Kaunas University of Technology

Faculty of Chemical Technology

Processing of *Phaeodactylum tricornutum* Biomass into Valuable Functional Ingredients by High-Pressure Extraction Techniques

Master's Final Degree Project

Nadiia Khakimova

Project author

Assoc. prof. Michail Syrpas

Supervisor

Kaunas, 2020

Kaunas University of Technology

Faculty of Chemical Technology

Processing of *Phaeodactylum tricornutum* Biomass into Valuable Functional Ingredients by High-Pressure Extraction Techniques

Master's Final Degree Project

Food Science and Safety (6211FX011)

Nadiia Khakimova

Project author

Assoc. prof. Michail Syrpas

Supervisor

Assoc. prof. Paulius Kraujalis

Reviewer

Kaunas, 2020

Kaunas University of Technology

Faculty of Chemical Technology

Nadiia Khakimova

Processing of *Phaeodactylum tricornutum* Biomass into Valuable Functional Ingredients by High-Pressure Extraction Techniques

Declaration of Academic Integrity

I confirm that the final project of mine, Nadiia Khakimova, on the topic „Processing of Phaeodactylum tricornutum Biomass into Valuable Functional Ingredients by High-Pressure Extraction Techniques“ 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.

I fully and completely understand that any discovery of any manifestations/case/facts of dishonesty inevitably results in me incurring a penalty according to the procedure(s) effective at Kaunas University of Technology.

Nadiia Khakimova

(name and surname filled in by hand) (signature)

Nadiia Khakimova. Processing of *Phaeodactylum tricornutum* Biomass into Valuable Functional Ingredients by High-Pressure Extraction Techniques. Master's Final Degree Project / supervisor Assoc. prof. Michail Syrpas; Faculty of Chemical Technology, Kaunas University of Technology.

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

Keywords: microalgae, *Phaeodactylum tricornutum*, supercritical carbon dioxide extraction, pressurized liquid extraction, response surface methodology, carotenoids.

Kaunas, 2020. 89 p.

Summary

Phaeodactylum tricornutum, the only species in the genus *Phaeodactylum*, belongs to a significant group of microalgae called diatoms. *P. tricornutum* is considered a promising feedstock of high-value products. Mainly, due to its ability to synthesize polyunsaturated fatty acids, like eicosapentaenoic acid, and carotenoids, especially fucoxanthin – a valuable pigment with multiple biological activities. This work aimed to develop multi-step *P. tricornutum* processing schemes for isolation of high-added value fractions using conventional and high-pressure extraction techniques.

Various standard methods achieved the determination of chemical composition. Conventional and high-pressure extraction techniques like solid-liquid extraction (SLE), supercritical fluid extraction (SFE), and pressurized liquid extraction (PLE) were applied using various solvents in order to obtain extracts of various polarity. The high-pressure extraction techniques, SFE and PLE, were optimized using response surface methodology (RSM) in order to maximize total yield, carotenoid, and fucoxanthin content.

In vitro antioxidant capacity of initial material, as well as all extracts were determined using the total phenolic content (by the Folin-Ciocalteu method), $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ assays. The activity of solid residues was tested with the same *in vitro* antioxidant capacity assays following the QUENCHER approach.

The qualitative determination of pigments and quantitative determination of fucoxanthin content was performed using HPLC-DAD. The fatty acid profile was determined using GC-FID. Moreover, for all extracts, the antimicrobial activity was using the disc diffusion assay, determining the minimal inhibitory concentration for the active ones.

This work demonstrates the feasibility of high-pressure techniques for the isolation of high-added value products from this rather underutilized feedstock.

Nadiia Khakimova. *Phaeodactylum tricornutum* biomasės perdirbimas į vertingus funkcionaliuosius komponentus taikant aukšto slėgio ekstrakcijos metodus. Magistro baigiamasis projektas / vadovas Doc. dr. Michail Syrpas; Kauno technologijos universitetas, Cheminės technologijos fakultetas.

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

Reikšminiai žodžiai: mikrodumbliai, *Phaeodactylum tricornutum*, superkritinis anglies dioksido ekstrahavimas, slėgio skysčio ekstrahavimas, atsako paviršiaus metodika, karotinoidai.

Kaunas, 2020. 89 p.

Santrauka

Phaeodactylum tricornutum yra vienintelė *Phaeodactylum* genties rūšis, priklausanti mikrodumblių, vadinamų diatomomis, grupei. Dėl savo unikalios sudėties *P. tricornutum* yra laikoma puikiu šaltiniu, siekiant gauti aukštos kokybės produktus. Šie mikrodumbliai geba sintetinti polinesočiąsias riebalų rūgštis, tokias kaip eikozapentaeno rūgštis, ir karotenoidus, ypač fukoksantiną – pigmentą, kuris pasižymi įvairiapusiu biologiniu aktyvumu. Šio darbo tikslas buvo sukurti daugiapakopes *P. tricornutum* perdirbimo schemas, skirtas išgauti aukštos prodėtinės vertės frakcijas, naudojant tradicinius ir didelio slėgio ekstrakcijos metodus.

Cheminė sudėtis nustatyta naudojant įvairius standartinius metodus. Norint gauti skirtingo poliškumo ekstraktus panaudoti tradiciniai ir didelio slėgio ekstrakcijos metodai, tokie kaip maceravimas (SLE), superkritinė ekstrakcija anglies dvidegieniu (SFE) ir ekstrakcija padidinto slėgio aplinkoje (PLE), naudojant įvairius tirpiklius. Didelio slėgio ekstrakcijos metodai (SFE ir PLE) optimizuoti taikant paviršiaus atsako metodologiją (RSM), siekiant gauti didžiausią išeigą, karotenoidų ir fukoksantino kiekį.

Antioksidacinis aktyvumas *(in vitro*) pradinėje žaliavoje bei visuose ekstraktuose įvertintas nustatant bendrą fenolinių junginių kiekį Folin-Ciocalteu‘s metodu bei naudojant DPPH$^{\bullet}$ radikalo ir ABTS$^{\bullet+}$ katijono radikalo blukinimo metodus. Antioksidacinis pajėgumas liekanose po atliktų ekstrakcijų nustatytas naudojant tuos pačius metodus, tačiau pritaikant QUENCHER procedūrą.

Pigmentų kokybinis bei fukoksantino kiekybinis įvertinimas atliktas naudojant HPLC-DAD. Riebalų rūgščių sudėtis nustatyta naudojant GC-FID. Visų ekstraktų antimikrobinis aktyvumas įvertintas naudojant difuzijos į agarą metodą bei nustatant minimalią slopinančią koncentraciją.

Šis darbas parodo didelio slėgio ekstrakcijos metodų, galinčių išskirti aukštos pridėtinės vertės produktus iš šios nepakankamai išnaudotos žaliavos, galimybes.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS AND TERMS

$ABTS^{\bullet+}$	2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid radical cation
ANOVA	Analysis of variance
CCD	Central composite experimental design
$DPPH^{\bullet}$	2,2-diphenyl-1-picrylhydrazyl radical
DW	Dry weight
GAE	Gallic acid equivalents
GC-FID	Gas chromatography with flame ionization detector
GRAS	Generally recognized as safe
HPLC-DAD	High performance liquid chromatography with diode-array detection
MeOH	Methanol
MIC	Minimal inhibitory concentration
PBS	Phosphate buffered saline
PLE	Pressurized liquid extraction
R^2	Determination coefficient
ROS	Reactive Oxygen Species
RSM	Response surface methodology
SFE	Supercritical fluid extraction
SFE-CO_2	Supercritical carbon dioxide extraction
SLE	Solid liquid extraction
SLE-HEX	Solid liquid extraction using hexane
SLE-AC	Solid liquid extraction using acetone
SLE-EtOH	Solid liquid extraction using ethanol
SLE-H_2O	Solid liquid extraction using water
Sox	Soxhlet extraction
TEAC	Trolox equivalent antioxidant capacity
TE	Trolox equivalent
TPC	Total phenolic content
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

INTRODUCTION

Food production will have to increase fivefold to keep up with the world population, projected to be at around 10 billion by the twenty-first century. Most of today's farmland is being rapidly diverted for other uses, further limiting the area that can be used to increase food production[86].

Recently, microalgae have drawn considerable interest worldwide, due to their substantial potential for use in renewable energy and they are considered as alternative feedstocks for production of valuable bioproducts, with potential applications in pharmaceuticals, nutraceuticals and cosmetics[73]. Microalgae are small unicellular microscopic organisms which abound in different ecosystems around the globe. They can be grown in open ponds or photobioreactors without arable land use.

Phaeodactylum tricornutum is a unicellular pennate diatom. It can exist in three possible morphotypes (fusiform, triradiate and oval) that are defined by the growing conditions. Due to its high polyunsaturated fatty acids and carotenoid content, especially fucoxanthin, the *P. tricornutum* is a promising natural source for these high-added value products. Commercial production of fucoxanthin from *P. tricornutum* biomass was already investigated in the literature[77], [108]. Nevertheless, recovery of these compounds requires the previous recovery from the biomass and further downstream processing. As most metabolites are located intracellularly, cell disruption is important to boost extraction with a suitable solvent as a pretreatment. Supercritical carbon dioxide extraction (SFE-CO_2) and pressurized liquid extraction (PLE) have a great potential to extract valuable metabolites from microalgae. Nowadays, besides the economic cost biorefinery approaches focus on the utilization of sustainable extraction techniques with small environmental impact.

Supercritical carbon dioxide extraction and pressurized liquid extraction are considered as green sustainable extraction techniques, for isolation of functional products from various sources including microalgae. The use of high pressure allows solvents to change their liquid state, above their boiling point at very high temperatures. These conditions speed up extraction by increasing the analytes' mass transfer rate from the material to the solvent, allowing to achieve high yields in shorter processing periods and a lower solvent usage compared with traditional extraction techniques. Extraction methods based on compressed fluids have already been shown to be effective solutions. Supercritical fluid and the pressurized liquid extraction, carried out with different green and safe solvents as ethanol or CO_2, can be sufficient for extracting a variety of compounds according to their polarity[44].

In current research, supercritical carbon dioxide extraction and pressurized liquid extraction were evaluated for the recovery of high-value compounds from *P. tricornutum* biomass, including fatty acids and carotenoids. Moreover, conventional and novel extraction techniques were compared, high-pressure techniques were optimized, obtained extracts were examined for their *in vitro* antioxidant capacity, fatty acid profile, carotenoid content and composition, together with determination of antimicrobial activity.

The research is aimed to perform chemical characterization of initial *Phaeodactylum tricornutum* biomass with further development and optimization of biorefining approaches, using high-pressure extraction techniques for the isolation of valuable compunds from *P. tricornutum* freeze-dried biomass. For realization of the goal, the following objectives were set:

1. To determine the chemical composition of *P. tricornutum* freeze-dried biomass.
2. To isolate polar and non-polar fractions from *P. tricornutum* biomass using SLE.
3. To optimize SFE-CO_2, in order to obtain the maximum yield, total carotenoid content and fucoxanthin in the extracts, evaluating the effect of three selected factors by response surface methodology (RSM).

4. To perform acetone-assisted PLE of SFE-CO_2 dry residue in order to extract remaining carotenoids and optimize the process, evaluating the effect of temperature and extraction time by RSM.
5. To evaluate PLE-ethanol for recovery of polar fractions from the residues after SFE-CO_2 and PLE-acetone, and optimize the process using RSM to optimize total yield, carotenoid content and TPC.
6. To measure total phenolic content (TPC) and *in vitro* radical scavenging capacity ($ABTS^{\bullet+}$ and $DPPH^{\bullet}$ assays) of the initial material, solid residues and extracts after solid-liquid extraction (SLE) and SFE-CO_2 (solid residues and extracts).
7. To perform of qualitative determination of pigments and quantitative determination of fucoxanthin in SFE-CO_2 and PLE extracts using high-performance liquid chromatography (HPLC-DAD).
8. To characterize the fatty acids profile, as their corresponding fatty acid methyl-esters, in non-polar extracts by means of gas chromatography with flame ionization detector (GC-FID).
9. To evaluate the antimicrobial activity of all obtained extracts using disc diffusive assay and to measure the minimal inhibitory concentration (MIC) of active extracts.

1. LITERATURE REVIEW

1.1 *Phaeodactylum tricornutum*

1.1.1. General characteristics

Phaeodactylum tricornutum belongs to a widespread, diverse group of eukaryotic microalgae called diatoms. Diatoms can be found primarily in saltwater, including alkaline salt lakes but also freshwater terrestrial environments. *P. tricornutum* is the only species from the genus *Phaeodactylum.* Diatoms are photosynthetic autotrophs; thus, they can fixate CO_2 and produce O_2 in the water; that is why they contribute significantly to the primary productivity of aquatic environments. Although they represent only 1% of photosynthetic biomass on earth, diatoms are responsible for almost half (around 45%) of global primary production, thus contributing significantly to the primary productivity of aquatic environments[124].

P. tricornutum is the only diatom that can be pleiomorphic, can exist in different morphotypes (oval, triradiate, and fusiform), which can be determined by growing conditions. This property is linked to the unusual nature of the cell wall, which does not contain a large amount of silica, similar to other diatoms, so the diatom does not require silicon for growth and can survive without developing silicified frustules. Nevertheless, typically, all three morphotypes have silicified structures which are located on the epitheca, cell walls consist of organic compounds of three layers: outer and inner organic layers and intermediate silicified layer with a thickness of approximately 7, 5 and 3 nm throughout the walls[13]. The microphotograph of *P. tricornutum* is presented in **Figure 1.1**.

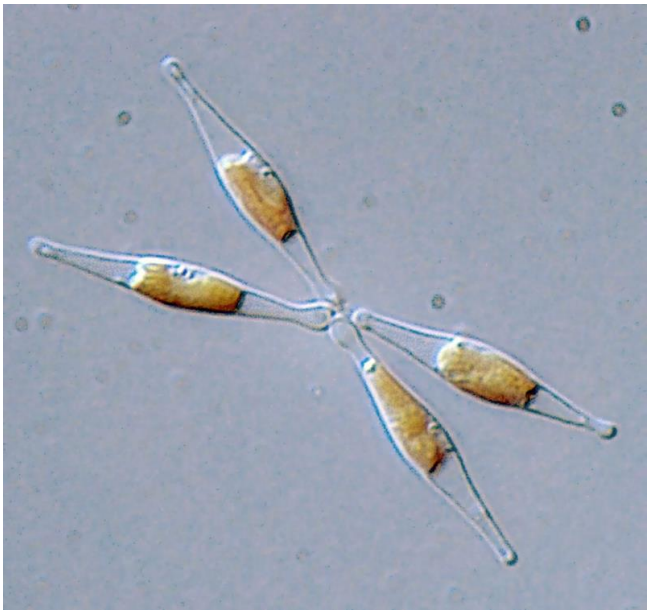


Figure 1.1. Microphotograph of *P. tricornutum.* Adapted from Diner R. E. et al.[34]

Despite the atypical structure, *P. tricornutum* is one of the diatom model species for research, as it was the second diatom which genome has been sequenced. Moreover, RNAi vectors are available, and a transformation protocol was developed[122].

Compared to other microalgae species that cannot grow at relatively high pH, *P. tricornutum* has an upper pH limit of 10.3 and can grow in salinity of 5–65 psu. The potential capacity to grow at alkaline pH environments is probably due to its carbon absorption profile since the alkalization of water causes an increase in the amount of bicarbonates in dissolved inorganic carbon. This may cause the ability to switch the specialization of its enzymatic systems depending on environmental conditions[118].

P. tricornutum is widely used in studying the biology of marine phytoplankton in areas of ecology, physiology, biochemistry, and molecular biology[103]. Also, the diatom has been used as a feed in aquaculture. Both biolistic and electroporation-mediated genetic engineering methods have been reported for *P. tricornutum*[9]. It has been proven to be a biofuel precursor[24], a potential source of protein and essential pharmaceuticals.

1.1.2. Chemical composition and bioactivity

The potential of *P. tricornutum* as a source of valuable compounds has recently gained broad interest. As it is known, microalgae can collect solar energy and consume nutrients while growing, with further nutrients' transformation into biomass, much of which are proteins, lipids, and carbohydrates[11]. With increasing interest in biorefinery approaches, the number of studies focusing on potential products and utilization of *P. tricornutum* biomass is continually growing. At the moment, *P. tricornutum* is considered to be an excellent natural source of polyunsaturated fatty acids, such as EPA eicosapentaenoic acid (EPA, C20:5_{n-3}) and docosahexaenoic acid (DHA, C22:6_{n-3}), as well as carotenoids, especially fucoxanthin[76]. It has also been recognized as a potential lipid source for biofuel production[16].

Previous research has shown that protein content in this microalga has varied depending on the cultivation conditions. The average values have been reported to be from 30% up to 50%[17], [54]. Brown et al. have investigated the amino acid profile of 15 different microalgae species and concluded that *P. tricornutum* contains high levels of essential amino acids[17]. The primary essential amino acids found in *P. tricornutum* are leucine, lysine, arginine, valine, threonine, and phenylalanine. The main non-essential amino acids present in this diatom are glutamic acid and aspartic acid. Moreover, evaluated essential amino acid index of *P. tricornutum* (0.9) was almost equal to that of an ideally balanced protein (egg albumin protein, 1.0)[150].

The carbohydrate content is also different depending on cultivation media. The diatom has shown to exhibit relatively high carbohydrate content, which was 47%[45], although some other studies have reported lower content of 26%[120]. According to different studies, the ash content constitutes up to 15% of the biomass[8], [45]. Whereas, the moisture content in dried biomass is relatively low, with maximum reported values up to 4%[8]. On the other hand, the lipid content of *P. tricornutum* can fluctuate from 10 to 60% of the dry weight, depending on growing conditions, metabolic status, the extraction method, and solvent used. The high oil content makes this diatom suitable for biodiesel production besides the production of bioactive compounds[8], [39], [128].

Fatty acids. Lipids derived from microalgae are multifarious in their biochemical structure and composition, they can be presented as neutral lipids (i.e. triacylglycerol) and polar lipids (i.e., phospholipids and glycolipids), forming fatty acid chains ranging from medium (C10-C14) to long (C16- 18) or very-long (>C20) and can be present in both – saturated or unsaturated forms[90]. Apart from the variety of compounds found in microalgae, omega-3, and other fatty acids are of significant

interest in the field of nutrition, considering their potential health benefits[32]. The diatom is rich in omega-3 polyunsaturated fatty acids (omega-3 PUFAs). Omega-3 PUFAs is a particular group of polyunsaturated fatty acids, where the first double bond is located between the third and fourth carbon atoms, counting from the methyl end of the fatty acid. Moreover, due to high PUFA content, *P. tricornutum* is extensively used as a feed in the aquaculture sector[139].

In 2018 the global value for omega-3 fatty acids was around 2.29 billion USD[37]. Dietary supplements represent 61% of global market segmentation of EPA/DHA fatty acids, followed pet foods 19%, food and beverage 14%, infant formula 3%. Fatty acids derived from microalgae make up 3% of the EPA/DHA market volume and approximately 18% of the value[157]. The amount of commercial production of DHA has increased with involving microalgae feedstocks in industrial production. Moreover, saltwater species have shown higher DHA content compared to freshwater microalgae. Although fish is the commercial source of PUFAs, it cannot supply the global demand. Moreover, during recent years global fish feedstock is decreasing and certain concerns have been raised due to the poor oxidative stability of fish oil which makes it unpleasant, as well as potential danger of contaminants such as mercury[58]. Considering this fact, microalgae become the second alternative as a feedstock of commercial production of PUFAs.

Although the importance of PUFAs on human health is widely accepted, according to recent research, developed countries consume five times less PUFAs than the recommended dose[7]. During clinical studies, EPA has shown a positive effect in the treatment of various diseases, from migraine and psoriasis to inflammatory and heart diseases[138]. DHA has shown anticarcinogenic activity, the ability to lower blood cholesterol levels, immuno-enhancing, and detox effects[58]. In addition, several studies have shown positive metabolic effects of PUFAs on obesity, as the reduction of inflammation markers in plasma of obese women[58], the reduction of accumulation of fat in mice fatty tissues[126] or the *in vitro* suppression of differentiation of adipocytes[91]. The structures of DHA and EPA are shown in **Figure 1.2.**

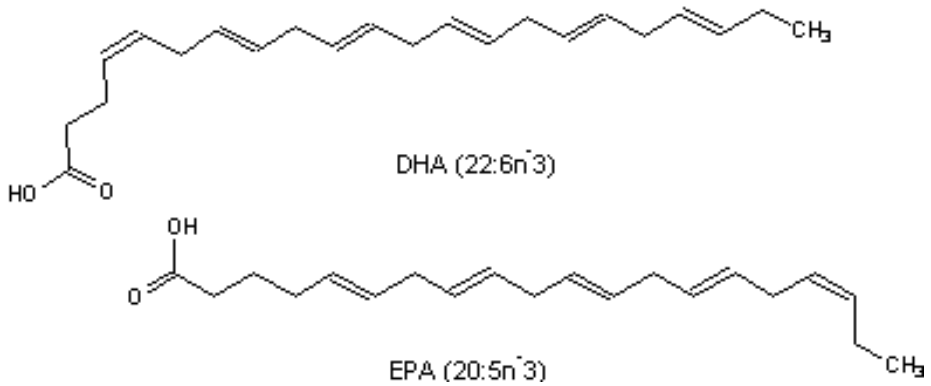


Figure 1.2. Structures of DHA and EPA. Adapted from Links R. et al. [93]

Studies that evaluated *P. tricornutum* fatty acid profile have concluded that diatom mostly contains EPA, DHA, palmitic acid, and some short-chain polyunsaturated fatty acids[8], [127], [128]. Palmitic acid (C16:0) was found to be the most abundant saturated fatty acid from *P. tricornutum*. Among monounsaturated fatty acids, palmitoleic acid (C16:1) and oleic acid (C18:1) were reported in high amounts (more than 100 mg/g^{-1}) in *P. tricornutum*[39], [41].

Carotenoids. Carotenoids are a large class of organic pigments that are nowadays highly demanded by industry because of several proven human health benefits. They exhibit a wide array of bioactivities from antioxidant capacity to neuroprotective and anti-obesity effects[112]. Carotenoids can be classified into two main groups, the carotenes, which are hydrocarbons (i.e. β-carotene, lycopene), and the xanthophylls, which contain oxygen atoms (i.e. zeaxanthin, fucoxanthin)[27]. The main natural carotenoid sources are plants and algae, as well as several bacteria and fungi. The carotenoids global market has reached $1.5 billion in 2017 and is predicted to increase to $2.0 billion by 2022, with 5.7% of annual growth rate in the period of 2017-2022[105].

A large group of photosynthetic pigments that are involved in photoprotection is localized in the chloroplasts of *P. tricornutum*, including β-carotene, diatoxanthin, diadinoxanthin and fucoxanthin. β-carotene is known for its efficiency as a natural quencher, which can scavenge reactive oxygen species (ROS), peroxyl radicals, and singlet oxygen. Together with α-tocopherol, it is involved in the inhibition of lipid peroxidation [110].

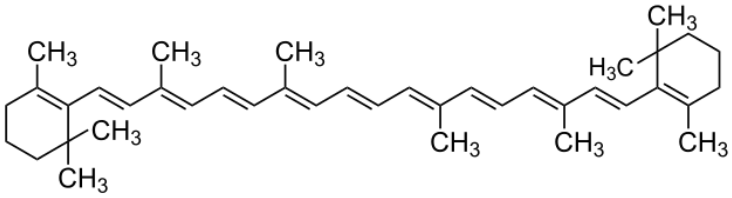


Figure 1.3. The structure of β-carotene

Also, β-carotene stimulates gap junctions, which provide intercellular communication, responsible for cell growth, apoptosis, and differentiation[141]. The structure of β-carotene is presented in **Figure 1.3**.

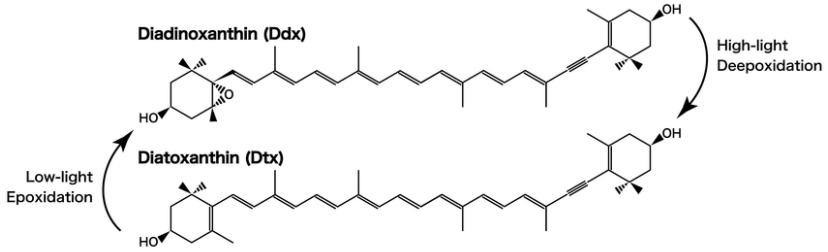


Figure 1.4. Molecular structures of the xanthophyll cycle conversion of diadinoxanthin to diatoxanthin when exposed to the light[147]

Diatoxanthin and diadinoxanthin are the other main xanthophylls in the diatom, apart from fucoxanthin. Their main role is the protection from the harmful effects of light saturation. Diadinoxanthin is the precursor of diatoxanthin, which is usually stored in an inactive form and can be transformed into the active form within a couple of minutes when exposed to light stress conditions (this process is shown on **Figure 1.4**). Diatoxanthin also can scatter energy by using non-photochemical quenching[36]. Despite the photoprotective importance for the cells, no further biological activity, nutritional importance, or health benefits of diatoxanthin and diadinoxanthin were proven[83].

1.1.3. Fucoxanthin

P. tricornutum contains up to 30 mg/g total carotenoids of dry biomass, mainly fucoxanthin, which ranges from 13 to 26.1 mg/g of dry biomass[29], [78]. Fucoxanthin, the primary marine carotenoid of *P. tricornutum*, is widespread in macro- and microalgae and, in several cases, provides more than 10% of the total natural carotenoid production[59]. Fucoxanthin is located in the thylakoids of chloroplasts and is able to generate a light-harvesting complex with chlorophyll a/c[155]. It is a xanthophyll, that absorbs light in the blue-green to the yellow range of the visible spectrum (450 to 540 nm with a peak approximately at 510-525 nm)[63]. When fucoxanthin is bound to proteins, the absorption spectrum of fucoxanthin changes from 450-540 nm to 390-580 nm, that is required for successful photosynthesis in the oceans, thus making it more useful for aquatic environments[116]. The structure of fucoxanthin is shown on **Figure 1.5**.

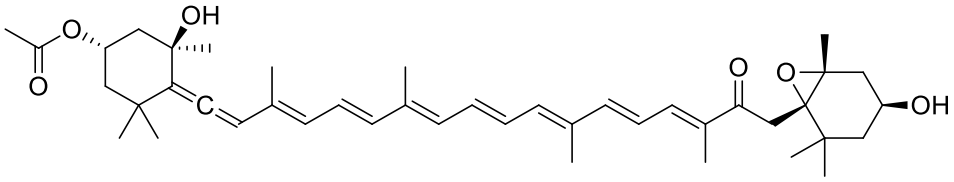


Figure 1.5. Fucoxanthin structure

Fucoxanthin's lipophilicity and its interaction with the intestinal cells' phospholipid membrane determine the cellular uptake after ingestion[144]. Therefore, the bioactivity of this carotenoid depends on its digestibility, which starts in the gastrointestinal tract by enzyme lipase that hydrolyzes it, or cholesterol esterase that deacetylates it to fucoxanthinol. **Figure 1.6** shows how fucoxanthin is being dehydrogenated into amarouciaxanthin A in the liver[5], [28], [114].

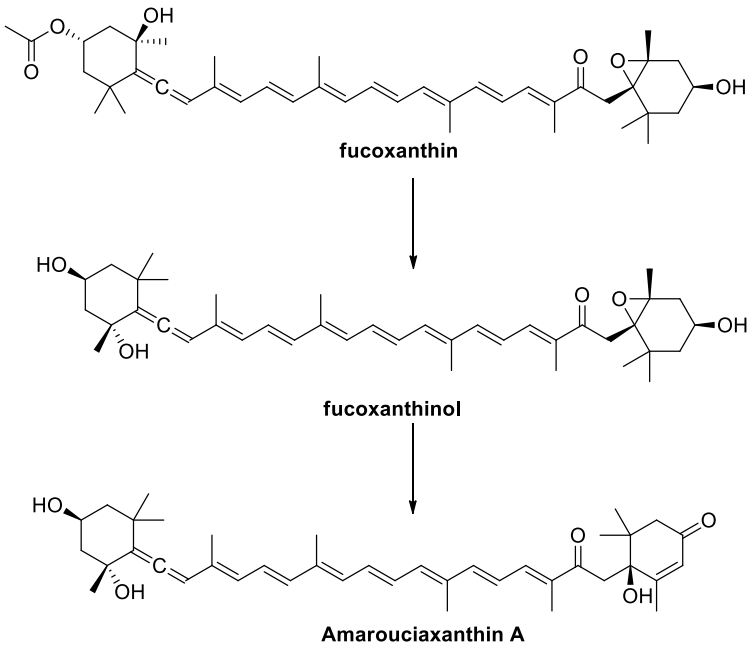


Figure 1.6. Metabolic pathway of fucoxanthin during digestion and intestinal absorption in mice. Adapted from Asai et al.[5]

The fucoxanthin has been found to have several therapeutic activities, including anticancer, antihypertensive, anti-inflammatory, and anti-obesity effects[43], [53], [92]. In the study conducted by Neumann *et al.*, the antioxidative, antiproliferative, and anti-inflammatory effects of fucoxanthin derived from *P. tricornutum* were investigated. At concentrations up to 50 µg/mL, fucoxanthin

showed no cytotoxic effect, although, at higher concentrations, it showed a cytotoxic effect on the mouse macrophage cell line RAW 264.7. It also has exhibited stronger antioxidant capacity during the FRAP analysis as compared to β-carotene. No anti-inflammatory effects of fucoxanthin derived from *P. tricornutum* were proven; however, anti-proliferative and antioxidant effects were reported[108]. These results allow concluding that fucoxanthin derived from diatom biomass helps to inhibit the effects of oxidative stress associated diseases and might potentially facilitate traditional cancer treatment[108].

For now, the majority of studies that have elucidated the beneficial health effects of fucoxanthin were using macroalgae-derived fucoxanthin, while the diatom *P. tricornutum* contains several times more fucoxanthin than macroalgae, it could become a new promising source of this marine carotenoid. Additionally, the diatoms can be cultivated in artificial photobioreactors; thus their production could not be linked to fixed season and environmental conditions as than macroalgae[142]. *P. tricornutum* is already produced in different types of closed and open photobioreactors. Hence, it could be a suitable feedstock for scaling-up microalgal fucoxanthin production[106], [107], [137].

