student test ($p < 0.0001$, F=61.70), whereas the "*lack of fit*" was not significant ($p > 0.05$) relative to the pure error, with a p-value of 0.8991. The obtained results showed that A τ was not significant ($p > 0.05$) and τ^2 were significant ($p < 0.05$) on the phycoerythrin yield from SLE with UAE extract. The factor with the largest effect was amplitude A with F value of 0.18, followed by extraction time τ (F = 34.65). Interaction between the time and amplitude (A τ) had not significant effects on phycoerythrin yield in a linear manner ($p > 0.05$).

The adequacy of the model can be also deduced from the determination coefficient (R^2 = 0.9778), which indicates good fit of the model to the experimental data. Moreover, this design could be used for modelling design space as the adjusted R-squared value of 0.9620 (a measure of the amount of variation about the mean) and predicted R-squared of 0.9445 are in reasonable agreement. The coefficient of variation (CV) of the model was 4.75 %, meaning that model can be considered as

reasonably reproducible. The close fit between experimental and predicted values within the investigated experimental range complemented the information, provided by the statistical indicators (Figure A.17.). Second order polynomial regression model, describing relationship between dependent and independent variables (P, T, τ), is summarized in equation 27:

$$Yield_{PE} = -5.24 + 2.76 * \tau + 0.16 * A + 2.04 * \tau A - 0.15 * \tau^2 - 2.89 * A^2 \quad (27)$$

Analysis (Table A.17.) of total phycobiliproteins yield quadratic regression model showed that the model itself was significant according to the Student test (p = 0.0001, F=38.10), whereas the "*lack of fit*" was not significant (p > 0.05) relative to the pure error, with a p-value of 0.8521. The obtained results showed that A τ was not significant (p > 0.05) and τ^2 were significant (p < 0.05) on the total yield from SLE with UAE extract. The factor with the largest effect was amplitude A with F value of 0.74, followed by extraction time τ (F = 27.36). Interaction between the time and amplitude (A τ) had not significant effects on total yield in a linear manner (p > 0.05).

The adequacy of the model can be also deduced from the determination coefficient (R^2 = 0.9646), which indicates good fit of the model to the experimental data. Moreover, this design could be used for modelling design space as the adjusted R-squared value of 0.9392 (a measure of the amount of variation about the mean) and predicted R-squared of 0.8985 are in reasonable agreement. The coefficient of variation (CV) of the model was 3.46 %, meaning that model can be considered as reasonably reproducible. The close fit between experimental and predicted values within the investigated experimental range complemented the information, provided by the statistical indicators (Figure A.18.). Second order polynomial regression model, describing relationship between dependent and independent variables (P, T, τ), is summarized in equation 28:

$$Yield_{TOTAL} = 13.46 + 13.88 * \tau + 0.98 * A - 5.56 * \tau A - 0.78 * \tau^2 - 3.27 * A^2 \quad (28)$$

Comparing all four phycobiliprotein extractions with UAE determined that the highest yield of total phycobiliproteins content was received using freeze-thaw with liquid nitrogen combined with UAE (139.28 mg/g DW) and the lowest yield was received using UAE (131.12 mg/g DW). The highest phycocyanin yield was received using freeze-thaw with liquid nitrogen combined with UAE (53.78 mg/g DW) and the lowest yield was received using solid-liquid extraction combined with UAE (46.24 mg/g DW).

Ultrasound assisted extraction was used for the extraction of algal lipid [115], carbohydrates [116], phenolic compounds [117], phycocyanins [114],[118]. Tavanandi et al. reported that using untrsonication, combined methods of freeze-thawing and ultrasonication, maceration and ultrasonication, and homogenization and ultrasonication was received 51.51, 109.03, 99.31, 91.00

mg/g DW yields of phycocyanin from *Arthospira platensis*, respectively and using phosphate buffer as a solvent [114]. Costa Ores et al. used ultrasonic homogenization to extract phycocyanin (90 mg/g) and allophycocyanin (70 mg/g) from *S. platensis* [119]. Extractions depends not only on the amplitude or time, but also on the chosen solvent, for example allophycocyanin was extracted in greater quantity followed by phycocyanin from *Spirulina platensis* using protic ionic liquids [120]. Guldhe et al. agreed that UAE has cavitational effect, which increases the extraction of chemical compounds from microalgal cells by alleviating solvent passing through cell disruption [121]. Also it is known that ultrasonication cooling is neede to prevent overheating and proteins denaturation [122]. The data reported in this study fall in a range with other reaserchers results, however, comparison of the results values is complicated, due to most of these studies are focused on specific microalgal species grown under optimal comditions.

3.2.6. *In vitro* antioxidant activity assessment of phycobiliproteins extracts

In the next step, antioxidant activity values was assessed. As reported in Table 17, TPC and $TEAC_{ABTS}$ values of protein extracts were determined from extracts after SLE, freeze-thaw, homogenization, SLE with UAE, freeze-thaw with UAE and homogenization with UAE. The highest antioxidant activity was in SLE combined with UAE extract (58.40 mg GAE/g for TPC and 47.33 mg TE/g for $TEAC_{ABTS}$) and the lowest in freeze-thaw extract (38.84 mg GAE/g) for TPC and in homogenizer combined with UAE extract (42.53 mg TE/g) for $TEAC_{ABTS}$.

Table 17. Total phenolic content (TPC), and $TEAC_{ABTS}$ scavenging capacity of protein extracts from wild cyanobacterial biomass after SLE, freeze-thaw extraction, homogenization, SLE combined with UAE for 2 minutes, freeze-thaw extraction combined with UAE for 2 minutes and homogenization combined with UAE for 2 minutes

***In vitro* antioxidant capacity**	**Phycobiliprotein extracts**					
	SLE	Freeze-thaw	Homogenizer	SLE and UAE 2’	Freeze-thaw and UAE 2’	Homogenizer and UAE 2’
TPC, mg GAE/g:						
mg /g DW[1**]	41.78±2.19[a]	38.84±2.37[a]	44.75±3.06[b]	58.40±4.13[c]	40.77±2.55[a]	45.67±3.34[b]
$TEAC_{ABTS}$, mg TE/g:						
mg /g DW[1**]	44.45±1.64[b]	43.43±0.75[ab]	44.57±0.53[b]	47.33±0.49[c]	45.53±0.79[b]	42.53±0.41[a]

Data expressed as mean value ± standard deviation of five replicates

This is in agreement with other studies, for example, Ilter et al. reported that TPC values of solid-liquid extraction was 162.93 mg GAE/L, of ultrasound extraction was 163.24 mg GAE/L, and $TEAC_{ABTS}$ values was 37.09 mM tolox/ml and 33.17 mM tolox/ml, respectively from frozen, dried

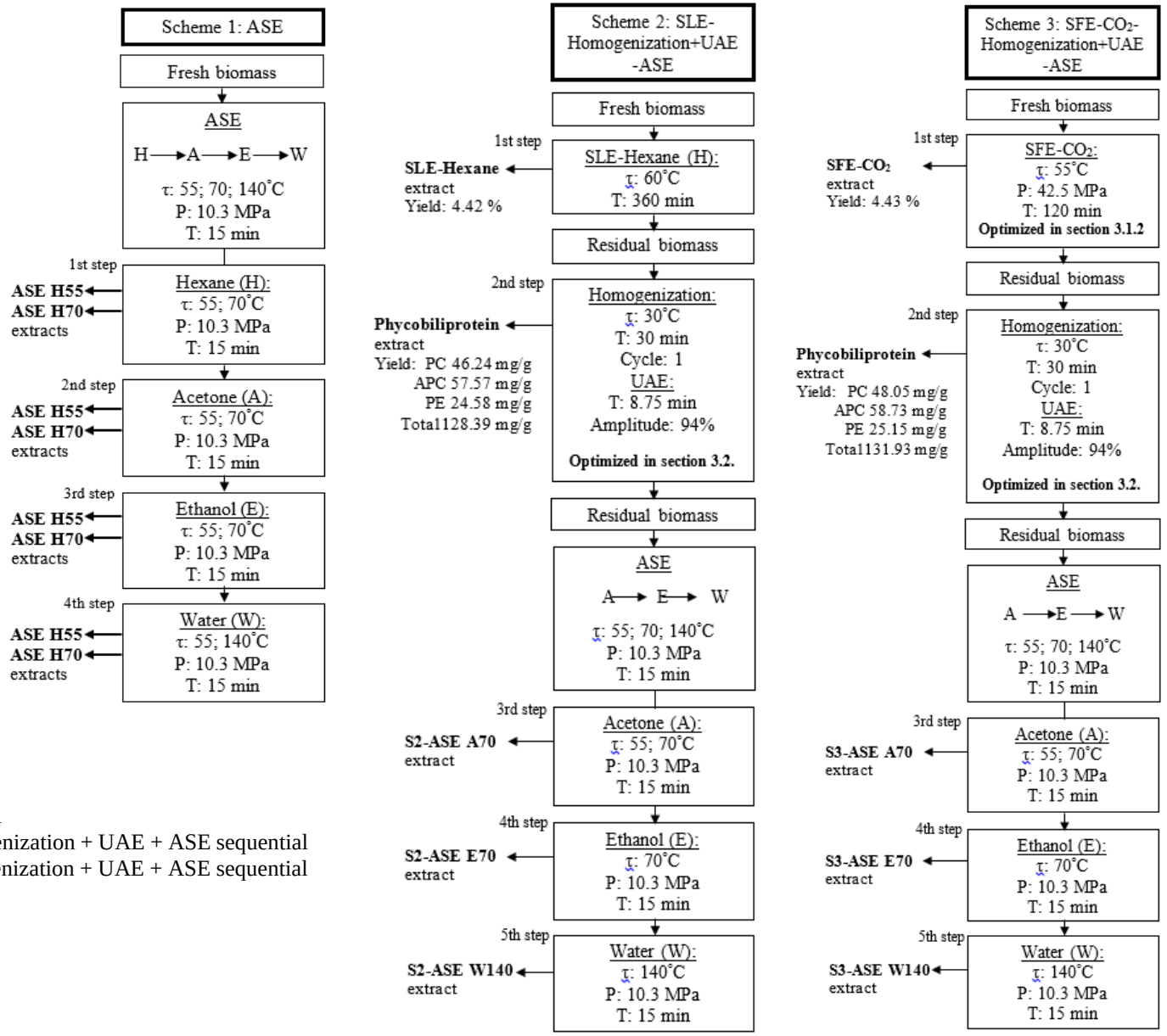
fresh *S. platensis* biomass [112]. Nakagawa et al. reported that TPC values of phycocyanin, extracted from *S. maxima* using mortar and pestle method, was ~17 mg GAE/g DW [123].

3.3. Development of multistep valorization (biorefinering) for wild cyanobacteria biomass utilization

3.3.1. Development of biorefining schemes

The objective of this part of the research was to develop consecutive multistep fractionation scheme (Figure 16), using solvents of increasing polarity (hexane; acetone; ethanol; water) by means of ASE at different temperatures. Secondly, to apply the most efficient ASE conditions for isolation of polar extracts as a third step of wild cyanobacterial biomass valorization after lipophilic fraction removal (1st step, Schemes 2 and 3) and phycobiliprotein removal (2nd step, Schemes 2 and 3). It should be noted that optimal extraction conditions for step 1 of Scheme 2 (SFE-CO_2) were already discussed in section 3.1.2 of this thesis. Similarly, reasoning for applied conditions in step 2 (homogenizer + UAE) for phycobiliprotein removal (schemes 2 and 3) are discussed in section 3.2. Although, freeze/thaw cycles followed by UAE showed the maximum phycobiliprotein yield, for this section homogenization followed by UAE was chosen as a more industrially relevant and energy efficient process than freeze/thaw cycles. Moreover, in comparison to SFE-CO_2 the efficiency of SLE (Scheme 2) and ASE (Scheme 1) with hexane to remove lipophilic products were also evaluated.

SFE-CO_2 under optimal conditions (55 °C, 42.5 MPa and 120 min) showed the highest yield (4.43 %) of lipophilic extract comparing with SLE (4.42 % (Scheme 2)) and Soxhlet (3.6 %) extracts. The phycobiliprotein removal from wild cyanobacteria biomass in Scheme 2 and 3 showed that 3 % higher content of total phycobiliproteins was received in Scheme 3 from biomass after removal of lipophilic fraction by SFE-CO_2 under optimal conditions



Scheme 1: ASE sequential
Scheme 2: SLE + Homogenization + UAE + ASE sequential
Scheme 3: SFE + Homogenization + UAE + ASE sequential

Figure 16. Multistep biorefinering schemes for wild cyanobacteria valorization

3.3.2. Accelerated solvent extraction from wild cyanobacteria biomass

ASE was performed with three cyanobacterial biomasses – fresh wild cyanobacterial biomass, biomass after removed lipophilic fraction by SLE method and removed phycobiliproteins by homogenization combined with UAE, and biomass after SFE and removed phycobiliproteins by homogenization combined with UAE as showed in Schemes 1, 2 and 3.

Table 18. Extracts content (%) after accelerated solvent extraction – Scheme 1, 2 and 3

Solvent	Temperature °C	Extract yield %		
		Scheme 1	Scheme 2	Scheme 3
Hexane	55	6.94 ± 0.02	na	na
	70	4.58 ± 0.11	na	na
Acetone	55	6.16 ± 0.03	na	na
	70	7.34 ± 0.05	4.17 ± 0.16	1.09 ± 0.09
Ethanol	55	7.50 ± 0.05	na	na
	70	7.85 ± 0.21	3.16 ± 0.10	2.26 ± 0.18
Water	55	4.53 ± 0.19	na	na
	140	6.46 ± 0.17	2.15 ± 0.12	1.07 ± 0.09

SFE – supercritical fluid extraction; SLE – solid liquid extraction; UAE – ultrasound assisted extraction; na – not applicable. Results expressed as % of biomass sample. Values are reported as mean value ± standard deviation. In all cases relative standard deviation did not exceed 10%.

Based on the results (Table 18), from fresh cyanobacterial biomass (Scheme 1) the highest extraction yield was obtained by ethanol at 70 °C and the lowest yield – by water at 55 °C. All extractions performed in higher temperatures gave higher yields, except extraction with hexane, where yield after extraction at 70 °C was 1.5 times lower than after extraction at 55 °C.

Biomass in Schemes 2 and 3 was extracted using acetone, ethanol and water respectively at 70 °C, 70 °C and 140 °C. The results showed that after phycobiliproteins and lipophilic fraction removal the yields of extracts decreased. ASE results from Scheme 3 showed that after extractions with acetone, ethanol and water extracted yields were respectively 6.7, 3.5 and 6 times lower comparing with yields from fresh biomass after ASE (Scheme 1). ASE results from Scheme 2 showed that after extractions with acetone, ethanol and water extracted yields were respectively 1.7, 2.5 and 3 times lower comparing with yields from fresh biomass after ASE (Scheme 1). Scheme 2 ASE results showed that after extractions with acetone, ethanol and water extracted yields were respectively 3.8, 1.4 and 2 times higher comparing with yields after ASE from Scheme 3. ASE results from Scheme 1 showed that yields of lipophilic extracts with hexane at 55 °C (4.58 %) and with hexane at 70 °C (6.94 %) were the highest lipophilic fraction yields comparing with SFE-CO_2 under optimal conditions,

SLE and Soxhlet. The water extracts amount of Schemes 1 and 2 was too low to determine in vitro antioxidant activity.

Results after accelerated solvent extraction is in range with previous studies. Rodriguez-Meizoso et al. submitted a study in which was specified yields of extracts by accelerated solvent extraction from *Phormidium* species. Extraction was performed with three solvents, hexane, ethanol and water, in different temperature from 50 until 200 °C. Results showed that yield after extraction with hexane at 50 °C was 0.47 % DW and at 100 °C it was 1.68 % DW, yield after extraction with ethanol at 50 °C was 5.28 % DW and at 100 °C it was 8.48 % DW, yield after extraction with water at 50 °C was 0.71% DW, at 100 °C it was 2.01 % DW and at 150 °C it was 6.42 % DW. All extractions was performed 20 minutes [124]. Moreover, Herrero et al. reported that after ASE from *S. platensis* at 60 °C using hexane as a solvent yield of extract was 0.58 % DW, at 60 °C using ethanol as a solvent yield of extract was 7.21 % DW and at 170 °C using water as a solvent 10.12 % DW [125]. Accelerated solvent extraction takes less time and solvent, and extraction yields are higher compared to traditional techniques such as Soxhlet or SLE.

3.3.3. Fatty acid profile of ASE extracts from wild cyanobacteria

In the next step, the fatty acid profile of the ASE (Scheme 1) and SLE extracts (Scheme 2) from wild cyanobacterial biomass was measured by GC-FID. Four saturated, four monounsaturated and three polyunsaturated fatty acids were identified in these extracts. Extract after ASE with hexane at 55 °C had more SFAs and MUFAs than extract after ASE with hexane at 70 °C but had less PUFAs because in the extract after ASE with hexane at 70 °C was more α-Linolenic acid extracted. Extract after SLE with hexane had more fatty acids than others extracts.

There were three most abundant fatty acids in all extracts from Schemes 1 and 2 – first α-Linolenic acid (~ 34 %), second palmitic acid (~17 %) and third myristic acid (~10 %) (Table 19). The best yield of fatty acids was in the extract after solid-liquid extraction with hexane followed by extract after ASE extraction with hexane at 70 °C. Tang et al. reported that after accelerated solvent extraction, using methanol and chloroform mix (2:1) as a solvent, three main fatty acids in C. vulgaris extract was C18:1 (3.2 %), C16:0 (2.4 %) and C18:3. The highest yield of - α-Linolenic acid was found in C. zofingiensis extract [126]. Moreover, Pieber et al. reported that after ASE extraction with hexane at 60 °C 21 % palmitoleic, 16 % palmitic and 14.9 % EPA fatty acids was obtained from *Nannochloropsis oculata* [127]. The data reported in this study fall in a range with other reaserchers results.

Table 19. Fatty acid content (% and mg/g DW) of ASE and SLE extractions from wild cyanobacterial biomass, obtained under biorefing Schemes 1 and 2

Fatty acid	Profile, % and mg/g of the total GC-FID peak area					
	Scheme 1				Scheme 2	
	ASE H55		ASE H70		SLE hexane	
	%	mg/g	%	mg/g	%	mg/g
C12:0	0.31±0.06^{a}	1.81±0.37^{a}	1.58±0.02^{b}	11.40±0.12^{b}	0.87±0.00^{a}	10.42±0.01^{a}
C14:0	9.90±0.55^{d}	70.05±3.87^{d}	8.98±0.38^{f}	68.35±2.91^{f}	10.32±0.29^{d}	122.87±3.46^{d}
C14:1	1.03±0.06^{ab}	6.09±0.34^{ab}	0.85±0.03^{ab}	6.14±0.18^{ab}	0.76±0.04^{a}	9.18±0.52^{a}
C16:0	16.6±1.20^{e}	117.39±8.46^{e}	15.23±0.49^{g}	115.93±3.71^{g}	19.34±0.82^{e}	230.20±9.78^{e}
C16:1	1.66±0.15^{ab}	11.77±1.09^{ab}	1.61±0.05^{b}	12.25±0.41^{b}	1.85±0.11^{ab}	21.98±1.25^{ab}
C18:0	2.63±0.19^{b}	18.60±1.32^{b}	2.63±0.05^{c}	20.05±0.42^{c}	3.09±0.21^{bc}	36.77±2.55^{b}
C18:1n9t	4.30±0.30^{bc}	30.44±2.10^{bc}	4.37±0.09^{d}	33.25±0.69^{d}	4.57±0.10^{c}	54.37±1.15^{c}
C18:1n9c	1.05±0.18^{ab}	7.40±1.24^{ab}	1.11±0.01^{ab}	8.47±0.11^{ab}	1.10±0.11^{a}	13.04±1.36^{a}
C18:2n6c	7.44±0.52^{cd}	52.62±3.7^{cd}	7.87±0.12^{e}	59.91±0.95^{e}	8.73±0.40^{d}	103.91±4.79^{d}
C18:3n3	31.93±2.24^{f}	225.86±15.80^{f}	34.28±0.48^{h}	260.85±3.68^{h}	36.15±0.97^{f}	430.00±11.59^{f}
C20:3n3	_nd	_nd	0.41±0.01^{a}	2.96±0.11^{a}	0.79±0.06^{a}	9.51±0.69^{a}
SFAs	29.44	207.85	28.42	215.73	33.62	400.26
MUFAs	8.04	55.70	7.94	60.11	8.28	98.57
PUFAs	39.37	278.48	42.56	323.72	45.67	543.42

C12:0 – Lauric acid; C14:0 – Myristic acid; C14:1 – Myristoleic acid; C16:0 – Palmitic acid; C16:1 – Palmitoleic acid; C18:0 – Stearic acid; C18:1n9t – Elaidic acid; C18:1n9c – Oleic acid; C18:2n6c – Linoleic acid; C18:3n3 - α-Linolenic acid; C20:3n3 – cis-11,14,17-Eicosatrienoic acid; ASE – accelerated solvent extraction; SLE – solid-liquid extraction; H55 – solvent hexane at 55 °C; H70 – solvent hexane at 70 °C; results expressed as % and mg/g DW of biomass sample. Values are reported as mean value ± standard deviation. In all cases relative standard deviation did not exceed 10%. Different letters in each column indicate statistically significant differences.

Three most abundant fatty acids in all lipophilic extracts was polyunsaturated α-Linolenic acid and saturated palmitic and myristic acids. The highest content of all theese fatty acids was found in SLE extract (36 % of α-Linolenic acid, 19 % of palmitic acid and 10 % of myristic acid), followed by extracts from Scheme 1, Soxhlet and SFE-CO_2 under optimal conditions. Yield of α-Linolenic acid was 9 % lower in SFE-CO_2 extract under optimal conditions compared to yield from SLE extract.

3.3.4. *In vitro* antioxidant activity assessment of ASE and SLE extracts

TPC and $TEAC_{ABTS}$ values of ASE extracts (Scheme 1) reported in Table 20. The highest TPC and $TEAC_{ABTS}$ antioxidant activity was in E70 extract (32.85 mg GAE/g, 175.23 mg TE/g), and the lowest TPC was in H70 (27.58 mg GAE/g) and $TEAC_{ABTS}$ antioxidant activity was in H55 extract

(42.59 mg GAE/g). The best antioxidant properties had an extract obtained from fresh wild cyanobacterial biomass after ASE extraction when ethanol was used as a solvent.

Table 20. Total phenolic content (TPC), DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ scavenging capacity of ASE extracts from wild cyanobacterial biomass, obtained under biorefining Scheme 1

***In vitro* antioxidant capacity**	**ASE extracts**				
	Scheme 1				
	H55	H70	A70	E70	W140
TPC, mg GAE/g:					
mg /g extract*	31.40±0.81^{c}	27.58±0.71^{a}	29.97±0.31^{b}	32.85±0.68^{d}	30.31±1.33^{bc}
mg /g DW[1**]	2.18±0.06^{c}	1.26±0.03^{a}	2.20±0.02^{c}	2.58±0.05^{d}	1.96±0.08^{b}
$TEAC_{ABTS}$, mg TE/g:					
mg /g extract*	42.59±2.42^{a}	48.03±3.04^{b}	91.81±3.52^{c}	175.23±3.13^{d}	49.90±0.42^{b}
mg /g DW[1**]	2.96±0.17^{b}	2.20±0.14^{a}	6.74±0.26^{c}	13.76±0.25^{d}	3.22±0.03^{b}

H55 – solvent hexane at 55 °C; H70 – solvent hexane at 70 °C; A70 – solvent acetone at 70 °C; E70 – solvent ethanol at 70 °C; W140 – solvent water at 140 °C. %. Data expressed as mean value ± standard deviation of five replicates. *: Different superscript letters within the same line for individual in vitro antioxidant activity assessment assays indicate significant differences (one-way ANOVA and Tukey's test, $p < 0.05$).

In addition, calculated Pearson correlation coefficients between different antioxidant activity indices indicate the presence of a positive correlation (0.7554) between TPC and ABTS$^{\bullet+}$ scavenging assays (Table 21).

Table 21. Analysis of correlation between different antioxidant activity indices of extracts after ASE

Antioxidant activity indices	**TPC**	**ABTS$^{\bullet+}$**
TPC	1	0.7554
ABTS$^{\bullet+}$		1

Expressed as mg GAE/g DW or mg TE/g DW; correlation coefficients were calculated using Exel.

TPC and $TEAC_{ABTS}$ values of ASE extracts (Shemes 2 and 3) reported in Table 22. TPC antioxidant activity varied from 29.99 to 33.67 mg GAE/g and $TEAC_{ABTS}$ antioxidant activity varied from 67.20 to 145.19 mg TE/g. The removal of the lipophilic fraction from the wild cyanobacterial biomass by SFE or SLE methods, and the phycobiliproteins by homogenization and UAE methods showed that the activity of TPC antioxidants was almost unchanged, and the activity of $TEAC_{ABTS}$ antioxidant decreased 26.46 % of acetone extract and 35.29 % of ethanolic extract. In addition, calculated Pearson correlation coefficients between different antioxidant activity indices indicate the presence of a positive correlation (0.7350) between TPC and ABTS$^{\bullet+}$ scavenging assays (Table 23).

Table 22. Total phenolic content (TPC), DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ scavenging capacity of ASE extracts from wild cyanobacteria, obtained under biorefining Schemes 2 and 3

***In vitro* antioxidant capacity**	**ASE extracts**				
	Scheme 2			**Scheme 3**	
	SLE	S2 A70	S2 E70	S3 A70	S3 E70
TPC, mg GAE/g:					
mg/g extract*	30.49±1.34^{a}	32.56±0.43^{b}	33.67±0.52^{b}	29.99±0.53^{a}	33.45±0.26^{b}
mg /g DW[1**]	1.35±0.06^{d}	1.36±0.02^{d}	1.06±0.02^{c}	0.33 ± 0.01^{a}	0.76±0.01^{b}
$TEAC_{ABTS}$, mg TE/g:					
mg/g extract*	85.10±5.76^{b}	67.84±3.76^{a}	145.19±3.97^{c}	67.20±4.37^{a}	81.58±2.64^{b}
mg /g DW[1**]	3.76±0.25^{d}	2.83±0.16^{c}	4.59±0.13^{e}	0.73±0.05^{a}	1.84±0.06^{b}

A70 – acetone at 70 °C; E70 – ethanol at 70 °C. Data expressed as mean value ± standard deviation of five replicates. *: Different superscript letters within the same line for individual in vitro antioxidant activity assessment assays indicate significant differences (one-way ANOVA and Tukey's test, $p < 0.05$).

Table 23. Analysis of correlation between different antioxidant activity indices of extracts after ASE with removed lipophilic and phycobiliproteins fractions

Antioxidant activity indices	**TPC**	**ABTS$^{\bullet+}$**
TPC	1	0.7350
ABTS$^{\bullet+}$		1

Expressed as mg GAE/g DW or mg TE/g DW; correlation coefficients were calculated using Exel.

In a recent report of Li et al. the TPC and $TEAC_{ABTS}$ content of 23 microalgae strains was evaluated in three different polarity extracts (hexane, ethyl acetate and water), including several cyanobacteria. For the hexane fractions, the antioxidant capacities ranged from 0.01 to 11.41 μmol Trolox/g of $TEAC_{ABTS}$ and from 2.12 to 39.87 mg GAE/g of TPC, for the ethyl acetate fractions - ranged from 0.01 to 16.00 μmol Trolox/g of $TEAC_{ABTS}$ and from 0.01 to 9.80 mg GAE/g of TPC, for the water fractions – ranged from 0.01 to 9.23 μmol Trolox/g of $TEAC_{ABTS}$ and from 0.97 to 10.68 mg GAE/g of TPC [128]. The same experiment was confirmed by Hajimahmoodi et al., when the TPC content of 12 microalgae strains was evaluated and the combined TPC of three different polarity extracts (hexane, ethyl acetate and water) varied between 1.49 to 16.65 mg of GAE/g of DW [129], which falls in the range of the data reported in this study too. However, direct comparison of these values is rather complex, since most of these studies focus on specific strains grown under optimal conditions.

The TPC antioxidant activity was highest in SFE-CO_2 extract under optimal conditions compared with all extrats from Schemes 1, 2 and 3. Moreover, $TEAC_{ABTS}$ results showed that the highest antioxidant activity was in ethanolic extracts from Schemes 1 and 2 (13.76 and 4.59 mg TE/g

DW respectively). $TEAC_{ABTS}$ results of lipophilic extracts showed that the highest antioxidant activity was in SLE (3.9 mg TE/g DW) compared with SFE-CO_2 under optimal conditions (2.97 mg TE/g DW), Soxhlet extracts (2.33 mg TE/g DW) and extracts from Scheme 1 (2.96 and 2.20 mg TE/g DW of H55 and H70 respectively).

3.3.5. Preliminary phytochemical characterization by means of UPLC-TOF-MS of non-polar and polar fractions obtained under different biorefinery schemes

All lipophilic (Table 24), acetone (Table 25), ethanolic (Table 26) and water (Table 27) extracts were studied in UPLC-TOF-MS to determine the compounds present in them. Experiment was done in positive and negative ionization and identification of compounds was performed using Metlin and ChemSpider data bases.

α-Linolenic acid gave an *m/z* of 279.2323 in positive ionization and 277.2170 in negative ionization and was found in lipophilic, acetone and ethanol extracts. These results are in a range with Herrero et al. research in which reported linolenic, palmitic, stearic acids (as most abbundant in the extracts from cyanobacteria) and their complementary compounds with glycerol and hexose [130].

Ergosterol and brassicasterol gave an m/z of 377.1416 and 379.1573 in negative ionization respectively and were found in lipophilic and acetone extracts. Stigmasterol was found in lipophilic and ethanolic extracts and gave *m/z* of 413.3989 in positive ionization and 393.1054 in negative ionization. Kohlhase et al. reported about sterols found by GC/MS. Ergosterol was found in *A. viguieri*, *A. solitaria* and *N. harveyana* cyanobacterias in low quantities, brassicasterol was found in the same three cyanobacterias as ergosterol and in *A. cylindrica* and *N. carneum*. Stigmasterol was also find in all five tested cyanobacteria species [131].

Carotenoids were found in all extracts. β-carotene gave *m/z* of 537.3553 in positive ionization and 535.1535 in negative ionization, zeaxanthin gave *m/z* of 593.2761 in positive ionization, β-cryptoxanthin gave *m/z* of 575.4513 and 533.2968 in positive and negative ionizations respectively and astaxanthin gave *m/z* of 595.2405 in negative ionization. Goodwin et al. tested seven cyanobacteria species and reported that three major carotenoids was β-carotene, echinenone and myxoxanthophyll and that zeaxanthin was presented in small amounts [132].

Chlorophyll a was found in ethanolic and water extracts and gave *m/z* of 893.2762 in positive ionization and 873.4355 in negative ionization.

Table 24. Compounds found in lipophilic extracts from wild cyanobacterial biomass under different biorefining schemes

Time	Meas. m/z	Δppm	Suggested formula	Name*		SFE	Soxhlet	SLE	S1- H55	S2-H70
Positive										
4.7-4.8	230.2476	0	$C_{14}H_{28}O$	Tetradecenone, Xestoaminol	M+H	+				
	274.2744	1	$C_{16}H_{36}NO_2$	Sphinganine	M+H	+	+	+	+	
5.0-5.2	155.1068	0	$C_9H_{15}O_2$	Nonadienoic acid	M+H	+	+			+
	275.2007	0	$C_{18}H_{27}O_2$	Octadecapentaenoic acid	M+H	+	+			+
	333.2040	1	$C_{18}H_{30}O_4$	Hydroperoxy-octadecatrienoic acid	M+Na	+	+			+
7.0-7.2	458.2874	5	$C_{23}H_{36}NO_3$	Docosahexaenoyl glutamic acid	M+H	+	+	+	+	+
	295.2272	1	$C_{18}H_{31}O_3$	Octadecatrienoic acid	M+H				+	
7.5-7.6	123.1170	1	C_9H_{15}	4-Hydroxybenzaldehyd	M+H				+	
	279.2323	1	$C_{18}H_{31}O_2$	α-Linolenic acid	M+H		+	+	+	+
8.4-8.6	293.2109	0	$C_{18}H_{29}O_3$	Hydroxy-hexadecenoic acid	M+Na	+				
	537.3553	0	$C_{40}H_{57}$	β-carotene	M+H				+	
Negative										
3.8-3.9	355.2120	0	$C_{18}H_{31}O_2$	Octadecadienoate	M-H	+				
5.2-5.4	267.1960	0	$C_{16}H_{27}O_3$	Hexadecanedioic acid	M-H	+				
	311.2224	1	$C_{18}H_{31}O_2$	Octadecadienoic acid	M-H	+				
5.9-6.0	309.2067	1	$C_{18}H_{29}O_4$	Hydroperoxy-octadecatrienoic acid	M-H	+	+		+	
6.2-6.3	358.2316	0	$C_{22}H_{32}NOS$	Curacin D	M-H			+		+
6.6-6.9	249.1859	0	$C_{16}H_{25}O_2$	Hexadecatrienoic acid	M-H	+	+	+	+	+
	499.3787	1	$C_{32}H_{51}O_4$	Oleananoic acid	M-H	+	+		+	+
7.6-7.7	277.2168	1	$C_{18}H_{29}O_2$	α-Linolenic acid	M-H	+	+	+	+	+
	377.1416	0	$C_{28}H_{43}$	Ergosterol	M-H2O-H	+	+		+	+
	393.1054	0	$C_{29}H_{47}O$	Stigmasterol	M-H			+		
8.1-8.2	379.1573	0	$C_{28}H_{45}$	Brassicasterol	M-H2O-H	+	+		+	+

*Name and average mass of compounds was suggested by ChemSpider chemical structure database.

Table 25. Compounds found in acetonic extracts from wild cyanobacterial biomass under different biorefining schemes

Time	Meas. m/z	Δppm	Suggested formula	Name*		S1-A55	S1- A70	S2-A70	S3-A70
Positive									
4.7-4.8	230.2474	0	$C_{14}H_{28}O$	Tetradecenone, Xestoaminol	M+H			+	
	274.2742	0	$C_{16}H_{36}NO_2$	Sphinganine	M+H	+	+	+	+
7.5-7.6	279.2318	0	$C_{18}H_{31}O_2$	α-Linolenic acid	M+H			+	+
8.2-8.3	593.2761	0	$C_{40}H_{56}O_2$	Zeaxanthin	M+Na			+	+
Negative									
5.1-5.2	309.2070	1	$C_{18}H_{29}O_4$	hydroperoxy-octadecatrienoic acid	M-H	+	+	+	+
	372.2383	0	$C_{23}H_{35}NOS$	Curacin A	M-H	+			
5.7-5.9	533.2968	0	$C_{40}H_{54}$	β-cryptoxanthin	M-H2O-H	+			+
6.0-6.1	358.2597	0	$C_{22}H_{32}NOS$	Curacin D	M+H	+		+	+
6.6-6.8	205.1597	0	$C_{11}H_9O4$	Sinapic acid	M-H20-H	+	+	+	+
6.8-6.9	249.1857	0	$C_{16}H_{25}O_2$	Hexadecatrienoic acid	M-H	+	+	+	+
7.5-7.7	277.2170	0	$C_{18}H_{29}O_2$	α-Linolenic acid	M-H	+	+	+	
	377.1421	0	$C_{28}H_{43}$	Ergosterol	M-H2O-H	+	+	+	
8.0-8.2	379.1580	0	$C_{28}H_{45}$	Brassicasterol	M-H2O-H			+	+

*Name and average mass of compounds was suggested by ChemSpider chemical structure database.

Table 26. Compounds found in ethanolic extracts from wild cyanobacterial biomass under different biorefining schemes

Time	Meas. m/z	Δppm	Suggested formula	Name*		S1-E55	S1-E70	S2-E70	S3-E70
Positive									
0.8-1.0	137.0455	2	C_8H_5O	PAA (auxin)	M+H	+		+	+
	537.1689	0	$C_{40}H_{57}$	β-carotene	M+H			+	+
4.7-4.9	274.2745	1	$C_{16}H_{36}NO_2$	Sphinganine	M+H	+	+	+	+
7.0-7.1	254.2480	0	$C_{16}H_{32}NO$	Palmitoleamide	M+H			+	
7.5-7.6	279.2318	0	$C_{18}H_{31}O_2$	α-Linolenic acid	M+H				+
7.7-7.8	413.3989	0	$C_{29}H_{49}O$	Stigmasterol	M+H	+		+	
	575.4513	0	$C_{40}H_{55}O$	β-cryptoxanthin	M+Na	+		+	
	871.5738	0	$C_{55}H_{75}N_4O_5$	Pheophytin	M+H		+		
	893.6589	0	$C_{66}H_{85}O$	Chlorophyll a	M+H				+
8.2-8.3	593.2762	0	$C_{40}H_{56}O_2$	Zeaxanthin	M+Na	+	+	+	+
Negative									
0.3-0.4	631.2202	0	$C_7H_{19}N_{32}O_5$	Sialyllactosamine	M-H			+	
0.8-1.0	535.1535	0	$C_{40}H_{55}$	β-carotene	M-H			+	+
	595.2405	0	$C_{40}H_{51}O_4$	Astaxanthin	M-H	+			
	873.4355	0	$C_{52}H_{69}MgN_4O_4$	Chlorophyll a	M-H2O-H	+			
6.0-6.1	358.2602	0	$C_{22}H_{32}NOS$	Curacin D	M-H	+	+	+	+
6.6-6.9	205.1595	1	$C_{11}H_9O_4$	Sinapic acid	M-H2O-H	+	+	+	
	249.1859	0	$C_{16}H_{25}O_2$	Hexadecatrienoic acid	M-H			+	+
7.5-7.6	277.2172	0	$C_{18}H_{29}O_2$	α-Linolenic acid	M-H			+	+

*Name and average mass of compounds was suggested by ChemSpider chemical structure database.