1.2. Extraction techniques for bioactive compounds recovery from microalgae

In many companies producing algae-based products, biomass is harvested, dried, ground, or formulated into pellets, or microalgae are the final product itself[25]. In order to isolate various bioactive algal compounds such as omega-3 fatty acids, minerals, vitamins, and carotenoids, different polarity solvents would be required. Carbohydrates for example, are polar, and they are soluble in polar solvents like acetone, ethanol and water. PUFAs and carotenoids are mainly non-polar substances, so they are better extracted with non-polar hexane, supercritical CO_2 and semi-polar acetone[19]. Majority of bioactive compounds are susceptible to high temperatures. That is why choosing proper extraction techniques, solvent and extraction conditions depending on obtaining desirable compounds is a crucial part during the recovery process. The most common extraction techniques applied for algae are Soxhlet extraction, microwave-assisted extraction (MAE), ultrasound-assisted extraction (UAE), solid-liquid extraction (SLE), pressurized liquid extraction (PLE), supercritical fluid extraction (SFE), enzyme-assisted extraction (EAE)[70]. A comparison of total oil yield in different microalgal species using SFE, PLE, and MAE is represented in **Table 1.1**.

Table 1.1. Comparison of total oil yield of high-value metabolites extracted from different microalgae through SFE, PLE and MAE

Algae species	SFE, oil yield (%)	PLE, oil yield (%)	MAE, oil yield (%)	References
Arthrospira platensis	4.07	10.95	4.27	[6], [10]
Chlorella vulgaris	4.9	39.31	31.9	[6], [22]
Scenedesmus sp.	6.2	–	28.33	[6], [161]
Nannochloropsis gaditana	12.9	36	40	[128]

Algae species	SFE, oil yield (%)	PLE, oil yield (%)	MAE, oil yield (%)	References
Monoraphidium sp.	29.46	20	–	[42]
Pavlova sp.	12.9	–	–	[23]
Phaeodactylum tricornutum	14.7	34.3	28.5	[39], [48]
Schizochytrium limacinum	33.9	–	24	[6], [148]
Haematococcus pluvialis	28.2	15.81	14.26	[6], [121]

SFE – supercritical fluid extraction, PLE – pressurized liquid extraction, MAE – microwave assisted extraction

Microalgal biomass typically undergoes a pretreatment step before extraction. After gathering from natural ecosystems or photobioreactors, biomass is washed, and all possible impurities and residues are removed. Then, biomass is dried and crushed in order to increase the surface-to-volume ratio. These procedures also allow better matrix penetration by a solvent that results in a more efficient extraction process[57], [159].

1.3. Conventional and novel extraction techniques

1.3.1. Conventional extraction techniques

Several conventional extraction techniques based on mechanical and chemical processes have been suggested for the recovery of bioactive compounds from different sources, including microalgae. The most widely utilized in the literature include maceration, infusion, decoction, Soxhlet extraction, and hydrodistillation[131], [136]. In order to maximize yield and get pure and high-quality substances, the choice of the extraction method always depends on the nature and polarity of the target compound. Typically, conventional extraction methods have low processing cost, and they are easy in operation. However, due to several phases in the system, a mass transfer resistance might occur, which limits some conventional extraction techniques as Soxhlet[68].

Moreover, conventional techniques require longer extraction time, do not require high purity solvents, they show lower extraction yield, have decreased selectivity, and may cause thermal decomposition of bioactive compounds. Above all, most of the organic solvents used in conventional extraction techniques are flammable and volatile, energy-intensive, cause environmental pollution, contribute to the greenhouse effect, climate change, and can harm people[140]. That is why, due to these limitations and the growing need for producing bioactive compounds novel, cost-effective, fast, eco-friendly, and safe methods are needed to be implemented.

1.3.2. Supercritical fluid extraction (SFE)

The first characterization of supercritical fluid behavior was reported by *Hannay and Hogarth* in 1879[143]. A substance behaves as a supercritical fluid when its pressure and temperature increase above its critical point. Under these conditions, the supercritical fluid has a similar viscosity to a gas,

the density becomes similar to a liquid, and its diffusivity is an average between gas and liquid. Because of high diffusivity and low viscosity, supercritical fluids can extract more compounds and exhibit wider transport properties compared to conventional solvents[131]. Supercritical fluid extraction is now widely applied to recover a variety of bioactive compounds from food and plant sources. Carbon dioxide has a Generally Recognized as Safe (GRAS) status in the food industry. Supercritical carbon dioxide extraction (SFE-CO_2) has also been suggested for the recovery of high-value compounds from micro- and macro algae (*Rhodophyceae, Phaeophyceae, Chlorophyceae*)[100]. SFE-CO_2 typically results in high extraction yields and fast extraction rate, also with minimal or no consumption of toxic organic solvents, which makes it a green and sustainable extraction method with no harmful effect on people and the environment[4]. Other solvents as propane, butane, dimethyl ester were also introduced for usage in SFE. Despite their recognized toxicity, the quantities used during SFE extractions are too low compared to other extraction techniques[75], [133].

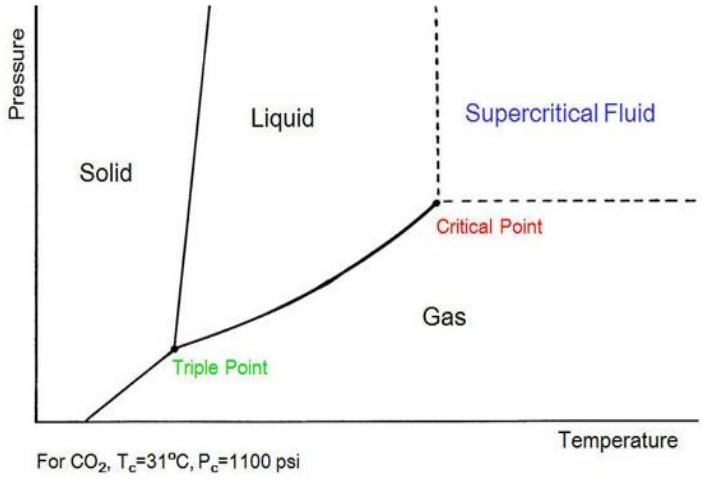


Figure 1.7. Phase diagram for carbon dioxide[40]

At room temperature CO_2 turns back to gas, so obtained extract is entirely solvent-free. Moreover, supercritical conditions for carbon dioxide are relatively low, with a critical temperature of 31.2°C and pressure 7.38 MPa[132]. Properties of gas, liquid, and supercritical fluid are presented in **Table 1.2**.

Table 1.2. Comparison of total oil yield of high-value metabolites extracted from different microalgae through SFE, PLE and MAE

	Density (g/mL)	**Diffusivity (cm^2/s)**	**Dynamic Viscosity (g/cm s)**
Gas	10^{-3}	10^{-1}	10^{-2}
Liquid	1.0	$5*10^{-6}$	10^{-4}
Supercritical Fluid	$3*10^{-1}$	10^{-3}	10^{-2}

The schematic diagram of the supercritical CO2 extraction process is shown in **Figure 1.8**. The system consists of a CO_2 pump, supply pump, extraction column, modifier pump, and co-solvent pump (if necessary), pre-heat and mixture vessels, and pressure release valves. Also, the system contains pressure and temperature controllers and, if needed, one or several fractionation cells. Due to the high cost of CO_2, a recycling system is usually added[104].

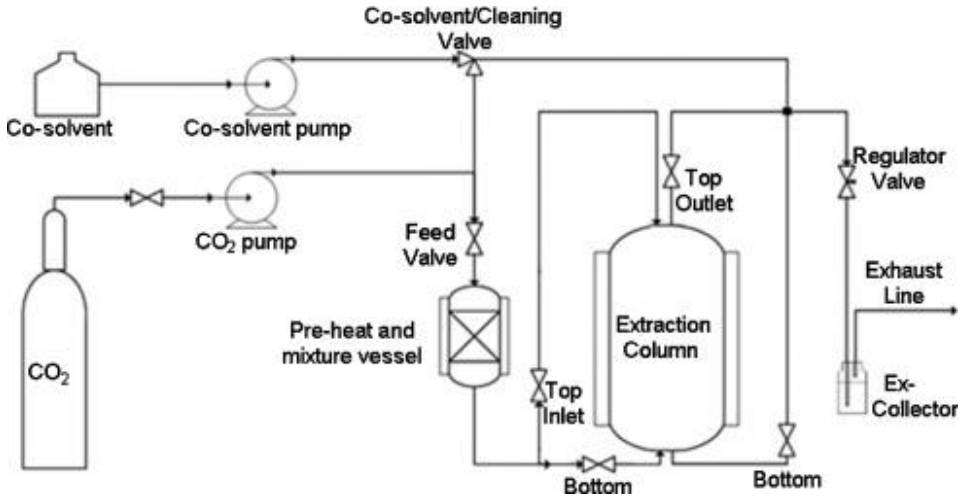


Figure 1.8. Schematic diagram of supercritical CO_2 extraction process. Adapted from Martins et al.[104]

Because supercritical carbon dioxide is a highly non-polar solvent, in certain cases, there is a requirement to introduce some modifiers, also called co-solvents, to the system to increase solubility and allow the extraction of more polar compounds such as xanthophylls, phenols, and flavonoids. The most common co-solvents are methanol and ethanol. In microalgal extraction, the addition of small amounts of ethanol helped to swell the algal matrix[100]. Moreover, carotenoids containing hydroxyl groups are extracted more efficiently because of forming hydrogen bonds with ethanol[18], [71], [99]. As a result, decreased CO_2 density by ethanol leads to higher carotenoid yield compared to pure CO_2[79]. On the other hand, pure CO_2 exhibits higher selectivity compared to combination with co-solvents, which can lead to extraction of not only non-polar fat components and carotenoids but also polar compounds that sometimes can be undesired. Particle size also contributes to the mass transfer and extraction yield, so in order to increase extraction efficiency, deflocculating agents, and crushing of material can be used[109], [153].

Temperature and pressure have a significant effect on SFE extraction kinetics. By applying different conditions, the solvent's characteristics such as density, viscosity, and diffusivity can be tuned. As a result, dissolving properties of the fluid can be controlled depending on the nature of the target compounds. With pressure increasing to its critical point, the solubility of extracting substances increases as well, and the temperature increases. When the extraction process runs under high pressure, the temperature decrease leads to increasing the solubility of target compounds[131]. Overall, according to studies on SFE extraction of carotenoids from microalgae, raising the temperature while pressure was low has led to lower extraction yield due to decreased density of CO_2 and carotenoids' solubility. However, if the carotenoid is sufficiently thermostable, further raising of pressure and temperature results in higher extraction yields[18], [20], [79], [99], [125], [161] Extraction efficiency is also determined by polarity, solubility, chemical and thermal stability of the extracting material. Generally, for carotenoid and lipid compounds extraction from microalgae, pressure ranges from 10 to 30 MPa, and temperature ranges of 40 to 50 ºC are being used[66].

Advantages of SFE: Compared to other extraction techniques, the main advantage of SFE is high extraction efficiency, which results in higher yields within a relatively short time. Because the fluid is continuously moving through the material, complete or quantitative extraction of compounds is ensured. SFE extraction is easily controlled by changing temperature, pressure, or modifiers used. Dissolved solutes can be depressurized, and the concentration process can be avoided if pure CO_2 is used. Because of the possibility of extraction under low temperatures, thermolabile compounds can

also be extracted. CO_2 used as a solvent can be recycled, it is recognized as GRAS, and extracts are free from residuals solvents. SFE can be applied from a laboratory scale to pilot plant and industrial-scale production[132].

Disadvantages of SFE: Because of CO_2's non non-polar nature, the quantitative extraction of polar substances from solid matrices can be difficult. Meanwhile, the addition of modifiers decreases selectivity[67], [101]. Deficient interaction between material and supercritical CO2 can occur. SFE requires high investments and operation costs because high pressure is used during the extraction process. Moreover, safety measures should be considered while working with high-pressure techniques to avoid damage to people and the environment[95], [129].

1.3.3. Pressurized liquid extraction

The first use of pressurized liquid extraction was mentioned in 1996 by Richter *et al*.[96]. PLE is also known as accelerated solvent extraction, enhanced solvent extraction, high-pressure solvent extraction, and pressurized fluid extraction. The higher extraction efficiency is achieved when high pressure brings solvents above the temperature of its boiling point, as the high temperature increases mass transfer rate and solubility [97,98]. Also, raising the temperature helps to obtain more effective matrix penetration and, as a result, better extraction rate due to reduced surface tension and viscosity of solvents. Because the amount of solvents used in PLE is much lower as compared to other extraction techniques, it is generally recognized as a green technique [99].

A typical PLE system is presented in **Figure 1.9.** It consists of solvent reservoirs, solvent pump, nitrogen tank, heating oven and pumps, pressure relief valve, extraction cell, collection and waste vessels, temperature, and pressure controllers. The prepared material is introduced to the extraction cell. The cell is filled with the heated solvent, and pressure and temperature are set to enhance the extraction. Extraction can be static, dynamic, or combined. Static mode allows extracting more analytes, while dynamic mode has increased mass transfer. When the extraction is finished, the excess solvent is removed by purging nitrogen into the system. Afterward, the extract is collected into the vial. In order to prevent over-pressurization of the cell, a pressure valve is installed[72]. Time of extraction, number of cycles, type of solvent, temperature, and pressure can be changed depending on the material and desired to extract compounds and yield. The rate of extraction depends on the nature of the material and location of bioactive compounds within the matrix[55].

The selection of solvent strictly controls the nature of the recovered analytes. Unlike SFE, PLE can easily extract both polar and non-polar substances. The most common solvents used in PLE are acetone, ethanol, water, or mixture of them. Hexane can also be used to extract lipophilic compounds. Also, methane, propane, ethyl acetate, dichloromethane and ionic liquids can be used[62], [117], [119]. In cases when hot water is used as the only solvent to replace other organic solvents, the process is called pressurized hot water extraction or subcritical water extraction[35].

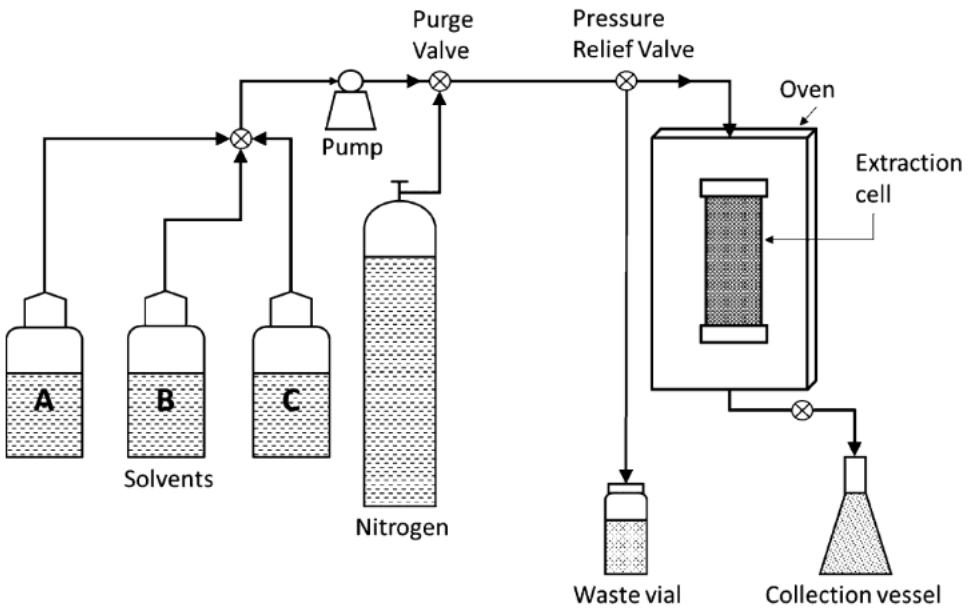


Figure 1.9. Schematic diagram of pressurized liquid extraction. Adapted from Kadam et al.[131]

PLE has been used for the recovery of lipids and a wide range of bioactive substances from algae and microalgae. Generally, the temperature in PLE varies from 50 to 200°C, and the pressure range is 3.5−20 MPa. PLE extraction of zeaxanthin from *Chlorella ellipsoidea* was performed using ethanol, hexane and 2-propanol as solvents[82]. The same method was used for zeaxanthin recovery from *Chlorella vulgaris* with ethanol. Hexane and 2-propanol, required higher temperatures to obtain the same result[22]. Rodriguez-Meizoso *et al.* studying carotenoid recovery from *Phormidium* sp. with PLE using ethanol, also observed that under the temperature range of 50–100°C and the pressure of 10.3MPa extraction yields increased[123]. *P. tricornutum* was also studied for fucoxanthin recovery using PLE with optimum conditions of 50°C, 100% EtOH and 20 min extraction time. PLE showed a higher fucoxanthin extraction yield as compared to MAE[47].

Advantages of PLE. Compared to other extraction techniques, PLE requires much less time to perform one extraction. Moreover, it drastically reduces the amount of solvent used for extraction. It also allows us to use a wide range of solvents and have a fully automated process with the possibility to control extraction parameters[134].

Disadvantages of PLE. Because of the high temperatures used in extraction, PLE is not the best option to recover thermolabile compounds. It also does not have high selectivity. As well as SFE, PLE should be carefully monitored upon using for safety reasons because of the high pressure used throughout the process[65].

1.4. Processing and technology of *P. tricornutum*

P. tricornutum has been mostly targeted as a feedstock of bioactive compounds such as PUFAs and fucoxanthin. The most common extraction techniques applied were SLE and PLE using n-hexane, acetone, ethanol and water, and SFE-CO_2. Kim *et al.* has conducted research using MAE, UAE, PLE, and Soxhlet extraction with a range of solvents as hexane, acetone, ethanol, water, and mixes in different proportions. Moreover, carotenoid content was evaluated using HPLC (high performance liquid chromatography), and EPA content was compared with several micro- and macroalgae

species. The major carotenoid in *P. tricornutum* was found to be fucoxanthin, followed by several chlorophyll-type *a*, c_1, c_2 carotenoids and diatoxanthin The highest fucoxanthin content was obtained by using acetone and ethanol as solvent. There was no statistically significant difference between fucoxanthin yield in the different extraction methods, although the highest yield (16.51 mg/g DW) was under PLE extraction. Also, fucoxanthin yield increased with increasing the pressure and gradually decreased with increasing the temperature[47], [77].

Gilbert-López *et al.* have optimized PLE and MAE using ethanol, water alone or in different ratios for the recovery of bioactive compounds from *P. tricornutum*. In general, the extracts' composition was similar in both extraction techniques. However, extraction yield, carotenoids, and PUFAs recovery were shown to be higher in PLE[47]. Only one recent research has reported data on the extraction of *P. tricornutum* and several other microalgal biomass using supercritical CO_2 and subcritical n-butane. Extraction conditions of SFE-CO_2 for *P. tricornutum* were 30 MPa, 40°C, extraction time was 2 hours and CO_2 flow rate was 9mL min^{-1}. The maximum lipid extraction yield was 14.7 mg g^{-1} DW with further evaluation of extracts on fatty acids profile and total carotenoid content. As it was summarized, SFE-CO_2 has the potential to be commercially applied for fucoxanthin production from *P. tricornutum*[56], [85].

Currently, there is lack of data in the literature on PLE extraction potential and influence of different conditions on *P. tricornutum* biomass, with additional evaluation of extracts for antioxidant capacity, total phenolic content, fatty acids and carotenoids, as well as other possible bioactivities. Moreover, there is no data on the optimization of SFE-CO_2 for the diatom with further integration of the biorefinery concept. Thus, the optimization of SFE-CO_2 and further biorefinery of *P. tricornutum* biomass applying PLE to the SFE-CO_2 residues was performed in the current research.

1.5. Biorefinery concept

Microalgae biomass consists of a wide range of valuable compounds, which makes it a suitable feedstock for producing bio-products such as nutraceuticals, pharmaceuticals, as well as a potential biodiesel resource[74]. Nowadays, the main challenge is high production costs because of the expenses linked with microalgae cultivation. The key issue is in downstream autotrophic cultures and the impossibility of simultaneously optimizing process recovery which accounts for 40% of the total cost. This happens mainly due to the diluted nature of the multiple microalgal components and the challenges associated with their separation from the biomass[46]. The main bottlenecks in microalgae production are enhancing the growth rate and product synthesis of microalgae, dewatering the cultivation of algae for the production of biomass and biomass pretreating[73]. Thus, integration of the biorefinery concept could provide an opportunity to significantly decrease production costs by producing multiple components from microalgae biomass[151].

The idea of microalgae biorefinery is not recent and has been discussed for several years now. The concept implies the conversion of biomass into several high-cost products by sustainable production with minimal waste and maximum utilization of raw materials, which makes the whole process cost-effective[30]. **Figure 1.10** illustrates possible algae biorefinery pathways. Pressurized fluids can be coupled with the production, so yield is expected to be much higher compared to conventional production, and by using green techniques, no damage to people and the environment will be caused.

Moreover, as it was reported the current microalgal biomass production cost which is about \$4.92 kg^{-1}, is expected to be reduced down to \$0.50 kg^{-1} within a biorefinery approach [84].

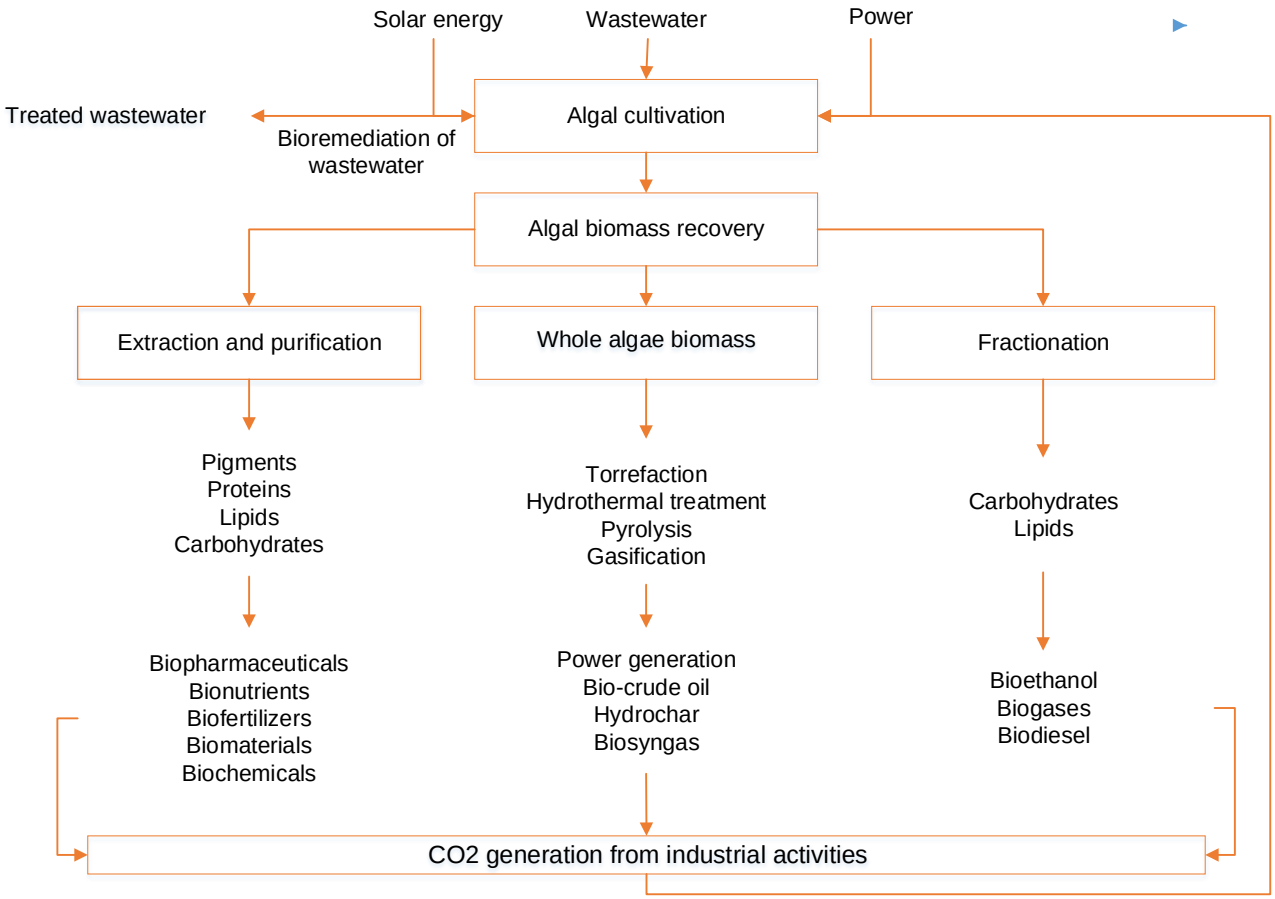


Figure 1.10. Possible algae biorefinery pathways. Adapted from [74]

For now, there are only a few studies on the biorefinery concept for *P. tricornutum*[12], [52]. Considering this fact, current research was conducted to investigate bioactivity of high-value compounds derived from *P. tricornutum*, optimize the SFE-CO_2 extraction with further application of PLE on solid residues to maximize full recovery of compounds.

2. MATERIALS AND METHODS

2.1. Algal material

P. tricornutum freeze-dried biomass (Phytobloom Prof Phaeodactylum ®) was obtained from Necton SA, Portugal. The material was stored in the freezer (-18 ℃) until further analysis.

2.2. Chemicals and reagents

Acetone, Analytical/HPLC grade hexane, 2,2'-azinobis-3-ethylbenzothiazoline-6-sulfonic acid radical cation (ABTS●+, 99 %), catalytic tablet (K2SO4, CuSO4), Conc. H_2SO_4, NaOH, H_3BO_4, Na_2CO_3, 2,2-Diphenyl-1-picrylhydrazyl hydrate (DPPH●, 99%), 3,4,5-trihydroxybenzoic acid (gallic acid, 99 %), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, 97 %), analytical/HPLC grade methanol (Sigma-Aldrich, Poole, UK), hexane (PENTA Chemikalien, Mainaschaff, Germany), Folin-Ciocalteu's phenol reagent 2M, nitrogen liquid(AGA SIA, Riga, Latvia), carbon dioxide, nitrogen gases (99.9%, Gaschema, Jonava region, Lithuania), cotton-wool (Bella-cotton, Poland), microcrystalline cellulose 20 μm (Sigma-Aldrich, St. Louis, MO, USA), NaCl, KCl, KH_2PO_4, $K_2S_2O_8$ (Lach-Ner, Brno, Czech Republic), Na_2HPO_4 (Merck KGaA, Darmstadt, Germany), sodium carbonate (99.5 %, AppliChem, Darmstadt, Germany), ultrapure water obtained by Millipore purification system (Billerica, MA, USA), ethanol (99.5%) (VWR Chemicals, Fontenay-sous-Bois, France), commercial β-carotene and fucoxanthin standards (Sigma-Aldrich, USA), ASE filters (Glass Fiber (X) Cellulose and diatomaceous earth (100% SiO2) from Dionex Corporation (Sunnyvale, CA, USA), ethanol (99.5%) (VWR Chemicals, Fontenay-sous-Bois, France).

2.3. Determination of chemical composition

2.3.1. Determination of Ash Content

P. tricornutum freeze-dried biomass 1±0.1 g was placed in a dry, constant weight crucible. Experiments were performed in triplicates. Crucibles with ground samples were placed on the electric hotplate and heated until the smoke stopped to form. After this, crucibles with samples were transferred and kept in the muffle under 600-650 ℃ temperature. Ash (mineral) content (%) was calculated using **Equation 2.1** below and expressed in a g/100 g of dry weight (DW):

$$(k1-k2)/(k1-k) * 100, \text{ g/100g of DW; (Eq. } \mathbf{2.1}\text{)}$$

where: k – the weight of the crucible, g; k1 – the weight of the crucible with the sample before drying, g; k2 – the weight of the crucible with the sample after drying, g.

2.3.2. Determination of oil content by Soxhlet-Hex extraction

P. tricornutum freeze-dried biomass was previously manually crushed in a mortar for 5 min. Afterward, 2±0.01 g were placed in cellulose extraction thimbles. Soxhlet extraction was performed in automated Soxhlet extractor EZ100H (Behr Labor-Technik, Düsseldorf, Germany). Cellulose extraction thimbles were inserted into an inner tube of the Soxhlet extraction apparatus for the extraction. All extractions were performed in triplicates, and the non-polar fraction was isolated using hexane solvent. Total extraction time was 360 min, extraction rate one cycle was 3 min, and the

temperature was 80º C. Hexane was evaporated in a Büchi V-850 Rotavapor R-210 (Flawil, Switzerland). After the organic solvent evaporation, extracts were kept under nitrogen flow for 10 to 15 min to evaporate residues of hexane. Extract yields were determined gravimetrically (±0.001 g) and expressed as a % of dry weight.

2.3.3. Determination of nitrogen content by Kjeldahl method

P. tricornutum freeze-dried biomass was weighed (of 1±0.01 g) to the special Kjeldahl flask. 20 ml of concentrated $H2SO_4$ was added, as well as the tablet of catalyst (K_2SO_3 3.4 g, $CuSO_4$ 0,4 g), and the mixture was heated for 2 hours until it became transparent. The heating intensity was 60%. Then the solution was distilled using an automatic steam distillation system under the following conditions – 3 s NaOH, 3 s H_3BO_4, the time of distillation was 300 min, the intensity of the steam was 80 %. After distillation, the solution was collected into the flask, followed by the addition of the Toshiro indicator and titration with a 0,1 N HCl solution until the color changes from light green to violet. 20 ml concentrated H_2SO_4 was used as a control sample and analyzed in the same manner as described above. The nitrogen content was calculated using the following **Equation 2.2** (with expression in a percentage):

$$\%N=(VA-VB) * NHCl * 1{,}4007/W; \text{ (Eq. } \mathbf{2.2}\text{)}$$

where: VA – volume of standard HCl required for the sample, ml; VB - volume of standard HCl required for the blank, ml; NHCl – normality of acid standard; 1,4007 – miliequivalent weight of N*100; W – weight of the sample, g.

2.3.4. Determination of water content

The weight of 2±0.05 g of *P. tricornutum* freeze-dried biomass was placed in a dry, constant weight glasses with a cap and rod. Experiments were performed in duplicates. Glasses with a cap, rod, and samples were weighted gravimetrically (±0.001 g), periodically stirred, and dried in the oven at the 100-105 °C temperature. After every 24 h, samples were cooled in the desiccator for 30 minutes and then weighted gravimetrically (±0.001 g) until the constant weight. After 48h of drying, the water content (%) was calculated using **Equation 2.3** below and expressed in a g/100 g of dry weight (DW).

$$X=(m1-m2)/(m1-m) * 100; \text{ g/100 g of DW; (Eq. } \mathbf{2.3}\text{)}$$

where: m – weight of the glass with a cap and rod, g; m1 – weight of the glass with a cap, rod and the sample before drying, g; m2 – weight of the glass with a cap, rod and the sample after drying, g.

2.4. Conventional extraction techniques. Solid-liquid extraction (SLE)

Solid-liquid stepwise extractions with different solvents (hexane, acetone, ethanol, and water) were performed in a thermostatically controlled shaker. All extractions were performed in triplicates. *P. tricornutum* freeze-dried biomass with a weight of 10g was loaded into dry plastic bottles. 10 ml of hexane was poured on the biomass. After each step fresh solvent was added at the ratio of 1:1 (g biomass: ml of solvent). Bottles with mixture were shaken at 900 rpm. Every extraction took 360 min and was performed at 40°C. After each extraction step, the extraction bottles were cooled down, centrifuged (9000 rpm, 10 min), and the mixture was filtered (Whatman filter paper 1). All the organic solvents were evaporated in a Büchi V-850 Rotavapor R-210 (Flawil, Switzerland), and water extracts

were freeze-dried. After organic solvent evaporation, extracts were kept under the nitrogen flow for 15 min to evaporate residues of organic solvents. All extracts were kept at -20 ºC protected from light until the next analysis was performed. SLE-HEX, SLE-AC, SLE-EtOH, SLE-H_2O extracts yields were determined gravimetrically (±0.001 g) and expressed in a % of dry weight DW.

2.5. Isolation of bioactive compounds from *P. tricornutum* by high-pressure extraction techniques

2.5.1. Lab-scale supercritical CO_2 extraction (SFE-CO_2)

Supercritical CO_2 extraction (SFE-CO_2) was performed in a Helix extraction system (Applied Separation, Allentown, PA, USA) using 2.500 ± 0.001 g of *P. tricornutum* freeze-dried biomass (previously manually crushed in a mortar for 5 min), which was placed in a 50 mL cylindrical extractor (14mm inner diameter and 320mm length) between two layers of the cotton wool to avoid particle carry-over to the system. The surrounding heating cover controlled cylindrical extractor temperature. The biomass was extracted at the following conditions: pressure 150–450 bars, temperature 30–70 °C, the flow of EtOH as a co-solvent 5–10%, dynamic extraction (continuous flow of supercritical CO_2) was constant – 90 min. The static extraction was conducted with pressurizing supercritical CO_2 for 10 min in a sealed extraction vessel prior to each dynamic extraction.

2.5.2. Pressurized liquid extraction (PLE)

Pressurized liquid extraction (PLE) was performed in ASE-350 extraction system (Thermo Scientific Dionex, Sunnyvale, CA, USA) 3.000 ± 0.001 g of *P. tricornutum* residual biomass after SFE-CO_2 optimal conditions, which were mixed with 7.500 g quartz sand (1/2.5, w/w) and placed to 66 mL stainless-steel extraction cells with cellulose filters in each end, before to the extraction experiments[47]. The biomass was stepwise extracted with acetone and ethanol at the following conditions: temperature 40–100°C pressure 103 bars (constant for ASE-350), dynamic extraction time 15–45 min (5, 10 and 15 min×3 cycles), pre-heating time 5 min, cell flush volume 50% and purge time 120 s with nitrogen to collect the extracts in the vials. Selection of temperature higher than 100°C was avoided, as it may damage sensitive bioactive compounds such as carotenoids and induce oxidation of polyunsaturated fatty acids, while extraction time was based on previously performed studies demonstrating that it is sufficient for the recovery of carotenoids and polyunsaturated fatty acids from microalgae[47]. All the organic solvents were evaporated in a Büchi V-850 Rotavapor R-210 (Flawil, Switzerland), and water extracts were freeze-dried. After organic solvent evaporation, extracts were kept under the nitrogen flow for 15 min to evaporate residues of organic solvents. All extracts were kept at -20 ºC protected from light until the next analysis was performed. SLE-HEX, SLE-AC, SLE-EtOH, SLE-H_2O extracts yields were determined gravimetrically (±0.001 g) and expressed in a % of dry weight DW.

2.5.3. Experimental design

Central composite design (CCD) and response surface methodology (RSM) were used for the experimental design setup of SFE-CO_2. For SFE-CO_2, three independent variables and their variation levels were chosen, namely pressure (150–450 bars), temperature (30–70 °C), and the amount of ethanol as co-solvent (5–10%). The response factors (RF) were the total yield of SFE−CO_2 extract,

total carotenoid content, and amount of fucoxanthin. The complete design consisted of 18 experimental runs (8 factorial, 6 axial, and 4 center points) and was established using the Design-Expert 12 software (Stat–Ease Inc., Minneapolis, MN). The multiple regression equation was applied in order to fit the second-order polynomial equation, expressing the yield of SFE-CO_2 extract as a function of independent variables. Values were evaluated using version 8.0.7.1 of the Design-Expert trial program. Model statistical significance and each variable were calculated using the Student test (p-value) at a likelihood level of 5% ($p<0.05$). The suitability of the model was determined by comparing the coefficient of 'lack of fit' and the obtained Fisher test value (F-value).

PLE was applied to SFE-CO_2 optimal conditions residues. Central composite design (CCD) and response surface methodology (RSM) were used to design the experiment. For PLE, two independent variables and their variation levels were chosen – time per cycle (5–15min) and temperature (40–100°C). Total yield, total carotenoid content and total phenolic content were chosen to be response factors. The complete design consisted of 12 experimental runs (in duplicates) and was established using the Design-Expert trial version 8.0.7.1 software (Stat–Ease Inc., Minneapolis, MN). All models were evaluated considering the percent variation explained by the residual standard deviation (RSD), determination coefficient (R^2) and lack-of-fit test for the model from the analysis of variance table, as the significance criteria. Response surfaces were obtained by accepting significances at $p \leq 0.05$, by the combination of two experimental factors, looking to maximize the yield and total carotenoid content. Biorefining scheme for *P. tricornutum* is shows on the **Figure 2.3.**

2.6. *In vitro* antioxidant capacity

2.6.1. Measurement of total phenolics content (Folin-Ciocalteu assay)

Total phenolics content (TPC) of the SLE and SFE-CO_2 residual biomass and extracts of *P. tricornutum* were determined by using the Folin-Ciocalteu's assay with some modifications[26]. The working solution was prepared by diluting commercial Folin-Ciocalteu's reagent in distilled water (1:9, v/v). 750 µL diluted Folin-Ciocalteu's solution was mixed with 150 µL of the sample. MeOH was used for the blank. After 3 minutes, 600µL of Na_2CO_3 (75g/L) was added into the solution, left in the dark for 120 min at 25 °C. For QUENCHER approach TPC of the initial residual biomass after SLE and SFE-CO_2 was measured by its direct application to the solid particles[130]. Microcrystalline cellulose was used as blank. 750 µL diluted Folin-Ciocalteu's solution was mixed with 10 mg of sample and 150 µL distilled water in a test tube. After 3 minutes, 600 µL of Na_2CO_3 (75g/L) was added to neutralize the mixture, shake in the dark for 120 min at 25 °C, centrifuged at 4500 rpm 5 min. Absorbance was measured at 760 nm with Spectronic Genesys 8 spectrophotometer (Thermo Spectronic, Rochester, NY, USA).

Gallic acid solutions were used to obtain the calibration curve in the 20µg/ml to 80µg/mL concentration range. Extracts (**Equation 2.4**) and QUENCHER (**Equation 2.5**) calibration curve equations:

$$f(x)=0.0115x+0.034;\ R^2=0.9991;\ \text{(Eq. 2.4)}$$

$$f(x)=0.012x+0.0147;\ R^2=0.9999;\ \text{(Eq. 2.5)}$$

TPC was expressed in an mg of gallic acid equivalents (GAE) per g of extract or DW of the material. All analysis was performed in six replicates.

2.6.2. $ABTS^{\bullet+}$ cation radical assay

The $ABTS^{\bullet+}$ cation radical assay was adapted from Re et al. with some modifications[3]. The phosphate-buffer saline (PBS; 75 mmol/L, pH 7.4) was prepared by dissolving 8.18 g NaCl, 1.42 g Na2HPO4, 0.27 g KH_2PO_4, 0.15 g KCL in 1 L distilled water. The $ABTS^{\bullet+}$ radical solution was prepared by reacting 50 mL of $ABTS^{\bullet+}$ (2 mmol/L PBS) and 200 L $K_2S_2O_8$ (70 mmol/L) and left for 15-16 hours in the dark at 25 °C before use. Afterward, the working solution was prepared by diluting $ABTS^{\bullet+}$ radical solution with PBS to obtain 0.700 (±0.01) AU at 734 nm. 1500 L of $ABTS^{\bullet+}$ solution was mixed with 25µ L of *P. tricornutum* SLE and SFE-CO_2 extracts or MeOH (blank) in an Eppendorf vial and left for 2 hours in the dark at 25 °C.

QUENCHER. For SLE and SFE-CO_2 solid residues quencher analysis, 10 mg of sample or cellulose (blank) was mixed with 1500µL of $ABTS^{\bullet+}$ solution and 25 µL MeOH, vortexed for 15 s, shaken at 250 rpm for 2 hours in the dark at 25 °C, centrifuged at 4500 rpm 5 min. Absorbance was measured at 734 nm with Spectronic Genesys 8 spectrophotometer (Thermo Spectronic, Rochester, NY). Trolox solutions (25µL) at various concentrations (0-1500 mmol/L) were used to obtain the calibration curve. The following extracts (**Equation 2.6**) and QUENCHER (**Equation 2.7**) calibration curve equations were obtained:

$$f(x)=0.058x-0.4155;\ R^2=0.9978;\ \text{(Eq. } \mathbf{2.6}\text{)}$$

$$f(x)=0.0545x+3.6261;\ R^2=0.9945;\ \text{(Eq. } \mathbf{2.7}\text{)}$$

TEAC of samples was calculated through dose-response curves for Trolox. Results were expressed as TEAC values are expressed in mg of Trolox per g of extract or DW of plant material. All analysis was performed in six replicates.

2.6.3. $DPPH^{\bullet}$ radical scavenging assay

$DPPH^{\bullet}$ radical scavenging assay method was adapted from Brand-Williams et al. with some modifications[94]. The working solution was prepared by mixing 1000 L $DPPH^{\bullet}$ methanolic solution (~ 89.7 mol/L, final absorption 0.800 ± 0.1 AU at 517 nm) and 500µ L of SLE extracts or MeOH (blank). The mixtures were kept for 2 hours in the dark at 25 °C.

QUENCHER. For initial biomass material, SLE residues and SFE-CO2 quencher analysis 10 mg of sample or cellulose (blank) was mixed with 500 L MeOH and 1000 L 89.7 mol/L $DPPH^{\bullet}$ methanolic solution, vortexed for 15 s, shaken at 250 rpm for 2 hours in the dark at 25 °C, centrifuged at 4500 rpm 5 min. The absorbance of all the samples was measured at 517 nm with Spectronic Genesys 8 spectrophotometer (Thermo Spectronic, Rochester, NY, USA). Trolox solutions (500 L) in various concentrations (0-50 mol/L MeOH) were used to obtain the calibration curve. Extracts (**Equation 2.8**) and QUENCHER (**Equation 2.9**) calibration curves equitation:

$$f(x)=1.5798x+0.0986;\ R^2=0.986;\ \text{(Eq. } \mathbf{2.8}\text{)}$$

$$f(x)=1.4312x-1.6651;\ R^2=0.9979;\ \text{(Eq. } \mathbf{2.9}\text{)}$$

TEAC of samples was calculated using dose-response curves for Trolox. Results were expressed as TEAC values in mg of Trolox per g of extract or DW plant material. All analysis was performed in six replicates.

2.7. Chemical characterization of *P. tricornutum*

2.7.1. Chemical characterization of carotenoids by HPLC– DAD

The carotenoid profile of *P. tricornutum* extracts was determined by HPLC–DAD (high-performance liquid chromatography with diode-array detector) according to a method previously described[21]. HPLC analyses of the extracts were conducted using an Agilent 1200 series liquid chromatograph (Santa Clara, CA, USA) equipped with a diode-array detector. Reversed-phase column was Agilent, Eclipse Plus C18 (5.0 µm; 3.0 x 250 mm), the detection was performed at 440 and 460 nm, spectra recorded in the range of 350 to 600 nm. Mobile phase: A - acetone: water (75: 25, v/v) and B - acetone: methanol (75: 25, v/v): 0-25% B to 10 min, then up to 100% B to 35 min, 100% B to 45 min, 0% B to 65 min, post-time 15 min. The flow was 1.5 ml/min, and the column temperature was 30 °C, with an injection volume of 10 µl. For the calibration curve, six different concentrations of fucoxanthin in ethanol, ranging from 0.005 to 0.05 mg/mL, were analyzed using the HPLC–DAD instrument.

2.7.2. Analysis of fatty acid composition by means of GC-FID

Fatty acid methyl esters were prepared from three non-polar extracts (Soxhlet, SFE-CO_2, and PLE-acetone) using a method based on 14% boron trifluoride/methanol solution, as recommended method for this type of substrates[64].

Based on the Park and Goins method[113], the lipid extraction was omitted and in situ transesterification (ISTE) was performed by heating lipid-containing samples at 90 °C for 10 min after adding 0.5 N NaOH in methanol for methanolysis and continued heating another 10 min for further methylation after adding 14% BF_3 in methanol. Nitrogen gas was used for drying and removing solvents from fatty acid methyl esters. Obtained samples were analyzed by a GC Agilent 7890A system with FID, auto-injection module for liquid, equipped with fused silica capillary column (SP-2560, 100 m x 0.25mm, I.D., 0.20 µm). Helium was used as a carrier gas (purity > 99.9997 vol %, flow rate = 1.26 ml/min). The fatty acids peaks were identified by comparison of retention times with retention times of standards from Supelco 37 component FAMEs mix and with data from an internal data library, based on previous experiments. Results were expressed as a percentage of the total GC-FID peak area.

2.7.3. Evaluation of the antimicrobial activity of extracts using the disc-diffusion assay and determination of minimal inhibitory concentration (MIC)

P. tricornutum extracts after SFE-CO2, two-step PLE (acetone, ethanol) and SLE (hexane, acetone, ethanol, water) in duplicates were evaluated for their antimicrobial effects against eight pathogenic bacteria (*B. cereus, S. aureus, L. monocytogenes, E. coli, Salmonella spp, E. faecalis, P. aeruginosa* and fungus *Aspergillus niger*). Bacteria were streaked onto Lysogeny broth (LB) agar plates, which were subsequently incubated at 28 °C for 24 h. The bacterial solution was uniformly spread onto the LB agar plates using sterile glass beads. 7mm GF/F filters (Whatman, Maidstone, United Kingdom)

were put onto the LB agar plates seeded with the bacteria. 30 µL of each extract, with a concentration of 1mg/mL (MeOH), were pipetted onto the filters with an equivalent volume of methanol (and water for SLE-water extract) as a negative control. After 24 h of incubation at 28 °C, the diameters of inhibition zone were evaluated, and extracts that showed antimicrobial activity were selected for MIC analysis.

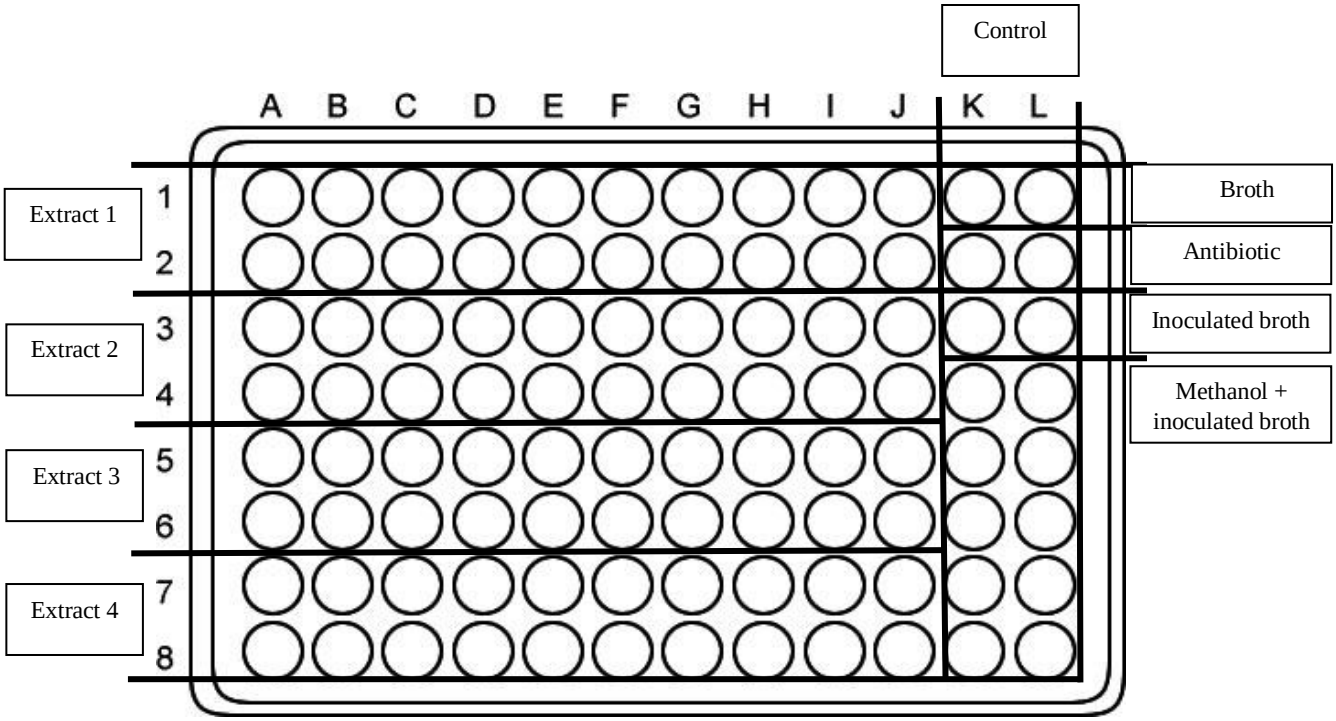


Figure 2.1. Scheme of microdilution plate

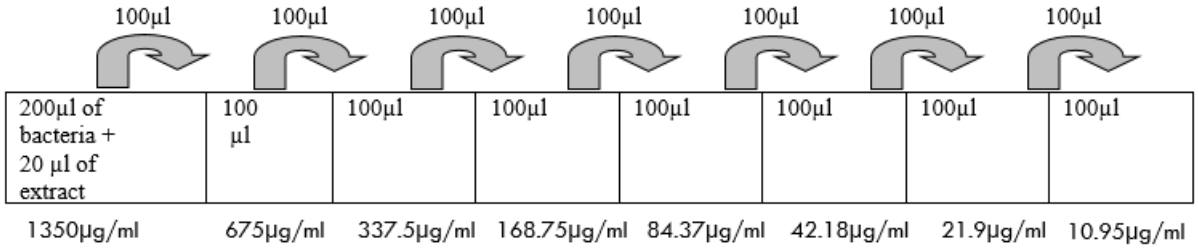


Figure 2.2. Order of dilution

Microdilution method. In the first column (A) 200 µL of each bacterium were added, and to all other columns (B-J) 100 µL of bacteria were added. After, 20 µL of each extract was added to column 1 (A). A series of double dilutions by mixing 100 µL from row A to row B, from row B to row C, etc. were made. Finally, excessive100 µL from the last row E was removed. For the control, 100 µL of non-inoculated broth was added to row 1 of fields K and L. In row 2 100 µL of antibiotic + 100 µL of inoculated broth was added. 100 µL of inoculated broth was added to row 3 (to evaluate growth control without extract). To the row 1, 100 µL of agar broth was added to the appropriate places for the negative control, and 100 µL of bacteria + 10 µL of methanol were added for solvent control.

2.8. Statistical analysis

GraphPad Prism 8 software was used for a one-way analysis of variance (ANOVA), followed by the Tukey's test in order to compare mean values and their significance (p-value < 0.05). Microsoft Excel 2016 was used for calculating mean values and standard deviations.

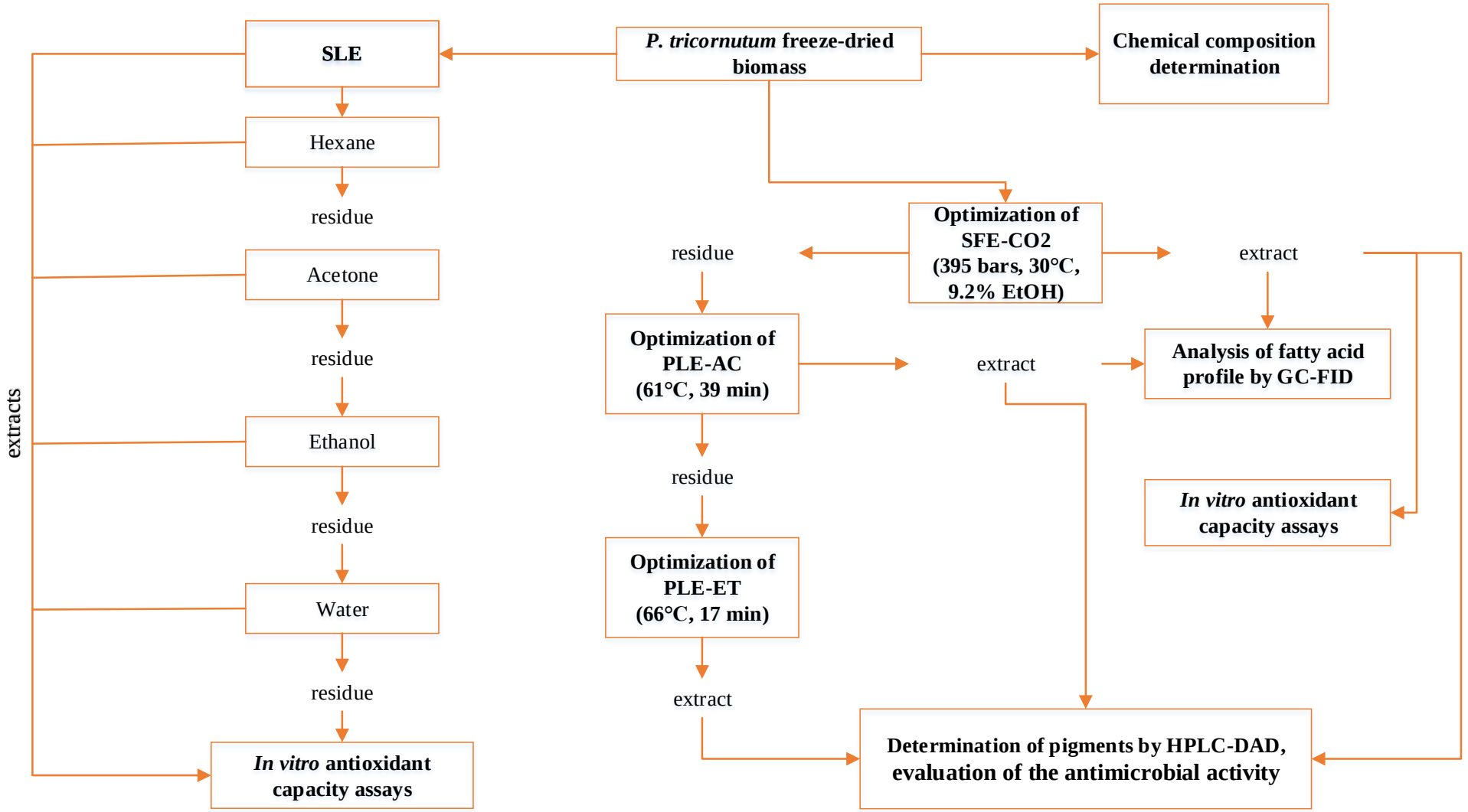


Figure 2.3. Biorefining scheme for *P. tricornutum*

3. RESULTS AND DISCUSSION

3.1. Chemical composition

During the first step of the research chemical composition of microalgae, *P. tricornutum* was determined. The results are presented in **Table 3.1.** The protein content was 43.54%. Obtained protein content was in the range with results reported by German-Baez *et al*., where protein content was determined to be 37%[45]. Oil content in the biomass was 10.1% using Soxhlet extraction with hexane, which was in range with literature data. Ryckebosch *et al.* have reported the oil content in *P. tricornutum* to be 17.8%[128], while there are additional studies that defined total oil content in freeze-dried biomass to be up to 44.8%, depending on the cultivation conditions and extraction method[8]. Biomass ash content was 8.16%, which is almost half lower than reported average data of 14-15% in the researches available[8], [45], [137]. Residual moisture was found to be 3.97%, which is in the range with the only recent research, where the water content in *P. tricornutum* was evaluated and amounted to be 2.9%[8].

Table 3.1. Chemical composition of *P. tricornutum*

Compounds	Values (%)
Protein content	43.54 ± 0.34
Oil content	10.1 ± 0.13
Ash content	8.16 ± 0.042
Water content	3.97 ± 0.16

*values are expressed as mean ± standard deviation (n = 3)

3.2. Conventional extraction techniques. Solid-liquid extraction (SLE).

Conventional stepwise solid-liquid extraction was performed in order to evaluate the influence of four different polarity solvents on the extraction yield under the same conditions (temperature and extraction time). The results are presented in **Figure 3.1.** Obtained results allow concluding that type of solvent has a crucial impact on the extraction yield of *P. tricornutum* biomass. The highest extraction yield was obtained using water as a solvent, followed by ethanol, while acetone showed the lowest yield.

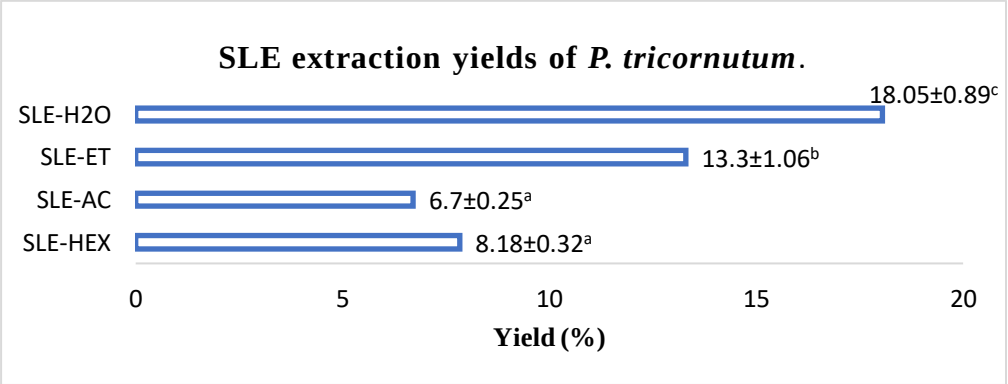


Figure 3.1. SLE extraction yields of *P. tricornutum* (values are expressed as mean ± standard deviation n = 3). Different lowercase superscript letters indicate significant differences within the same row at $p < 0.05$ (ANOVA, $p < 0.05$). SLE-HEX – solid liquid extraction with hexane, SLE-AC – solid liquid extraction with acetone SLE-ET – solid liquid extraction with ethanol SLE-H_2O – solid liquid extraction with water

In the study of Ryckebosch *et al.* influence of different solvents on the SLE extraction yield of *P. tricornutum* free-dried biomass was investigated[128]. Hexane has shown the lowest yield compared to hexane/isopropanol or more polar chloroform/methanol mixtures; hexane yield was 6%, which is lower compared to the results obtained in the present study. During Soxhlet extraction, the total lipophilic yield was 10.1%; however, in this case, the temperature should be considered as a factor that enhances extraction. During Soxhlet extraction, the temperature was ~60°C, while during SLE, it was 40°C. Overall, non-polar lipid compounds and lipophilic, insoluble in water carotenoids are definitely extracted better using non-polar solvents such as hexane or semi-polar acetone. At the same time, *P. tricornutum* shows a significant amount of polar compounds probably polysaccharides, proteins and carotenoids. These types of compounds were extracted better with polar solvents such as ethanol and water.

3.3. High-pressure extraction techniques: Optimization of SFE-CO_2

Supercritical carbon dioxide extraction was applied to *P. tricornutum* freeze-dried biomass in order to obtain fucoxanthin-rich fractions. As raw biomass contains about 10.1% of oil (**Table 3.1**) and SFE-CO_2 have demonstrated high selectivity in extracting non-polar compounds such as fatty acids and carotenoids[80], [125], current extraction method has been chosen for optimization of non-polar compounds recovery from *P. tricornutum.*

Ethanol as co-solvent was applied, as during several extractions without ethanol amount of yield was not higher than 3.6%, which is 3-4 times lower compared to co-solvent assisted extraction (data is shown in **Table 3.2**). *P. tricornutum* contains mostly xanthophyll carotenoid fucoxanthin, which has low solubility in very non-polar carbon dioxide. Ethanol helps to increase carotenoid solubility by swelling microalgae cell-matrix and forming hydrogen bonds with carotenoids that contain hydroxyl as their functional group. The same tendency was reported several times in the literature, where co-solvent assisted SFE-CO_2 was applied to microalgae extraction, such as *Scenedesmus sp.*, *Synechococcus sp.*, *Haematococcus pluvialis,* and *Chlorella vulgaris*[20], [80], [99].

Table 3.2. Total oil yield during SFE-CO_2 extraction of *P. tricornutum* biomass without applying the co-solvent

Conditions (temperature/pressure)	Total oil yield (g/100 g DW)
30°C/150 bars	2.76±0.30[a]
50°C/450 bars	3.24±0.23[a]
70°C/500 bars	3.63±0.17[a]

*values are expressed as mean ± standard deviation (n = 2). Different lowercase superscript letters indicate significant differences within the same column at $p < 0.05$ (ANOVA, $p < 0.05$)

For optimization of extraction parameters, Response Surface Methodology (RSM) and Central Composite Design (CCD) were used. The three independent optimization parameters were the temperature (30–70°C), pressure (150–450 bars), and the amount of ethanol as a co-solvent (5–10%). Yield, total carotenoid content and fucoxanthin content were three independent response factors.

During the optimization of the process and maximizing extraction yield, time remained constant (90 min), as during several trial extractions runs it has been proven that during 180 min extraction time amount of yield stops to change significantly after 60 min (**Figure 3.2**). The extraction initially goes faster, as the extract moves from solid particles to the surface before being dissolved in the supercritical fluid. When the extract concentration at the surface of the material drops to zero, the thermodynamic equilibrium is reached and the total yield achieves its maximum[38].