Table 27. Compounds found in water extracts from wild cyanobacterial biomass under different biorefining schemes

Time	Meas. m/z	Δppm	Suggested formula	Name*		S1-W55	S1-W140	S2-W140	S3-W140
Positive									
0.3-0.5	134.0448	0	$C_4H_8NO_4$	Aspartic acid	M+H	+		+	
0.9-1.0	268.1038	4	$C_9H_{18}NO_8$	Neuraminic acid	M+H		+		
	537.1687	0	$C_{40}H_{57}$	β-carotene	M+H		+		+
2.3-2.4	227.1755	0	$C_{10}H_8CLNO_2$	4-CL-IAA (auxin)	M+NH4				+
	679.5121	0	$C_{36}H_{63}N_5O_6$	Ceramide	M+NH4	+	+	+	+
4.7-4.9	230.2483	0	$C_{14}H_{28}O$	Tetradecenone, Xestoaminol	M+H				+
	274.2745	1	$C_{16}H_{36}NO_2$	Sphinganine	M+H	+	+	+	+
7.7-7.8	577.4675	0	$C_{32}H_{65}O_8$	Hexacosanetriol	M+H	+			+
	893.6593		$C_{66}H_{85}O$	Chlorophyll a	M+H				+
8.2-8.5	593.2768		$C_{40}H_{56}O_2$	Zeaxanthin	M+Na	+			+
Negative									
0.4-0.6	132.0304	1	$C_4H_6NO_4$	Aspartic acid	M-H		+		
	133.0143	0	C_8H_5O	PAA (auxin)	M-H2O-H			+	
	179.0562	0	$C_6H_{11}O_6$	Syringic acid /caffeic acid	M-H20-H/ M-H				+
	191.0198	0	$C_6H_7O_7$	Citric acid	M-H	+	+	+	
	549.1681	1	$C_{40}H_{53}O$	Plectaniaxanthin	M-H20-H	+	+		
	631.2201	0	$C_{23}H_{39}N_2O_{18}$	Sialyllactosamine	M-H	+	+		+
0.8-1.0	289.0679	0	$C_{10}H_{13}N_2O_8$	Catechin	M-H				+
1.5-1.6	313.1040	4	$C_{13}H_{17}N_2O_7$	Dihydropteroic acid	M-H		+		
5.5-5.6	595.2397	0	$C_{40}H_{51}O_4$	Astaxanthin	M-H		+		
5.7-5.9	533.2966	0	$C_{40}H_{53}$	β-cryptoxanthin	M-H2O-H				+
6.0-6.2	358.2602	0	$C_{22}H_{32}NOS$	Curacin D	M-H			+	+
6.3-6.4	309.1739	1	$C_7H_{21}N_{10}O_4$	hydroperoxy-octadecatrienoic acid	M-H	+			+
6.6-6.8	205.1597	0	$C_{11}H_9O4$	Sinapic acid	M-H2O-H	+	+	+	
	249.1860	0	$C_{16}H_{25}O_2$	Hexadecatrienoic acid	M-H				+

*Name and average mass of compounds was suggested by ChemSpider chemical structure database.

Curacin A was found in ASE A55 extract (Scheme 1) and gave *m/z* of 372.2383 in positive ionization. Curacin D gave *m/z* of 358.2316 in negative ionization and was found in all extracts.Curacin can be produced by cyanobacteria naturaly and has been characterized as antiproliferative cytotoxic compound with anticancer activity [133], [134]. Singh et al reported that Curacin A was found in *L. majuscula* [104] and Esquuenazi et al. reported about the same result in acetone extract from *L. majuscula* [135]. 4-hydroxybenzaldehyd gave *m/z* of 123.1170 in positive ionization and was found in ASE with hexane at 55 °C extract. Klejdus et al. reported that 4-hydroxybenzaldehyd was found in *S. spongiosa* and in *S. platensis* in low concentrations in methanol:water (1:1, v:v) extract [136]. Auxin PAA was found in ethanolic and water extracts and gave *m/z* of 137.0455 in positive ionization and 133.0143 in negative ionization. Auxin 4-CL-IAA was found in water extract after ASE (Scheme 3) and it gave *m/z* of 277.1755 in positive ionization. Hussain et al. reported that auxin IAA was found in 5 from 13 cyanobacteria species [137]. A full analysis of the compounds found in the extracts is given in the annexes in Tables A.18-21.

3.3.6. Evaluation of applied biorefinery schemes

The highest yield of all extracts was obtained from Scheme 1 (28.59 %), followed by Scheme 2 (26.74 %) and Scheme 3 (22.04 %). The total yield of Scheme 1 was 21 and 30 % higher compared with Scheme 2 and 3 respectively.

ASE extracts with hexane at 55 °C (4.58 %) and with hexane at 70 °C (6.94 %) from Scheme 1 were the highest lipophilic fraction yields comparing with SFE-CO_2 under optimal conditions (4.43 %), SLE (4.42 %). Compared *in vitro* antioxidant activity results of lipophilic fraction showed that the most effective extract was SFE-CO_2 under optimal conditions and its antioxidant capacity of TPC was 2 times higher compared with results of hexane extracts. $TEAC_{ABTS}$ antioxidant activity of lipophilic fraction showed the highest activity in SLE extract (Scheme 2).

Yields of acetone extracts was compared and results showed that yields of Scheme 1 was 1.76 and 5.65 times higher than yield of Schemes 2 and 3, respectively. The TPC antioxidant activity was the same in all acetone extracts (~30 mg GEA/g extract) and $TEAC_{ABTS}$ activity was the same in acetone extracts of Schemes 2 and 3 (67 mg TE/g extract) and results of Scheme 1 was 1.4 time higher. Yields of ethanol extracts was compared and results showed that yields of Scheme 1 was 2.48 and 3.47 times higher than yield of Schemes 2 and 3, respectively. The TPC antioxidant activity was the same in all acetone extracts (~33 mg GEA/g extract) and $TEAC_{ABTS}$ activity was highest in ethanol extract of Scheme 1 (175.23 mg TE/g extract).

Total phycobiliproteins content of Schemes 2 and 3 were 6.43 and 3.85 % lower compared with the yield after extraction with homogenization combined with UAE under optimal conditions.

CONCLUSIONS

1. The following chemical composition was determined for the wild cyanobacteria biomass utilized in this study: lipid content 4 %, nitrogen content 67 %, mineral content 5 %, and moisture content 6 %. Crude wild cyanobacteria biomass showed antioxidant activity: TPC was 23.49 g GAE/ mg DW, $TEAC_{ABTS}$ was 57.14 mg TE/g DW and $TEAC_{DPPH}$ 5.04 mg TE/g DW.
2. Response surface methodoly indicated 55 °C, 425 bar and 120 min as optimal extraction conditions. Under these conditions the SFE-CO_2 extract yield was 4.43 g/100g which was 20 % higher compared to Soxhlet extract (3.6 g/100g). SFE-CO_2 proved to be more efficient (120%) and three times faster compared to conventional Soxhlet extraction.
3. GC-FID revealed that α-linolenic acid was the most abundant fatty acid in SFE-CO_2 lipophilic extract obtained under optimal conditions (27 %) and in Soxhlet extract (34 %), followed by palmitic acid (16 and 15 %), oleic acid (6 and 8 %) and myristic acid (6 and 7 %) respectively. HPLC-FL revealed that a-tocopherol content was 293.73 μg/g of SFE-CO_2 extract and 326.68 μg/g for Soxhlet extract. The highest antioxidant activity value was obtained for the wild cyanobacterial biomass prior any extraction (23.49 mg GAE/g DW from TPC). After removing the lipophilic fraction by SFE-CO_2 or Soxhlet extraction, the total phenolic contect in residual biomass was reduced by 32 and 31 % respectively. Moreover, SFE-CO_2 extract under optimal conditions showed higher total phenolic content (2.93 mg GAE/g DW) and radical scavenging capacity (2.97 mg TE/g DW for ABTS and 2.45 mg TE/g DW for DPPH) than the Soxhlet extract (2.06 mg GAE/g DW for TPC, 2.33 mg TE/g DW for ABTS and 1.81 mg TE/g DW for DPPH).
4. Several conventional (freeze-thaw, homogenization, SLE) and the same techniques completed with UAE were chosen for aquaeous extraction of phycobiliproteins. Response surface methodology indicated several optimal extraction conditions as a response to PC, APC, PE and total phycobiliprotein yield content. UAE significantly increased the phycobiliproteins content of extracts as compared to the conventional extraction methods. The highest phycobiliproteins content (139.28 mg/g DW) was obtained with freeze-thaw combined with UAE extraction under optimal conditions (one freeze-thaw cycle followed by UAE extraction for 9.39 min and 99 % of amplitude). The highest antioxidant activity of conventional extractions was shown in homogenization (44.75 mg GAE/g DW for TPC and 44.57 mg TE/g DW for ABTS) and the lowest antioxidant activity was reported in freeze-thaw extract (38.84 mg GAE/g DW for TPC and 43.43 mg TE/g DW for $TEAC_{ABTS}$). The highest antioxidant activity of combined extractions was shown in SLE + UAE extract (58.40 mg GAE/g DW for TPC and 47.33 mg TE/g DW for $TEAC_{ABTS}$)

and the lowest antioxidant activity was reported in freeze-thaw + UAE (40.77 mg GAE/g DW for TPC) and homogenization + UAE extract (42.53 mg TE/g DW for TEAC).

5. ASE conditions were evaluated with two different temperatures at each solvent (hexane, acetone, ethanol, water). The highest yield of non-polar fraction of ASE was with hexane at 55 °C, for the polar fractions - acetone at 70 °C, ethanol at 70 °C and water at 140 °C. The highest temperature showed the highest yield in all polar extracts. Specifically, for acetone extract (7.34 %), ethanol (7.85 %), water (6.46 %). The same conditions for polar fractions were also applied in Schemes 2 and 3 after lipophilic and phycobiliprotein content removal. The yields of polar extracts in Scheme 2 were higher in all cases than yields of polar extracts in Scheme 3. Acetone extract from Scheme 2 had 3.8 times higher yield comparing with acetone extract from Scheme 3. Also, ethanol and water extracts from Scheme 2 had 1.4 and 2 times higher yields than the same extracts from Scheme 3, respectively. Non-polar extracts from Scheme 1 in two different temperatures had the highest yields (at 55 °C the yield was 6.94 % and at 70 °C it was 4.58 %) comparing with SFE-CO_2 (4.42 %), SLE (4.43 %) and Soxhlet (3.6 %).
6. All lipophilic extracts were evaluated by determination of fatty acids profile using GC-FID. Results showed that 4 saturated fatty acids were found in all lipophilic extracts, but SFE-CO_2 extract under optimal conditions had 2 saturated fatty acids more (Arachdic and Behenic fatty acids). 4 monounsaturated and 3 polyunsaturated fatty acids were found in all analysed lipophilic extracts. Three most abundant fatty acids in all lipophilic extracts were α-Linolenic acid, palmitic and myristic acids. The highest content of all theese fatty acids was found in SLE extract (36 % of α-Linolenic acid, 19 % of palmitic acid and 10 % of myristic acid), followed by extracts from Scheme 1 (32 and 34 % of α-Linolenic acid, 17 and 15 % of palmitic acid and 9 and 9 % of myristic acid in extract with hexane at 55 °C and extract with hexane at 70 °C respectively), Soxhlet (34 % of α-Linolenic acid, 15 % of palmitic acid and 7 % of myristic acid) and SFE-CO_2 under optimal conditions (27 % of α-Linolenic acid, 16 % of palmitic acid and 6 % of myristic acid). Yield of α-Linolenic acid was 9 % lower in SFE-CO_2 extract under optimal conditions compared to yield from SLE extract.
7. Lipophilic, acetone, ethanolic, water extracts after ASE were evaluated by determination of *in vitro* antioxidant activity. The TPC antioxidant activity from Scheme 1 was highest in hexane extractact at 55 °C (2.18 mg GAE/g DW) of non-polar extracts and in ethanol extract at 70 °C (2.58 mg GAE/g DW) of polar extracts. The TPC antioxidant activity from Schemes 2 and 3 was highest in SLE (Scheme 2) and acetone extracts – 1.35 and 1.36 mg GAE/g DW, respectively. The $TEAC_{ABTS}$ antioxidant activity from Scheme 1 was highest in hexane extractact at 55 °C (2.96 mg TE/g DW) of non-polar extracts and in ethanol extract at 70 °C (13.76 mg TE/g DW) followed by

acetone at 70 °C (6.74 mg TE/g DW) of polar extracts. The $TEAC_{ABTS}$ antioxidant activity from Scheme 2 and 3 was highest in ethanol extractact (4.59 mg TE/g DW).

8. UPLC-TOF-MS revealed that α-Linolenic acid was found in all extracts. The main carotenoids found in cyanobacteria was β-carotene, zeaxanthin, β-cryptoxanthin and astaxanthin. Also, ergosterol, bassicasterol and stigmasterol were found in lipophilic extracts from wild cyanobacteria. Moreover, UPLC-TOF-MS showed that wild cyanobacteria produces auxins.
9. Conclusively, it was shown that wild cyanobacteria could be utilized as feedstock for productions of high-added value ingredients. The suggested methodologies, as such or with slight modifications could be applied for biorefinery of wild cyanobacteria.

LIST OF LITERATURE REFERENCES

[1] O. P. Sharma, “Textbook of Algae.” p. 396, 1986.

[2] B. Qiu, J. Liu, Z. Liu, and S. Liu, “Distribution and ecology of the edible cyanobacterium Ge-Xian-Mi (Nostoc) in rice fields of Hefeng County in China,” *J. Appl. Phycol.*, vol. 14, no. 5, pp. 423–429, 2002.

[3] V. P. Patil and S. N. Nandan, “Biodiversity of Cyanophyceae from Amaravati Dam of Dhule district (Maharashtra),” *J. Exp. Sci.*, vol. 2, no. 3, pp. 33–36, 2011.

[4] A. De Los Ríos, M. Grube, L. G. Sancho, and C. Ascaso, “Ultrastructural and genetic characteristics of endolithic cyanobacterial biofilms colonizing Antarctic granite rocks,” *FEMS Microbiol. Ecol.*, vol. 59, no. 2, pp. 386–395, 2007.

[5] K. D. Jöhnk, J. Huisman, J. Sharples, B. Sommeijer, P. M. Visser, and J. M. Stroom, “Summer heatwaves promote blooms of harmful cyanobacteria,” *Glob. Chang. Biol.*, vol. 14, no. 3, pp. 495–512, 2008.

[6] “Big Toxic Algal Bloom Again in Forecast for Lake Erie - Circle of Blue.” .

[7] M. Bresciani *et al.*, “Monitoring blooms and surface accumulation of cyanobacteria in the Curonian Lagoon by combining MERIS and ASAR data,” *Remote Sens. Environ.*, vol. 146, pp. 124–135, 2014.

[8] S. P. Cuellar-Bermudez, I. Aguilar-Hernandez, D. L. Cardenas-Chavez, N. Ornelas-Soto, M. A. Romero-Ogawa, and R. Parra-Saldivar, “Extraction and purification of high-value metabolites from microalgae: Essential lipids, astaxanthin and phycobiliproteins,” *Microb. Biotechnol.*, vol. 8, no. 2, pp. 190–209, 2015.

[9] L. Machu *et al.*, “Phenolic content and antioxidant capacity in algal food products,” *Molecules*, vol. 20, no. 1, pp. 1118–1133, 2015.

[10] A. P. Batista, L. Gouveia, N. M. Bandarra, J. M. Franco, and A. Raymundo, “Comparison of microalgal biomass profiles as novel functional ingredient for food products,” *Algal Res.*, vol. 2, no. 2, pp. 164–173, 2013.

[11] J. Singh and S. Gu, “Commercialization potential of microalgae for biofuels production,” *Renew. Sustain. Energy Rev.*, vol. 14, no. 9, pp. 2596–2610, 2010.

[12] R. Halim, M. K. Danquah, and P. A. Webley, “Extraction of oil from microalgae for biodiesel production: A review,” *Biotechnol. Adv.*, vol. 30, no. 3, pp. 709–732, 2012.

[13] G. A. Codd, “Cyanobacterial toxins, the perception of water quality, and the prioritisation of eutrophication control,” *Ecol. Eng.*, vol. 16, no. 1, pp. 51–60, 2000.

[14] B. Luckas *et al.*, “Overview of key phytoplankton toxins and their recent occurrence in the north and baltic seas,” *Environ. Toxicol.*, vol. 20, no. 1, pp. 1–17, 2005.

[15] W. Stahl and H. Sies, “Bioactivity and protective effects of natural carotenoids,” *Biochim. Biophys. Acta - Mol. Basis Dis.*, vol. 1740, no. 2, pp. 101–107, 2005.

[16] F. S. Steinhoff, M. Karlberg, M. Graeve, and A. Wulff, “Cyanobacteria in Scandinavian coastal waters - A potential source for biofuels and fatty acids?,” *Algal Res.*, vol. 5, no. 1, pp. 42–51, 2014.

[17] A. Paldavičiene, H. Mazur-Marzec, and A. Razinkovas, “Toxic cyanobacteria blooms in the Lithuanian part of the Curonian Lagoon,” *Oceanologia*, vol. 51, no. 2, pp. 203–216, 2009.

[18] S. Šulčius *et al.*, “Increased risk of exposure to microcystins in the scum of the filamentous cyanobacterium Aphanizomenon flos-aquae accumulated on the western shoreline of the Curonian Lagoon,” *Mar. Pollut. Bull.*, vol. 99, no. 1–2, pp. 264–270, 2015.

[19] W. W. Carmichael, C. Drapeau, and D. M. Anderson, “Harvesting of Aphanizomenon flos-aquae Ralfs ex Born. & Flah. var. flos-aquae (Cyanobacteria) from Klamath Lake for human dietary use,” *J. Appl. Phycol.*, vol. 12, no. 6, pp. 585–895, 2000.

[20] L. Axelsson, M. Franzén, M. Ostwald, G. Berndes, G. Lakshmi, and N. H. Ravindranath, “Perspective: Jatropha cultivation in southern India: Assessing farmers’ experiences,” *Biofuels, Bioprod. Biorefining*, vol. 6, no. 3, pp. 246–256, 2012.

[21] D. B. Stengel, S. Connan, and Z. A. Popper, “Algal chemodiversity and bioactivity: Sources of natural variability and implications for commercial application,” *Biotechnol. Adv.*, vol. 29, no. 5, pp. 483–501, 2011.

[22] L. Gouveia, A. P. Batista, I. Sousa, A. Raymundo, and N. M. Bandarra, *Microalgae in Novel Food Product*. 2008.

[23] L. Wang, B. Pan, J. Sheng, J. Xu, and Q. Hu, “Antioxidant activity of Spirulina platensis extracts by supercritical carbon dioxide extraction,” *Food Chem.*, vol. 105, no. 1, pp. 36–41, 2007.

[24] A. Dalle Zotte *et al.*, “Dietary Spirulina (Arthrospira platensis) and Thyme (Thymus vulgaris) supplementation to growing rabbits: Effects on raw and cooked meat quality, nutrient true retention and oxidative stability,” *Meat Sci.*, vol. 98, no. 2, pp. 94–103, 2014.

[25] D. F. Horrobin, M. S. Manku, H. Hillman, A. Iain, and M. Glen, “Fatty acid levels in the brains of schizophrenics and normal controls,” *Biol. Psychiatry*, vol. 30, no. 8, pp. 795–805, 1991.

[26] Y. Amagai *et al.*, “Dihomo-γ-linolenic acid prevents the development of atopic dermatitis through prostaglandin D<inf>1</inf> production in NC/Tnd mice,” *J. Dermatol. Sci.*, vol. 79, no. 1, pp. 30–37, 2015.

[27] Y. J. Zhou, Y. L. Song, H. Zhou, and Y. Li, “Linoleic acid activates GPR40/FFA1 and phospholipase C to increase [Ca2+]irelease and insulin secretion in islet beta-cells,” *Chinese Med. Sci. J.*, vol. 27, no. 1, pp. 18–23, 2012.