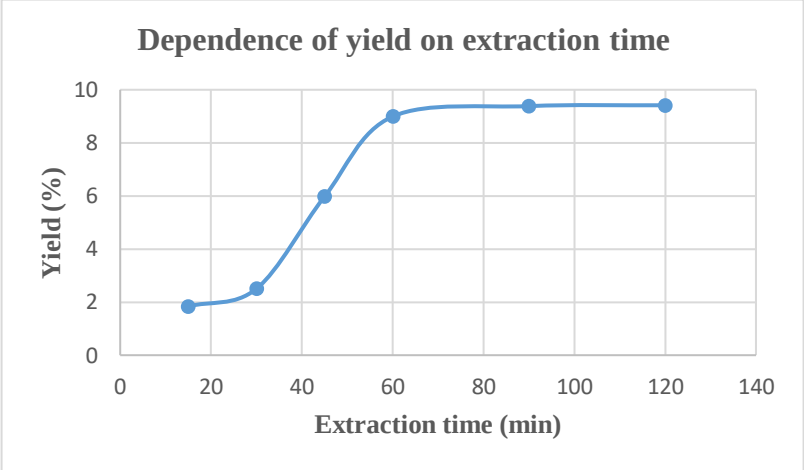


Figure 3.2. Kinetics of change in yield as a function of extraction time (300 bars, 50°C, 7.5% EtOH)

Table 3.3. presents SFE-CO2 extract yields (g/100 g DW), total carotenoid content (mg/g extract), and fucoxanthin content (mg/g extract) of extracted *P. tricornutum* biomass. Overall, the yield obtained ranged from 4.8 to 11.7% with the maximum value (11.7%) under 450 bars, 70°C and 10% of ethanol as co-solvent. Total carotenoid content ranged from 14 to 40 mg/g extract with the maximum amount obtained under 450 bars, 30°C and 10% of co-solvent. In order to determine the model adequacy for both yield and total carotenoid content, predicted and actual values were compared (**Figure 3.4, 3.6**).

Table 3.3. Central composite design matrix for SFE-CO2 optimization for *P. tricornutum* biomass and values of observed responses: SFE-CO2 extract yields (g/100 g DW), total carotenoid content (mg/g extract) and fucoxanthin content (mg/g extract)

Run	Pressure (bar)	Temperature (°C)	Co-solvent (%)	Yield** (g/ 100 g DW)	Total carotenoid content** (mg β-carotene/g extract)	Fucoxanthin content (mg/g extract)
1	300	50	7.5	9.02±0.12	33.31±0.08	32.36
2	450	70	5	7.70±0.07	28.91±0	19.84
3	450	30	10	10.64±0.56	40.60±0.25	48.00
4	150	50	7.5	6.64±0.56	16.62±0.13	10.11
5	450	50	7.5	9.81±0.19	38.56±0.13	20.98
6	150	70	5	5.62±0.03	15.48±0.04	9.00
7	150	30	10	6.89±0.12	17.34±0.08	25.72
8	300	50	7.5	9.11±0.53	34.25±0.16	32.36

	Pressure	Temperature	Co-solvent	Yield**	Total carotenoid content**	Fucoxanthin content
9	450	70	10	11.73±0.75	34.73±0.25	22.54
10	300	30	7.5	9.19±0.34	36.88±0.17	49.77
11	150	70	10	8.41±0.52	14.64±0	9.13
12	450	30	5	8.61±0.33	32.81±0.04	28.66
13	300	70	7.5	9.84±0.28	32.51±0.18	39.15
14	150	30	5	4.88±0.29	16.60±0.04	16.03
15	300	50	7.5	9.47±0.31	34.73±0.09	38.02
16	300	50	5	7.42±0.07	26.21±0.17	25.76
17	300	50	10	10.9±0.57	31.49±0.17	41.30
18	300	50	7.5	9.22±0.70	35.25±0.21	34.20
Optimal conditions	395	30	9.2	9.80±0.22	35.14±2.45	47.70

*optimal conditions were suggested by Design-Expert 7.0 software
**data is given as mean ± standard deviation (n = 2)

A. Total oil yield

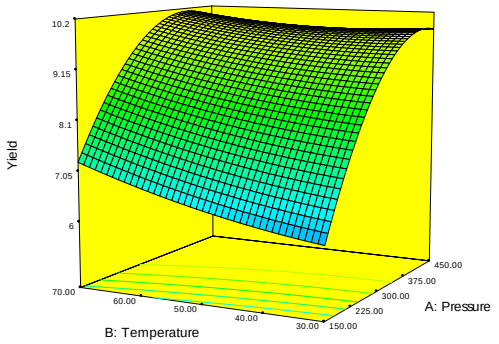
Figure 3.3 shows the effects of independent variables (temperature, pressure, and co-solvent) on SFE-CO2 extract yields (g/100 g DW) from *P. tricornutum* biomass. In general, the greatest impact on both yield and total carotenoid content had pressure, followed by the amount of co-solvent used. The obtained yield was in a range with that reported previously in the literature (14.7%), where SFE-CO_2 extraction of *P. tricornutum* was run under 40°C, and 300 bar, flow rate of CO_2 was 9mL/min^{-1} and extraction time was 2 hours[39]. Overall, actual values of yield (9.80%) were in good relation with predicted ones (9.69%) as well as obtained values were close to 45° line, which indicates good points distribution and confirms that the model fits well.

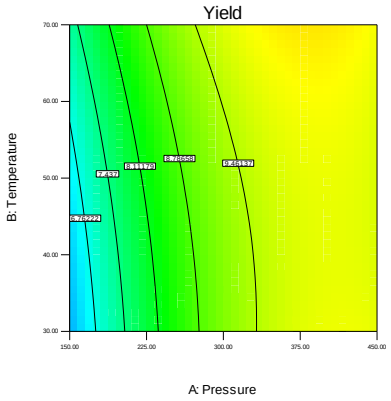
The regression equation obtained using CCD for yield (**Equation 3.1**).

$$\text{Yield, \%} = 9.29 + 1.61 * A + 0.32 * B + 1.44 * C - 0.27 * A * B + 0.14 * A * C + 0.34 * B * C - 1.15 * A^2 + 0.14 * B^2 - 0.22 * C^2, \text{ (Eq. } \mathbf{3.1}\text{)},$$

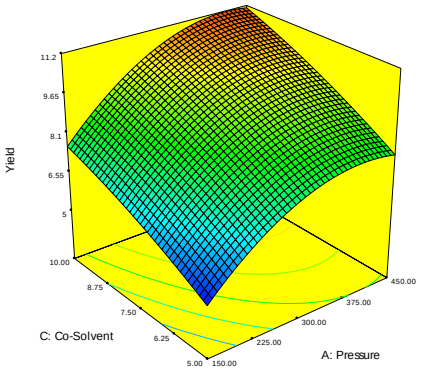
where A – pressure; B – temperature; C – co-solvent.

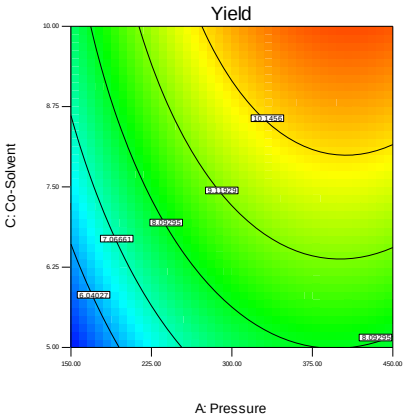
A: Effect of temperature and pressure at constant co-solvent, %.



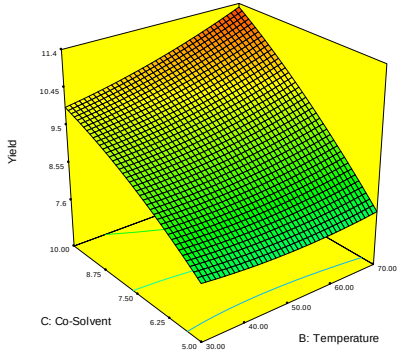


B: Effect of co-solvent and pressure at a constant temperature





C: Effect of co-solvent and temperature at constant pressure



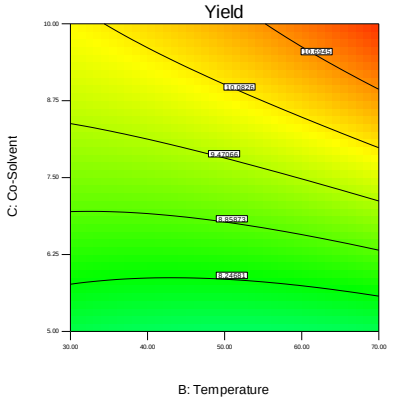


Figure 3 3. Response surface 3D and 2D plots showing the effects of independent variables (temperature, pressure, and co-solvent) on SFE-CO2 extract yields (g/100 g DW) from *P. tricornutum* biomass (A: effect of temperature and pressure at a constant time; B: effect of co-solvent and pressure at constant temperature: C: effect of co-solvent and temperature at constant pressure

Analysis of variance (ANOVA) of the regression parameters for the response surface quadratic model for SFE-CO_2 for extract yield (g/100 g) is presented in **Table 3.4.** The model was evaluated on the basis of the statistical significance of each effect on the response by comparing an estimate of the experimental error with the mean square. In the current report, five effects have statistically significant values ($p < 0.05$). It can be concluded from the data that pressure and co-solvent had the most significant effect on the extraction yield, while temperature had the least significant effect. The R-squared of 98.8% shows that the model explains 98.8% of the extraction yield variability, and the predicted R-Squared of 87.3% is in reasonable agreement with the adjusted R-Squared of 97.6%. The Model F-value of 79.03 implies the model is significant, as there is only a 0.01% chance that a "Model F-value" this large could occur due to noise. An adequate precision ratio of 32.252 indicates an adequate signal, so the model can be used to navigate the design space.

Table 3.4. Analysis of variance of the regression parameters for response surface quadratic model for SFE-CO_2 optimization for *P. tricornutum* biomass (Response factor: SFE-CO_2 extract yield, g/100 g)

Source	**Sum of squares**	**df**	**Mean square**	**F value**	**p-value**
Model	55.65	9	6.18	79.03	< 0.0001*
Pressure (P, bars)	25.82	1	25.82	330.09	< 0.0001*
Temperature (T, °C)	1	1	1	12.84	0.0071
Co-Solvent (C, %)	20.68	1	20.68	264.31	< 0.0001*
PT	0.56	1	0.56	7.18	0.0279
PC	0.15	1	0.15	1.93	0.2019
TC	0.92	1	0.92	11.82	0.0088*
P^2	3.6	1	3.6	45.99	0.0001*
T^2	0.051	1	0.051	0.66	0.4414
C^2	0.13	1	0.13	1.64	0.2366
Residual	0.63	8	0.078		
Lack of Fit	0.51	5	0.1	2.7	0.2213**
Pure Error	0.11	3	0.038		
Total	56.27	17			

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

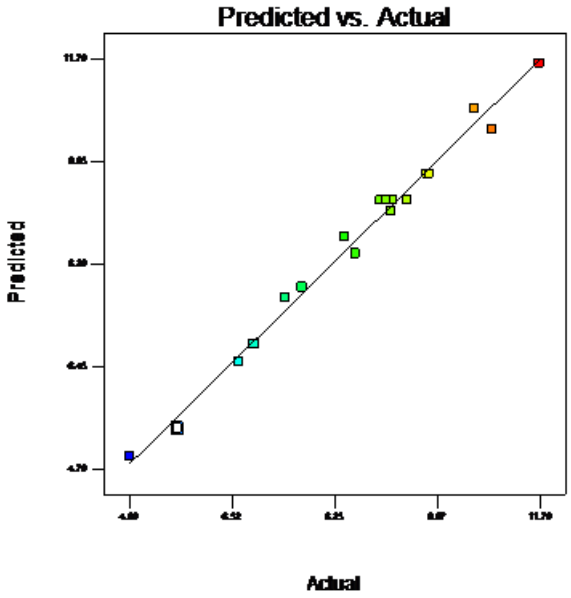


Figure 3.4. Comparison between predicted and observed SFE-CO2 extract yields (g/100 g DW) from *P. tricornutum* biomass

B. Total carotenoid content

Response surface 3D and 2D plots showing the effects of independent variables (temperature, pressure, and co-solvent) on SFE-CO2 total carotenoid content (mg/g extract) from *P. tricornutum* biomass are shown on **Figure 3.5.** The greatest impact on the total carotenoid content had pressure, while co-solvent and temperature had almost the same effect. While increasing the pressure and the amount of co-solvent helped to increase total carotenoid contend in extracts, the temperature had the opposite effect. The highest amount of carotenoids was obtained under the minimum extraction temperature of 30°, which most likely happens because carotenoids are thermolabile compounds, as they can degrade easily in response to heat.

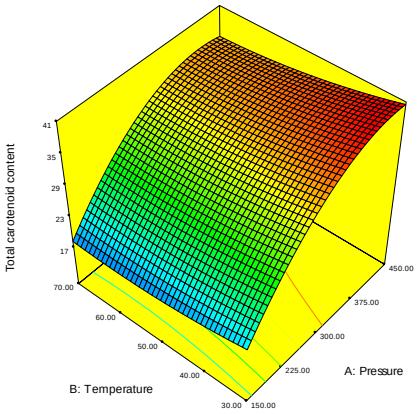
The regression equation obtained using CCD for total carotenoid content (**Equation 3.2**).

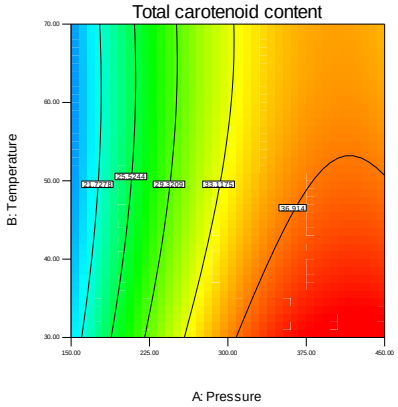
$$\text{Total carotenoid content, mg/g extract} = 33.58 + 9.40 * A - 1.80 * B + 1.94 * C - 0.75 * A * B + 1.75 * A * C - 0.50 * B * C - 5.97 * A^2 + 1.03 * B^2 - 4.27 * C^2, \text{ (Eq. 3.2)}$$

where A – pressure; B – temperature; C – co-solvent.

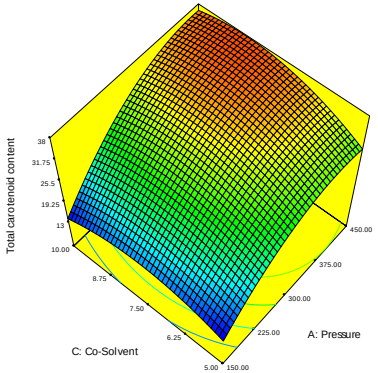
In the only reported study with supercritical extraction used by Feller *et al.*, total carotenoid content after SFE-CO_2 extraction of *P. tricornutum* biomass was much lower – 2.6 mg/g extract weight. However, only one extraction condition was tested (40°C, 300 bar, CO_2 flows rate was 9mL/min^{-1} and extraction time 2 hours), and no co-solvent was used.

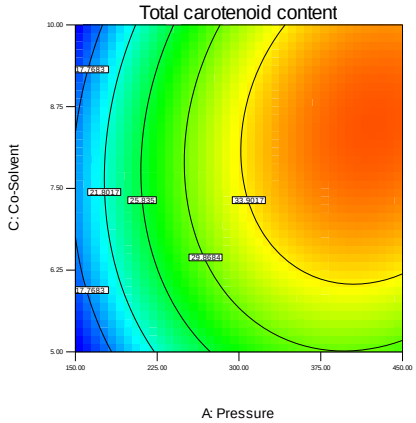
A: Effect of temperature and pressure at constant co-solvent, %



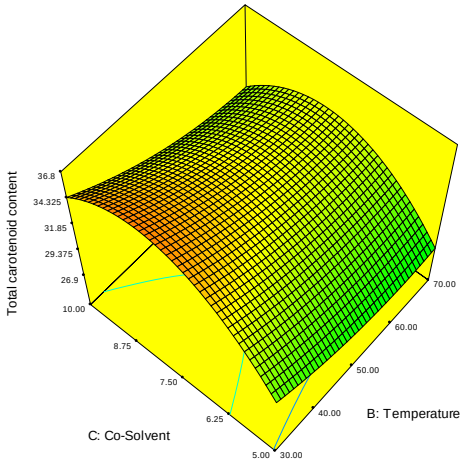


B: Effect of co-solvent and pressure at a constant temperature





C: Effect of co-solvent and temperature at constant pressure



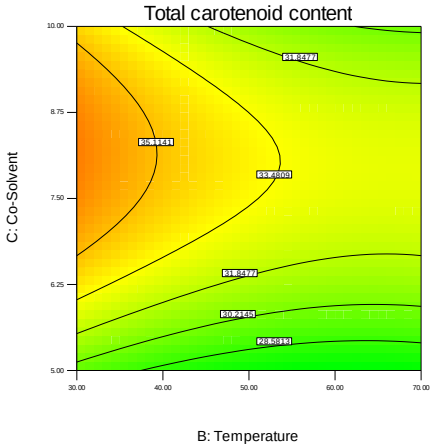


Figure 3 5. Response surface 3D and 2D plots showing the effects of independent variables (temperature, pressure, and co-solvent) on SFE-CO2 total carotenoid content (mg/g extract) from *P. tricornutum* biomass (A: effect of temperature and pressure at a constant time; B: effect of co-solvent and pressure at constant temperature: C: effect of co-solvent and temperature at constant pressure

Analysis of variance (ANOVA) of the regression parameters for the response surface quadratic model for SFE-CO_2 for total carotenoid content (mg/g DW) is presented in **Table 3.5.** For the second response, seven effects had p-values less than 0.05, thus were statistically significant. The R-squared value shows that the model explains 98.9% of the total carotenoid content variability, and the predicted R-Squared of 93.7% is in reasonable agreement with the adjusted R-Squared of 97.7%. The Model F-value of 82.17 implies the model is significant, as well as obtained an adequate Precision ratio of 27.193 indicates that the model can be used to navigate the design space.

Table 3.5. Analysis of variance of the regression parameters for response surface quadratic model for SFE-CO2 optimization for *P. tricornutum* biomass (Response factor: Total carotenoid content, mg/g extract)

Source	**Sum of squares**	**df**	**Mean square**	**F value**	**p-value**
Model	1302.71	9	144.75	82.17	< 0.0001*
Pressure (P, bars)	883.6	1	883.6	501.64	< 0.0001*
Temperature (T, °C)	32.4	1	32.4	18.39	0.0027*
Co-Solvent (C, %)	37.64	1	37.64	21.37	0.0017*
PT	4.5	1	4.5	2.55	0.1486
PC	24.5	1	24.5	13.91	0.0058*
TC	2	1	2	1.14	0.3177
P^2	96.74	1	96.74	54.92	< 0.0001*
T^2	2.85	1	2.85	1.62	0.2393
C^2	49.52	1	49.52	28.11	0.0007*
Residual	14.09	8	1.76		
Lack of Fit	11.72	5	2.34	2.97	0.1995**
Pure Error	2.37	3	0.79		
Total	1316.81	17			

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

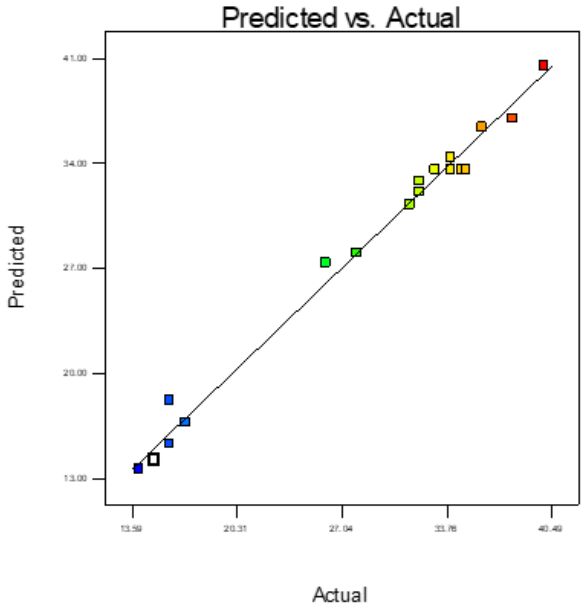


Figure 3.6. Comparison between predicted and observed SFE-CO2 total carotenoid content (mg/g extract) from *P. tricornutum* biomass

Figure 3.6 shows that predicted and actual values are close to the 45° line, which indicates good points distribution and confirms that the model fits well. Real values (35.14 mg/g extract) of optimal conditions for total carotenoid content were in good relation with predicted ones (35.6 mg/g extract).

C. Fucoxanthin content

Response surface 3D and 2D plots showing the effects of independent variables (temperature, pressure, and co-solvent) on SFE-CO2 fucoxanthin content (mg/g extract) from *P. tricornutum* biomass are shown on **Figure 3.7.** The greatest impact on the fucoxanthin content had pressure and temperature, whereas co-solvent had twice less impact. While increasing the amount of co-solvent helped to increase fucoxanthin content in extracts, gradual increasing the temperature had the opposite effect. Increasing the pressure had a positive impact on fucoxanthin content but only till a certain point, as further increasing the pressure above approximately 370 bars resulted in a decline of fucoxanthin content. The highest amount of fucoxanthin (49.7 mg/g extract) was obtained under the minimum extraction temperature of 30°, 300 bars, and 7.5 % of EtOH, which is most probably associated with the combination of low temperature, increased pressure and amount of co-solvent.

The regression equation obtained using CCD for fucoxanthin content (**Equation 3.3**).

Fucoxanthin content, mg/g extract = 34.62 + 6.92 * A - 6.83 * B +4.60 * C - 1.49 * A * B + 1.56 * A * C - 3.26 * B * C - 19.99 * A^2 + 9.36 * B^2 - 1.69 * C^2, (Eq. **3.3**)

where A – pressure; B – temperature; C – co-solvent.

Although, there is no study on the impact of different SFE conditions on the fucoxanthin content; in the study by Gilbert-López, PLE and MAE were optimized with a further determination of fucoxanthin content by HPLC-DAD[48]. Recovery of total fucoxanthin in PLE under optimal extraction conditions (50 °C, 100% EtOH, 20 min) and MAE under optimal extraction conditions (30 °C, 100% EtOH, 2 min) was 32.29 and 31.6 mg fucoxanthin/g extract respectively. Referent acetone extraction performed for 24 h in an orbital shaker at the room temperature was 59.47 mg/g extract. In

the current research, the fucoxanthin content obtained under optimal conditions during SFE-CO_2 was 47.7 mg/g extract (predicted variation 4.6%), which is higher than in the results obtained by Gilbert-López for PLE and MAE (maximum values were 32.29 and 31.6 respectively), that allows concluding that SFE-CO_2 extraction is more efficient in extracting main carotenoid fucoxanthin compared to PLE and MAE. This is due to the naturally lipophilic fucoxanthin, which results in its higher solubility in CO_2 combined with only CO_2 or polar solvents themselves[39].

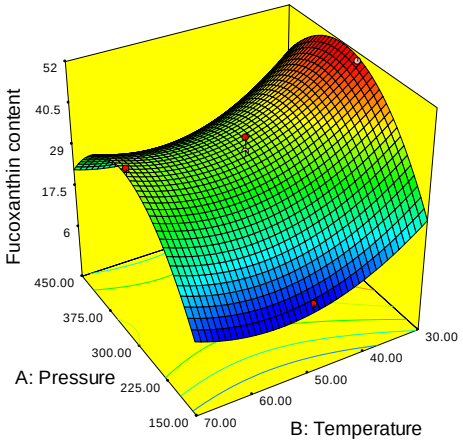
Analysis of variance (ANOVA) of the regression parameters for response surface quadratic model for SFE-CO_2 for fucoxanthin content (mg/g extract) from *P. tricornutum* biomass is presented in **Table 3.6.** In the third response seven parameters showed a statistically significant effect ($p < 0.05$). The R-squared of 97.3% shows that the model explains 97.3% of the fucoxanthin content variability, and the predicted R-Squared of 79.6% is in reasonable agreement with the adjusted R-Squared of 94.4%. The Model F-value of 32.91 implies that the model is significant, as well as obtained an adequate Precision ratio of 19.4 indicates that the model can be used to navigate the design space.

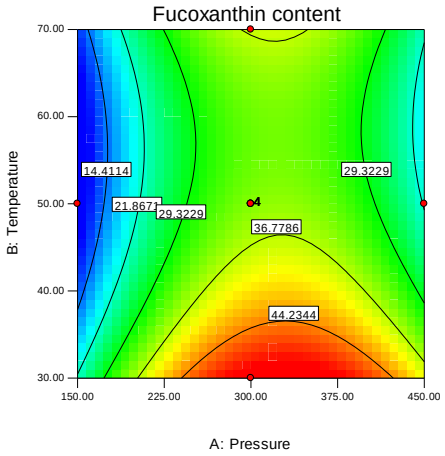
Table 3.6. Analysis of variance of the regression parameters for response surface quadratic model for SFE-CO_2 optimization for *P. tricornutum* biomass (Response factor: Fucoxanthin content, mg/g extract)

Source	Sum of squares	df	Mean square	F value	p-value
Model	2630.35	9	292.26	32.91	< 0.0001*
Pressure (P, bars)	478.45	1	478.45	53.88	< 0.0001*
Temperature (T, °C)	466.08	1	466.08	52.49	< 0.0001*
Co-Solvent (C, %)	211.88	1	211.88	23.86	0.0012*
PT	17.79	1	17.79	2.00	0.1947
PC	19.44	1	19.44	2.19	0.1773
TC	84.96	1	84.96	9.57	0.0148*
P^2	1083.07	1	1083.07	121.97	< 0.0001*
T^2	237.26	1	237.26	26.72	0.0009*
C^2	7.76	1	7.76	0.87	0.3771
Residual	71.04	8	8.88		
Lack of Fit	49.23	5	9.85	1.35	0.4272**
Pure Error	21.81	3	7.27		
Total	2701.39	17			

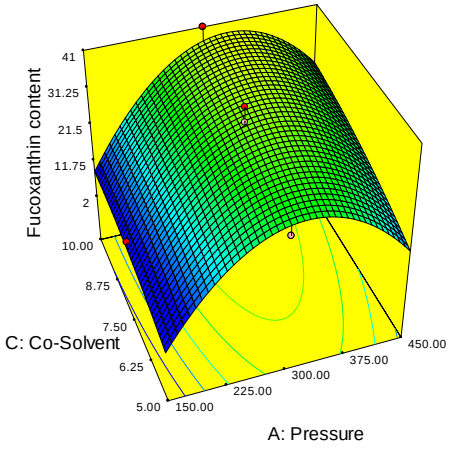
*: significant; **: not significant; df: degree of freedom; F: Fisher value.; MS: mean square; RF: response factor; SS: sum of square.

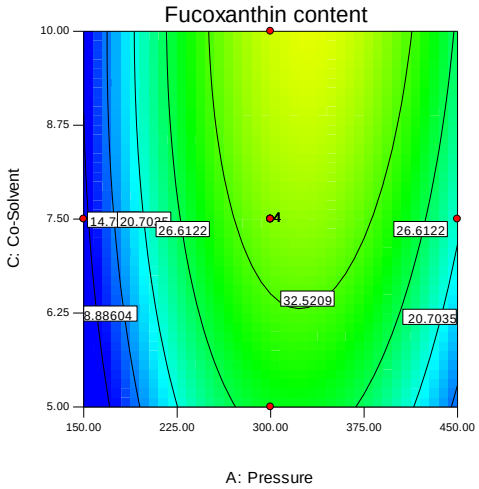
A: Effect of temperature and pressure at constant co-solvent, %



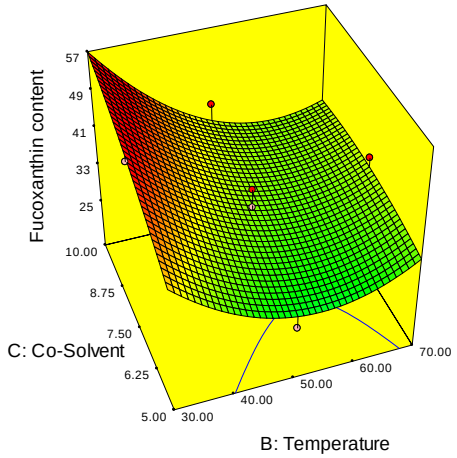


B: Effect of co-solvent and pressure at a constant temperature, C





C: Effect of co-solvent and temperature at constant pressure, %



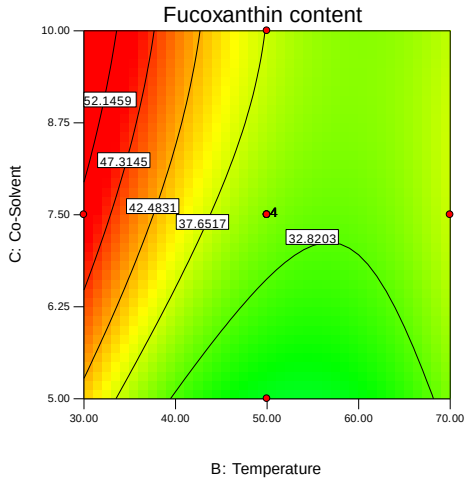


Figure 3.7. Response surface 3D and 2D plots showing the effects of independent variables (temperature, pressure, and co-solvent) on SFE-CO2 fucoxanthin content (mg/g extract) from *P. tricornutum* biomass (A: effect of temperature and pressure at a constant time; B: effect of co-solvent and pressure at constant temperature: C: effect of co-solvent and temperature at constant pressure

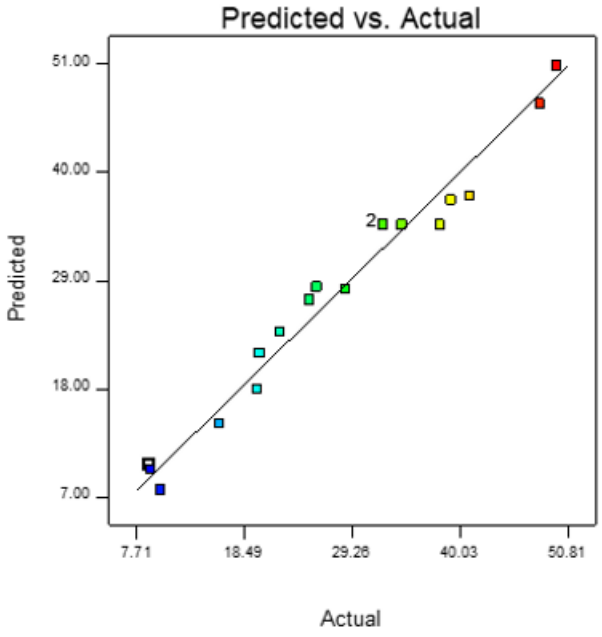


Figure 3.8. Comparison between predicted and observed SFE-CO2 fucoxanthin content (mg/ g extract) from *P. tricornutum* biomass

Figure 3.8 shows that predicted and actual values indicate good points distribution and confirm that the model fits well. Real values (47.7 mg fucoxanthin/g extract) of optimal conditions for total carotenoid content were in good relation with predicted ones (50 mg fucoxanthin/g extract).

3.3.1 Validation of the predictive model of multiple response optimization for SFE-CO_2

In order to validate the optimization conditions obtained by RSM, experiments under optimal conditions in duplicate were carried out, and the results are shown in **Table 3.7**. Multiple response optimization was performed to maximize the total oil yield and total carotenoid content, to extract the maximum amount of fucoxanthin. All response variables were considered equally important. The optimum conditions proposed by the model were 395 bars pressure, 30°C temperature, and 9.2% of EtOH as a co-solvent.

Table 3.7. Validation results of SFE-CO_2 optimal conditions. Predicted and actual values of extraction

Parameter under optimal conditions (395 bars, 30°C, 9.2% EtOH)	**Predicted**	**Actual**	**Variation, %**
Yield, g/100 g DW	9.69	9.80±0.22	-1.18
Total carotenoid content, mg/g extract	35.6	35.14±2.45	+1.28
Fucoxanthin content, mg/g extract	50.0	47.70	+4.60

*values are expressed as mean ± standard deviation (n = 2)

In all three responses, the variation between predicted and actual values was less than 5%, which indicates the good validity of the suggested models. Both values for total carotenoid content and fucoxanthin content were lower than predicted, with fucoxanthin content having the highest variation of 4.6%. The actual total oil yield values were higher than the predicted ones.

Overall, current optimization shows that these regression equation model can be used to optimize oil, total carotenoid and fucoxanthin extraction by SFE-CO2 from freeze-dried *P. tricornutum* biomass. Based on ANOVA 's assessment of the total carotenoid content and fucoxanthin, the pressure was found to be the most important parameter of all the extraction parameters, followed by the temperature; whereas the amount of co-solvent was the least important factor. At the same time, the pressure was also the main factor for the total oil yield, the amount of co-solvent was the second crucial parameter for the efficient extraction process and the temperature had the lowest impact.

3.4. High-pressure extraction techniques. Optimization of Pressurized liquid extraction (PLE)

The PLE optimization was performed on SFE-CO_2 residues of *P. tricornutum* biomass extracted under optimal conditions, as described in section 3.3. As stepwise solid-liquid extraction has shown that only a part of antioxidant-rich fractions has been removed after using non-polar solvent, a similar approach was followed for the sequential high-pressure extraction. Towards this end, a sequential PLE extraction with acetone followed by ethanol was performed on the residues after SFE, aiming to extract the maximum of active polar substances. Multiple studies have shown that *P. tricornutum* contains significant amounts of polar compounds such as polysaccharides and proteins that are better extracted using more polar solvents. In contrast, phenolic compounds and carotenoids (which are mostly related to the antioxidant capacity) are often extracted with pure ethanol[14], [29], [45], [102]. Central Composite Design (CCD) and Response Surface Methodology (RSM) were applied to study the effect of two parameters extraction time (15 to 45 min) and temperature (40–100°C) on the optimization process. Yield, total carotenoid content and total phenolic content were chosen as three independent response factors. In PLE optimization in contrary to SLE, only acetone and ethanol were chosen as solvents with gradually increasing polarity, excluding water. This was done because during several trial extraction runs on the last step of PLE extraction with water, biomass had formed sticky gel and could not pass through the system filter.

3.4.1. PLE-acetone optimization of *P. tricornutum* SFE-CO2 residue

Three independent responses (total yield, total carotenoid content and TPC) were evaluated by the impact of temperature and pressure on them. **Table 3.8.** presents PLE extract yields (g/100 g DW), total carotenoid content (mg/g extract), and total phenolic content (mg GAE/g DW) of *P. tricornutum* biomass residues after SFE-CO_2. Overall, the yield obtained ranged from 4.8 to 10.65% with the maximum yield obtained under 100°C and 45 min of extraction time. Total carotenoid content ranged from 8.4 to 17.92 mg/g extract with the maximum amount obtained under 40°C and 45 min of extraction time. Total phenolic content varied from 2.2 to 9.7 mg GAE/g DW with the maximum amount obtained under 100°C and 15 min of extraction time. In order to determine the model adequacy for all responses, predicted and actual values were compared (**Figure 3.10, 3.12, 3.14**). As a result, optimum conditions were suggested to be 61°C and 39 min of extraction time.

Table 3.8. Central composite design matrix for PLE-acetone optimization for *P. tricornutum* SFE-CO_2 residual biomass and values of observed responses: PLE extract yields (g/100 g DW), total carotenoid content (mg/g DW) and total phenolic content (mg GAE/g DW)

Run	Temperature (°C)	Time (min)	Yield (g/ 100 g DW)	Total carotenoid content (mg/g extract)	Total phenolic content (mg GAE/g DW)
1	40	45	3.83	17.92	3.38
2	70	30	8.43	10.32	5.97
3	40	15	4.41	11.47	3.12
4	100	15	9.29	19.81	9.76
5	70	30	8.44	10.41	4.25
6	100	30	9.40	14.30	7.66
7	70	30	8.01	10.04	4.48
8	40	30	4.32	11.36	2.41
9	70	30	8.98	8.40	5.19
10	70	15	8.01	13.89	6.41
11	100	45	10.65	11.72	8.68
12	70	45	7.72	12.77	5.82
Optimal conditions	61	39	7.60	10.9	4.68

*optimal conditions were suggested by Design-Expert 7.0 software

A. Effect of time and temperature on the PLE extraction yield of *P. tricornutum* SFE-CO_2 residue

The highest PLE extraction yield (10.65 g/100g DW) was obtained under the maximum tested temperature and extraction time, 100°C and 45 min whereas, the lowest (3.8 g/100g DW) under 40°C and 45 min. At the central point (70°C and 30 min), yield ranged from 8.4 to 8.98 g/100g DW. Extraction time had a minor effect as with constant temperature and different extraction time changes in yield were not significant. Overall, the yield was positively influenced by the temperature, which can be explained by more efficient mass transfer, reduction in solvent viscosity, which results in better matrix penetration, and increased solubility of compounds[1]. In the work of Gilbert-López *et al.*, PLE optimization for freeze-dried *P. tricornutum* biomass has also shown higher yields under increased temperature (ranged from 50 to 170°C). Moreover, the highest extraction yield (57.84%) was obtained with a mixture of water: ethanol (50:50) than with the pure solvent[48].

Figure 3.9. shows the effects of independent variables (temperature and extraction time) on PLE extract yields (g/100 g DW) from *P. tricornutum* SFE-CO_2 residues.

The regression equation obtained using CCD for yield (**Equation 3.4**).

$$\text{Yield, \%} = 8.33 + 2.79 * A + 0.090 * B + 0.51 * A * B - 1.22 * A^2 - 0.22 * B^2, \text{ (Eq. } \mathbf{3.4}\text{)},$$

where A – temperature; B – time.

The temperature turned out to be the most significant independent variable in the evaluated central composite design for PLE acetone optimization for *P. tricornutum* residual biomass after SFE-CO_2 optimal conditions (extraction yield, g/100 g DW) (**Table 3.9**). It means that the extraction temperature had a substantially greater impact on the yield compared to extraction time. ANOVA showed that the model is significant according to the Student test ($p<0.05$), while the lack of fit is not significant. The R-squared of 97.7% shows that the model explains adequately the yield variability, and the predicted R-Squared of 86.9% is in reasonable agreement with the adjusted R-Squared of 95.8%. An adequate precision of 20.7 indicates an adequate signal of the model. Thus, it can be used to navigate through the design space.

Table 3.9. Analysis of variance of the regression parameters for response surface quadratic model for PLE optimization for *P. tricornutum* residual biomass after SFE-CO_2 optimal conditions (Response factor: Extraction yield, g/100 g DW)

Source	**Sum of squares**	**df**	**Mean square**	**F value**	**p-value**
Model	53.03	5	10.61	52.15	< 0.0001*
A-Temperature	46.76	1	46.76	229.93	< 0.0001*
B-Time	0.049	1	0.049	0.24	0.6423
AB	1.05	1	1.05	5.17	0.0634
A^2	3.99	1	3.99	19.64	0.0044*
B^2	0.13	1	0.13	0.63	0.4585
Residual	1.22	6	0.20		
Lack of Fit	0.74	3	0.25	1.56	0.3625**
Pure Error	0.48	3	0.16		
Total	54.25	11			

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

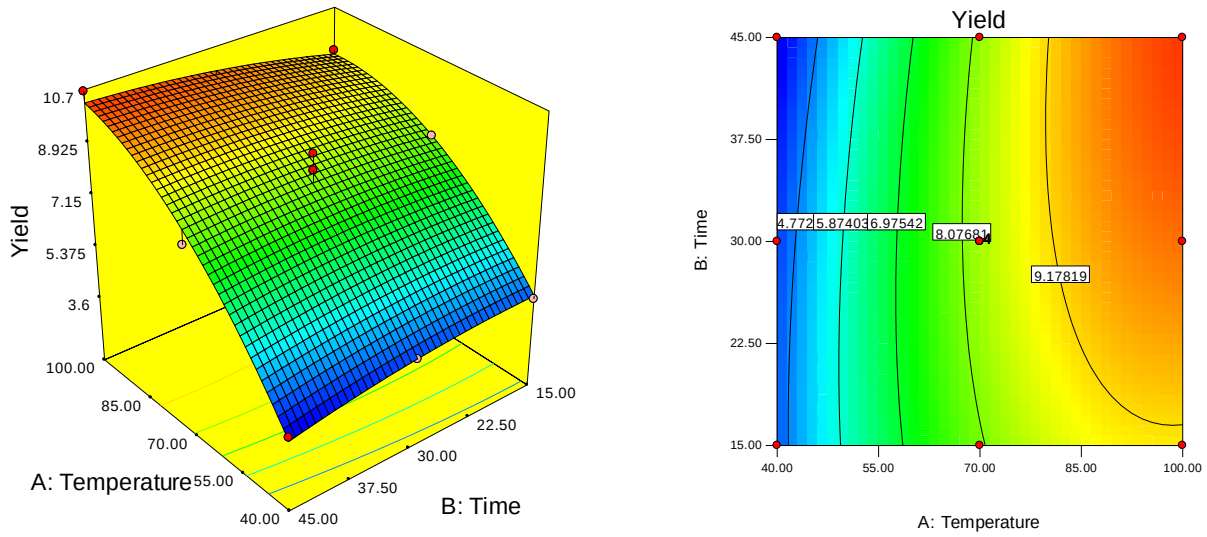


Figure 3.9. Response surface 3D and 2D plots showing the effects of independent variables (temperature and extraction time) on PLE extract yield (g/100 g DW) from *P. tricornutum* biomass

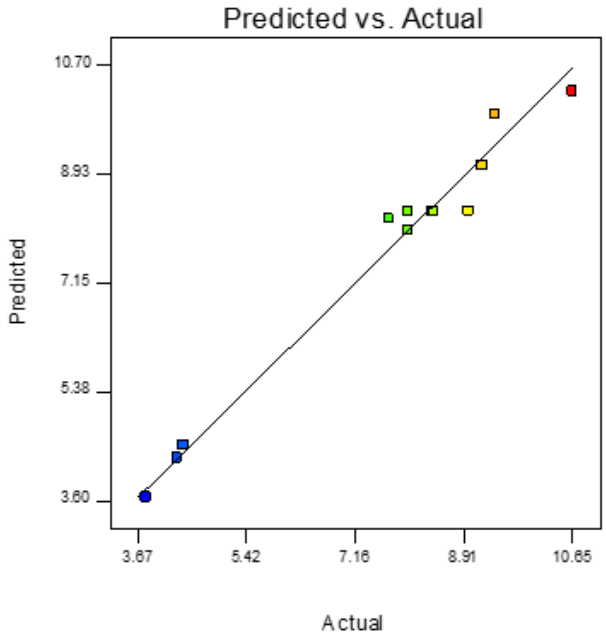


Figure 3.10. Comparison between predicted and observed PLE extraction yield, (g/100 g DW) from *P. tricornutum* biomass SFE-CO_2 residues

Predicted and actual values are shown in **Figure 3.10.** Predicted and actual values are close to the 45° line. Real values (7.6 mg g/100g DW) under optimal conditions for total yield are in good relation with predicted ones (7.26 mg g/100g DW). Thus, it shows a normal distribution and confirms that the model is a perfect fit.

B. Effect of time and temperature on the PLE total carotenoid content of *P. tricornutum* SFE-CO_2 residue

Total carotenoid content values of PLE ranged between 8.4 to 19.8 mg/g extract. The highest value (19.8 mg/g extract) was obtained under 100°C and 15 min extraction time, while the lowest (8.4mg/g extract) was obtained under 70°C and 30 min. Values at the central points were around 10 mg/g extract. Overall, the temperature had a positive effect on the total carotenoid content, while increasing extraction time at the constant temperature has resulted in a decreased amount of carotenoids. This could be explained by the thermolabile nature of carotenoids and other polar substances well extracted by acetone. While increased temperature results in better solubility and fast mass transfer, prolonged extraction time causes carotenoid degradation[102]. A similar tendency was observed in recent works, where *P. tricornutum* and *P. cruentum* were undergoing PLE extraction in order to evaluate them as potential carotenoid sources[44], [47]. In both cases, total carotenoid content increased with temperature until a certain point; meanwhile, further elevation of temperature (150°C and above) and extraction time caused a decline in carotenoid content. However, data comparison on carotenoid content with other studies must be made with caution, as composition and carotenoid content of different microalgae species could be greatly influenced by cultivation conditions such as light and oxygen intensity and nutrient availability[49].

Figure 3.11. shows the effects of independent variables (temperature and extraction time) on PLE total carotenoid content (mg/g extract) from *P. tricornutum* SFE-CO_2 residues.

The regression equation obtained using CCD for total carotenoid content (**Equation 3.5**).

$$\text{Carotenoid content, mg/g extract} = 9.98 + 0.84*A - 0.46*B - 3.63*A*B + 2.47*A^2 + 2.97*B^2, \quad \text{(Eq. 3.5)},$$

where A – temperature; B – time.

Analysis of variance (ANOVA) of the regression parameters for response surface quadratic model for PLE optimization for *P. tricornutum* residual biomass after SFE-CO_2 optimal conditions (total carotenoid content, mg/g extract) is presented in the **Table 3.10.** For this response, four effects had P-values less than 0.05, indicating their significance. The R-squared shows that the developed model can explain 96.1% of the total carotenoid content variability, and the predicted R-Squared is in reasonable agreement with the adjusted R-Squared (81.5% and 92.9% respectively). The Model F-value of 29.85 indicates that the model is significant, as well as the obtained Precision ratio of 16.5 indicates an adequate signal and that the current model can be used to navigate the design space.

Table 3.10. Analysis of variance of the regression parameters for response surface quadratic model for PLE optimization for *P. tricornutum* residual biomass after SFE-CO_2 optimal conditions (Response factor: Total carotenoid content, mg/g extract)

Source	Sum of squares	df	Mean square	F value	p-value
Model	117.65	5	23.53	29.85	0.0004*

Source	Sum of squares	df	Mean square	F value	p-value
A-Temperature	4.28	1	4.28	5.44	0.0585
B-Time	1.26	1	1.26	1.60	0.2529
AB	52.78	1	52.78	66.96	0.0002*
A^2	16.24	1	16.24	20.60	0.0039*
B^2	23.48	1	23.48	29.79	0.0016*
Residual	4.73	6	0.79		
Lack of Fit	2.07	3	0.69	0.78	0.5793**
Pure Error	2.66	3	0.89		
Total	122.38	11			

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

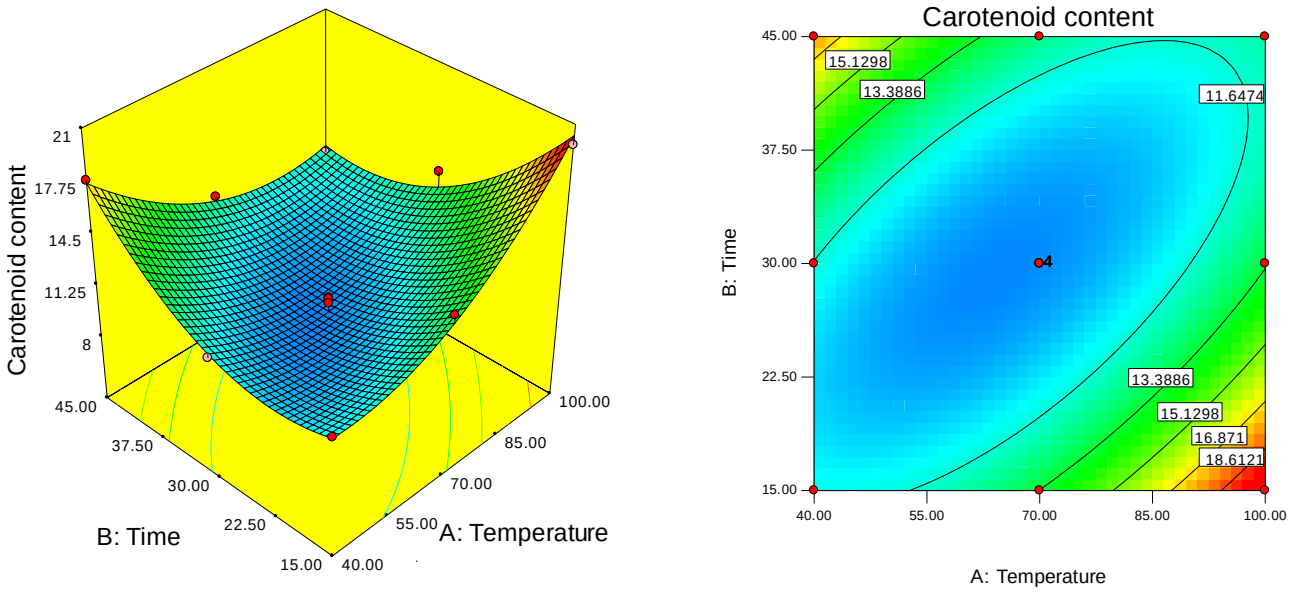


Figure 3.11. Response surface 3D and 2D plots showing the effects of independent variables (temperature and extraction time) on PLE total carotenoid content (mg/g extract) from *P. tricornutum* residual biomass

As the main target of the PLE optimization was maximizing all parameters, the model suggested optimal conditions were 61°C and 39 min extraction time. Under optimal conditions, the obtained value for total carotenoid content was 10.9 mg/g extract. Predicted and actual values can be seen in **Figure 3.12**. Predicted and actual figures showed good distribution along 45° line, which confirms that the model fits well. Optimal conditions actual values (10.9 mg/g extract) for total carotenoid content were in reasonable relation with predicted values (11.41 mg g/ extract).

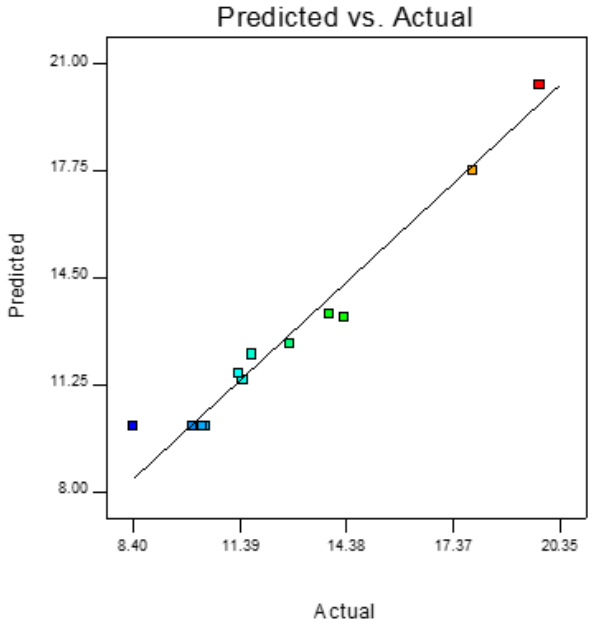


Figure 3.12. Comparison between predicted and observed PLE total carotenoid content (mg/g extract) from *P. tricornutum* biomass SFE-CO_2 residues

C. Effect of time and temperature on the PLE-acetone total phenolic content of *P. tricornutum* SFE-CO_2 residue

As it is known, carotenoids can contribute significantly to antioxidant capacity in microalgal biomass. It was proven in several studies that both total carotenoid and total phenolic content contribute significantly to the antioxidant properties of extracts[41], [60], [81]. The highest TPC content (9.7 mg GAE/g DW) was observed under the same conditions as the highest carotenoid content – 100°C and 15 min extraction time. The lowest TPC content was found to be under 40°C and 30 min. In general, the temperature had the maximum positive effect on phenolic content, while time did not affect substantially. In the study of Gilbert-López *et al.,* TPC during optimization of PLE for *P. tricornutum* biomass was dependent on the solvent composition. Ethanol: water mixtures, as well as pure solvents, were used. The program suggested optimum conditions to maximize TPC were 100% EtOH at 50 °C[47]. In the current research, TPC content under optimal conditions (61°C and 39 min) was 4.68 mg GAE/g DW, which was in range with data obtained in the literature, considering that part of active antioxidant compounds, including carotenoids, were removed during the previous step of SFE-CO_2. The total phenolic content of the PLE extracts is expressed as milligrams of gallic acid equivalents per gram dry weight. According to ANOVA, one effect, the quadratic variable of temperature, had a p-value <0.05.

The results from CCD can be used to compose the second-order polynomial model equation (**Equation 3.6**).

$$\text{Carotenoid content, mg/g extract} = 9.98 + 0.84*A - 0.46*B - 3.63*A*B + 2.47*A^2 + 2.97*B^2, \quad \text{(Eq. 3.6)},$$

where A – temperature; B – time.

Figure 3.13. shows the effects of independent variables (temperature and extraction time) on PLE total phenolic content (mg GAE/g DW) from *P. tricornutum* SFE-CO_2 residues.

Analysis of variance (ANOVA) of the regression parameters for response surface quadratic model for PLE optimization for *P. tricornutum* residual biomass after SFE-CO_2 optimal conditions (total phenolic content mg GAE/g DW) is presented in the **Table 3.11.** For the response, three effects had p-values less than 0.05. An adequate precision of 18.2 indicates an adequate signal of the model and that it can be used to navigate through the design space. The Model F-value of 32.53 indicates that the model is significant.

Table 3.11. Analysis of variance of the regression parameters for response surface quadratic model for PLE optimization for *P. tricornutum* residual biomass after SFE-CO_2 optimal conditions (Response factor: Total phenolic content mg GAE/g DW)

Source	Sum of squares	df	Mean square	F value	p-value
Model	54.39	5	10.88	32.53	0.0003*
A-Temperature	49.28	1	49.28	147.35	< 0.0001*
B-Time	0.33	1	0.33	0.99	0.3592
AB	0.45	1	0.45	1.34	0.2911
A^2	0.021	1	0.021	0.064	0.8093
B^2	3.65	1	3.65	10.92	0.0163*
Residual	2.01	6	0.33		
Lack of Fit	0.19	3	0.063	0.10	0.9519**
Pure Error	1.82	3	0.61		
Total	56.40	11			

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

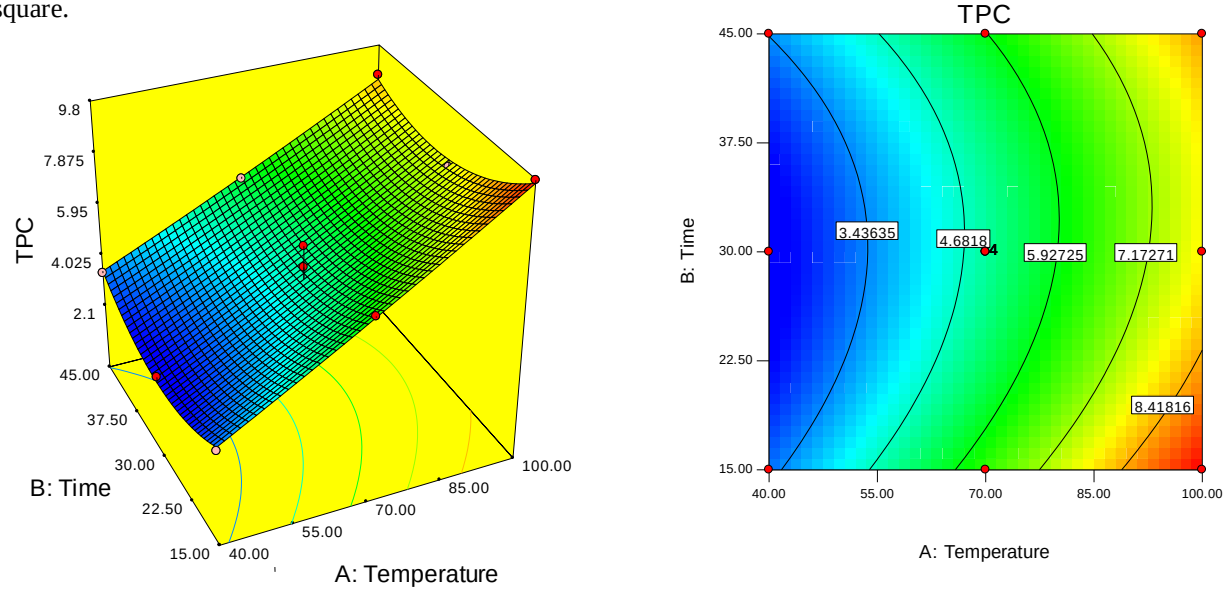


Figure 3.13. Response surface 3D and 2D plots showing the effects of independent variables (temperature and extraction time) on PLE total carotenoid content (mg/g extract) from *P. tricornutum* residual biomass

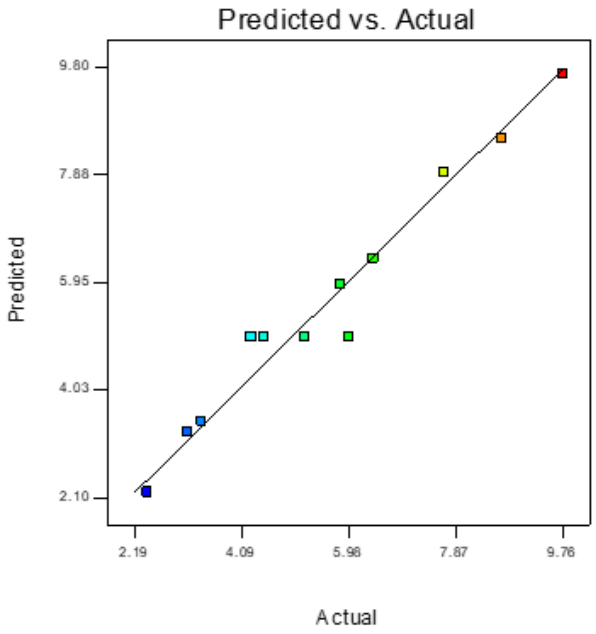


Figure 3.14. Comparison between predicted and observed PLE total phenolic content (mg GAE/g DW) from *P. tricornutum* biomass SFE-CO2 residues

Predicted and actual values for total phenolic content can be seen in **Figure 3.14**. Both values are close to the 45° line, which indicates good points distribution and confirms that the model fits well. TPC actual values for the optimal conditions (4.68 mg GAE/g DW) were in reasonable relation with predicted values (4.46 mg GAE/g DW).

3.4.2. PLE-ethanol optimization of *P. tricornutum* PLE-acetone residue

The PLE-acetone residues of *P. tricornutum* biomass were used for the optimization of PLE-ethanol. A similar biorefining approach was discussed in recent works, indicating that due to its chemical composition, *P. tricornutum* can be used for an integrated biorefinery, to optimize environmental and economic process feasibility. Such an approach also considers residual biomass valorization on each step of high-components extraction in order to convert and obtain a wide range of different compounds and products[15], [156].

Table 3.12. presents PLE-ethanol extract yields (g/100 g DW), total carotenoid content (mg/g DW), and total phenolic content (mg GAE/g DW) of *P. tricornutum* biomass residues after PLE-acetone. In general, the yield obtained ranged from 9.8 to 17.2 g/100g DW, with the maximum value obtained under 100°C and 45 min of extraction time. Total carotenoid content ranged from 3 to 9.3 mg/g extract with the maximum amount under 40°C and 15 min of extraction time. Total phenolic content varied from 2.96 to 6.12 mg GAE/g DW with the maximum amount under 100°C and 45 min of extraction time. In order to determine the model adequacy for all responses, predicted, and actual values were compared (**Figures 3.15, 3.16, 3.17, 3.18, 3.19, 3.20**). The most significant effect on the parameters had temperature, while extraction time showed only minor effects. Just as during SFE-CO_2 and PLE-acetone optimization, the total extract yield increased with rising of the temperature, whereas the amount of total carotenoids dropped. Compared to PLE-acetone optimization results, PLE-ethanol has shown higher amounts of extracted yields but lower amounts of carotenoids and phenolic compounds. A similar trend can be

observed after SLE when the highest amount of yield was obtained during water extraction, followed by ethanol. The more polar nature of ethanol helps to extract comparatively more residual components compared to acetone. As a result, optimum conditions were suggested to be 66°C and 17 min of extraction time.