[28] S. J. Hur and Y. Park, “Effect of conjugated linoleic acid on bone formation and rheumatoid arthritis,” *Eur. J. Pharmacol.*, vol. 568, no. 1–3, pp. 16–24, 2007.

[29] R. L. Mendes, A. D. Reis, and A. F. Palavra, “Supercritical CO2extraction of γ-linolenic acid and other lipids from Arthrospira (Spirulina)maxima: Comparison with organic solvent extraction,” *Food Chem.*, vol. 99, no. 1, pp. 57–63, 2006.

[30] A. C. Guedes, H. M. Amaro, C. R. Barbosa, R. D. Pereira, and F. X. Malcata, “Fatty acid composition of several wild microalgae and cyanobacteria, with a focus on eicosapentaenoic, docosahexaenoic and α-linolenic acids for eventual dietary uses,” *Food Res. Int.*, vol. 44, no. 9, pp. 2721–2729, 2011.

[31] A. Pan *et al.*, “Alpha-linolenic acid and risk of cardiovascular disease: a systematic review and meta-analysis,” *Am J Clin Nutr*, vol. 96, no. 6, pp. 1262–1273, 2012.

[32] R. K. Saini and Y. S. Keum, “Tocopherols and tocotrienols in plants and their products: A review on methods of extraction, chromatographic separation, and detection,” *Food Res. Int.*, vol. 82, pp. 59–70, 2016.

[33] H. Ahsan, A. Ahad, J. Iqbal, and W. A. Siddiqui, “Pharmacological potential of tocotrienols: A review,” *Nutr. Metab.*, vol. 11, no. 1, pp. 1–22, 2014.

[34] T. J. Kizhakekuttu and M. E. Widlansky, “Natural antioxidants and hypertension: Promise and challenges,” *Cardiovasc. Ther.*, vol. 28, no. 4, pp. 20–32, 2010.

[35] M. Rashid Khan, H. Ahsan, S. Siddiqui, and W. A. Siddiqui, “Tocotrienols have a nephroprotective action against lipid-induced chronic renal dysfunction in rats,” *Ren. Fail.*, vol. 37, no. 1, pp. 136–143, 2015.

[36] E. Mocchegiani *et al.*, “Vitamin E-gene interactions in aging and inflammatory age-related diseases: Implications for treatment. A systematic review,” *Ageing Res. Rev.*, vol. 14, no. 1, pp. 81–101, 2014.

[37] A. Kamal-Eldin and E. Budilarto, *Tocopherols and tocotrienols as antioxidants for food preservation*. Elsevier Ltd, 2015.

[38] J. J. Thiele, S. N. Hsieh, and S. Ekanayake-Mudiyanselage, “Vitamin E: critical review of its current use in cosmetic and clinical dermatology.,” *Dermatologic Surg.*, vol. 31, no. 7 Pt 2, p. 805–813;, 2005.

[39] UCLA Chemistry & Biochemistry, “Illustrated Glossary of Organic Chemistry,” *UCLA Chemistry & Biochemistry*. p. 2016, 2016.

[40] J. A. Mendiola *et al.*, “Enrichment of vitamin E from Spirulina platensis microalga by SFE,” *J. Supercrit. Fluids*, vol. 43, no. 3, pp. 484–489, 2008.

[41] E. Fasano *et al.*, “Antioxidant and anti-Inflammatory effects of selected two human immortalized keratinocyte lines,” *Biomed Res. Int.*, vol. 2014, pp. 1–11, 2014.

[42] J. S. Park, J. H. Chyun, Y. K. Kim, L. L. Line, and B. P. Chew, “Astaxanthin decreased oxidative stress and inflammation and enhanced immune response in humans,” *Nutr. Metab.*, vol. 7, pp. 1–10, 2010.

[43] J. Virtamo *et al.*, "Effects of α-tocopherol and β-carotene supplementation on cancer incidence and mortality: 18-Year postintervention follow-up of the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study," *Int. J. Cancer*, vol. 135, no. 1, pp. 178–185, 2014.

[44] A. Shaish *et al.*, "9-cis β-carotene-rich powder of the alga Dunaliella bardawil increases plasma HDL-cholesterol in fibrate-treated patients," *Atherosclerosis*, vol. 189, no. 1, pp. 215–221, 2006.

[45] M. Gong and A. Bassi, "Carotenoids from microalgae: A review of recent developments," *Biotechnol. Adv.*, vol. 34, no. 8, pp. 1396–1412, 2016.

[46] S. Millao and E. Uquiche, "Extraction of oil and carotenoids from pelletized microalgae using supercritical carbon dioxide," *J. Supercrit. Fluids*, vol. 116, pp. 223–231, 2016.

[47] E. Christaki, E. Bonos, and P. Florou-Paneri, *Innovative Microalgae Pigments as Functional Ingredients in Nutrition*. Elsevier Inc., 2015.

[48] R. Chaiklahan, N. Chirasuwan, and B. Bunnag, "Stability of phycocyanin extracted from Spirulina sp.: Influence of temperature, pH and preservatives," *Process Biochem.*, vol. 47, no. 4, pp. 659–664, 2012.

[49] "Chemical structure of phycoerythrin bilin chromophore (open-chain." .

[50] D. A. Esquivel-Hernández *et al.*, "Advancement of green process through microwave-assisted extraction of bioactive metabolites from Arthrospira Platensis and bioactivity evaluation," *Bioresour. Technol.*, vol. 224, pp. 618–629, 2017.

[51] M. Cho, H. S. Lee, I. J. Kang, M. H. Won, and S. You, "Antioxidant properties of extract and fractions from Enteromorpha prolifera, a type of green seaweed," *Food Chem.*, vol. 127, no. 3, pp. 999–1006, 2011.

[52] O. P. Sharma and T. K. Bhat, "DPPH antioxidant assay revisited," *Food Chem.*, vol. 113, no. 4, pp. 1202–1205, 2009.

[53] F. Shahidi and Y. Zhong, "Measurement of antioxidant activity," *J. Funct. Foods*, vol. 18, pp. 757–781, 2015.

[54] O. Babić, D. Kovač, M. Rašeta, F. Šibul, Z. Svirčev, and J. Simeunović, "Evaluation of antioxidant activity and phenolic profile of filamentous terrestrial cyanobacterial strains isolated from forest ecosystem," *J. Appl. Phycol.*, vol. 28, no. 4, pp. 2333–2342, 2016.

[55] M. M. S. Ismaiel, Y. M. El-Ayouty, and M. D. Piercey-Normore, "Antioxidants characterization in selected cyanobacteria," *Ann. Microbiol.*, vol. 64, no. 3, pp. 1223–1230, 2014.

[56] I. Jerez-Martel, S. García-Poza, G. Rodríguez-Martel, M. Rico, C. Afonso-Olivares, and J. L. Gómez-Pinchetti, "Phenolic profile and antioxidant activity of crude extracts from microalgae and cyanobacteria strains," *J. Food Qual.*, vol. 2017, 2017.

[57] V. Gökmen, A. Serpen, and V. Fogliano, "Direct measurement of the total antioxidant capacity of foods: the 'QUENCHER' approach," *Trends Food Sci. Technol.*, vol. 20, no. 6–7, pp. 278–288, 2009.

[58] K. Goiris, W. Van Colen, I. Wilches, F. León-Tamariz, L. De Cooman, and K. Muylaert, "Impact of nutrient stress on antioxidant production in three species of microalgae," *Algal Res.*, vol. 7, pp. 51–57, 2015.

[59] S. K. Chandini, P. Ganesan, and N. Bhaskar, "In vitro antioxidant activities of three selected brown seaweeds of India," *Food Chem.*, vol. 107, no. 2, pp. 707–713, 2008.

[60] S. B. Kedare and R. P. Singh, "Genesis and development of DPPH method of antioxidant assay," *J. Food Sci. Technol.*, vol. 48, no. 4, pp. 412–422, 2011.

[61] W. Choochote, L. Suklampoo, and D. Ochaikul, "Evaluation of antioxidant capacities of green microalgae," *J. Appl. Phycol.*, vol. 26, no. 1, pp. 43–48, 2014.

[62] M. D. Luque de Castro and F. Priego-Capote, "Soxhlet extraction: Past and present panacea," *J. Chromatogr. A*, vol. 1217, no. 16, pp. 2383–2389, 2010.

[63] D. A. Esquivel-Hernández *et al.*, "Supercritical carbon dioxide and microwave-assisted extraction of functional lipophilic compounds from Arthrospira platensis," *Int. J. Mol. Sci.*, vol. 17, no. 5, 2016.

[64] M. A. Islam, K. Heimann, and R. J. Brown, "Microalgae biodiesel: Current status and future needs for engine performance and emissions," *Renew. Sustain. Energy Rev.*, vol. 79, no. January 2016, pp. 1160–1170, 2017.

[65] K. M. Sharif *et al.*, “Experimental design of supercritical fluid extraction - A review,” *J. Food Eng.*, vol. 124, pp. 105–116, 2014.

[66] M. Herrero, A. Cifuentes, and E. Ibañez, “Sub- and supercritical fluid extraction of functional ingredients from different natural sources: Plants, food-by-products, algae and microalgae - A review,” *Food Chem.*, vol. 98, no. 1, pp. 136–148, 2006.

[67] Q. Lang and C. M. Wai, “Supercritical fluid extraction in herbal and natural product studies — a practical review,” vol. 53, pp. 771–782, 2001.

[68] “Why and how are co-solvents sometimes used in SFE processes?,” *Supercritical Fluid Technologies Inc.* p. 2018, 2017.

[69] M. G. K.G., U. S. K, S. R, and R. G.A., “Supercritical CO2 extraction of functional compounds from Spirulina and their biological activity,” *J. Food Sci. Technol.*, vol. 52, no. June, pp. 3627–3633, 2014.

[70] M. Herrero, A. del P. Sánchez-Camargo, A. Cifuentes, and E. Ibáñez, “Plants, seaweeds, microalgae and food by-products as natural sources of functional ingredients obtained using pressurized liquid extraction and supercritical fluid extraction,” *TrAC - Trends Anal. Chem.*, vol. 71, pp. 26–38, 2015.

[71] M. Herrero, M. Castro-Puyana, J. A. Mendiola, and E. Ibañez, “Compressed fluids for the extraction of bioactive compounds,” *TrAC - Trends Anal. Chem.*, vol. 43, no. 2, pp. 67–83, 2013.

[72] Y. Picó, “Ultrasound-assisted extraction for food and environmental samples,” *TrAC - Trends Anal. Chem.*, vol. 43, pp. 84–99, 2013.

[73] M. Vinatoru, T. J. Mason, and I. Calinescu, “Ultrasonically assisted extraction (UAE) and microwave assisted extraction (MAE) of functional compounds from plant materials,” *TrAC - Trends Anal. Chem.*, vol. 97, pp. 159–178, 2017.

[74] S. Y. Lee, J. M. Cho, Y. K. Chang, and Y. K. Oh, “Cell disruption and lipid extraction for microalgal biorefineries: A review,” *Bioresour. Technol.*, vol. 244, pp. 1317–1328, 2017.

[75] V. Skorupskaite, V. Makareviciene, M. Ubartas, J. Karosiene, and M. Gumbyte, “Green algae Ankistrodesmus fusiformis cell disruption using different modes,” *Biomass and Bioenergy*, vol. 107, no. October, pp. 311–316, 2017.

[76] K. W. Chew *et al.*, “Microalgae biorefinery: High value products perspectives,” *Bioresour. Technol.*, vol. 229, pp. 53–62, 2017.

[77] F. Haque, S. Banayan, J. Yee, and Y. W. Chiang, “Extraction and applications of cyanotoxins and other cyanobacterial secondary metabolites,” *Chemosphere*, vol. 183, pp. 164–175, 2017.

[78] L. E. Gonzalez, G. C. Diaz, D. A. G. Aranda, Y. Cruz, and M. M. Fortes, “Biodiesel Production Based in Microalgae: A Biorefinery Approach,” *Nat. Sci.*, vol. 7, no. July, pp. 358–369, 2015.

[79] J. P. Berry, M. Gantar, M. H. Perez, G. Berry, and F. G. Noriega, “Cyanobacterial toxins as allelochemicals with potential applications as algaecides, herbicides and insecticides,” *Mar. Drugs*, vol. 6, no. 2, pp. 117–146, 2008.

[80] A. Hussain and S. Hasnain, “Phytostimulation and biofertilization in wheat by cyanobacteria,” *J. Ind. Microbiol. Biotechnol.*, vol. 38, no. 1, pp. 85–92, 2011.

[81] J. C. Quinn and R. Davis, “The potentials and challenges of algae based biofuels: A review of the techno-economic, life cycle, and resource assessment modeling,” *Bioresour. Technol.*, vol. 184, pp. 444–452, 2015.

[82] W. G. Sunda, “Feedback interactions between trace metal nutrients and phytoplankton in the ocean,” *Front. Microbiol.*, vol. 3, no. JUN, pp. 1–22, 2012.

[83] D. Schmid, S. Cornelia, and Z. Fred, “UV-A sunscreen from red algae for protection against premature skin aging,” *Cosmetics*, pp. 139–143, 2004.

[84] T. Soule, D. Shipe, and J. Lothamer, “Extracellular Polysaccharide Production in a Scytonemin-Deficient Mutant of Nostoc punctiforme Under UVA and Oxidative Stress,” *Curr. Microbiol.*, vol. 73, no. 4, pp. 455–462, 2016.

[85] A. Singh, M. B. Tyagi, and A. Kumar, “Cyanobacteria growing on tree barks possess high amount of sunscreen compound mycosporine-like amino acids (MAAs),” *Plant Physiol. Biochem.*, vol. 119, pp. 110–120, 2017.

[86] S. Nagarkar, G. A. Williams, G. Subramanian, and S. K. Saha, “Cyanobacteria-dominated biofilms: A high quality food resource for intertidal grazers,” *Hydrobiologia*, vol. 512, pp. 89–95, 2004.

[87] N. Querques, M. Cesta, R. M. Santos, Y. W. Chiang, and M. Road, “No Title.”

[88] V. Kraujalyte, P. R. Venskutonis, A. Pukalskas, L. Česoniene, and R. Daubaras, “Antioxidant properties and polyphenolic compositions of fruits from different European cranberrybush (Viburnum opulus L.) genotypes,” *Food Chem.*, vol. 141, no. 4, pp. 3695–3702, 2013.

[89] G. Panfili, A. Fratianni, and M. Irano, “Normal phase high-performance liquid chromatography method for the determination of tocopherols and tocotrienols in cereals,” *J. Agric. Food Chem.*, vol. 51, no. 14, pp. 3940–3944, 2003.

[90] V. Kitryte, A. Šaduikis, and P. R. Venskutonis, “Assessment of antioxidant capacity of brewer’s spent grain and its supercritical carbon dioxide extract as sources of valuable dietary ingredients,” *J. Food Eng.*, vol. 167, pp. 18–24, 2015.

[91] V. L. Singleton, R. Orthofer, and R. M. Lamuela-Raventós, “Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent,” *Methods Enzymol.*, vol. 299, no. 1974, pp. 152–178, 1998.

[92] A. Activity, A. An, I. Abts, and C. D. Assay, “Antioxidant Activity Applying an Improved Abts Radical,” vol. 26, no. 98, pp. 1231–1237, 1999.

[93] W. Brand-Williams, M. E. Cuvelier, and C. Berset, “Use of a free radical method to evaluate antioxidant activity,” *LWT - Food Sci. Technol.*, vol. 28, no. 1, pp. 25–30, 1995.

[94] K. H. Ogbonda, R. E. Aminigo, and G. O. Abu, “Influence of temperature and pH on biomass production and protein biosynthesis in a putative Spirulina sp.,” *Bioresour. Technol.*, vol. 98, no. 11, pp. 2207–2211, 2007.

[95] C. Safi *et al.*, “Aqueous extraction of proteins from microalgae: Effect of different cell disruption methods,” *Algal Res.*, vol. 3, no. 1, pp. 61–65, 2014.

[96] C. Crampon, O. Boutin, and E. Badens, “Supercritical carbon dioxide extraction of molecules of interest from microalgae and seaweeds,” *Ind. Eng. Chem. Res.*, vol. 50, no. 15, pp. 8941–8953, 2011.

[97] J. Lorenzen *et al.*, “Extraction of microalgae derived lipids with supercritical carbon dioxide in an industrial relevant pilot plant,” *Bioprocess Biosyst. Eng.*, vol. 40, no. 6, pp. 911–918, 2017.

[98] D. Pyo and H. Shin, “Supercritical fluid extraction of microcystins from cyanobacteria,” *Anal. Chem.*, vol. 71, no. 20, pp. 4772–4775, 1999.

[99] D. Pyo and C. Lim, “Supercritical fluid extraction of microcystin LR from cyanobacteria using aqueous acetonitrile modifier,” *J. Liq. Chromatogr. Relat. Technol.*, vol. 29, no. 18, pp. 2691–2700, 2006.

[100] V. Galhano *et al.*, “Morphological, biochemical and molecular characterization of Anabaena, Aphanizomenon and Nostoc strains (Cyanobacteria, Nostocales) isolated from Portuguese freshwater habitats,” *Hydrobiologia*, vol. 663, no. 1, pp. 187–203, 2011.

[101] T. Bauersachs, H. M. Talbot, F. Sidgwick, K. Sivonen, and L. Schwark, “Lipid biomarker signatures as tracers for harmful cyanobacterial blooms in the Baltic Sea,” *PLoS One*, vol. 12, no. 10, pp. 1–25, 2017.

[102] D. Los and K. Mironov, “Modes of Fatty Acid Desaturation in Cyanobacteria: An Update,” *Life*, vol. 5, no. 1, pp. 554–567, 2015.

[103] T. J. Johnson *et al.*, “Producing next-generation biofuels from filamentous cyanobacteria: An economic feasibility analysis,” *Algal Res.*, vol. 20, pp. 218–228, 2016.

[104] S. Singh, B. N. Kate, and U. C. Banecjee, “Bioactive compounds from cyanobacteria and microalgae: An overview,” *Crit. Rev. Biotechnol.*, vol. 25, no. 3, pp. 73–95, 2005.

[105] O. Mudimu, I. K. Koopmann, N. Rybalka, T. Friedl, R. Schulz, and W. Bilger, “Screening of microalgae and cyanobacteria strains for α-tocopherol content at different growth phases and the influence of nitrate reduction on α-tocopherol production,” *J. Appl. Phycol.*, vol. 29, no. 6, pp. 2867–2875, 2017.

[106] R. R. Sonani *et al.*, “Purification and antioxidant activity of phycocyanin from Synechococcus sp. R42DM

isolated from industrially polluted site," *Bioresour. Technol.*, vol. 245, no. August, pp. 325–331, 2017.

[107] D. Belhaj *et al.*, "Box-Behnken design for extraction optimization of crude polysaccharides from Tunisian Phormidium versicolor cyanobacteria (NCC 466): Partial characterization, in vitro antioxidant and antimicrobial activities," *Int. J. Biol. Macromol.*, vol. 105, pp. 1501–1510, 2017.

[108] M. F. G. Assunção *et al.*, "Screening microalgae as potential sources of antioxidants," *J. Appl. Phycol.*, vol. 29, no. 2, pp. 865–877, 2017.

[109] D. P. Singh, R. Prabha, S. Verma, K. K. Meena, and M. Yandigeri, "Antioxidant properties and polyphenolic content in terrestrial cyanobacteria," *3 Biotech*, vol. 7, no. 2, pp. 1–14, 2017.