Table 3.12. Central composite design matrix for PLE-ethanol optimization for *P. tricornutum* PLE-acetone residues and values of observed responses: PLE extract yields (g/100 g DW), total carotenoid content (mg/g extract) and total phenolic content (mg GAE/g DW)

Run	Temperature (°C)	Time (min)	Yield (g/ 100 g DW)	Total carotenoid content (mg/g extract)	Total phenolic content (mg GAE/g DW)
1	40	45	9.80	8.73	3.09
2	70	30	16.53	5.95	5.17
3	40	15	10.19	9.30	4.03
4	100	15	16.11	4.41	5.00
5	70	30	16.12	4.92	4.88
6	100	30	16.50	3.04	5.68
7	70	30	15.70	5.17	4.96
8	40	30	11.20	7.00	2.96
9	70	30	15.92	6.07	4.92
10	70	15	14.74	8.72	5.23
11	100	45	17.22	5.07	6.12
12	70	45	16.70	8.04	5.41
Optimal conditions	66	17	15.22	7.60	5.12

*optimal conditions were suggested by Design-Expert 7.0 software

A. Effect of time and temperature on the PLE-ethanol extraction yield of *P. tricornutum* PLE-acetone residue

The highest PLE-ethanol extraction yield (17.2 g/100g DW) after PLE-acetone biomass residues was obtained under maximum temperature and extraction time – 100°C and 45 min and the lowest (9.8 g/100g DW) under 40°C and 45 min. The maximum and minimum values were reached under the same extraction conditions as for PLE-acetone extraction. However, for the yield, PLE-ethanol extraction has shown higher yields as compared to the PLE-acetone yield. At the central point (70°C and 30 min), yield ranged from 15.7 to 16.53 g/100g DW. Extraction time had a minor effect, as at a constant temperature and different extraction time changes in yield were not significant. While temperature has shown the biggest positive impact on the yield, the same trend was observed during PLE-acetone optimization. These response surface plots are of great benefit in making the extraction more efficient; it can be done by increasing the extraction time at lower temperatures or applying high temperatures for a short extraction time.

Overall, total oil yields were higher during the second step of PLE with ethanol compared to the first step with acetone. The same trend was observed in the current research during SLE when the highest

extraction yield was obtained during the last step of extraction with water, followed by yields using ethanol, acetone, and hexane. In several studies, different microalgae species were subjected to PLE using different solvents such as ethanol, 2-propanol, hexane, petroleum ether, and water. In all cases, ethanol or ethanol/water mixtures showed the highest obtained yields[61], [82], [123], [135]. When non-polar solvents are applied, low efficiency was achieved because of the fact that usually polar compounds comprise the major part of microalgae biomass.

The results from CCD can be used to compose the second-order polynomial model equation (**Equation 3.7**).

$$\text{Yield, g/100g DW} = 16.09 + 3.12 * A + 0.47 * B + 0.35 * A * B - 2.30 * A^2 - 0.45 * B^2, \text{ (Eq. } \mathbf{3.7}\text{)},$$

where A – temperature; B – time.

Response surface quadratic model for PLE-ethanol optimization for *P. tricornutum* residual biomass after PLE-acetone (yield, g/100g DW) and its analysis of variance is presented in **Table 3.13.** For this response, three effects had P-values<0.05, and the model's lack of fit was not significant. For the extract yield, the importance of independent variables can be arranged in the following order: extraction temperature > extraction time. The Model F-value of 49.48 indicates that the model is significant, as well as the obtained adequate Precision ratio of 17.9 indicates that the model can be used to navigate the design space.

Table 3.13. Analysis of variance of the regression parameters for response surface quadratic model for PLE-ethanol optimization for *P. tricornutum* residual biomass after PLE-acetone (Response factor: Total yield g/100g DW)

Source	Sum of squares	df	Mean square	F value	p-value
Model	78.71	5	15.74	49.48	< 0.0001*
A-Temperature	58.28	1	58.28	183.18	< 0.0001*
B-Time	1.31	1	1.31	4.11	0.0891
AB	0.49	1	0.49	1.54	0.2609
A^2	14.15	1	14.15	44.48	0.0005*
B^2	0.55	1	0.55	1.73	0.2370
Residual	1.91	6	0.32		
Lack of Fit	1.53	3	0.51	4.05	0.1402**
Pure Error	0.38	3	0.13		
Total	80.62	11			

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

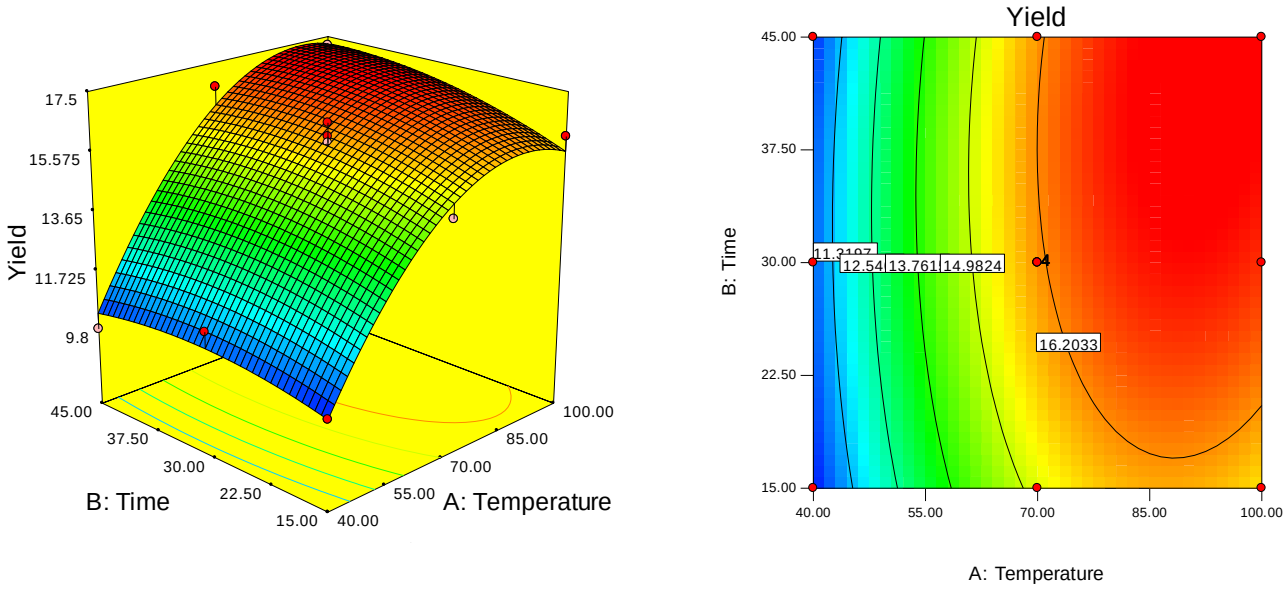


Figure 3.15. Response surface 3D and 2D plots showing the effects of independent variables (temperature and extraction time) on PLE-ethanol yield (g/100g DW) from *P. tricornutum* PLE-acetone residual biomass

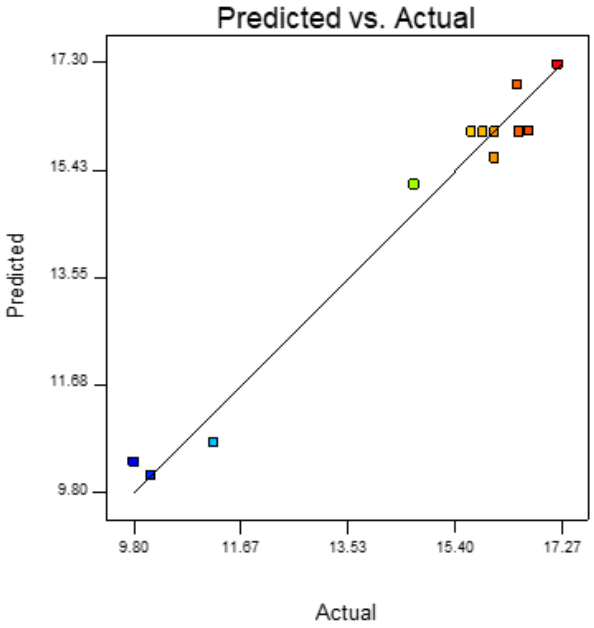


Figure 3.16. Comparison between predicted and observed PLE-ethanol total yield (g/100g DW) from *P. tricornutum* biomass SFE-acetone residues

Predicted and actual values for total yield can be seen in the **Figure 3.16**. Both values are close to the 45° line, which indicates good points distribution and confirms that the model fits well. The actual total yield values for the optimal conditions (15.22 g/100g DW) were in reasonable relation with predicted values (14.94 g/100g DW).

B. Effect of time and temperature on the PLE-ethanol total carotenoid content of *P. tricornutum* PLE-acetone residue

The highest PLE-ethanol carotenoid content (9.3 mg/g extract) after PLE-acetone biomass residues was obtained under minimum temperature and extraction time – 40°C and 15 min and the lowest (3.04 mg/g extract) under 100°C and 30 min. It could be explained by degradation of carotenoids under high temperatures. At the central point (70°C and 30 min), total carotenoid content ranged between 4.9 and 6 mg/g extract.

The model showed a higher significance of temperature as compared to extraction time. Typically, a longer extraction time stimulates a higher yield; however, in this case total carotenoid content decreased with longer extraction time (from 8.72 mg/g extract during 15 min extraction to 8.04 mg/g extract during 45 min extraction at 70°C and from 9.30 mg/g extract during 15 min to 8.73 mg/g extract during 45 min at 40°C). The same pattern was reported during several studies of PLE application on microalgae, aiming to extract algal carotenoids. Similarly, during lutein extraction from *Haematococcus pluvialis* and *Dunaliella salina* lower yields were obtained with longer time of extraction, as lutein was found to be less thermolabile than, for example, astaxanthin[31]. For fucoxanthin, short PLE extraction times (10 min at 100°C in ethanol) from *P. tricornutum* freeze-dried biomass, gave higher extraction yield compared to longer PLE extraction time or other methods (same carotenoid content was achieved either at room temperature with UAE or during Soxhlet extraction at 80°C, but after 30 min)[78].

Moreover, ethanol turned out to be one of the best solvents for various carotenoid extraction from microalgae, including fucoxanthin. Despite that major part of carotenoids was extracted during SFE-CO_2 and the first cycle of PLE-acetone, ethanol was able to extract remaining carotenoids from the residual biomass. Ethanol was also an efficient solvent in PLE of fucoxanthin from *Eisenia bicyclis* at 110°C at 100-200 bars and astaxanthin from *Haematococcus pluvialis* at 200°C and 103 bars[135]. During the extraction of carotenoids from *Spirulina platensis* the highest yields were achieved in ethanol, followed by water, hexane, and petroleum ether[61].

The regression equation obtained using CCD for total carotenoid content (**Equation 3.8**).

$$\text{Total carotenoid content, mg/g extract} = 5.66 - 2.10 * A - 0.12 * B + 0.29 * A * B - 0.99 * A^2 + 2.35 * B^2, \text{ (Eq. } \mathbf{3.8}\text{)},$$

C. Where A – temperature; B – time.

Analysis of variance (ANOVA) of the regression parameters for response surface quadratic model for PLE-ethanol optimization for *P. tricornutum* residual biomass after PLE-acetone (total carotenoid content, mg/g extract) is presented in the **Table 3.14.** For the response, four effects had P-values less than 0.05, indicating their significance from zero. The extraction temperature had a substantially bigger impact on the total carotenoid content than extraction time. The R-squared shows that the model explains 95.5% of the total yield variability, and the predicted R-Squared of 80% is in reasonable agreement with the adjusted R-Squared of 91.8%. The Model F-value of 25.89 indicates that the model is significant, as well as the Precision ratio of 17.32 indicates an adequate signal of the model and that it can be used to navigate the design space.

Table 3.14. Analysis of variance of the regression parameters for response surface quadratic model for PLE-ethanol optimization for *P. tricornutum* residual biomass after PLE-acetone (Response factor: total carotenoid content, mg/g extract)

Source	Sum of squares	df	Mean square	F value	p-value
Model	41.72	5	8.34	25.89	0.0005*
A-Temperature	26.42	1	26.42	81.96	0.0001*
B-Time	0.079	1	0.079	0.25	0.6374
AB	0.34	1	0.34	1.06	0.3426
A^2	2.60	1	2.60	8.07	0.0296*
B^2	14.76	1	14.76	45.78	0.0005*
Residual	1.93	6	0.32		
Lack of Fit	0.93	3	0.31	0.93	0.5248**
Pure Error	1.00	3	0.33		
Total	43.66	11			

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

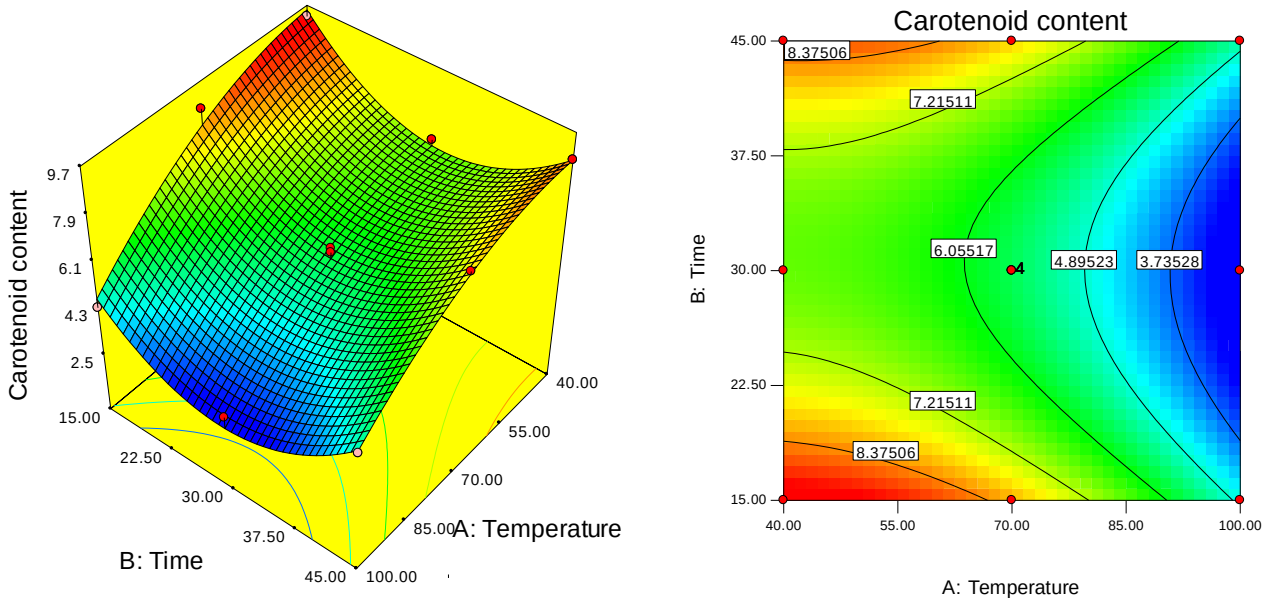


Figure 3.17. Response surface 3D and 2D plots showing the effects of independent variables (temperature and extraction time) on PLE-ethanol total carotenoid content (mg/g extract) from *P. tricornutum* PLE-acetone residual biomass

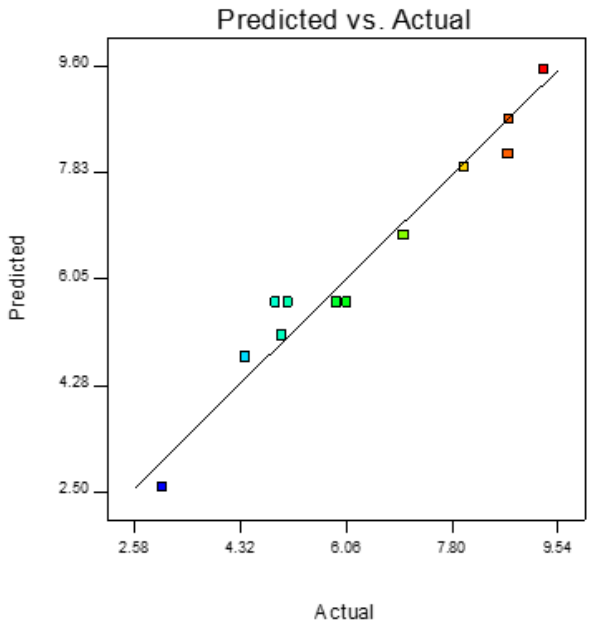


Figure 3.18. Comparison between predicted and observed PLE-ethanol total carotenoid content (mg/g extract) from *P. tricornutum* biomass SFE-acetone residues

C. Effect of time and temperature on the PLE-ethanol total phenolic content of *P. tricornutum* PLE-acetone residue

TPC in PLE-ethanol extracts was lower than during the previous step of PLE-acetone. Alike SLE, during PLE, the TPC decreased with further extraction steps, as active antioxidant compounds were being removed gradually with each extraction process. It can also be seen by the decrease in total carotenoids in each following extraction, as it was mentioned above, carotenoids contribute significantly to the antioxidant effects of the extracts. The highest total phenolic content (6.12 mg GAE/g DW) was obtained under 100°C and 45 min extraction time, while the lowest (2.96 mg GAE/g DW) under 40°C and 30 min.

In contrast to PLE-acetone extraction in PLE-ethanol, the highest TPC value did not correspond with the highest total carotenoids value. However, similarly to PLE-acetone, the antioxidant capacity of PLE-ethanol extracts was enhanced by increasing the temperature to 100°C, as in general, the solubility of polar compounds related to antioxidant capacity increases with the temperature[47]. Similar results were reported by during PLE extraction of active antioxidant compounds from *Phormidium* species and *Spirulina platensis* when the antioxidant capacity of ethanol extracts rose with the temperature increased to 150 °C[61], [123].

The regression equation obtained using CCD for total phenolic content (**Equation 3.9**).

$$\text{Total phenolic content, mg GAE/g DW} = 5.00 + 1.12 * A + 0.060 * B + 0.52 * A * B - 0.71 * A^2 + 0.29 * B^2, \text{ (Eq. } \mathbf{3.9}\text{)},$$

A. Where A – temperature; B – time.

Analysis of variance (ANOVA) of the regression parameters for response surface quadratic model for PLE-ethanol optimization for *P. tricornutum* residual biomass after PLE-acetone (total carotenoid

content, mg/g extract) is presented in the **Table 3.15.** For the current response, four effects are significant (p< 0/05). The R-squared showed that the model explains 97.7% of the total yield variability, and the predicted R-Squared of 80% is in reasonable agreement with the adjusted R-Squared of 95.8%. The Model F-value of 51.57 indicates that the model is significant, as well as obtained an adequate Precision ratio of 23.5 indicates that the model can be used to navigate the design space.

Table 3.15. Analysis of variance of the regression parameters for response surface quadratic model for PLE-ethanol optimization for *P. tricornutum* residual biomass after PLE-acetone (Response factor: total phenolic content, mg GAE/g DW)

Source	Sum of squares	df	Mean square	F value	p-value
Model	9.97	5	1.99	51.57	< 0.0001*
A-Temperature	7.53	1	7.53	194.72	< 0.0001*
B-Time	0.022	1	0.022	0.56	0.4830
AB	1.06	1	1.06	27.45	0.0019*
A^2	1.35	1	1.35	34.90	0.0010*
B^2	0.22	1	0.22	5.75	0.0534
Residual	0.23	6	0.039		
Lack of Fit	0.18	3	0.061	3.63	0.1588**
Pure Error	0.050	3	0.017		
Total	10.20	11			

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

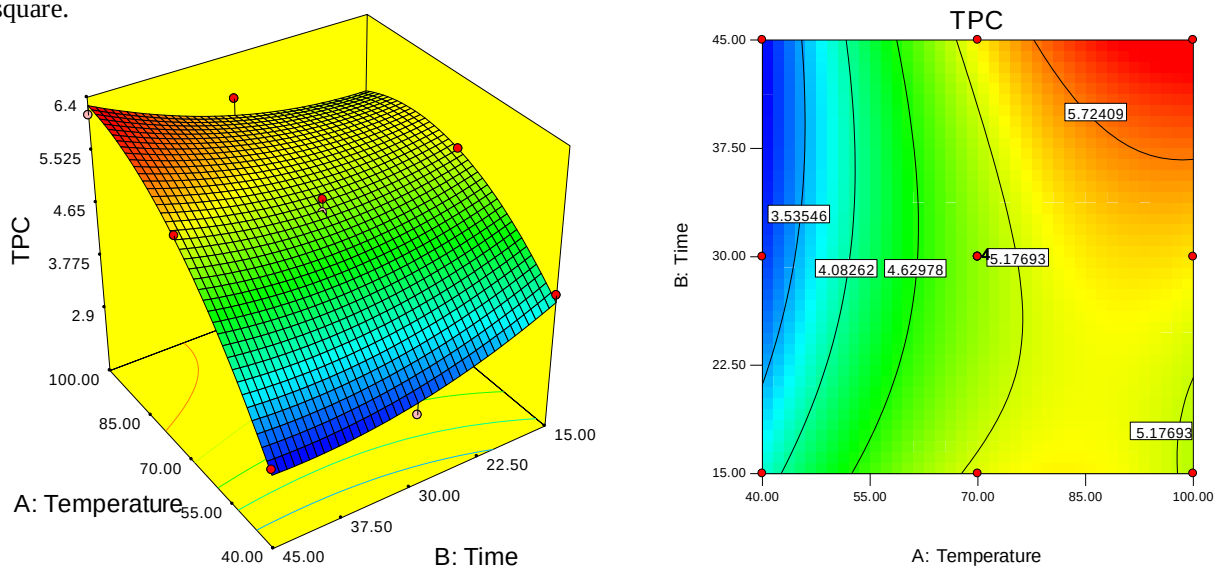


Figure 3.19. Response surface 3D and 2D plots showing the effects of independent variables (temperature and extraction time) on PLE-ethanol total phenolic content (mg GAE/g DW) from *P. tricornutum* PLE-acetone residual biomass

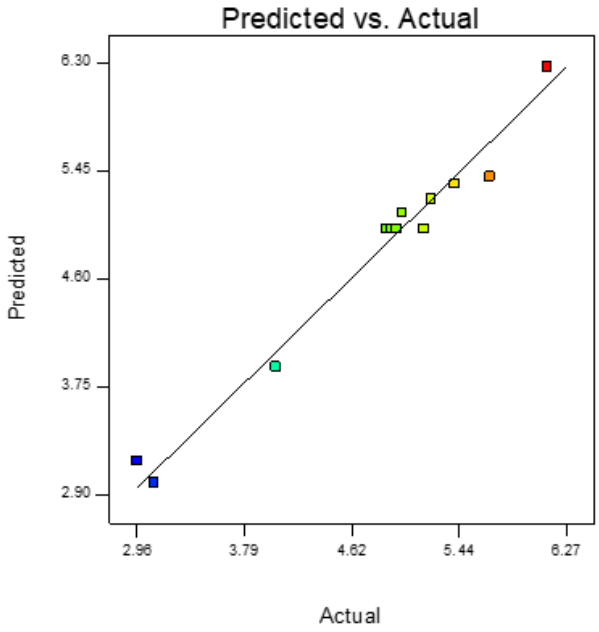


Figure 3.20. Comparison between predicted and observed PLE-ethanol total phenolic content (mg GAE/g DW) from *P. tricornutum biomass* SFE-acetone residues

3.4.3. Verification of the predictive model of multiple response optimization for PLE-acetone and PLE-ethanol from *P. tricornutum* SFE-CO_2 residue

Multiple response optimization was carried out in order to determine the most suitable extraction conditions to maximize the studied variables, specifically, extraction yield and total carotenoid content. Total phenolic content was used to determine the amount of reducing substances, and thus, the potential antioxidant capacity of the extracts. Since phenolic compounds are powerful scavengers of free radicals and the strong correlation between the amount of phenols and antioxidant capacity of *P. tricornutum* biomass is also reported in previous studies[8], [47]. Both extraction steps were performed in duplicates for each of the proposed optimum conditions. The validation results are shown in **Table 3.16**.

Table 3.16. Validation results of PLE-acetone and PLE-ethanol optimal conditions. Predicted and actual values of extraction

PLE-acetone optimal conditions (61°C, 39 min)	**Predicted**	**Actual**	**Variation, %**
Yield, g/ 100 g DW	7.26	7.6±0.35	-4.68
Total carotenoid content, mg/g extract	11.41	10.9±0.63	+4.46
TPC, mg GAE/g DW	4.46	4.68±0.29	-4.93
PLE-ethanol optimal conditions (66°C, 17 min)	**Predicted**	**Actual**	**Variation, %**
Yield, g/100 g DW	14.94	15.22±0.29	-1.8
Total carotenoid content, mg/g extract	7.80	7.6±0.42	+2.56
TPC, mg GAE/g DW	5.06	5.1±0.22	-0.79

* values are expressed as mean ± standard deviation (n = 2). PLE – pressurized liquid extraction, TPC – total phenolic content; DW – dry weight; GAE – gallic acid equivalence

For the PLE-acetone optimization, the optimal conditions were suggested to be 61°C and 39 min extraction time, which allowed to obtain relatively high yields of extracts, rich in total carotenoid content and high antioxidant capacity. The validity of the obtained model can be approved, as predicted, and actual values are in a good agreement. Moreover, both total yield and TPC values were higher than predicted. According to ANOVA, for TPC and total yield, the temperature was the determinant factor (had the biggest F-value) for these responses. While for the total carotenoid content, both temperature and extraction time were determinant, as with prolonging the extraction time at the high temperature, total carotenoid content was decreasing.

The model for PLE-ethanol optimization with two independent variables by CCD predicted that optimal parameters are 66°C and 17 min of extraction time. Under these optimal conditions, the actual values agreed with the values predicted by the model with minimal variation under 3%. Ethanol-assisted PLE allowed to obtain higher extraction yields compared to PLE-acetone, as well as to extract remaining carotenoids.

Table 3.17. Recoveries of fucoxanthin and total yield from *P. tricornutum* biomass

	mg fucoxanthin/g extract	**total yield g/100 g DW (optimal conditions)**
SFE-CO_2	47.70^{a}	9.80 ± 0.22^{a}
PLE-acetone	9.93^{b}	7.60 ± 0.35^{b}
PLE-ethanol	7.03^{b}	15.22 ± 0.29^{c}
Reference Soxhlet	22.33^{c}	10.10 ± 0.13^{a}

*different lowercase superscript letters indicate significant differences within the same column (ANOVA, $p < 0.05$). SFE-CO_2 – supercritical carbon dioxide extraction, PLE – pressurized liquid extraction, DW – dry weight

Overall, the highest total yield was obtained during PLE-ethanol extraction, which is in agreement with data obtained during stepwise SLE, where the highest yield was achieved using polar solvents, such as ethanol and water. Total lipophilic yield after SFE-CO_2 and Soxhlet extraction was not considerably different. However, time spent on SFE-CO_2 extraction was more than three times shorter compared to Soxhlet extraction, to obtain the same yields (1.5 and 6 hours respectively), as well as during SFE-CO_2 the usage of toxic solvents as hexane is eliminated.

According to the study by Gilbert-López, *P. tricornutum* contains approximately 60 mg/g extract of fucoxanthin. In the mentioned study *P. tricornutum* free-dried biomass was macerated in acetone during 24h at room temperature, and the average concentration of fucoxanthin in the extract was calculated as a reference value for total fucoxanthin in the biomass[48]. Current results demonstrated that SFE-CO_2 under optimal conditions had extracted most of the oils and hydrophobic carotenoids due to its non-polar nature, as well as a major part of fucoxanthin (47.7 mg/g extract) with the help of ethanol as a co-solvent. Afterwards, most of the remaining fucoxanthin was extracted by PLE with acetone (9.9 mg/g extract), followed by PLE-ethanol (7 mg/g extract). In total, combined fucoxanthin content after SFE-CO_2, PLE-acetone and PLE-ethanol accounts 64.67 mg/g extract, which is in a range with the amount of reference fucoxanthin (60 mg/g extract) reported by Gilbert-López. Compared to the amount of fucoxanthin after extracted using the Soxhlet method (22.3 mg/g extract), the recovery was almost 50% lower than during SFE-CO_2 (47.7 mg/g extract). Thus, it can be concluded that SFE-CO_2 followed by stepwise PLE with statistical methodology, is an efficient method for optimizing the extraction conditions for maximum

fucoxanthin recovery from brown microalgae *P. tricornutum* in a relatively short time with non-toxic solvents, suggesting further applications of this method for fucoxanthin extraction from microalgal biomass. As a result, the bio-refinery concept can be applied on SFE-CO2 by-products of *P. tricornutum* freeze-dried biomass in order to get valuable bioactive compounds by pressurized liquid extraction technique.

3.5. *In-vitro* antioxidant capacity

3.5.1. Total phenolic content and antioxidant capacity for initial biomass and SLE

Freeze-dried biomass of *P. tricornutum* was evaluated for its *in vitro* antioxidant capacity. **Table 3.18** shows total phenolic content and antioxidant capacity of *P. tricornutum* initial biomass, extracts, and solid residues after stepwise SLE – hexane, acetone, ethanol, and water. These solvents were chosen so that the solvent polarity would gradually increase from non-polar hexane to very polar water. Initial biomass and solid residues after each extraction step were evaluated for its antioxidant capacity using QUENCHER (QUick, Easy, New, CHEap, and Reproducible) approach. This approach allows us to measure total antioxidant capacity accurately and precisely in a single operation, including both, free and bound antioxidant compounds. The procedure is based on direct measurement of solid samples by combining, followed by a spectrometric measurement afterwards. The reaction between an antioxidant and a radical occurs at the interface when they come into contact, irrespectively of the hydrophobicity of the compound of interest[51]. QUENCHER method was specifically developed for widely used antioxidant assays such as TPC, $ABTS^{\bullet+,}$ and DPPH•.

The total phenolic content values are expressed as milligrams of gallic acid equivalence per gram of sample. The initial TPC value of raw biomass was 21.67±0.68, and it finally decreased to 6.66±0.42 after the last step of water extraction. Total phenols values for extracts ranged between 1.75±0.04 and 6.02±0.41. Overall, acetone showed the highest capability of extracting phenolic compounds, and hexane extracts exhibited the lowest antioxidant capacity.

For $ABTS^{\bullet+}$ radical scavenging assay values were expressed as Trolox equivalents – TEAC mg Trolox/g residue for dry biomass and TEAC mg Trolox/g DW extract for extracts. The values for SLE dry residues ranged from 64.58±4.82 to 19.03±2.57 mg Trolox/g residue. Hexane has shown the lowest capacity, while acetone extracts expressed the most potent antioxidant activities with values of 14.25±2.72 mg Trolox/g DW extract. The highest $ABTS^{\bullet+}$ values were observed in residual biomass after hexane extraction, compared to initial biomass.

Initial biomass and extracts were also evaluated on their antioxidant capacity using $DPPH^{\bullet}$ cation radical assay. For the initial biomass, values range from 7.84±0.62 to 1.02±0.17 mg Trolox/g residue after water extraction. Initial biomass has shown the most potent ability to scavenge $DPPH^{\bullet}$ free radicals. Whereas for extracts values ranged from 0.23±0.02 to 2.50±0.26 mg Trolox/g DW extract, with the highest activity observed after acetone-assisted extraction, and lowest after water extracts, followed by hexane extracts.