[110] J. Abalde, "Purification and characterization of phycocyanin from the marine cyanobacterium Synechococcus sp . 109201," *plant Sci.*, vol. 136, pp. 109–120, 1998.

[111] S. T. Silveira, J. F. M. Burkert, J. A. V Costa, C. A. V Burkert, and S. J. Kalil, "Optimization of phycocyanin extraction from Spirulina platensis using factorial design," *Bioresour. Technol.*, vol. 98, no. 8, pp. 1629–1634, 2007.

[112] S. Aky, Z. Demirel, M. Koç, and M. Conk-dalay, "Journal of Food Composition and Analysis Optimization of phycocyanin extraction from Spirulina platensis using di ff erent techniques," vol. 70, no. August 2017, pp. 78–88, 2018.

[113] C. C. Moraes, L. Sala, G. P. Cerveira, and S. J. Kalil, "C-Phycocyanin extraction from Spirulina platensis wet biomass," *Brazilian J. Chem. Eng.*, vol. 28, no. 1, pp. 45–49, 2011.

[114] H. A. Tavanandi, R. Mittal, J. Chandrasekhar, and K. S. M. S. Raghavarao, "Simple and efficient method for extraction of C-Phycocyanin from dry biomass of Arthospira platensis," *Algal Res.*, vol. 31, no. August 2017, pp. 239–251, 2018.

[115] S. P. Jeevan Kumar, G. Vijay Kumar, A. Dash, P. Scholz, and R. Banerjee, "Sustainable green solvents and techniques for lipid extraction from microalgae: A review," *Algal Res.*, vol. 21, pp. 138–147, 2017.

[116] A. F. Ferreira, A. P. S. Dias, C. M. Silva, and M. Costa, "Effect of low frequency ultrasound on microalgae solvent extraction: Analysis of products, energy consumption and emissions," *Algal Res.*, vol. 14, pp. 9–16, 2016.

[117] M. F. da Silva *et al.*, "Recovery of phenolic compounds of food concern from Arthrospira platensis by green extraction techniques," *Algal Res.*, vol. 25, no. April, pp. 391–401, 2017.

[118] C. Le Guillard *et al.*, "Ultrasound-assisted extraction of R-phycoerythrin from Grateloupia turuturu with and without enzyme addition," *Algal Res.*, vol. 12, pp. 522–528, 2015.

[119] J. da C. Ores, M. C. A. de Amarante, and S. J. Kalil, "Co-production of carbonic anhydrase and phycobiliproteins by Spirulina sp. and Synechococcus nidulans," *Bioresour. Technol.*, vol. 219, pp. 219–227, 2016.

[120] R. D. P. Rodrigues, F. C. de Castro, R. S. de Santiago-Aguiar, and M. V. P. Rocha, "Ultrasound-assisted extraction of phycobiliproteins from Spirulina (Arthrospira) platensis using protic ionic liquids as solvent," *Algal Res.*, vol. 31, no. November 2017, pp. 454–462, 2018.

[121] A. Guldhe, B. Singh, I. Rawat, K. Ramluckan, and F. Bux, "Efficacy of drying and cell disruption techniques on lipid recovery from microalgae for biodiesel production," *Fuel*, vol. 128, pp. 46–52, 2014.

[122] M. Vanthoor-Koopmans, R. H. Wijffels, M. J. Barbosa, and M. H. M. Eppink, "Biorefinery of microalgae for food and fuel," *Bioresour. Technol.*, vol. 135, pp. 142–149, 2013.

[123] K. Nakagawa, W. Ritcharoen, P. Sri-Uam, P. Pavasant, and S. Adachi, "Antioxidant properties of convective-air-dried Spirulina maxima: Evaluation of phycocyanin retention by a simple mathematical model of air-drying," *Food Bioprod. Process.*, vol. 100, pp. 292–302, 2016.

[124] I. Rodríguez-Meizoso *et al.*, "Pressurized fluid extraction of bioactive compounds from *Phormidium* species," *J. Agric. Food Chem.*, vol. 56, no. 10, pp. 3517–3523, 2008.

[125] M. Herrero, P. J. Martín-Álvarez, F. J. Señoráns, A. Cifuentes, and E. Ibáñez, "Optimization of accelerated solvent extraction of antioxidants from Spirulina platensis microalga," *Food Chem.*, vol. 93, no. 3, pp. 417–423, 2005.

[126] Y. Tang, Y. Zhang, J. N. Rosenberg, N. Sharif, M. J. Betenbaugh, and F. Wang, "Efficient lipid extraction and

quantification of fatty acids from algal biomass using accelerated solvent extraction (ASE)," *RSC Adv.*, vol. 6, no. 35, pp. 29127–29134, 2016.

[127] S. Pieber, S. Schober, and M. Mittelbach, "Pressurized fluid extraction of polyunsaturated fatty acids from the microalga Nannochloropsis oculata," *Biomass and Bioenergy*, vol. 47, pp. 474–482, 2012.

[128] H. Bin Li, K. W. Cheng, C. C. Wong, K. W. Fan, F. Chen, and Y. Jiang, "Evaluation of antioxidant capacity and total phenolic content of different fractions of selected microalgae," *Food Chem.*, vol. 102, no. 3, pp. 771–776, 2007.

[129] M. Hajimahmoodi, M. A. Faramarzi, N. Mohammadi, N. Soltani, M. R. Oveisi, and N. Nafissi-Varcheh, "Evaluation of antioxidant properties and total phenolic contents of some strains of microalgae," *J. Appl. Phycol.*, vol. 22, no. 1, pp. 43–50, 2010.

[130] T. Piper *et al.*, "Determination of 13C/12C ratios of endogenous urinary steroids: methd validation, reference population and application to doping control purposes," *Rapid Commun. Mass Spectrom.*, vol. 22, pp. 2161–2175, 2008.

[131] M. Kohlhase and P. Pohl, "Saturated and unsaturated sterols of nitrogen-fixing blue-green algae (cyanobacteria)," *Phytochemistry*, vol. 27, no. 6, pp. 1735–1740, 1988.

[132] B. Y. T. W. Goodwin, "The Nature and Distribution," no. May, pp. 467–473, 2016.

[133] R. Wagner *et al.*, "GNAT-Like Strategy for Polyketide," *Science (80-.).*, vol. 318, no. November, pp. 970–975, 2007.

[134] P. Verdier-Pinard *et al.*, "Structure-activity analysis of the interaction of curacin A, the potent colchicine site antimitotic agent, with tubulin and effects of analogs on the growth of MCF-7 breast cancer cells.," *Mol. Pharmacol.*, vol. 53, no. 1, pp. 62–76, 1998.

[135] E. Esquenazi, C. Coates, L. Simmons, D. Gonzalez, H. William, and P. C. Dorrestein, "Imaging," vol. 4, no. 6, pp. 562–570, 2010.

[136] B. Klejdus, J. Kopecký, L. Benešová, and J. Vacek, "Solid-phase/supercritical-fluid extraction for liquid chromatography of phenolic compounds in freshwater microalgae and selected cyanobacterial species," *J. Chromatogr. A*, vol. 1216, no. 5, pp. 763–771, 2009.

[137] A. Hussain, M. Krischke, T. Roitsch, and S. Hasnain, "Rapid determination of cytokinins and auxin in cyanobacteria," *Curr. Microbiol.*, vol. 61, no. 5, pp. 361–369, 2010.

APPENDICES

Table A.1. Analysis of variance of the regression parameters for response surface quadratic model for SFE-CO_2 optimisation for wild cyanobacteria biomass (Response factor: SFE-CO_2 extract yield, g/100 g)

Source	Sum of squares	df	Mean square	F value	p-value
Model	35.8	9	3.98	330.07	< 0.0001*
Pressure (P, MPa)	21.87	1	21.87	1814.93	< 0.0001*
Temperature (T, °C)	0.062	1	0.062	5.18	0.0461*
Time (τ, min)	1.43	1	1.43	118.55	< 0.0001*
P T	2.6	1	2.6	215.66	< 0.0001*
P τ	0.045	1	0.045	3.73	0.0821**
T τ	0.051	1	0.051	4.25	0.0663**
P^2	5.03	1	5.03	417.52	< 0.0001*
T^2	$7.65*10^{-3}$	1	$7.65*10^{-3}$	0.63	0.4443**
τ^2	$8.64*10^{-4}$	1	$8.64*10^{-4}$	0.072	0.7943**
Residual	0.12	10	0.012		
Lack of Fit	0.088	5	0.018	2.76	0.145**
Pure Error	0.032	5	$6.42**10^{-3}$		
Cor Total	35.92	19			

*: significant; **: not significant; df: degree of freedom; F: Fisher value.; MS: mean square; RF: response factor; SS: sum of square.

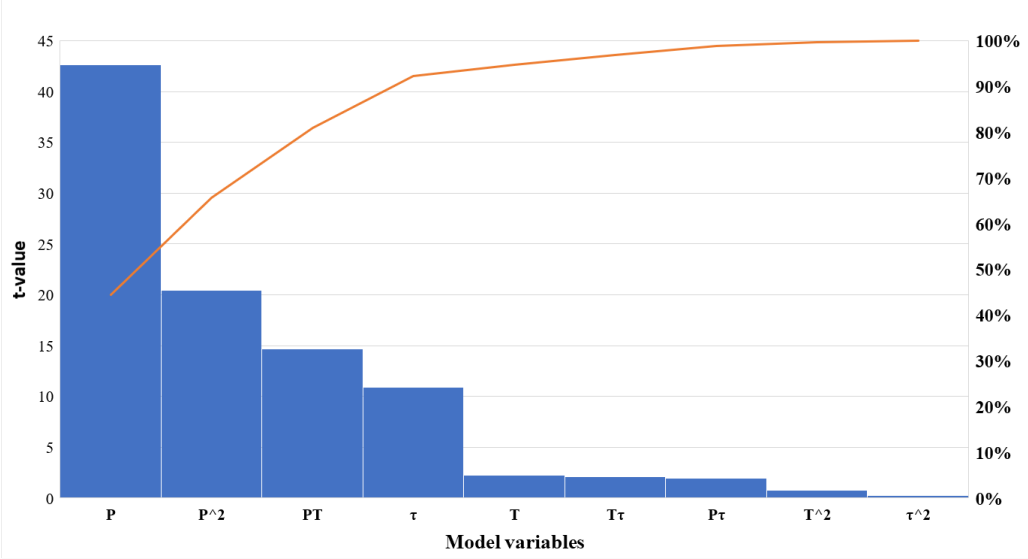


Figure A.1. Pareto chart (p=0.05) for the main effects of evaluated SFE-CO_2 parameters and interactions thereof on the total extraction yield: pressure (P), temperature (T) and time (τ)

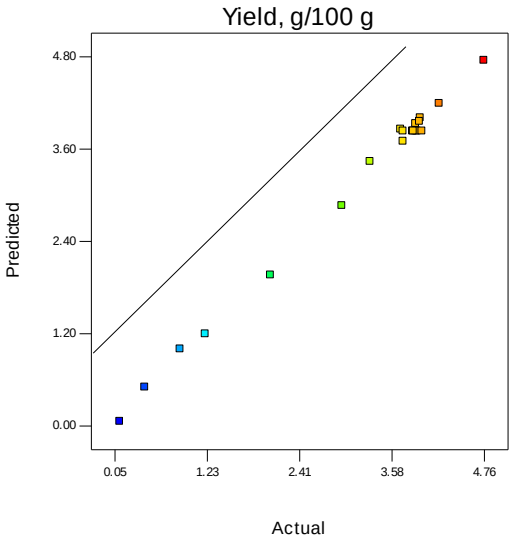


Figure A.2. Comparison between predicted and observed SFE-CO_2 extract yields (g/100 g DW) from wild cyanobacteria biomass

Table A.2. Analysis of variance of the regression parameters for response surface quadratic model for UAE optimisation for wild cyanobacteria biomass (Response factor: PC extract yield, mg/g)

Source	Sum of squares	df	Mean square	F value	p-value
Model	336.66	5	67.33	34.01	< 0.0001*
Time (τ, min)	107.66	1	107.66	54.38	0.0002*
Amplitude (A, %)	194.15	1	194.15	98.06	< 0.0001*
A τ	10.14	1	10.14	5.12	0.0581**
τ^2	2.99	1	2.99	1.51	0.2587**
A^2	13.33	1	13.33	6.73	0.0357*
Residual	13.86	7	1.98		
Lack of Fit	8.24	3	2.75	1.96	0.2626**
Pure Error	5.62	4	1.40		
Cor Total	350.52	12			

*: significant; **: not significant; df: degree of freedom; F: Fisher value.; MS: mean square; RF: response factor; SS: sum of square;

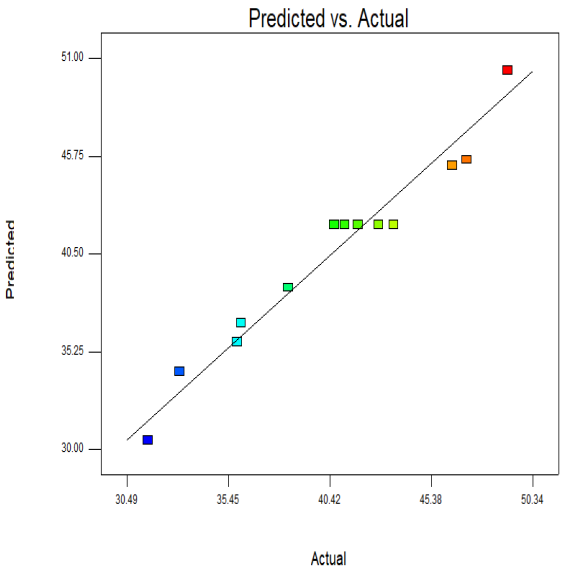


Figure A.3. Comparison between predicted and actual phycocyanin yields (mg/g DW) after UAE from wild cyanobacteria biomass

Table A.3. Analysis of variance of the regression parameters for response surface quadratic model for UAE optimisation for wild cyanobacteria biomass (Response factor: APC extract yield, mg/g)

Source	Sum of squares	df	Mean square	F value	p-value
Model	2358.57	5	471.71	18.71	0.0006*
Time (τ, min)	937.98	1	937.98	37.19	0.0005*
Amplitude (A, %)	1037.50	1	1037.50	41.14	0.0004*
A τ	7.35	1	7.35	0.29	0.6060**
τ^2	103.60	1	103.60	4.11	0.0823**
A^2	129.39	1	129.39	5.13	0.0579**
Residual	176.53	7	25.22		
Lack of Fit	157.06	3	52.35	10.76	0.0219*
Pure Error	19.47	4	4.87		
Cor Total	2535.10	12			

*: significant; **: not significant; df: degree of freedom; F: Fisher value.; MS: mean square; RF: response factor; SS: sum of square.

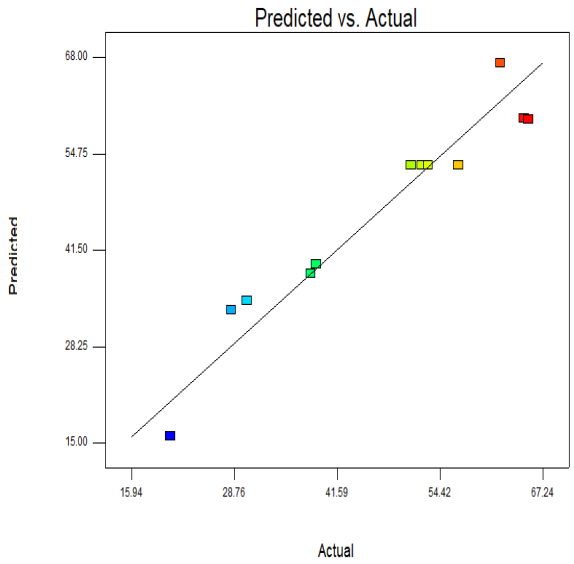


Figure A.4. Comparison between predicted and actual allophycocyanin yields (mg/g DW) after UAE from wild cyanobacteria biomass

Table A.4. Analysis of variance of the regression parameters for response surface quadratic model for UAE optimisation for wild cyanobacteria biomass (Response factor: PE extract yield, mg/g)

Source	Sum of squares	df	Mean square	F value	p-value
Model	395.41	5	79.08	27.01	0.0002*
Time (τ, min)	165.52	1	165.52	56.52	0.0001*
Amplitude (A, %)	173.71	1	173.71	59.32	0.0001*
A τ	1.24	1	1.24	0.42	0.5358**
τ^2	9.75	1	9.75	3.33	0.1108**
A^2	25.26	1	25.26	8.63	0.0218*
Residual	20.50	7	2.93		
Lack of Fit	11.65	3	3.88	1.75	0.2943**
Pure Error	8.85	4	2.21		
Cor Total	415.91	12			

*: significant; **: not significant; df: degree of freedom; F: Fisher value.; MS: mean square; RF: response factor; SS: sum of square;

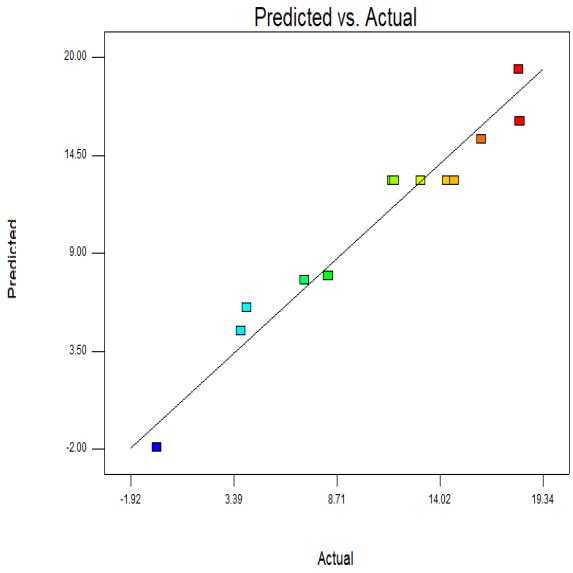


Figure A.5. Comparison between predicted and actual phycoerythrin yields (mg/g DW) after UAE from wild cyanobacteria biomass

Table A.5. Analysis of variance of the regression parameters for response surface quadratic model for UAE optimisation for wild cyanobacteria biomass (Response factor: total extract yield, mg/g)

Source	Sum of squares	df	Mean square	F value	p-value
Model	7473.40	5	1494.68	25.09	0.0002*
Time (τ, min)	2901.76	1	2901.76	48.70	0.0002*
Amplitude (A, %)	3519.30	1	3519.30	59.07	0.0001*
A τ	49.13	1	49.13	0.82	0.3940**
τ^2	225.91	1	225.91	3.79	0.0925**
A^2	402.08	1	402.08	6.75	0.0355*
Residual	417.07	7	59.58		
Lack of Fit	341.89	3	113.96	6.06	0.0571**
Pure Error	75.17	4	18.79		
Cor Total	7890.46	12			

*: significant; **: not significant; df: degree of freedom; F: Fisher value.; MS: mean square; RF: response factor; SS: sum of square;