In all cases, the antioxidant capacity of residual biomass was gradually decreasing, indicating that only a limited number of active antioxidant compounds are extracted from the microalgae biomass after each extraction step. It correlates with the general statement in other studies, that the most part of antioxidant active compounds is present in residual biomass in bound form and it is more difficult to extract, thus each extraction step removes only a particular part of active compounds[2].

P. tricornutum, like many other microalgae, has a wide range of bioactive constituents of different chemical composition and polarity, that exhibit antioxidant capacity. Because of the large number and different mechanisms of antioxidant action of these compounds, their antioxidant capacity determination can be complicated [50], [149]. The crucial factor in the antioxidant capacity of an extract plays the type of solvent used for extraction, as the polarity of the solvent determines the types of compounds that can be extracted using this solvent. Overall, as it was previously reported[97], the highest TPC and antioxidant capacity were found to be in the ethanolic extracts of microalgae than compared to hexane, ethyl acetate, water or water/ethanol mixtures. In the literature *in vitro* antioxidant capacity and total phenolic content of *P. tricornutum* were not studied using acetone-assisted extraction.

In a recent study by Valdez-Ortiz [154], *P. tricornutum* biomass was evaluated by its total phenolic content and antioxidant capacity for initial biomass and extracts after ethanolic extraction. $ABTS^{\bullet+}$ values were 67.93±0.36 mg Trolox/g DW for the initial biomass, 44.90±0.90 mg Trolox/g residue for the residual biomass after extraction with ethanol and 23.02±0.75 mg Trolox/g DW extract. These values are very close to the data obtained in the current research. In the literature $DPPH^{\bullet}$ assay was reported to show a total value of 9.54±0.13 mg Trolox/g DW for initial biomass, which is in good correlation with data obtained in the current work – 7.84±0.62. However, TPC content in the described study was significantly lower (2.90±0.02) than observed in the current work (21.67±0.68). In the study of Gilbert-López, microwave-assisted extraction with different %EtOH (0, 50, 100%) and temperature (30, 100, 170°C) was applied to freeze-dried *P. tricornutum* biomass with further TPC content determination[48]. The values ranged from 8.92 to 46.97 mg GAE/g extract, and the lowest content was obtained with water at 100°C and the highest with 100% EtOH under 30°C.

Table 3.18. Total phenolic content and antioxidant capacity of *P. tricornutum* initial biomass, extracts and solid residues after stepwise SLE – hexane (HEX), acetone (AC), ethanol (ET), water (W)

	P. tricornutum	**SLE-HEX**	**SLE-AC**	**SLE-EtOH**	**SLE-H_2O**
TPC, mg GAE/g DW (residue)	21.67±0.68[a]	20.20±1.66[a]	16.22±0.75[b]	8.89±1.21[c]	6.66±0.42[d]
TPC. mg GAE/g DW (extract)	NA	1.75±0.04[a]	6.02±0.41[b]	5.30±0.70[c]	2.12±0.12[a]
TPC, mg GAE/g extract	NA	21.8±0.22[a]	89.2±9.0[b]	45.1±6.0[c]	11.1±0.21[d]

$ABTS^{\bullet+}$, TEAC mg Trolox/g DW (residue)	***P. tricornutum***	**SLE-HEX**	**SLE-AC**	**SLE-EtOH**	**SLE-H_2O**
	61.85±4.22[a]	64.58±4.82[a]	41.05±1.00[b]	33.81±1.23[c]	19.03±2.57[d]
$ABTS^{\bullet+}$, TEAC mg Trolox/g DW (extract)					
	NA	1.56±0.009[a]	14.25±2.72[b]	8.56±0.46[c]	9.37±0.55[c]
$ABTS^{\bullet+}$, TEAC mg Trolox/g extract	NA	20.02±0.11[a]	170.0±8.9[b]	59.0±3.19[c]	39.1±0.73[d]

$DPPH^{\bullet}$, TEAC mg Trolox/g DW (residue)	***P. tricornutum***	**SLE-HEX**	**SLE-AC**	**SLE-EtOH**	**SLE-H_2O**
	7.84±0.62[a]	7.17±0.33[a]	5.35±0.49[b]	1.64±0.31[c]	1.02±0.17[c]
$DPPH^{\bullet}$, TEAC mg Trolox/g DW (extract)					
	NA	0.31±0.01[a]	2.50±0.26[b]	1.10±0.07[c]	0.23±0.02[a]
$DPPH^{\bullet}$, TEAC mg Trolox/g extract	NA	4.17±0.06[a]	38.6±1.68[v]	9.29±0.45[c]	1.34±0.02[d]

*values are expressed as mean ± standard deviation (n = 6). Different lowercase superscript letters indicate significant differences within the same row at $p < 0.05$ (ANOVA, $p < 0.05$). TPC – total phenolic content, DW – dry weight, GAE – gallic acid equivalence, TEAC – trolox equivalent antioxidant capacity

3.5.2. Total phenolic content and antioxidant capacity of SFE-CO_2 extracts and SFE-CO_2 residues

P. tricornutum residual biomass and extracts after supercritical carbon dioxide extraction were investigated for its total phenolic content and *in vitro* antioxidant capacity, in order to evaluate the effectiveness of antioxidants extraction by SFE-CO_2. The results are presented in **Table 3.19**. The values for TPC are expressed as milligrams of gallic acid equivalents per gram of sample. TPC value for residual biomass was 12.33±1.29 mg GAE/g DW, while the initial biomass value was 21.67±0.68 mg GAE/g DW. Compared to SLE-HEX, supercritical carbon dioxide extraction has shown lower antioxidant capacity in the residual biomass, concluding better removal of phenol-rich fractions during the extraction process.

The $ABTS^{\bullet+}$ radical scavenging assay values were expressed as Trolox equivalents – mg Trolox/g DW. The $ABTS^{\bullet+}$ values were 60.99±3.93 mg Trolox/g DW and 4.92±0.09 mg Trolox/g DW for residual biomass and extracts, respectively. The $ABTS^{\bullet+}$ values were in SFE-CO_2 extracts were not statistically significant compared to SLE-HEX. Thus, a smaller part of active antioxidant compounds was extracted and distributed in the extract from the initial biomass using SLE-HEX, when compared to supercritical extraction. The sample's antioxidant capacity was also evaluated by the ability to scavenge $DPPH^{\bullet}$ free radicals. The values of $DPPH^{\bullet}$ cation radical assay were 7.69±1.03 mg Trolox/g DW for the biomass and 0.28±0.06 mg Trolox/g DW for extracts. No significant changes in the antioxidant capacity of *P. tricornutum* biomass were observed prior and after non-polar fraction, removal using supercritical carbon

dioxide extraction; thus, indicating that the solid residues after SFE-CO_2 could be further utilized for recovery of antioxidant-rich fractions.

Table 3.19. Total phenolic content and antioxidant capacity of *P. tricornutum* residual biomass and extracts after supercritical fluid extraction under optimal conditions (395 bars, 30°C, 9.2% EtOH)

TPC, mg GAE/g DW (residue)	***P. tricornutum***	**SFE-CO_2**
	21,67±0,68[a]	12,33±1,29[b]
TPC, mg GAE/g DW (extract)		
	NA	2,9±0,14

$ABTS^{\bullet+}$, TEAC mg Trolox/g DW(residue)	***P. tricornutum***	**SFE-CO_2**
	61,85±4,22[a]	60,99±3,93[a]
$ABTS^{\bullet+}$, TEAC mg Trolox/g DW (extract)		
	NA	4,92±0,09

$DPPH^{\bullet}$, TEAC mg Trolox/g DW (residue)	***P. tricornutum***	**SFE-CO_2**
	7,84±0,62[a]	7,69±1,03[a]
$DPPH^{\bullet}$, TEAC mg Trolox/g DW (extract)		
	NA	0,28±0,06

*values are expressed as mean ± standard deviation (n = 6). Different lowercase superscript letters indicate significant differences within the same row at $p < 0.05$ (ANOVA, $p < 0.05$). SFE-CO_2 – supercritical carbon dioxide extraction, TPC – total phenolic content, DW – dry weight, GAE – gallic acid equivalence, TEAC – trolox equivalent antioxidant capacity

In the study of Feller *et al.*, *P. tricornutum* biomass was evaluated for $DPPH^{\bullet}$ cation radical assay after supercritical carbon dioxide extraction and compared with the values after subcritical n-butane extraction[39]. Among the two other microalgae studied, *P. tricornutum* SFE-CO_2 extracts showed the smallest $DPPH^{\bullet}$ scavenging activities (the scavenging ability was 12.0% and 9.0% using SFE-CO_2 and subcritical n-butane, respectively, which were statistically similar ($p< 0.05$)).

3.6. Chemical and microbiological characterization of *P. tricornutum* extracts

3.6.1. Analysis of carotenoids present in the extracts by HPLC-DAD

All SFE-CO_2 extracts, as well as Soxhlet and extracts obtained under PLE-acetone and PLE-ethanol optimal conditions, were analyzed by means of HPLC-DAD for qualitative and quantitative pigment determination. Overall, all chromatograms were identical regarding the number of peaks and types of carotenoids detected, with main differences in the peak area of eluted compounds, which indicates quantity of each carotenoid depending on extraction type and conditions. HPLC-DAD chromatogram of

SFE-CO_2 optimal conditions extract are shown in **Figure 3.21** Results of peak identification as well as the amount of each carotenoid in mg/g of fucoxanthin equivalence are shown in **Table 3.20**. The presence or absence of all main detected carotenoids in SFE-CO_2, PLE-acetone, PLE-ethanol, and Soxhlet extracts is shown in **Table 3.21**.

The key compounds found to be carotenoids, most notably isomers of fucoxanthin, being also the most abundant compounds of the all-E-fucoxanthin (peak 4) and fucoxanthin isomer (peak 6). The second most prominent peak on all chromatograms is found to be diatoxanthin (peak 8). Among the identified carotenoids were also found violaxanthin, diadinoxanthin, zeaxanthin, and β-carotene (peaks 5, 7, 9, and 12, respectively). Xanthophylls diadinoxanthin and diatoxanthin are involved in a specific molecular response to stressful light conditions called the diadinoxanthin cycle, which allows thermal scattering of excessive light energy and provides efficient photoprotection to the algal photosynthetic apparatus[87].

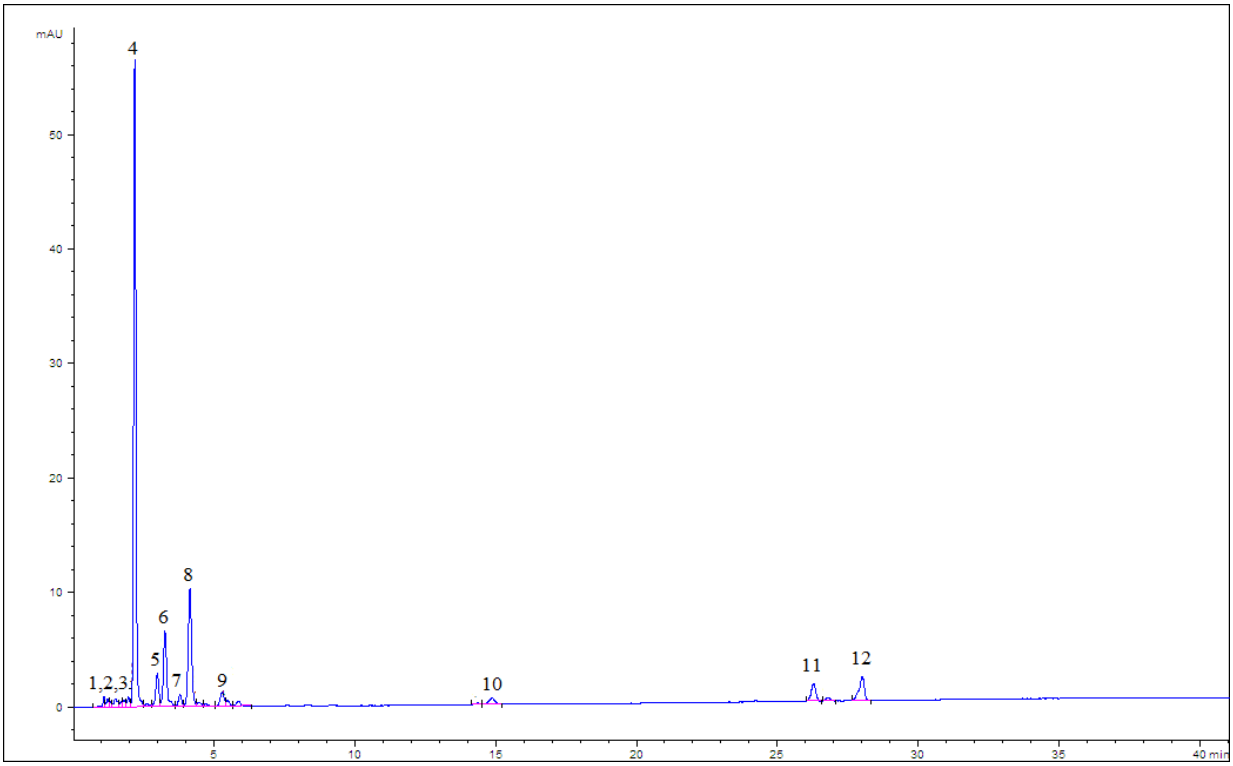


Figure 3.21. HPLC–DAD chromatograms (λ = 460 nm) corresponding to SFE-CO_2 extract under optimal conditions (30°C, 392 bars, 9.2% EtOH)

The other two carotenoids (peak 1, 3), as well as one carotenoid ester (peak 11), were detected in all extracts; however, they were not fully identified, as well as they were not identified in the literature[48], [83], [87]. The remaining minor peaks in the chromatogram (peaks 2 and 10) showed characteristic absorbance spectrum of chlorophylls, and hence those have been assigned as different chlorophyll-types.

Table 3.20. Main carotenoids identification in SFE-CO_2 extract under optimal conditions

Peak	RT	Carotenoid	λmax, (nm)[a]	λmax according to literature, (nm)[a]	mg FE/g extract	percent-size, %	Reference
1	1.083	n/i	398, 418	–	0.84	1.11	[83], [88]
2	1.473	Chlorophyll-type	430	430	0.96	1.28	[83]
3	1.690	n/i	397, 484	–	0.51	0.68	[83]
4	2.160	(all-E)-Fucoxanthin[b]	448	447	47.7	63.16	[47], [83], [88]
5	2.963	(all-E)-Violaxanthin	(428), 448, 466	(418)–442–474	2.48	3.28	[83], [88]
6	3.235	(13Z)-Fucoxanthin[b]	444, 466	334–442–(466)	5.69	7.54	[47], [83], [88]
7	3.768	(all-E)-Diadinoxanthin	(416), 434, 460	(426)–450–478	1.08	1.44	[83], [88]
8	4.119	(all-E)-Diatoxanthin	(436), 454, 482	(430)–454–482	9.0	11.92	[47], [83], [88]
9	5.262	(all-E)-Zeaxanthin	(432), 450, 478	(430)–454–482	1.45	1.92	[47], [83], [88]
10	14.852	Chlorophyll-type	466	440	0.98	1.31	[83], [88]
11	26.270	Carotenoid-ester	410	450	1.87	2.48	[47], [83], [88]
12	28.001	β-carotene[c]	450, 478	451, 478	2.88	3.82	[83], [88]
Total					75.49	100	

RT – retention time, n/i – not identified, FE – fucoxanthin equivalence

[a] – shoulder is reported in parenthesis; [b,c] – identified with a commercial standard

In SFE-CO_2 optimal conditions extract all fucoxanthin isomers accounted for about 70% of total carotenoids, while all-E diatoxanthin was also present in all extracts in a lower but considerable concentration of about 12%. All-E violaxanthin and β-carotene comprise a total of 3% each. Similar fucoxanthin levels in *P. tricornutum* extracts were also reported in the literature[87], [128], [160].

Table 3.21. Presence of the main carotenoids in SFE-CO_2, PLE, and Soxhlet extracts.

Carotenoid	SFE-CO_2	PLE-acetone	PLE-ethanol	Soxhlet
Chlorophyll-type	+	+	+	+
(all-E)-Fucoxanthin[b]	+	+	+	+
(all-E)-Violaxanthin	+	+	+	+
(13Z)-Fucoxanthin[b]	+	+	+	+
(all-E)-Diadinoxanthin	+	+	+	+
(all-E)-Diatoxanthin	+	+	+	+
(all-E)-Zeaxanthin	+	+	-	+
Chlorophyll-type	+	-	-	+
Carotenoid-ester	+	+	-	+
β-carotene[c]	+	-	-	+

SFE-CO_2 – supercritical carbon dioxide extraction, PLE – pressurized liquid extraction

As described above, due to its potential bioactivities, E-fucoxanthin was the principal pigment and the most valuable compound. Therefore, it was further quantified in the extracts (**Table 3.22**). The amount of fucoxanthin recovered during SFE-CO_2 optimal conditions was 4.7 mg/g DW, while further recovery from the same biomass under PLE extraction with acetone and ethanol was 0.76 and 1.1 mg/g DW respectively, bringing the total amount of biorefined fucoxanthin to 6.6 mg/g DW. Overall, about 73%

of total fucoxanthin present in the freeze-dried *P. tricornutum* biomass was recovered using SFE-CO_2. Soxhlet extraction itself recovered only 2.25 mg/g DW of fucoxanthin.

Table 3.22. Recoveries of fucoxanthin (in mg/ extract and mg/g DW) from *P. tricornutum* biomass after stepwise SFE-CO_2/PLE extraction and Soxhlet as a reference

	mg/g extract	**Recovery (%)**	**mg/g DW**
SFE-CO_2	47.70^a	73.75	4.74^a
PLE-acetone	9.93^b	15.36	0.76^b
PLE-ethanol	7.03^b	10.87	1.10^b
Total	**64.66**	**100**	**6.60**
Reference Soxhlet	22.33^c	34.53	2.25^c

*Different lowercase superscript letters indicate significant differences within the same column at $p < 0.05$ (ANOVA, $p < 0.05$). SFE-CO_2 – supercritical carbon dioxide extraction, PLE – pressurized liquid extraction, DW – dry weight

In the work of Wu *et al.,* six strains of *P. tricornutum* were investigated for their fucoxanthin content, with the highest obtained value of 5.5 mg/g DW[160]. These results indicate that SFE-CO_2 is a suitable method for fucoxanthin recovery from *P. tricornutum* biomass, allowing to extract a major part of fucoxanthin and being almost twice more efficient compared to Soxhlet.

3.6.2. Fatty acid profile of extracted oils

P. tricornutum extracts after Soxhlet extraction with hexane, SFE-CO_2, and PLE acetone under optimal conditions were analyzed by means of GC-FID for fatty acid profile determination. Overall, the total saturated (SFA), monounsaturated (MUFA), polyunsaturated (PUFA), unsaturated, and the ratio of saturated to unsaturated (SFA/UFA) fatty acid composition of extracts is reported in the **Table 3.23.** All extracts showed a predominance of PUFA (45–51%) over SFA (23-33%) and MUFA (25–34%).

As expected, the most abundant fatty acid in all samples, as well as out of all PUFA, was eicosapentaenoic acid (EPA, C20:5 n3). EPA accounted for approximately 40% of the total amount of all fatty acids in extracts after Soxhlet extraction and SFE-CO_2 and about 20% in PLE extract of residual biomass after SFE-CO_2. Another polyunsaturated fatty acid, namely linoleic acid (C18:2n-6), was also present in considerable quantities (4–6%) in all extracts. The second most abundant fatty acids were both MUFA, oleic acid (C18:1n-9) and palmitoleic acid (C16:1), each of them comprised almost 13% in all extracted samples. Among SFA, the main present acids were palmitic (C16:0) 6–13%, myristic (C14:0) 3–4%, and pentadecanoic acid (C15:0) 1–3%. In general, all extracted showed similar fatty acid profiles, mainly differ in percent-size of each acid. However, PLE-acetone extract showed significantly higher content of three particular fatty acids – erucic acid (C22:1n-9), which was only detected after PLE extraction, docosanoic acid (C22:0) and C20:3n3.

The results obtained in current research are in agreement with the previous studies of Wu *et al.,* Di Lena *et al.,* Banskota *et al.* and Feller *et al.* [8], [39], [89], [160], where extracts from *P. tricornutum* were mostly composed of EPA, palmitoleic acid, palmitic acid, and oleic acid. Moreover, results showed that only small amounts of DHA (C22:6n-3) were detected – the maximum of 0.28% in the present study and from 1.54 to 2.64% in the study of Fller *et al.,* where six strains of *P. tricornutum* were evaluated for their fatty acid profile[160]. The n-3/n-6 ratio, as an indicator of the nutritional value of food, is beneficial to be elevated in the human diet in order to avoid cardiovascular and chronic inflammatory diseases and

counteract the excessive intake of n-6 PUFA typical for modern Western diets[69]. The obtained n-3/n-6 ratio was favorably high (5.2) for Soxhlet, SFE-CO_2, and PLE-acetone extracts. These values are in line with the study of Di Lena *et al.*, where the n-3/n-6 ratio for *P. tricornutum* extracts was reported to be 5.01, suggesting the potential health properties of microalgal oils[89].

Table 3.23. Fatty acids composition of the algae biomass extracts using the four extraction methods (% of the total GC-FID peak area)

Fatty acid	Soxhlet	SFE-CO_2	PLE-AC
C8:0	0.49[a]	0.30[a]	0.45[a]
C11:0	0.49[a]	0.37[a]	0.67[b]
C12:0	0.82[a]	0.76[a]	0.48[b]
C13:0	1.30[a]	1.24[a]	0.60[b]
C14:0	3.41[a]	4.01[b]	4.44[b]
C14:1	0.29[a]	0.31[a]	0.00[b]
C15:0	3.43[a]	3.70[a]	2.04[b]
C16:0	7.52[a]	6.94[a]	8.40[b]
C16:1	12.41[a]	13.22[a]	12.55[a]
C17:0	0.47[a]	0.45[a]	0.00[b]
C18:0	1.56[a]	3.62[b]	3.89[b]
C18:1n9c	13.17[a]	9.81[b]	13.50[a]
C18:2n6c	5.02[a]	6.18[b]	5.60[b]
C20:0	0.13[a]	0.35[b]	0.00[c]
C18:3n6	0.36[a]	1.21[b]	0.00[c]
C20:1n9	0.00[a]	0.11[b]	0.00[a]
C18:3n3	0.55[a]	1.03[b]	0.64[a]
C21:0	1.35[a]	0.43[b]	0.00[c]
C20:2	0.18[a]	0.52[b]	0.43[b]
C22:0	0.29[a]	0.80[b]	3.67[c]
C20:3n6	0.94[a]	0.80[a]	1.39[b]
C22:1n9	0.00[a]	0.00[a a]	2.10[b]
C20:3n3	1.10[a]	0.09[b]	10.23[c]
C20:4n6	0.21[a]	0.21[a]	0.00[b]
C22:2n6	1.30[a]	1.24[a]	0.97[b]
C24:0	1.86[a]	0.41[b]	0.57[b]
C20:5n3	40.69[s]	39.44[a]	26.70[b]
C24:1	0.38[a]	2.18[b]	0.65[a]
C22:6n3	0.28[a]	0.26[a]	0.00[b]
SFA	23.12[a]	23.37[a]	25.22[b]
MUFA	26.25[a]	25.64[a]	28.81[b]
PUFA	50.63[a]	50.99[a]	45.97[b]
TOTAL UFA	76.88[a]	76.63[a]	74.78[b]
SFA/UFA	0.30[a]	0.30[a]	0.34[a]
n6	7.83[a]	9.64[b]	7.97[a]
n3	42.62[a]	40.83[b]	37.57[c]

*Different lowercase superscript letters indicate significant differences within the same row at $p < 0.05$ (ANOVA, $p < 0.05$); SFE – saturated fatty acids; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids; UFA – unsaturated fatty acids

3.6.3. Evaluation of the antimicrobial activity of *P. tricornutum* extracts after different extraction techniques

P. tricornutum extracts after SFE-CO_2, two-step PLE (acetone, ethanol) and SLE (hexane, acetone, ethanol, water) in duplicates were evaluated for their antimicrobial effects against eight pathogenic bacteria (*B. cereus, S. aureus, L. monocytogenes, E. coli, Salmonella* spp, *E. faecalis, P. aeruginosa* and

fungus *Aspergillus niger*) using broth microdilution method (data is shown in the **Table 3.24**). After performing the disk diffusion in order to evaluate the minimal inhibitory concentration (MIC) of each active extract, microdilution method was applied on SFE-CO_2 (optimal conditions) extract, as well as SLE-ethanol and SLE-H_2O that showed inhibition of two bacteria growth (*B. cereus, S. aureus*) at the concentration of 1 mg/ml. The antimicrobial activity of active extracts was quantified in μg/ml and the results are shown in **Table 3.25.**

Table 3.24. Inhibition activity of all tested extracts against eight pathogenic bacteria: a plus sign indicates the antimicrobial activity of each particular extract against bacteria

Bacteria	Extracts						
	SFE-CO_2	**PLE-acetone**	**PLE-ethanol**	**SLE-hexane**	**SLE-acetone**	**SLE-ethanol**	**SLE-water**
B. cereus	+	-	-	-	-	+	+
E. faecalis	-	-	-	-	-	-	-
L. monocytogenes	-	-	-	-	-	-	-
E. coli	-	-	-	-	-	-	-
Salmonella spp.	-	-	-	-	-	-	-
S. aureus	+	-	-	-	-	+	+
P. aeruginosa	-	-	-	-	-	-	-
Aspergillus niger	-	-	-	-	-	-	-

SFE-CO_2 – supercritical carbon dioxide extraction, PLE – pressurized liquid extraction, SLE – solid liquid extraction

Overall, MIC values ranged from 84 to 675 μg/ml, with SFE-CO_2 extract showing the highest antimicrobial activity, with significant difference in concentration, against both bacterial species, MIC for *B. cereus* was 84 μg/ml and for *S. aureus* 168 μg/ml. The second most active extract turned out to be SLE-ethanol with MIC of 337 μg/ml against *B. cereus* and 168 μg/ml against *S. aureus*; meanwhile, SLE-H_2O expressed the same MIC of 675 μg/ml against both pathogenic bacteria.

Table 3.25. Minimum inhibitory concentration (MIC, given as μg extract/ml) of SFE-CO_2 optimal conditions, SLE-ethanol and SLE-H_2O against *B. cereus, S. aureus*

	MIC (μg/ml)		
Bacterial species	**SFE-CO_2**	**SLE-ethanol**	**SLE-H_2O**
B. cereus	84.37^{a}	337.5^{a}	675^{a}
S. aureus	168.75^{a}	168.75^{b}	675^{a}

*Different lowercase superscript letters indicate significant differences within the same column at $p < 0.05$ (ANOVA, $p < 0.05$). SFE-CO_2 – supercritical carbon dioxide extraction, SLE – solid liquid extraction, MIC – minimal inhibitory concentration

In the work of Wang *et al., P. tricornutum* methanolic extract (freeze-fried biomass was macerated in 100% methanol for 10 min, with further centrifugation and solvent evaporation) was tested for its antimicrobial activity against gram-positive *Bacillus subtilis.* MIC was found to be 307.5 μg/ml, which is similar to the MIC of the ethanolic extract against *B. cereus* obtained in the current study[158].

Moreover, in the study by Maadane *et al., P. tricornutum* lyophilized biomass was macerated in ethanol for 3h, and extracts were evaluated against the bacteria*: E. coli, P. aeruginosa* and *S. aureus*, the yeast *Candida albicans* and the fungus *Aspergillus niger*. *P. tricornutum* showed a lot weaker inhibition activity and only against *E. coli, S. aureus C. albicans,* and *P. aeruginosa* with a significantly higher MIC ranged from 2.6 to 4.3 mg extract per ml[98].

Such antimicrobial activities against bacteria might occur due to a great part of dominant fatty acids, which were found to be present in all extracts, especially rich in SFE-CO_2. In polar extracts antimicrobial activity is mostly related to polar substances, such as carotenoids and polysaccharides[115]. This statement is in agreement with published observations[145], which confirms the antibacterial activity of the C16–C20 fatty acids found in extracts from the microalga *N. oculata*. It was also showed that EPA, the major fatty acid in all extracts of *P. tricornutum* has a significant antimicrobial effect[33]. Carotenoids as well demonstrated to have a considerable effect against both Gram-positive and Gram-negative bacteria[152]. According to the previous work[96], ethanolic extracts from *Dunaliella sp., P. tricornutum,* and *Tetraselmis sp.* contained a higher amount of carotenoids; thus the observed antimicrobial activities SLE water and ethanol extract extracts could be linked with their carotenoid content. Besides, the ethanol and water extracts from *P. tricornutum* contained a high amount of phenolic compounds (polyphenols), expressed as mg GAE/g DW. Hence, a part of the antimicrobial activity of extracts could be attributed to their total phenolic content, which is in agreement with data found in the literature[111].

Phytochemicals can work by inducing cellular membrane disruptions, interfering with certain microbial metabolic processes, modulating signal transduction, or gene expression. Fatty acids can initiate peroxidative processes and prevent bacterial fatty acids from being synthesized[33], [162]. Moreover, free fatty acids may cause leakage of molecules from microbial cells, inhibition of their respiration, or reduction of their nutrient uptake by interacting with cellular membranes of these cells[98], [146]. Carotenoids, fatty acids, and phenolic combinations are likely to interact independently or synergistically, indicating the existence of pharmaceutically promising antibacterial compounds in the obtained extracts.