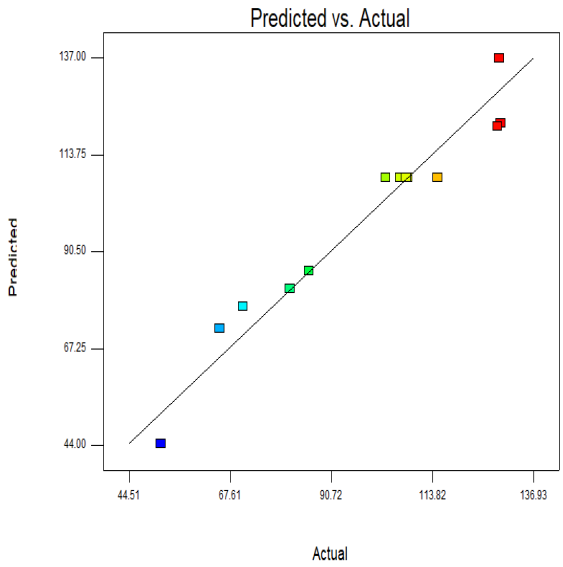


Figure A.6. Comparison between predicted and actual total phycobiliproteins yields (mg/g DW) after UAE from wild cyanobacteria biomass

Table A.6. Analysis of variance of the regression parameters for response surface quadratic model for combined freeze-thaw and UAE optimisation for wild cyanobacteria biomass (Response factor: PC extract yield, mg/g)

Source	Sum of squares	df	Mean square	F value	p-value
Model	141.74	5	28.35	42.54	<0.0001*
Time (τ, min)	67.23	1	67.23	100.88	<0.0001*
Amplitude (A, %)	58.52	1	58.52	87.81	<0.0001*
A τ	4.53	1	4.53	6.79	0.0351*
τ^2	11.43	1	11.43	17.15	0.0043*
A^2	2.18	1	2.18	3.27	0.1137**
Residual	4.66	7	0.67		
Lack of Fit	2.22	3	0.74	1.21	0.4143**
Pure Error	2.45	4	0.61		
Cor Total	146.40	12			

*: significant; **: not significant; df: degree of freedom; F: Fisher value.; MS: mean square; RF: response factor; SS: sum of square;

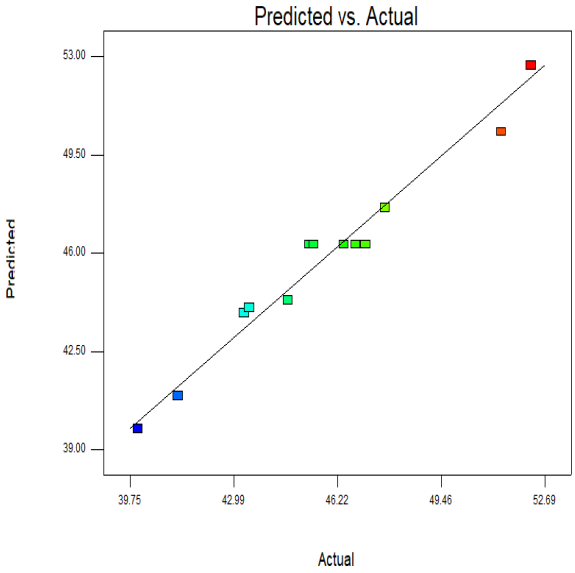


Figure A.7. Comparison between predicted and actual phycocyanin yields (mg/g DW) after combined freeze-thaw and UAE extractions from wild cyanobacteria biomass

Table A.7. Analysis of variance of the regression parameters for response surface quadratic model for combined freeze-thaw and UAE optimisation for wild cyanobacteria biomass (Response factor: APC extract yield, mg/g)

Source	Sum of squares	df	Mean square	F value	p-value
Model	892.93	5	178.59	37.02	<0.0001*
Time (τ, min)	558.40	1	558.40	115.74	<0.0001*
Amplitude (A, %)	141.69	1	141.69	29.37	0.0010*
A τ	0.028	1	0.028	5.882E-003	0.9410**
τ^2	69.02	1	69.02	14.31	0.0069*
A^2	50.73	1	50.73	10.51	0.0142*
Residual	33.77	7	4.82		
Lack of Fit	31.24	3	10.41	16.44	0.0103*
Pure Error	2.53	4	0.63		
Cor Total	926.70	12			

*: significant; **: not significant; df: degree of freedom; F: Fisher value.; MS: mean square; RF: response factor; SS: sum of square;

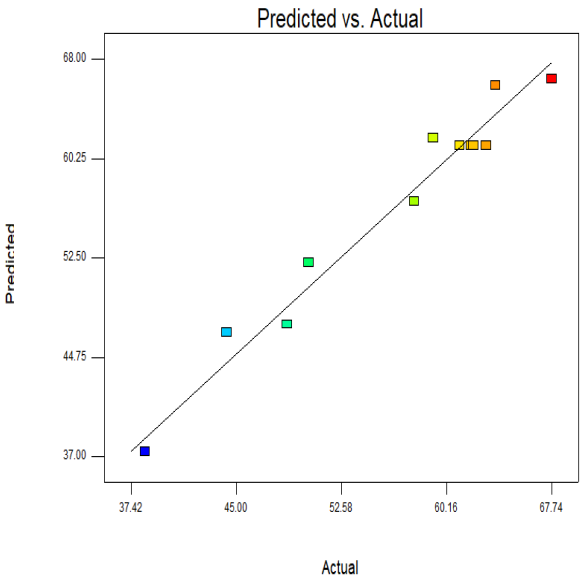


Figure A.8. Comparison between predicted and actual allphycocyanin yields (mg/g DW) after combined freeze-thaw and UAE extractions from wild cyanobacteria biomass

Table A.8. Analysis of variance of the regression parameters for response surface quadratic model for combined freeze-thaw and UAE optimisation for wild cyanobacteria biomass (Response factor: PE extract yield, mg/g)

Source	Sum of squares	df	Mean square	F value	p-value
Model	159.88	5	31.98	32.47	0.0001*
Time (τ, min)	86.88	1	86.88	88.23	<0.0001*
Amplitude (A, %)	38.68	1	38.68	39.28	0.0004*
A τ	0.092	1	0.092	0.094	0.7685**
τ^2	19.27	1	19.27	19.57	0.0031*
A^2	3.63	1	3.63	3.68	0.0964**
Residual	6.89	7	0.98		
Lack of Fit	5.57	3	1.86	5.60	0.0648**
Pure Error	1.33	4	0.33		
Cor Total	166.77	12			

*: significant; **: not significant; df: degree of freedom; F: Fisher value.; MS: mean square; RF: response factor; SS: sum of square;

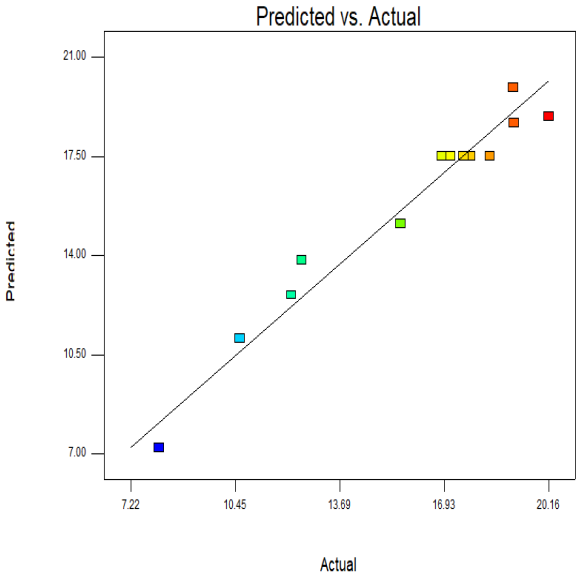


Figure A.9. Comparison between predicted and actual phycoerythrin yields (mg/g DW) after combined freeze-thaw and UAE extractions from wild cyanobacteria biomass

Table A.9. Analysis of variance of the regression parameters for response surface quadratic model for combined freeze-thaw and UAE optimisation for wild cyanobacteria biomass (Response factor: total extract yield, mg/g)

Source	Sum of squares	df	Mean square	F value	p-value
Model	2837.58	5	567.52	78.24	<0.0001*
Time (τ, min)	1693.36	1	1693.36	233.44	<0.0001*
Amplitude (A, %)	664.19	1	664.19	91.56	<0.0001*
A τ	2.74	1	2.74	0.38	0.5582**
τ^2	258.50	1	258.50	35.64	0.0006*
A^2	57.03	1	57.03	7.86	0.0264*
Residual	50.78	7	7.25		
Lack of Fit	35.51	3	11.84	3.10	0.1514**
Pure Error	15.27	4	3.82		
Cor Total	2888.36	12			

*: significant; **: not significant; df: degree of freedom; F: Fisher value.; MS: mean square; RF: response factor; SS: sum of square;

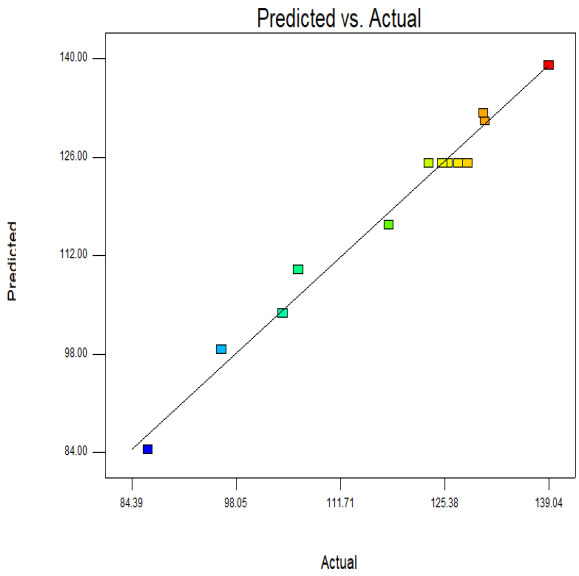


Figure A.10. Comparison between predicted and actual total phycobiliproteins yields (mg/g DW) after combined freeze-thaw and UAE extractions from wild cyanobacteria biomass

Table A.10. Analysis of variance of the regression parameters for response surface quadratic model for combined homogenizer and UAE optimisation for wild cyanobacteria biomass (Response factor: PC extract yield, mg/g)

Source	Sum of squares	df	Mean square	F value	p-value
Model	125.53	5	25.11	21.10	0.0004*
Time (τ, min)	55.93	1	55.93	47.01	0.0002*
Amplitude (A, %)	47.72	1	47.72	40.11	0.0004*
A τ	0.63	1	0.63	0.53	0.4903**
τ^2	7.52	1	7.52	6.32	0.0402*
A^2	5.68	1	5.68	4.77	0.0652**
Residual	8.33	7	1.19		
Lack of Fit	3.96	3	1.32	1.21	0.4138**
Pure Error	4.37	4	1.09		
Cor Total	133.86	12			

*: significant; **: not significant; df: degree of freedom; F: Fisher value.; MS: mean square; RF: response factor; SS: sum of square;

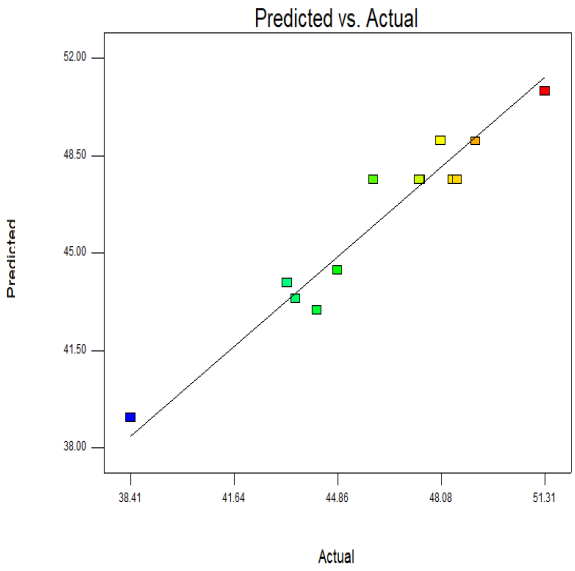


Figure A.11. Comparison between predicted and actual phycocyanin yields (mg/g DW) after combined homogenizer and UAE extractions from wild cyanobacteria biomass

Table A.11. Analysis of variance of the regression parameters for response surface quadratic model for combined homogenizer and UAE optimisation for wild cyanobacteria biomass (Response factor: APC extract yield, mg/g)

Source	Sum of squares	df	Mean square	F value	p-value
Model	874.13	5	174.83	41.48	<0.0001*
Time (τ, min)	358.53	1	358.53	85.07	<0.0001*
Amplitude (A, %)	190.18	1	190.18	45.12	0.0003*
A τ	31.27	1	31.27	7.42	0.0296*
τ^2	111.26	1	111.26	26.40	0.0013*
A^2	72.00	1	72.00	17.08	0.0044*
Residual	29.50	7	4.21		
Lack of Fit	18.48	3	6.16	2.24	0.2263**
Pure Error	11.02	4	2.75		
Cor Total	903.63	12			

*: significant; **: not significant; df: degree of freedom; F: Fisher value.; MS: mean square; RF: response factor; SS: sum of square;

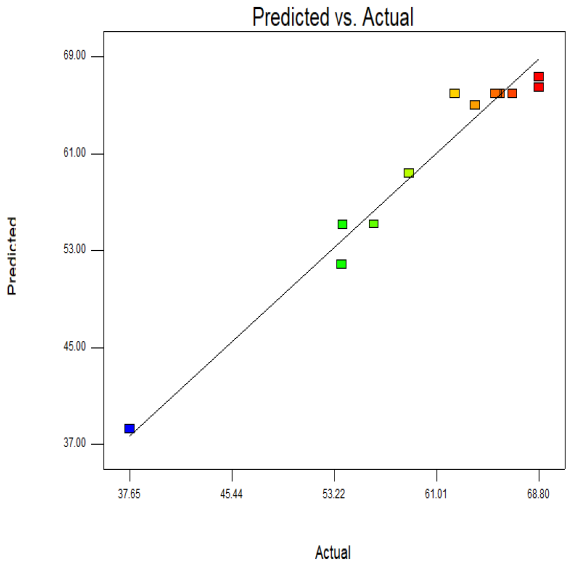


Figure A.12. Comparison between predicted and actual allophycocyanin yields (mg/g DW) after combined homogenizer and UAE extractions from wild cyanobacteria biomass

Table A.12. Analysis of variance of the regression parameters for response surface quadratic model for combined homogenizer and UAE optimisation for wild cyanobacteria biomass (Response factor: PE extract yield, mg/g)

Source	Sum of squares	df	Mean square	F value	p-value
Model	136.71	5	27.34	18.19	0.0007*
Time (τ, min)	64.26	1	64.26	42.74	0.0003*
Amplitude (A, %)	46.46	1	46.46	30.90	0.0009*
A τ	0.64	1	0.64	0.43	0.5334**
τ^2	11.67	1	11.67	7.76	0.0271*
A^2	4.49	1	4.49	2.98	0.1278**
Residual	10.53	7	1.50		
Lack of Fit	5.58	3	1.86	1.51	0.3416**
Pure Error	4.94	4	1.24		
Cor Total	147.24	12			

*: significant; **: not significant; df: degree of freedom; F: Fisher value.; MS: mean square; RF: response factor; SS: sum of square;

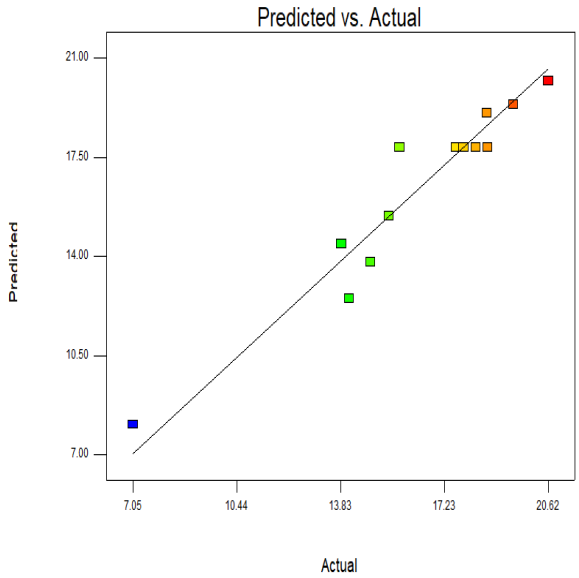


Figure A.13. Comparison between predicted and actual phycoerythrin yields (mg/g DW) after combined homogenizer and UAE extractions from wild cyanobacteria biomass

Table A.13. Analysis of variance of the regression parameters for response surface quadratic model for combined homogenizer and UAE optimisation for wild cyanobacteria biomass (Response factor: total extract yield, mg/g)

Source	Sum of squares	df	Mean square	F value	p-value
Model	2690.90	5	538.18	34.78	<0.0001*
Time (τ, min)	1185.38	1	1185.38	76.60	<0.0001*
Amplitude (A, %)	757.06	1	757.06	48.92	0.0002*
A τ	31.37	1	31.37	2.03	0.1975**
τ^2	279.09	1	279.09	18.03	0.0038*
A^2	168.64	1	168.64	10.90	0.0131*
Residual	108.33	7	15.48		
Lack of Fit	51.45	3	17.15	1.21	0.4148**
Pure Error	56.87	4	14.22		
Cor Total	2799.22	12			

*: significant; **: not significant; df: degree of freedom; F: Fisher value.; MS: mean square; RF: response factor; SS: sum of square;

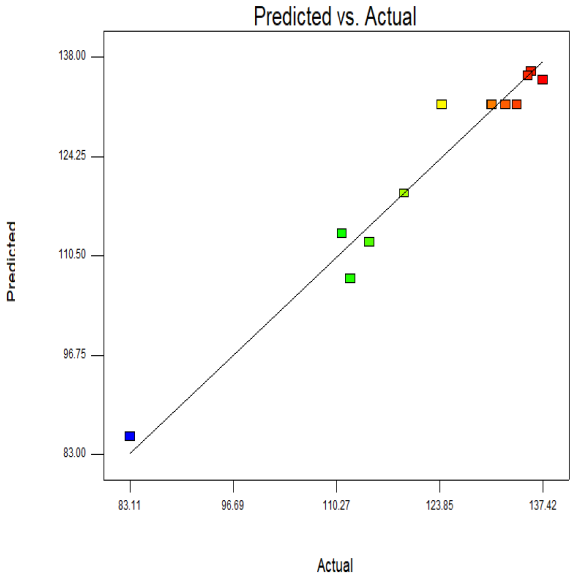


Figure A.14. Comparison between predicted and actual total phycobiliproteins yields (mg/g DW) after combined homogenizer and UAE extractions from wild cyanobacteria biomass

Table A.14. Analysis of variance of the regression parameters for response surface quadratic model for combined SLE and UAE optimisation for wild cyanobacteria biomass (Response factor: PC extract yield, mg/g)

Source	Sum of squares	df	Mean square	F value	p-value
Model	144.29	5	28.86	20.74	0.0005*
Time (τ, min)	89.28	1	89.28	64.16	<0.0001*
Amplitude (A, %)	28.64	1	28.64	20.59	0.0027*
A τ	0.15	1	0.15	0.10	0.7561**
τ^2	21.95	1	21.95	15.78	0.0054*
A^2	0.016	1	0.016	0.011	0.9184**
Residual	9.74	7	1.39		
Lack of Fit	3.13	3	1.04	0.63	0.6328**
Pure Error	6.61	4	1.65		
Cor Total	154.03	12			

*: significant; **: not significant; df: degree of freedom; F: Fisher value.; MS: mean square; RF: response factor; SS: sum of square;

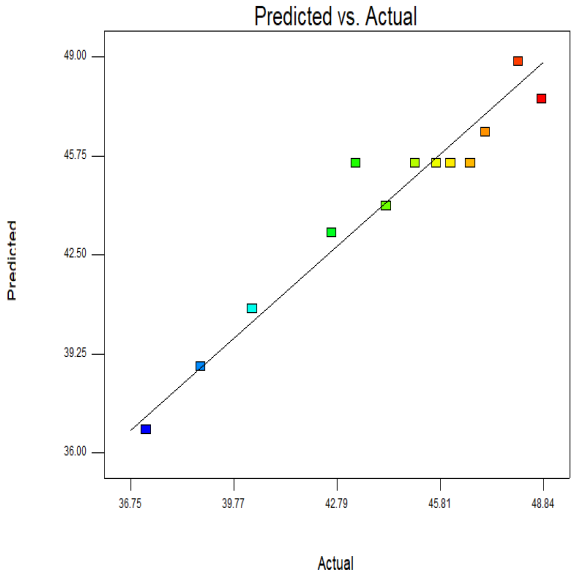


Figure A.15. Comparison between predicted and actual phycocyanin yields (mg/g DW) after combined SLE and UAE extractions from wild cyanobacteria biomass

Table A.15. Analysis of variance of the regression parameters for response surface quadratic model for combined SLE and UAE optimisation for wild cyanobacteria biomass (Response factor: APC extract yield, mg/g)

Source	Sum of squares	df	Mean square	F value	p-value
Model	927.51	5	185.50	35.16	<0.0001*
Time (τ, min)	482.66	1	482.66	91.48	<0.0001*
Amplitude (A, %)	235.66	1	235.66	44.66	0.0003*
A τ	2.35	1	2.35	0.45	0.5259**
τ^2	141.12	1	141.12	26.75	0.0013*
A^2	8.82	1	8.82	1.67	0.2371**
Residual	36.93	7	5.28		
Lack of Fit	6.69	3	2.23	0.29	0.8282**
Pure Error	30.24	4	7.56		
Cor Total	964.45	12			

*: significant; **: not significant; df: degree of freedom; F: Fisher value.; MS: mean square; RF: response factor; SS: sum of square;

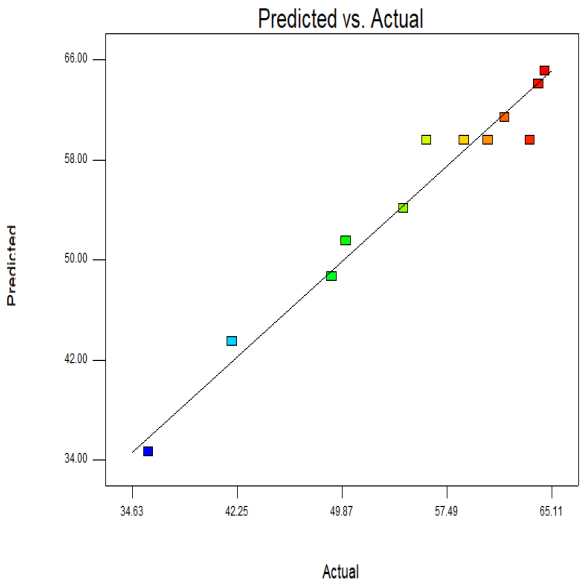


Figure A.16. Comparison between predicted and actual allphycocyanin yields (mg/g DW) after combined SLE and UAE extractions from wild cyanobacteria biomass

Table A.16. Analysis of variance of the regression parameters for response surface quadratic model for combined SLE and UAE optimisation for wild cyanobacteria biomass (Response factor: PE extract yield, mg/g)

Source	Sum of squares	df	Mean square	F value	p-value
Model	151.16	5	30.23	61.70	<0.0001*
Time (τ, min)	81.17	1	81.17	165.66	<0.0001*
Amplitude (A, %)	48.92	1	48.92	99.85	<0.0001*
A τ	1.668E-003	1	1.668E-003	3.404E-003	0.9551**
τ^2	16.98	1	16.98	34.65	0.0006*
A^2	0.090	1	0.090	0.18	0.6812**
Residual	3.43	7	0.49		
Lack of Fit	0.42	3	0.14	0.19	0.8991**
Pure Error	3.01	4	0.75		
Cor Total	154.59	12			

*: significant; **: not significant; df: degree of freedom; F: Fisher value.; MS: mean square; RF: response factor; SS: sum of square;

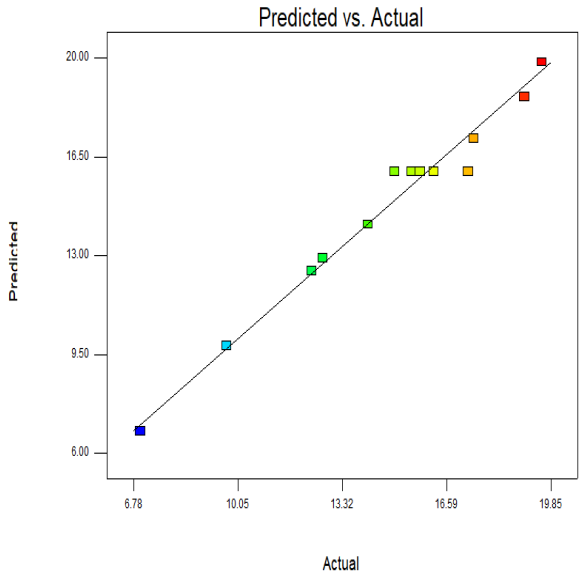


Figure A.17. Comparison between predicted and actual phycoerythrin yields (mg/g DW) after combined SLE and UAE extractions from wild cyanobacteria biomass

Table A.17. Analysis of variance of the regression parameters for response surface quadratic model for combined SLE and UAE optimisation for wild cyanobacteria biomass (Response factor: total extract yield, mg/g)

Source	Sum of squares	df	Mean square	F value	p-value
Model	2979.42	5	595.88	38.10	<0.0001*
Time (τ, min)	1634.42	1	1634.42	104.50	<0.0001*
Amplitude (A, %)	767.18	1	767.18	49.05	0.0002*
A τ	1.23	1	1.23	0.079	0.7868**
τ^2	427.88	1	427.88	27.36	0.0012*
A^2	11.53	1	11.53	0.74	0.4191**
Residual	109.48	7	15.64		
Lack of Fit	17.80	3	5.93	0.026	0.8521**
Pure Error	91.68	4	22.92		
Cor Total	3088.90	12			

*: significant; **: not significant; df: degree of freedom; F: Fisher value.; MS: mean square; RF: response factor; SS: sum of square;

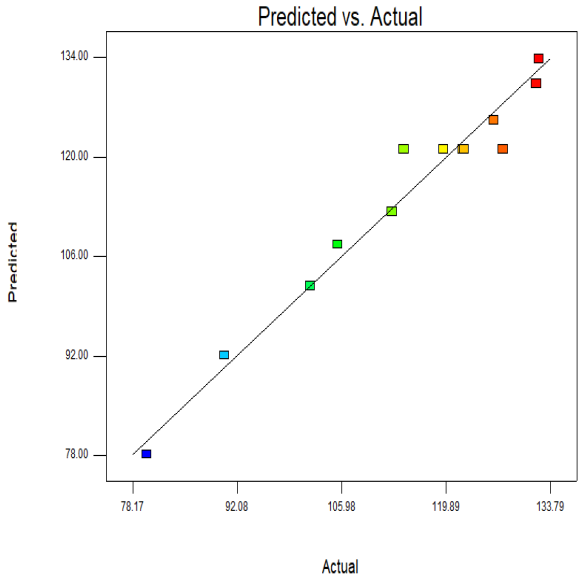


Figure A.18. Comparison between predicted and actual total phycobiliproteins yields (mg/g DW) after combined SLE and UAE extractions from wild cyanobacteria biomass

Table A.18. Compounds found in lipophilic extracts from wild cyanobacterial biomass

Time	Meas.m/z	Δppm	Suggested formula	Name*		SFE	Soxhlet	SLE	S1-H55	S1-H70
Positive										
4.0-4.1	181.1225	0	$C_{11}H_{17}O_2$	Fatty acid	M+H	+	+	+	+	+
	383.2193	0	$C_{22}H_{32}O_4$	Monoacylglycerophosphate	M+H	+	+	+	+	+
4.7-4.8	230.2476	0	$C_{14}H_{28}O$	Tetradecenone, Xestoaminol	M+H	+				
	274.2744	1	$C_{16}H_{36}NO_2$	Sphinganine	M+H	+	+	+	+	
	318.2999	1	$C_{18}H_{40}NO_3$	Phytosphingosine	M+H			+	+	
5.0-5.2	155.1068	0	$C_9H_{15}O_2$	Nonadienoic acid	M+H	+	+			+
	275.2007	0	$C_{18}H_{27}O_2$	Octadecapentaenoic acid	M+H	+	+			+
	293.2113	0	$C_{18}H_{29}O_3$	20 matches	M+H		+			
	333.2040	1	$C_{18}H_{30}O_4$	Hydroperoxy-octadecatrienoic acid	M+Na	+	+			+
	643.4184	6	$C_{34}H_{58}O_8$	Glycerophosphate	M+H	+	+			+
7.0-7.2	179.1434	2	$C_{12}H_{19}O$	Phenol	M+H	+	+	+	+	+
	458.2874	5	$C_{23}H_{36}NO_3$	Docosahexaenoyl glutamic acid	M+H	+	+	+	+	+
	585.4163	4	$C_{37}H_{53}N_4O_2$	Campesteryl glucoside	M+Na	+	+	+	+	+
	871.5736	3	$C_{47}H_{83}O_{12}P$	Glycerophospholipid	M+H	+				
	877.6190	2	$C_{54}H_{85}O_9$	Inositol phospholipid	M+H					+
7.3-7.4	280.2634	0	$C_{18}H_{34}NO$	3 matches	M+H			+		
	295.2272	1	$C_{18}H_{31}O_3$	Octadecatrienoic acid	M+H				+	
7.5-7.6	123.1170	1	C_9H_{15}	4-Hydroxybenzaldehyd	M+H				+	
	279.2323	1	$C_{18}H_{31}O_2$	α-Linolenic acid	M+H		+	+	+	+
	435.0773	6	$C_{13}H_{24}N_4O_5S_3$	Tetrapeptide	M+Na		+	+	+	+
	453.2823		$C_{20}H_{41}N_2O_9$	Unknown				+		
	506.5303		$C_{34}H_{68}NO$	Unknown			+			
	557.4564		$C_{36}H_{61}O_4$	Unknown					+	+
	871.5724	3	$C_{47}H_{83}O_{12}P$	Glycerophospholipid	M+H	+		+		
7.7-7.8	256.2640	0	$C_{16}H_{34}NO$	Palmitamide	M+H	+	+	+	+	+
	367.1894	3	$C_{21}H_{28}O_4$	Dehydrocorticosterone	M+Na	+				
	539.3341	0	$C_{26}H_{51}O_9P$	Glycerophospholipid	M+H					+

7.9-8.1	282.2799	0	$C_{18}H_{32}O$	Octadecadienal	M+NH4	+	+	+		
	353.2666	1	$C_{19}H_{38}O_4$	Monoacylglyceride	M+H	+			+	
	563.5511		$C_{36}H_{71}N_2O_2$	Unknown				+	+	
8.4-8.6	257.2478	1	$C_{16}H_{33}O_2$	Fatty acid	M+H		+			
	284.2951	0	$C_{18}H_{38}NO$	Stearamide	M+H	+			+	
	293.2109	0	$C_{18}H_{29}O_3$	Hydroxy-hexadecenoic acid	M+Na	+				
	506.5306		$C_{34}H_{68}NO$	Unknown		+	+			
	537.3553	0	$C_{40}H_{57}$	β-carotene	M+H				+	
	625.4077	0	$C_{31}H_{61}O_{10}P$	Glycerophospholipid	M+H	+			+	
	871.5741	0	$C_{55}H_{75}N_4O_5$	Pheophytin A	M+H	+	+			
9.2-9.3	338.3423	0	$C_{22}H_{44}NO$	Docosenamide	M+H	+		+	+	+
	506.5305		$C_{34}H_{68}NO$	Unknown		+				
	565.3872	1	$C_{32}H_{49}N_6O_3$	Canthaxanthin	M+H				+	
Negative										
3.8-3.9	355.2120	0	$C_{18}H_{31}O_2$	Octadecadienoate	M-H	+				
4.7-4.8	293.1523	5	$C_{16}H_{17}N_6$	Amino acid	M-H			+		+
5.2-5.4	194.0821	2	$C_{10}H_{12}NO_3$	Amines	M-H	+	+	+	+	+
	267.1960	0	$C_{16}H_{27}O_3$	Hexadecanedioic acid	M-H	+				
	269.1309		$C_{16}H_{17}N_2O_2$	Unknown					+	
	311.2224	1	$C_{18}H_{31}O_2$	Octadecadienoic acid	M-H	+				
	376.2701		$C_{19}H_{38}NO_6$	Unknown					+	
5.9-6.0	309.2067	1	$C_{18}H_{29}O_4$	hydroperoxy-octadecatrienoic acid	M-H	+	+		+	
	361.1702		$C_{10}H_{21}N_{10}O_5$	Unknown				+		
	529.3849	1	$C_{28}H_{53}N_2O_7$	Anhydroeschscholtzxanthin	M-H				+	
	797.5406	8	$C_{44}H_{79}O_{10}P$	Glycerophospholipid	M-H				+	
6.2-6.3	221.1547	0	$C_{14}H_{21}O_2$	13 matches	M-H					+
	295.2040	0	$C_{16}H_{27}N_2O_3$	Retinoid	M-H			+		+
	358.2316	0	$C_{22}H_{32}NOS$	Curacin D	M-H			+		+
	817.4454		$C_{52}H_{53}N_{10}$	Unknown				+		
6.6-6.9	249.1859	0	$C_{16}H_{25}O_2$	Hexadecatrienoic acid	M-H	+	+	+	+	+
	307.2272	2	$C_{18}H_{31}O_2$	Octadecadienoate	M-H	+				

	347.1714	0	$C_{16}H_{27}O_8$	Glucoside	M-H	+				+
	349.1111	0	$C_{22}H_{13}N_4O$	13 matches	M-H	+	+	+	+	
	397.2257	0	$C_{28}H_{45}O$	Brassicasterol	M-H	+				
	499.3787	1	$C_{32}H_{51}O_4$	Oleananoic acid	M-H	+	+		+	+
7.3-7.4	251.2010	2	$C_{16}H_{27}O_2$	Hexadecadienoic acid	M-H	+	+	+	+	+
	351.1260		$C_{22}H_{15}N_4O$	Unknown		+	+		+	+
	349.0832	0	$C_{19}H_{13}N_2O_5$	13 matches	M-H			+		
	503.4093	1	$C_{32}H_{58}O_5$	Diacylglycerol	M-H2O-H	+	+		+	+
	797.5399	8	$C_{44}H_{79}O_{10}P$	Glycerophospholipid	M-H	+			+	
7.6-7.7	277.2168	1	$C_{18}H_{29}O_2$	α-Linolenic acid	M-H	+	+	+	+	+
	377.1416	0	$C_{28}H_{43}O$	Ergosterol	M-H2O-H	+	+		+	+
	393.1054	0	$C_{29}H_{47}O$	Stigmasterol	M-H			+		
	555.4407	1	$C_{32}H_{55}N_6O_2$	Diacylglycerol	M-H	+	+	+	+	+
7.8-7.8	227.2009	0	$C_{14}H_{27}O_2$	29 matches	M-H		+			+
	327.1259	0	$C_{20}H_{15}N_4O$	13 matches	M-H		+		+	+
	329.1296		$C_6H_{13}N_{14}O_3$	Unknown						+
	538.3324	4	$C_{26}H_{54}NO_6PS$	Phosphocholine	M-H				+	+
	797.5400	8	$C_{44}H_{79}O_{10}P$	Glycerophospholipid	M-H				+	
8.1-8.2	279.2323	2	$C_{18}H_{31}O_2$	Fatty acids	M-H	+	+	+	+	+
	379.1573	0	$C_{28}H_{45}O$	Brassicasterol	M-H2O-H	+	+		+	+
	429.1940		$C_{25}H_{25}N_4O_3$	Unknown		+			+	+
	559.4725	0	$C_{36}H_{63}O_4$	Diacylglycerol	M-H	+		+	+	+
	581.4533	0	$C_{40}H_{53}O_3$	Violaxanthin	M-H2O-H				+	
8.5-8.7	255.2324	2	$C_{16}H_{31}O_2$	Palmitic acid	M-H	+	+	+	+	+
	355.1574	0	$C_{22}H_{19}N_4O$	17 matches	M-H	+	+	+	+	+
	511.4719		$C_{32}H_{63}O_4$	Unknown		+	+		+	+
	631.4197		$C_{18}H_{51}N_{18}O_7$	Unknown					+	+

*Name and average mass of compounds was suggested by Metlin and ChemSpider chemical structure databases.

Table A.19. Compounds found in acetone extracts from wild cyanobacterial biomass

Time	Meas. m/z	Δppm	Suggested formula	Name*		S1-A55	S1-A70	S2-A70	S3-A70
Positive									
4.7-4.8	230.2474	0	$C_{14}H_{28}O$	Tetradecenone, Xestoaminol	M+H			+	
	274.2742	0	$C_{16}H_{36}NO_2$	Sphinganine	M+H	+	+	+	+
	318.3001	0	$C_{18}H_{40}NO_3$	Phytosphingosine	M+H			+	+
7.0-7.1	254.2477	0	$C_{16}H_{32}NO$	Palmitoleamide	M+H			+	
7.5-7.6	279.2318	0	$C_{18}H_{31}O_2$	α-Linolenic acid	M+H			+	+
	435.0762	6	$C_{13}H_{24}N_4O_5S_3$	Tetrapeptide	M+Na			+	
	871.5726	3	$C_{47}H_{83}O_{12}P$	Glycerophospholipid	M+H			+	
7.7-7.8	256.2637	0	$C_{16}H_{34}NO$	Palmitamide	M+H	+	+	+	+
7.9-8.1	282.2797	0	$C_{18}H_{32}O$	Octadecadienal	M+NH4	+	+	+	+
	563.5512		$C_{36}H_{71}N_2O_2$	Unknown		+	+	+	+
8.2-8.3	593.2761	0	$C_{40}H_{56}O_2$	Zeaxanthin	M+Na			+	+
	871.5731	3	$C_{47}H_{83}O_{12}P$	Glycerophospholipid	M+H			+	
8.4-8.6	257.2474	0	$C_{16}H_{33}O_2$	Fatty acid	M+H				+
	284.2947	0	$C_{18}H_{38}NO$	Stearamide	M+H			+	+
	395.2199	0	$C_{21}H_{27}N_6O_2$	11 matches	M+H			+	+
	459.4403		$C_{28}H_{59}O_4$	Unknown				+	+
9.2-9.4	338.3425	0	$C_{22}H_{44}NO$	Docosenamide	M+H	+	+	+	+
Negative									
4.2-4.4	309.2066	1	$C_{18}H_{29}O_4$	hydroperoxy-octadecatrienoic acid	M-H				+
4.7-4.8	207.1392	0	$C_{13}H_{19}O_2$	14 matches	M-H	+			
	293.1760	0	$C_{18}H_{21}N_4$	7 matches	M-H	+	+		
4.9-5.0	269.1308		$C_{16}H_{17}N_2O_2$	Unknown		+	+		
5.1-5.2	309.2070	1	$C_{18}H_{29}O_4$	hydroperoxy-octadecatrienoic acid	M-H	+	+	+	+
	372.2383	0	$C_{23}H_{35}NOS$	Curacin A	M-H	+			
5.2-5.3	194.0823	0	$C_{10}H_{12}NO_3$	Amine	M-H	+	+	+	+
5.7-5.9	361.1994		$C_{16}H_{29}N_2O_7$	Unknown		+	+	+	+
	533.2968	0	$C_{40}H_{53}$	β-cryptoxanthin	M-H2O-H	+			+
	358.2597	0	$C_{22}H_{32}NOS$	Curacin D	M+H	+		+	+
	559.3120	2	$C_{29}H_{46}N_4O_8$	Glycerol	M-H2O-H	+		+	+
6.1-6.3	221.1543	0	$C_{14}H_{21}O_2$	13 matches	M-H	+	+		+

	295.2273	0	$C_{18}H_{31}O_3$	Octadecatrienoic acid	M+H	+	+		+
	743.4872	0	$C_{40}H_{73}O_{10}P$	Glycerophoslipid	M-H				+
6.6-6.8	205.1597	0	$C_{11}H_9O4$	Sinapic acid	M-H2O-H	+	+	+	+
	269.2117		$C_{16}H_{29}O_3$	Unknown					+
	397.2260	0	$C_{28}H_{45}O$	Brassicasterol	M-H				+
6.8-6.9	249.1857	0	$C_{16}H_{25}O_2$	Hexadecatrienoic acid	M-H	+	+	+	+
	347.1716	0	$C_{17}H_{23}N_4O_4$	Glucoside	M-H			+	
	349.1105	0	$C_9H_{21}N_2O_{12}$	13 matches	M-H	+	+	+	+
	537.3276		$C_{26}H_{49}O_{11}$	Unknown		+	+	+	+
7.3-7.4	251.2018	2	$C_{16}H_{27}O_2$	Hexadecadienoic acid	M-H			+	+
	351.1264		$C_{22}H_{15}N_4O$	Unknown				+	+
	743.4878	0	$C_{40}H_{73}O_{10}P$	Glycerophoslipid	M-H				+
7.5-7.7	277.2170	0	$C_{18}H_{29}O_2$	α-Linolenic acid	M-H	+	+	+	
	377.1421	0	$C_{28}H_{43}O$	Ergosterol	M-H2O-H	+	+	+	
	555.4415	1	$C_{21}H_{55}N_{12}O_5$	Diacylglycerol	M-H	+	+	+	+
8.0-8.2	279.2332	2	$C_{18}H_{31}O_2$	Fatty acid	M-H			+	+
	379.1580	0	$C_{28}H_{45}O$	Brassicasterol	M-H2O-H			+	+
	429.1943		$C_{10}H_{21}N_{16}O_4$	Unknown					+
8.5-8.7	255.2330	0	$C_{16}H_{31}O_2$	Palmitic acid	M+Na			+	+
	355.1581	0	$C_{22}H_{19}N_4O$	17 matches	M-H			+	+
	511.4732		$C_{32}H_{63}O_4$	Unknown				+	+

*Name and average mass of compounds was suggested by Metlin and ChemSpider chemical structure databases.

Table A.20. Compounds found in ethanol extracts from wild cyanobacterial biomass

Time	Meas. m/z	Δppm	Suggested formula	Name*		S1-E55	S1-E70	S2-E70	S3-E70
Positive									
0.3-0.5	112.0506	0	$C_4H_6N_3O$	Cytosine	M+H				+
	163.0598	1	$C_6H_{11}O_5$	25 matches	M+H	+	+		
	244.0928	0	$C_9H_{14}N_3O_5$	Amino acid	M+H				+
	255.1072	0	$C_9H_{19}O_8$	Galactosylglycerol	M+H	+			
	439.1418	6	$C_{26}H_{15}N_8$	Villinol	M+H	+	+		
	487.1781	6	$C_{18}H_{27}N_6O_{10}$	Flavonoid	M+H				+
	531.1887	4	$C_{16}H_{31}N_6O_{14}$	Glycoside	M+H	+	+		+
0.8-1.0	137.0455	2	C_8H_5O	PAA (auxin)	M+H	+		+	+
	268.1039	0	$C_{10}H_{14}N_5O_4$	Nucleoside	M+H	+	+	+	+
	360.1650	0	$C_{16}H_{26}NO_8$	9 matches	M+H	+	+		+
	537.1689	0	$C_{20}H_{25}N_8O_{10}$	β-carotene	M+H			+	+
2.6-2.8	340.2600	3	$C_{21}H_{35}NO$	Amine	M+Na	+	+	+	+
	679.5118	0	$C_{36}H_{63}N_5O_6$	Ceramide	M+NH4	+	+	+	+
2.9-3.0	100.0755	0	$C_5H_{10}NO$	5 matches	M+H				+
	453.3436		$C_{24}H_{45}N_4O_4$	Unknown					+
4.7-4.9	274.2745	1	$C_{16}H_{36}NO_2$	Sphinganine	M+H	+	+	+	+
7.0-7.1	254.2480	0	$C_{16}H_{32}NO$	Palmitoleamide	M+H			+	
7.5-7.6	279.2318	0	$C_{18}H_{31}O_2$	α-Linolenic acid	M+H				+
	526.4831		$C_{32}H_{64}NO_4$	Unknown					+
	870.5942	4	$C_{49}H_{86}NO_8P$	Glycerophospholipid	M+H	+			+
7.7-7.8	256.2636	0	$C_{16}H_{34}NO$	Palmitamide	M+H	+	+	+	+
	415.4151		$C_{26}H_{55}O_3$	Unknown			+		+
	413.3989	0	$C_{29}H_{49}O$	Stigmasterol	M+H	+		+	
	575.4513	0	$C_{40}H_{56}O$	β-cryptoxanthin	M+Na	+		+	
	708.5414	0	$C_{41}H_{74}NO_8$	Cyclitol	M+H	+	+	+	+
	871.5738	0	$C_{55}H_{75}N_4O_5$	Pheophytin	M+H		+		
	893.6589	0	$C_{66}H_{85}O$	Chlorophyll a	M+H				+
7.9-8.0	282.2796	0	$C_{18}H_{32}O$	Octadecadienal	M+NH4	+	+	+	
	284.2948	0	$C_{18}H_{38}NO$	Stearamide	M+H				+
	563.5511		$C_{36}H_{71}N_2O_2$	Unknown		+		+	
8.2-8.3	512.5036	0	$C_{32}H_{66}NO_3$	Dihydroceramide	M+H	+			
	593.2762	0	$C_{40}H_{56}O_2$	Zeaxanthin	M+Na	+	+	+	+

	871.5736	3	$C_{47}H_{83}O_{12}P$	Glycerophospholipid	M+H		+	+	
8.4-8.5	395.2201	0	$C_{21}H_{27}N_6O_2$	11 matches	M+H				+
	338.3421	1	$C_{22}H_{44}NO$	Docosenamide	M+H	+		+	+
	427.2510		$C_{27}H_{31}N_4O$	Unknown			+		
Negative									
0.3-0.4	253.0929	0	$C_9H_{17}O_8$	Glycerol	M-H			+	
	631.2202	0	$C_7H_{19}N_{32}O_5$	Sialyllactosamine	M-H			+	
0.8-1.0	243.0621	0	$C_9H_{11}N_2O_6$	Uridine	M-H		+	+	+
	267.0734	0	$C_{10}H_{11}N_4O_5$	Amine	M-H			+	
	535.1535	0	$C_{40}H_{55}$	β-carotene	M-H			+	+
1.1-1.2	130.0874	0	$C_6H_{12}NO_2$	Amine	M-H				+
4.7-4.8	207.1380	0	$C_9H_{15}N_6$	14 matches	M-H	+			+
	293.1757	0	$C_{17}H_{25}O_4$	7 matches	M-H	+	+	+	+
	869.4030		$C_{39}H_{65}O_{21}$	Unknown		+	+		
4.9-5.0	269.1307		$C_{16}H_{17}N_2O_2$	Unknown		+	+		+
	390.2499	0	$C_{19}H_{36}NO_7$	Hydroxyphernylretinamide	M-H	+	+		+
	593.1295	0	$C_{14}H_{25}N_8O_{18}$	Glycoside	M-H	+			
	761.3228		$C_{16}H_{45}N_{18}O_{17}$	Unknown		+	+		
5.2-5.3	194.0822	0	$C_{10}H_{12}NO_3$	Amines	M-H	+	+	+	+
5.5-5.6	455.2411	0	$C_{20}H_{41}O_9P$	Phosphoglycerol	M-H	+			
	595.2405	0	$C_{40}H_{51}O_4$	Astaxanthin	M-H	+			
	873.4355	0	$C_{52}H_{69}MgN_4O_4$	Chlorophyll a	M-H2O-H	+			
5.7-5.7	553.2682	1	$C_{30}H_{33}N_8O_3$	Glycerol	M-H	+		+	
6.0-6.1	358.2602	0	$C_{22}H_{32}NOS$	Curacin D	M-H	+	+	+	+
	377.1614	0	$C_{13}H_{26}N_6O_5S_1$	Tripeptide	M-H				+
	559.3124	2	$C_{29}H_{46}N_4O_8$	Glycerol	M-H2O-H	+	+	+	
	699.3816		$C_{30}H_{47}N_{14}O_6$	Unknown		+			+
6.2-6.5	221.1550	1	$C_{14}H_{21}O_2$	Acetate	M-H			+	+
	295.2278	0	$C_{18}H_{31}O_3$	Octadecatrienoic acid	M+H			+	+
6.6-6.9	205.1595	1	$C_{11}H_9O_4$	Sinapic acid	M-H2O-H	+	+	+	
	249.1859	0	$C_{16}H_{25}O_2$	Hexadecatrienoic acid	M-H			+	+
	311.2013	1	$C_{21}H_{27}O_2$	Steroid	M-H				+
	347.1716	1	$C_{16}H_{27}O_8$	Glycoside	M-H	+	+	+	
	483.2730	0	$C_{22}H_{45}O_9P$	PG: phosphatidic acid + glycerol + palmitic acid	M-H	+	+	+	+

	537.3280		$C_{26}H_{49}O_{11}$	Unknown				+	+
7.5-7.6	277.2172	0	$C_{18}H_{29}O_2$	α-Linolenic acid	M-H			+	+
	377.1424	0	$C_{28}H_{43}O$	Ergosterol	M-H2O-H			+	+
	555.4418	0	$C_{29}H_{55}N_{22}O_2$	Diacylglycerol	M-H			+	+
7.6-7.9	621.4581		$C_{33}H_{65}O_{10}$	Unknown		+	+	+	+
	914.5847		$C_{46}H_{72}N_{15}O_5$	Unknown		+	+	+	+
8.0-8.1	279.2329	2	$C_{18}H_{31}O_2$	Fatty acid	M-H			+	
	609.5103	0	$C_{37}H_{69}O_6$	Diacylglycerol	M-H			+	
8.5-8.6	255.2331	0	$C_{16}H_{31}O_2$	Palmitic acid	M+Na			+	+
	355.1583	0	$C_{22}H_{19}N_4O$	17 matches	M-H				+

*Name and average mass of compounds was suggested by Metlin and ChemSpider chemical structure databases.

Table A.21. Compounds found in water extracts from wild cyanobacterial biomass

Time	Meas. m/z	Δppm	Suggested formula	Name*		S1-W55	S1-W140	S2-W140	S3-W140
Positive									
0.3-0.5	112.0505	0	$C_4H_6N_3O$	Cytosine	M+H				+
	134.0448	0	$C_4H_8NO_4$	Aspartic acid	M+H	+		+	
	175.1186	2	C_8H_5O	PAA (auxin)	M+Na		+		
	255.1070	1	$C_9H_{19}O_8$	Galactosylglycerol	M+H	+		+	
	281.1405	2	$C_{16}H_{17}N_4O$	Aminonaphtalene	M+H		+		+
	487.1774	6	$C_{17}H_{31}N_2O_{14}$	Flavonoids	M+H		+		+
	561.2731		$C_{32}H_{33}N_8O_2$	Unknown			+		+
0.7-0.8	347.1449	0	$C_{14}H_{23}N_2O_8$	Glucosides	M-H	+			
0.9-1.0	86.0965	0	$C_5H_{12}N$	Piperidine	M+H				+
	132.1020	0	$C_6H_{14}NO_2$	Amino acid	M+H				+
	152.0565	0	$C_5H_6N_5O$	Nucleobases	M+H		+		+
	268.1038	4	$C_9H_{18}NO_8$	Neuraminic acid	M+H		+		
	284.0991	0	$C_{10}H_{14}N_5O_5$	Guanosine	M+H				+
	537.1687	0	$C_{40}H_{57}$	β-carotene	M+H		+		+
1.2-1.3	166.0863	0	$C_9H_{12}NO_2$	Amino acids	M+H				+
1.5-1.5	100.0756	0	$C_5H_{10}NO$	5 matches	M+H			+	
2.3-2.4	227.1755	0	$C_{10}H_8CLNO_2$	4-CL-IAA (auxin)	M+NH4				+
	453.3438		$C_{24}H_{45}N_4O_4$	Unknown					+
2.7-2.8	340.2598	3	$C_{21}H_{35}NO$	Amine	M+Na		+	+	+
	679.5121	0	$C_{36}H_{63}N_5O_6$	Ceramide	M+NH4	+	+	+	+
	701.4942		$C_{37}H_{69}N_2O_{10}$	Unknown		+	+	+	+
4.7-4.9	230.2483	0	$C_{14}H_{28}O$	Tetradecenone, Xestoaminol	M+H				+
	274.2745	1	$C_{16}H_{36}NO_2$	Sphinganine	M+H	+	+	+	+
	318.3002	0	$C_{18}H_{40}NO_3$	Phytospingosine	M+H		+	+	
7.7-7.8	256.2637	0	$C_{16}H_{34}NO$	Palmitamide	M+H	+	+	+	+
	415.4142		$C_{26}H_{55}O_3$	Unknown		+			+
	577.4675	0	$C_{32}H_{65}O_8$	Hexacosanetriol	M+H	+			+
	708.5416	0	$C_{41}H_{74}NO_8$	Cystitol	M+H	+	+	+	
	871.5727	3	$C_{47}H_{83}O_{12}P$	Glycerophospholipid	M+H			+	
	893.6593	0	$C_{66}H_{85}O$	Chlorophyll a	M+H				+

	1153.9302		$C_{66}H_{121}N_8O_8$	Unknown					+
7.8-8.0	282.2797	1	$C_{18}H_{32}O$	Octadecadienal	M+NH4	+	+	+	+
	563.5514		$C_{36}H_{71}N_2O_2$	Unknown		+	+	+	+
8.2-8.5	284.2949	0	$C_{18}H_{38}NO$	Streamide	M+H	+			
	339.3261	1	$C_{22}H_{43}O_2$	26 matches	M+H				+
	459.4417		$C_{29}H_{55}N_4$	Unknown					+
	593.2768	0	$C_{40}H_{56}O_2$	Zeaxanthin	M+Na	+			+
9.1-9.1	469.3436	2	$C_{29}H_{45}N_2O_3$	Tryptophan	M+H	+			
9.2-9.3	124.0870		$C_6H_{10}N_3$	Unknown		+		+	
	338.3423	1	$C_{22}H_{44}NO$	Docosenamide	M+H	+		+	
	427.2510		$C_{27}H_{31}N_4O$	Unknown				+	
	492.5138		$C_{33}H_{66}NO$	Unknown		+		+	
Negative									
0.4-0.6	132.0304	1	$C_4H_6NO_4$	Aspartic acid	M-H		+		
	133.0143	0	C_8H_5O	PAA (auxin)	M-H2O-H			+	
	179.0562	0	$C_6H_{11}O_6$	Syringic acid /caffeic	M-H2O-H/ M-H				+
	191.0198	0	$C_6H_7O_7$	Citric acid	M-H	+	+	+	
	253.0928	0	$C_9H_{17}O_8$	Glycerol	M-H	+	+	+	+
	317.0546		$C_{14}H_5N_8O_2$	Unknown		+		+	+
	387.1146	7	$C_{13}H_{23}O_{13}$	Fructoselysine 6-phosphate	M-H	+	+	+	+
	461.1516		$C_{14}H_{17}N_{14}O_5$	Unknown			+	+	+
	549.1681	1	$C_{40}H_{53}O$	plectaniaxanthin	M-H20-H	+	+		
	559.2595	5	$C_{20}H_{35}N_{10}O_9$	Tetrapeptide	M-H			+	+
	631.2201	0	$C_{23}H_{39}N_2O_{18}$	Sialyllactosamine	M-H	+	+		+
0.7-0.8	128.0354	0	$C_5H_6NO_3$	7 matches	M-H	+	+		+
0.8-1.0	267.0735	0	$C_{10}H_{11}N_4O_5$	Amine	M-H			+	
	289.0679	0	$C_{10}H_{13}N_2O_8$	Catechin	M-H				+
	453.1726		$C_{17}H_{29}N_2O_{12}$	Unknown					+
1.5-1.6	313.1040	4	$C_{13}H_{17}N_2O_7$	Dihydropteroic acid	M-H		+		
4.7-4.8	207.1387	1	$C_{13}H_{19}O_2$	14 matches	M-H			+	+
	293.1756	0	$C_{17}H_{25}O_4$	7 matches	M-H	+			+
	390.2496	0	$C_{19}H_{36}NO_7$	Hydroxyphernylretinamide	M-H			+	+
5.2-5.3	194.0822	0	$C_{10}H_{12}NO_3$	Amine	M-H	+	+	+	+

	358.2594	0	$C_{22}H_{32}NOS$	Curacin D	M-H				+
5.5-5.6	455.2406	0	$C_{20}H_{41}O_9P$	Phosphoglycerol	M-H		+		
	595.2397	0	$C_{40}H_{51}O_4$	Astaxanthin	M-H		+		
5.7-5.9	361.1996		$C_{16}H_{29}N_2O_7$	Unknown					+
	533.2966	0	$C_{40}H_{53}$	β-cryptoxanthin	M-H2O-H				+
6.0-6.2	221.1548	0	$C_{14}H_{21}O_2$	13 matches	M-H				+
	358.2602	0	$C_{22}H_{32}NOS$	Curacin D	M-H			+	+
6.3-6.4	309.1739	1	$C_7H_{21}N_{10}O_4$	hydroperoxy-octadecatrienoic acid	M-H	+			+
6.4-6.6	483.2724	0	$C_{22}H_{45}O_9P$	PG: phosphatidic acid + glycerol + palmitic acid	M-H	+	+		+
6.6-6.8	205.1597	0	$C_{11}H_9O4$	Sinapic acid	M-H2O-H	+	+	+	
	249.1860	0	$C_{16}H_{25}O_2$	Hexadecatrienoic acids	M-H				+
	301.1658	0	$C_{15}H_{25}O_6$	Tributyrin	M-H			+	
	347.1714	0	$C_{17}H_{23}N_4O_4$	Glycoside	M-H	+	+	+	+
	483.2726	0	$C_{22}H_{45}O_9P$	PG: phosphatidic acid + glycerol + palmitic acid	M-H	+	+	+	+
	656.2510		$C_{38}H_{34}N_5O_6$	Unknown				+	+
7.6-7.7	621.4579		$C_{33}H_{65}O_{10}$	Unknown		+		+	+

*Name and average mass of compounds was suggested by Metlin and ChemSpider chemical structure databases.