CONCLUSIONS

1. Chemical composition of *Phaeodactylum tricornutum* freeze-dried biomass was determined. Protein content was 43.54±0.34%, total oil content was 10.1±0.13%, ash content was 8.16±0.042% and water content was 3.97±0.16%.
2. Stepwise SLE extraction was performed using hexane, acetone, ethanol and water. The highest yield (18.05±0.89%) was obtained using water as solvent, following by ethanol (13.3±1.06%), hexane (8.18±0.32%) and acetone (6.7±0.25%).
3. SFE-CO_2 was optimized and the effect of pressure, temperature and the amount of co-solvent on extraction yield, total carotenoid and fucoxanthin content was investigated. Obtained optimal SFE-CO_2 extraction conditions were 395 bars, 30°C and 9.2% EtOH. Total oil yield under optimal conditions was 9.8±0.22%, total carotenoid content 35.14±2.45 mg/g extract and fucoxanthin content was 4.7 mg/g DW. With variation of actual and predicted values of maximum ±4% for each response.
4. The influence of temperature and extraction time on PLE-acetone was evaluated and the extraction was optimized. Suggested optimal conditions were 61°C and 39 min. Total yield, carotenoid contend and phenolic content under optimal conditions were 7.6±0.63 g/100g DW, 10.9±0.63 mg/g extract and 4.68±0.29 mg GAE/g DW respectively.
5. PLE ethanol-assisted extraction of the dry residues after SFE-CO_2 and PLE-acetone was evaluated and optimized using RSM. Optimal conditions were 66°C and 17 min of extraction time. Yield obtained under optimal conditions was 15.2±0.29 g/100g DW, total carotenoid content was 7.6±0.42mg/g extract and TPC was 5.1±0.22 mg GAE/g DW.
6. During SLE, the initial TPC value of raw biomass was 21.67±0.68 total phenols values for extracts ranged between 1.75±0.04 and 6.02±0.41, with acetone showing the highest capability of extracting phenolic compounds and hexane the lowest. The values of during $ABTS^{\bullet+}$ for dry residues ranged from 64.58±4.82 to 19.03±2.57 mg Trolox/g residue and acetone extracts expressed the strongest antioxidant activities (14.25±2.72 mg Trolox/g DW extract). In residual biomass, the highest $ABTS^{\bullet+}$ values were observed after hexane extraction. Values of $DPPH^{\bullet}$ scavenging assay for the initial biomass and dry residues ranged from 7.84±0.62 to 1.02±0.17 mg Trolox/g. For extracts values ranged from 0.23±0.02 to 2.50±0.26 mg Trolox/g DW extract, with the highest antioxidant capacity found in acetone and ethanol extracts.

   For SFE-CO_2 TPC value of residual biomass was 12.33±1.29 mg GAE/g DW, while initial biomass value was 21.67±0.68 mg GAE/g DW. The $ABTS^{\bullet+}$ values were 60.99±3.93 mg Trolox/g DW and 4.92±0.09 mg Trolox/g DW for residual biomass and extracts respectively. The values of $DPPH^{\bullet}$ cation radical assay were 7.69±1.03 mg Trolox/g DW for the biomass and 0.28±0.06 mg Trolox/g DW for extracts. Overall, SFE-CO_2 extracts showed low TPC and radical scavenging capacity, indicating that non-polar CO_2 does not extract the major antioxidant-rich fractions.
7. HPLC-DAD was performed for qualitative and quantitative carotenoid determination. Overall, the main carotenoid in non-polar extracts was found to be fucoxanthin and its isomers, with the highest recovery of approximately 73% of total fucoxanthin content, applying SFE-CO_2. The amount of fucoxanthin recovered during SFE-CO_2 under optimal conditions was 4.7 mg/g DW, while under PLE with acetone and ethanol was 0.76 and 1.1 mg/g DW respectively, bringing the total amount of biorefined fucoxanthin to 6.5 mg/g DW. Soxhlet extraction itself recovered only 2.25 mg/g DW of fucoxanthin.

8. GC-FID was applied to *P. tricornutum* Soxhlet, SFE-CO_2 and PLE-acetone extracts for fatty acid profile determination. Eicosapentaenoic acid (EPA, C20:5 n3) comprised of approximately 40% of the total amount of all fatty acids in extracts after Soxhlet extraction and SFE-CO_2 and about 20% in PLE-acetone extract. The second the most abundant fatty acids were oleic acid (C18:1n-9) and palmitoleic acid (C16:1), each of them comprised almost 13% in all extracted samples, following by polyunsaturated linoleic acid (C18:2n-6), which accounted for 4–6%. Palmitic (C16:0) 6–13%, myristic (C14:0) 3–4% and pentadecanoic acid (C15:0) 1–3% were the main SFE acids in all samples.
9. All extracts were tested for their antimicrobial activity against eight pathogenic bacteria (*B. cereus, S. aureus, L. monocytogenes, E. coli, Salmonella* spp, *E. faecalis, P. aeruginosa* and fungus *Aspergillus niger*) using broth microdilution method. Overall, only SFE-CO_2, SLE-ethanol and SLE-H_2O showed antimicrobial activity against 2 bacteria – *B. cereus, S. aureus*. MIC in SFE-CO_2 extract against *B. cereus* was 84 μg/ml and against *S. aureus* 168 μg/ml. For SLE-ethanol MIC was 337 μg/ml against *B. cereus* and 168 μg/ml against *S. aureus*; meanwhile, SLE-H_2O expressed the same MIC of 675 μg/ml against both pathogenic bacteria.

LIST OF REFERENCES

1. A. MUSTAFA AND C. TURNER: Pressurized liquid extraction as a green approach in food and herbal plants extraction: A review. In: *Analytica Chimica Acta* Bd. 703 (2011), Nr. 1, S. 8–18

2. ADOM KK AND LIU RH.: Antioxidant activity of grains. In: *Journal of Agricultural and Food Chemistry* Bd. 50 (2012), S. 6182–6187

3. ANTIOXIDANT ACTIVITY APPLYING AN IMPROVED ABTS RADICAL. In: *A. Activity, A. An, I. Abts, and C. D. Assay*. Bd. 26, 1999, S. 1231–1237

4. ARAVENA RI DEL VALLE J.: Effect of microalgae preconditioning on supercritical CO2 extraction of astaxanthin from haematococcus pluvialis. In: *10th International Conference of Supercritical Fluids*. USA

5. ASAI, A., SUGAWARA, T., ONO, H., & NAGAO, A.: Biotransformation of fucoxanthinol into amarouciaxanthin a in mice and HepG2 cells: Formation and cytotoxicity of fucoxanthin metabolites. In: *Drug Metabolism and Disposition* Bd. 32 (2004), Nr. 2, S. 205–211

6. AXELSSON, LISA ; FRANZÉN, MARIA ; OSTWALD, MADELENE ; BERNDES, GÖRAN ; LAKSHMI, G. ; RAVINDRANATH, N. H.: Perspective: Jatropha cultivation in southern India: Assessing farmers' experiences. In: *Biofuels, Bioproducts and Biorefining* Bd. 6 (2012), Nr. 3, S. 246–256

7. B.J. MEYER: Are we consuming enough long chain omega-3 polyunsaturated fatty acids for optimal health? In: *Prostaglandins, Leukotrienes Essent, Fat. Acids* Bd. 85 (2011), S. 75–280

8. BANSKOTA, ARJUN H. ; SPERKER, SANDRA ; STEFANOVA, ROUMIANA ; MCGINN, PATRICK J. ; O'LEARY, STEPHEN J.B.: Antioxidant properties and lipid composition of selected microalgae. In: *Journal of Applied Phycology* Bd. 31, Journal of Applied Phycology (2019), Nr. 1, S. 309–318

9. BAÑUELOS-HERNÁNDEZ, BERNARDO ; BELTRÁN-LÓPEZ, JOSUÉ I. ; ROSALES-MENDOZA, SERGIO: Production of Biopharmaceuticals in Microalgae. In: *Handbook of Marine Microalgae: Biotechnology Advances* (2015), S. 281–298 — ISBN 9780128011249

10. BARKIA, INES ; SAARI, NAZAMID ; MANNING, SCHONNA R.: Microalgae for high-value products towards human health and nutrition. In: *Marine Drugs* Bd. 17 (2019), Nr. 5, S. 1–29

11. BECKER E. W.: Microalgae as a source of protein. In: *Biotechnology Advances* (2007), Nr. 25, S. 207–210

12. BIMAKR, MANDANA ; ABDUL RAHMAN, RUSSLY ; TAIP, FARAH ; LING, TAU ; GANJLOO, ALI ; SELAMAT, JINAP ; ABDUL HAMID, AZIZAH: Supercritical Carbon Dioxide (SC-CO2) Extraction of Bioactive Flavonoid Compounds from Spearmint (Mentha Spicata L.) Leaves. In: *European Journal of Scientific Research* Bd. 33 (2009), S. 679–690

13. BOROWITZKA, M. A. & VOLCANI, B. E.: The polymorphic diatom Phaeodactylum tricornutum: ultrastructure of its morphotypes. In: *Journal of Phycology* (1978), Nr. 14, S. 10–21

14. BOROWITZKA, MICHAEL A.: High-value products from microalgae-their development and commercialisation. In: *Journal of Applied Phycology* Bd. 25 (2013), Nr. 3, S. 743–756 — ISBN 0921-8971

15. BRANCO-VIEIRA, MONIQUE ; MARTIN, SERGIO SAN ; AGURTO, CRISTIAN ; FREITAS, MARCOS A.V. ; MATA, TERESA M. ; MARTINS, ANTÓNIO A. ; CAETANO, NÍDIA: Biochemical characterization of Phaeodactylum tricornutum for microalgae-based biorefinery. In: *Energy Procedia* Bd. 153, Elsevier B.V. (2018), Nr. November, S. 466–470

16. BRANCO-VIEIRA, MONIQUE ; MARTIN, SERGIO SAN ; AGURTO, CRISTIAN ; SANTOS, MARCO A. ; FREITAS, MARCOS A.V. ; CAETANO, NÍDIA S.: Analyzing Phaeodactylum tricornutum lipid profile for biodiesel production. In: *Energy Procedia* Bd. 136, Elsevier B.V. (2017), S. 369–373

17. BROWN, MR.: The amino acid and sugar composition of 16 species of microalgae used in mariculture. In: *Journal of Experimental Marine Biology and Ecology* (1991), Nr. 145, S. 79–99

18. BUSTAMANTE A, ROBERTS P, ARAVENA R AND DEL VALLE JM: Supercritical extraction of astaxanthin from haematococcus pluvialis using ethanol-modified CO2, experiments and modelling. In: *11th International Conference of Engineering and Food, Athens, Greece*, 2011

19. C. M. GALANAKIS, G. LABORATORIES, V. GOULAS, S. TSAKONA, G. MANGANARIS: A Knowledge Base for The Recovery of Natural Phenols with Different Solvents. In: *International Journal of Food Properties* (2015)

20. CARDOSO LC, SERRANO CM, RODRIGUEZ RODRIGUEZ M, MARTINEZ DE LA OSSA EJ AND LUBIAN LM: Extraction of Carotenoids and Fatty Acids from Microalgae Using Supercritical Technology. In: *Journal of Analytical Chemistry* (2012), Nr. 3, S. 877–883

21. CASTRO-PUYANA, M., HERRERO, M., URRETA, I., MENDIOLA, J. A., CIFUENTES, A., IBÁÑEZ, E., & SUÁREZ-ALVAREZ, S.: Optimization of clean extraction methods to isolate carotenoids from themicroalga Neochloris oleoabundans and subsequent chemical characterization using liquid chromatography tandemmass spectrometry. In: *Analytical and Bioanalytical Chemistry* (2013), Nr. 406, S. 4607–4616

22. CHA KH, LEE HJ, KOO SY, SONG DG, LEE DU AND PAN CH: Optimization of pressurized liquid extraction of carotenoids and chlorophylls from chlorella vulgaris. In: *Journal of Agricultural and Food Chemistry* (2010), Nr. 58, S. 793–797

23. CHENG C-H, DU T-B, PI H-C, JANG S-M, LIN Y-H, LEE H-T.: Comparative study of lipid extraction from microalgae by organic solvent and supercritical CO 2. In: *Bioresource Technology* Bd. 102 (2011), Nr. 21, S. 10151–10153

24. CHISTI, Y.: Biodiesel from microalgae. In: *Biotechnology Advances Advances* (2007), Nr. 25, S. 294–306

25. COONEY, MICHAEL ; YOUNG, GREG ; NAGLE, NICK: Extraction of bio-oils from microalgae. In: *Separation and Purification Reviews* Bd. 38 (2009), Nr. 4, S. 291–325 — ISBN 15422119 (ISSN)

26. D. KIM, S. WEON, AND C. Y. LEE: Antioxidant capacity of phenolic phytochemicals from various cultivars of plums (2003), Nr. 81, S. 321–326

27. D.B. RODRIGUEZ-AMAYA: Carotenoids and chronic diseases, food carotenoids. In: *John Wiley & Sons Ltd* (2015), S. 282–304

28. DAS, S. K., REN, R., HASHIMOTO, T., & KANAZAWA, K.: Fucoxanthin induces apoptosis in osteoclast-like cells differentiated from RAW264.7 cells. In: *Journal of Agricultural and Food Chemistry* Bd. 58 (2010), Nr. 10, S. 6090–6095

29. DELBRUT, ANTOINE ; ALBINA, PIERRE ; LAPIERRE, THÉO ; PRADELLES, RÉMI ; DUBREUCQ, ERIC: Fucoxanthin and polyunsaturated fatty acids co-extraction by a green process. In: *Molecules* Bd. 23 (2018), Nr. 4, S. 1–15

30. DEMIRBAS, A.: Biorefineries: current activities and future developments. In: *Energy Conversion and Management* Bd. 50 (2009), Nr. 11, S. 2782–2801

31. DENERY JR, DRAGUL K, TANG CS AND LI QX: Pressurized fluid extraction of carotenoids from haematococcus pluvialis and Dunaliella salina and kavalactones from Piper methysticum. In: *Analytica Chimica Acta* (2004), Nr. 501, S. 175–181

32. DERWENSKUS, FELIX ; METZ, FELIX ; GILLE, ANDREA ; SCHMID-STAIGER, ULRIKE ; BRIVIBA, KARLIS ; SCHLIEßMANN, URSULA ; HIRTH, THOMAS: Pressurized extraction of unsaturated fatty acids and carotenoids from wet Chlorella vulgaris and Phaeodactylum tricornutum biomass using subcritical liquids. In: *GCB Bioenergy* Bd. 11 (2019), Nr. 1, S. 335–344

33. DESBOIS, A.P., & SMITH, V.J.: Antibacterial free fatty acids: activities, mechanisms of action and biotechnological potential. In: *Applied Microbiology and Biotechnology* (2010), Nr. 85, S. 1629–1642

34. DINER RE, SCHWENCK SM, MCCROW JP, ZHENG H, ALLEN AELLIS: Genetic manipulation of competition for nitrate between heterotrophic bacteria and diatoms. In: *Frontiers in Microbiology* Bd. 7 (2016)

35. ESKILSSON, C. S.; HARTONEN, K.; MATHIASSON, L.; RIEKKOLA, M.-L.: Pressurized hot water extraction of insecticides from process dust−comparison with supercritical fluid extraction. In: *Journal of Separation Science*

(2004), Nr. 27, S. 59– 64

36. FALKOWSKI, PAUL G ; RAVEN, JOHN A: *Aquatic Photosynthesis*. STU-Stud. : Princeton University Press, 2007 — ISBN 9780691115511

37. FAO: *The State of World Fisheries and Aquaculture 2018*. Bd. 3, 2018 — ISBN 9789251305621

38. FARÍAS-CAMPOMANES, ANGELA M. ; ROSTAGNO, MAURICIO A. ; COAQUIRA-QUISPE, JOEL J. ; MEIRELES, M. ANGELA A.: Supercritical fluid extraction of polyphenols from lees: Overall extraction curve, kinetic data and composition of the extracts. In: *Bioresources and Bioprocessing* Bd. 2, Springer Berlin Heidelberg (2015), Nr. 1

39. FELLER, RAFAEL ; MATOS, ÂNGELO P. ; MAZZUTTI, SIMONE ; MOECKE, ELISA H.S. ; TRES, MARCUS V. ; DERNER, ROBERTO B. ; OLIVEIRA, JOSÉ V. ; JUNIOR, AGENOR F.: Polyunsaturated Ω-3 and Ω-6 fatty acids, total carotenoids and antioxidant activity of three marine microalgae extracts obtained by supercritical CO2 and subcritical n-butane. In: *Journal of Supercritical Fluids* Bd. 133 (2018), Nr. November 2017, S. 437–443

40. FLUIDS, SUPERCRITICAL: Supercritical fluid chromatography (2020), S. 2–7

41. FOO, SU CHERN ; YUSOFF, FATIMAH MD ; ISMAIL, MAZNAH ; BASRI, MAHIRAN ; YAU, SOOK KUN ; KHONG, NICHOLAS M.H. ; CHAN, KIM WEI ; EBRAHIMI, MAHDI: Antioxidant capacities of fucoxanthin-producing algae as influenced by their carotenoid and phenolic contents. In: *Journal of Biotechnology* Bd. 241, Elsevier B.V. (2017), S. 175–183

42. FUJII K: Process integration of supercritical carbon dioxide extraction and acid treatment for astaxanthin extraction from a vegetative microalga. In: *Food and Bioproducts Processing* Bd. 90 (2012), Nr. 4, S. 762–766

43. FUNG, A.; HAMID, N.; LU, J.: Fucoxanthin content and antioxidant properties of Undaria pinnatifida. In: *Food Chemistry* (2013), Nr. 136, S. 1055–1062

44. GALLEGO, ROCÍO ; MARTÍNEZ, MARINA ; CIFUENTES, ALEJANDRO ; IBÁÑEZ, ELENA ; HERRERO, MIGUEL: Development of a Green Downstream Process for the Valorization of Porphyridium cruentum Biomass. In: *Molecules* (2019)

45. GERMAN-BÁEZ, L. J. ; VALDEZ-FLORES, M. A. ; FÉLIX-MEDINA, J. V. ; NORZAGARAY-VALENZUELA, C. D. ; SANTOS-BALLARDO, D. U. ; REYES-MORENO, C. ; SHELTON, L. M. ; VALDEZ-ORTIZ, A.: Chemical composition and physicochemical properties of Phaeodactylum tricornutum microalgal residual biomass. In: *Food Science and Technology International* Bd. 23 (2017), Nr. 8, S. 681–689

46. GIFUNI, IMMA ; POLLIO, ANTONINO ; SAFI, CARL ; MARZOCCHELLA, ANTONIO ; OLIVIERI, GIUSEPPE: Current Bottlenecks and Challenges of the Microalgal Biorefinery. In: *Trends in Biotechnology* Bd. 37, Elsevier Ltd (2019), Nr. 3, S. 242–252

47. GILBERT-LÓPEZ, BIENVENIDA ; BARRANCO, ALMUDENA ; HERRERO, MIGUEL ; CIFUENTES, ALEJANDRO ; IBÁÑEZ, ELENA: Development of new green processes for the recovery of bioactives from Phaeodactylum tricornutum. In: *Food Research International* Bd. 99, Elsevier Ltd (2017), S. 1056–1065

48. GILBERT-LÓPEZ, BIENVENIDA ; BARRANCO, ALMUDENA ; HERRERO, MIGUEL ; CIFUENTES, ALEJANDRO ; IBÁÑEZ, ELENA: Development of new green processes for the recovery of bioactives from Phaeodactylum tricornutum. In: *Food Research International* Bd. 99, Elsevier Ltd (2017), S. 1056–1065

49. GOIRIS K, MUYLAERT K, FRAEYE I AND FOUBERT I.: Antioxidant potential of microalgae in relation to their phenolic and carotenoid content. In: *Applied Phycology* (2012), Nr. 24, S. 1477–1486

50. GOIRIS K, MUYLAERT K, FRAEYE I AND FOUBERT I.: Antioxidant potential of microalgae in relation to their phenolic and carotenoid content. In: *Journal of Applied Phycology* (2012), Nr. 24, S. 1477–1486

51. GÖKMEN, VURAL ; SERPEN, ARDA ; FOGLIANO, VINCENZO: Direct measurement of the total antioxidant capacity of foods: the „QUENCHER“ approach. In: *Trends in Food Science and Technology* Bd. 20 (2009), Nr. 6–7, S. 278–288

52. GOYENECHE, ROSARIO ; FANOVICH, ALEJANDRA ; RODRIGUEZ RODRIGUES, CHRISTIAN ; NICOLAO, MARÍA ; DI

SCALA, KARINA: Supercritical CO2 extraction of bioactive compounds from radish leaves: Yield, antioxidant capacity and cytotoxicity. In: *The Journal of Supercritical Fluids* Bd. 135 (2018)

53. GRASA-LÓPEZ, A.; MILIAR-GARCÍA, Á.; QUEVEDO-CORONA, L.; PANIAGUA-CASTRO, N.; ESCALONA-CARDOSO, G.; REYES-MALDONADO, E.; JARAMILLO-FLORES, M.-E.: Undaria pinnatifida and fucoxanthin ameliorate lipogenesis and markers of both inflammation and cardiovascular dysfunction in an animal model of diet-induced obesity. In: *Marine Drugs* Bd. 14 (2016), Nr. 148

54. GUIL-GUERRERO, J. L. ; NAVARRO-JUÁREZ, R. ; LÓPEZ-MARTÍNEZ, J. C. ; CAMPRA-MADRID, P. ; REBOLLOSO-FUENTES, M. M.: Functional properties of the biomass of three microalgal species. In: *Journal of Food Engineering* Bd. 65 (2004), Nr. 4, S. 511–517

55. H. SUN, X. GE, Y. LV, AND A. WANG: Application of accelerated solvent extraction in the analysis of organic contaminants, bioactive and nutritional compounds in food and feed. In: *Journal of Chromatography A* Bd. 1237 (2012), S. 1–23

56. H.W. YEN, S.C. YANG, C.H. CHEN, JESISCA, J.S. CHANG: Supercritical fluid extraction of valuable compounds from microalgal biomass. In: *Bioresource Technology* (2015), Nr. 184, S. 291–296

57. HAHN, T.; LANG, S.; ULBER, R.; MUFFLER, K.: Novel procedures for the extraction of fucoidan from brown algae. In: *Process Biochemistry* (2012), Nr. 47, S. 1691−1698

58. HARUN, RAZIF ; SINGH, MANJINDER ; FORDE, GARETH M. ; DANQUAH, MICHAEL K.: Bioprocess engineering of microalgae to produce a variety of consumer products. In: *Renewable and Sustainable Energy Reviews* Bd. 14 (2010), Nr. 3, S. 1037–1047 — ISBN 13640321 (ISSN)

59. HAUGAN, J. A., & LIAAEN-JENSEN, S.: *Phytochemistry*, 1992

60. HERRERO, M., JAIME, L., MARTÍN-ALVAREZ, P. J., CIFUENTES, A., & IBÁÑEZ, E.: Optimization of the extraction of antioxidants from Dunaliella salina microalga by pressurized liquids. In: *Journal of Agricultural and Food Chemistry* (2006), Nr. 54, S. 5597–5603

61. HERRERO M, MARTIN-ALVAREZ PJ, SENORANS FJ, CIFUENTES A AND IBANEZ E: Optimization of accelerated solvent extraction of antioxidants from Spirulina platensis microalga. In: *FOOD CHEMISTRY* (2005), Nr. 93, S. 417–423

62. HOWARD, Z. XU AND L. R.: Analysis of Antioxidant-Rich Phytochemicals. In: *John Wiley & Sons Ltd* (2012)

63. HU T, LIU D, CHEN Y, WU J, WANG S: Antioxidant activity of sulfated polysaccharide fractions extracted from Undaria pinnitafida in vitro. In: *International Journal of Biological Macromolecules* (2010), Nr. 46 (2), S. 193–8

64. I. VEREŠBARANJI: Animal and vegetable fats and oils – Preparation of methyl esters of fatty acids. In: *InKontrolakvalitetasemenauljarica Ed. by Saveznizavodzastandardizaciju*. Belgrade, 1996

65. IBAÑEZ E., HERRERO M., MENDIOLA J.A., CASTRO-PUYANA M.: Extraction and Characterization of Bioactive Compounds with Health Benefits from Marine Resources: Macro and Micro Algae, Cyanobacteria, and Invertebrates. In: *Marine Bioactive Compounds* (2012)

66. J. A. MENDIOLA, M. HERRERO, A. CIFUENTES AND E. IBAÑEZ: Use of compressed fluids for sample preparation: Food applications. In: *Journal of Chromatography* Bd. 1152 (2007), Nr. 1–2, S. 234–246

67. J., PAWLISZYN: Kinetic model of supercritical fluid extraction. In: *Journal of Chromatographic Science* (1993), Nr. 31, S. 31–7

68. JADHAV, D.; B. N., R.; GOGATE, P. R.; RATHOD, V. K.: Extraction of vanillin from vanilla pods: a comparison study of conventional soxhlet and ultrasound assisted extraction. In: *Journal of Food Engineering* (2009), Nr. 93, S. 421−426

69. K.C. MAKI, F. EREN, M.E. CASSENS, M.R. DICKLIN, M.H. DAVIDSON: Ω-6 polyunsaturated fatty acids and cardiometabolic health: current evidence, controversies, and research gaps. In: *Advances in Nutrition* Bd. 9 (2018), S. 688–700

70. KADAM, SHEKHAR U. ; TIWARI, BRIJESH K. ; O'DONNELL, COLM P.: Application of novel extraction technologies for bioactives from marine algae. In: *Journal of Agricultural and Food Chemistry* Bd. 61 (2013), Nr. 20, S. 4667–4675 — ISBN 0021-8561

71. KANG CD AND SIM SJ: Selective extraction of free astaxanthin from haematococcus culture using a tandem organic solvent system. In: *Biotechnology Progress* (2007), Nr. 23, S. 866–871

72. KAUFMANN, B.; CHRISTEN, P.: Recent extraction techniques for natural products: microwave-assisted extraction and pressurised solvent extraction. In: *Phytochemical Analysis* (2002), Nr. 13, S. 105−113

73. KHAN, MUHAMMAD IMRAN ; SHIN, JIN HYUK ; KIM, JONG DEOG: The promising future of microalgae: Current status, challenges, and optimization of a sustainable and renewable industry for biofuels, feed, and other products. In: *Microbial Cell Factories* Bd. 17, BioMed Central (2018), Nr. 1, S. 1–21

74. KHOO, CHOON GEK ; DASAN, YALEENI KANNA ; LAM, MAN KEE ; LEE, KEAT TEONG: Algae biorefinery: Review on a broad spectrum of downstream processes and products. In: *Bioresource Technology* Bd. 292 (2019), Nr. June, S. 121964

75. KHOSRAVI-DARANI, K.: Research activities on supercritical fluid science in food biotechnology. In: *Critical Reviews in Food Science and Nutrition* (2010), Nr. 50, S. 479−488

76. KIM, S. K., & PANGESTUTI, R.: Biological activities and potential health benefits of fucoxanthin derived from marine brown algae. In: *Advances in Food and Nutrition Research* Bd. 64 (2011), S. 111–128

77. KIM, SANG MIN ; JUNG, YU JIN ; KWON, OH NAM ; CHA, KWANG HYUN ; UM, BYUNG HUN ; CHUNG, DONGHWA ; PAN, CHEOL HO: A potential commercial source of fucoxanthin extracted from the microalga Phaeodactylum tricornutum. In: *Applied Biochemistry and Biotechnology* Bd. 166 (2012), Nr. 7, S. 1843–1855 — ISBN 0273-2289

78. KIM, SANG MIN ; JUNG, YU JIN ; KWON, OH NAM ; CHA, KWANG HYUN ; UM, BYUNG HUN ; CHUNG, DONGHWA ; PAN, CHEOL HO: A potential commercial source of fucoxanthin extracted from the microalga Phaeodactylum tricornutum. In: *Applied Biochemistry and Biotechnology* Bd. 166 (2012), Nr. 7, S. 1843–1855

79. KITADA K, MACHMUDAH S, SASAKI M, GOTO M, NAKASHIMA Y, KUMAMOTO S AND HASEGAWA T: Supercritical CO2 extraction of pigment components with pharmaceutical importance from chlorella vulgaris. In: *Journal of Chemical Technology and Biotechnology* (2009), Nr. 84, S. 657–661

80. KITADA K, MACHMUDAH S, SASAKI M, GOTO M, NAKASHIMA Y, KUMAMOTO S AND HASEGAWA T: Supercritical CO2 extraction of pigment com- ponents with pharmaceutical importance from chlorella vulgaris. In: *Journal of Chemical Technology and Biotechnology* (2009), Nr. 84, S. 657–661

81. KOBAYASHI M, KAKIZONO T, NISHIO N, NAGAI S, KURIMURA Y, TSUJI Y: Antioxidant role of astaxanthin in the green alga Haematococcus pluvialis. In: *Applied Microbiology and Biotechnology* (1997), Nr. 48, S. 351–356

82. KOO SY, CHA KH, SONG DG, CHUNG D AND PAN CH: Optimization of pressurized liquid extraction of zeaxanthin from Chlorella ellipsoidea. In: *Applied Phycology* Bd. 24 (2012), Nr. 7, S. 25–730

83. KUCZYNSKA, PAULINA ; JEMIOLA-RZEMINSKA, MALGORZATA ; STRZALKA, KAZIMIERZ: Photosynthetic pigments in diatoms. In: *Marine Drugs* Bd. 13 (2015), Nr. 9, S. 5847–5881

84. KUMAR, DIPESH ; SINGH, BHASKAR: Biomass and Bioenergy Algal biorefinery : An integrated approach for sustainable biodiesel production. In: *Biomass and Bioenergy* Bd. 131, Elsevier Ltd (2019), Nr. 42, S. 105398

85. KUMAR, MUKESH ; SHARMA, MAHENDRA PAL: Potential assessment of microalgal oils for biodiesel production: A review. In: *Journal of Materials and Environmental Science* Bd. 5 (2014), Nr. 3, S. 757–766

86. LEE, B. H. (BYONG H.): *Fundamentals of Food Biotechnology*. Bd. 91. Second., 2015 — ISBN 9781118384954

87. DI LENA, GABRIELLA ; CASINI, IRENE ; LUCARINI, MASSIMO ; LOMBARDI-BOCCIA, GINEVRA: Carotenoid profiling of five microalgae species from large-scale production. In: *Food Research International* Bd. 120, Elsevier (2019), Nr. July 2018, S. 810–818

88. Di Lena, Gabriella ; Casini, Irene ; Lucarini, Massimo ; Lombardi-Boccia, Ginevra: Carotenoid profiling of five microalgae species from large-scale production. In: *Food Research International* Bd. 120 (2019), Nr. November 2018, S. 810–818

89. Di Lena, Gabriella ; Casini, Irene ; Lucarini, Massimo ; Sanchez del Pulgar, Josè ; Aguzzi, Altero ; Caproni, Roberto ; Gabrielli, Paolo ; Lombardi-Boccia, Ginevra: Chemical characterization and nutritional evaluation of microalgal biomass from large-scale production: a comparative study of five species. In: *European Food Research and Technology* Bd. 246, Springer Berlin Heidelberg (2020), Nr. 2, S. 323–332 — ISBN 0021701903346

90. Li, Y., Ghasemi Naghdi, F., Garg, S., Adarme-Vega, T., Thurecht, K. J., Ghafor, W., … Schenk, P. M.: A comparative study: the impact of different lipid extraction methods on current microalgal lipid research. In: *Microbial Cell Factories* Bd. 13 (2014), Nr. 1, S. 14

91. Li, Y.; Rong, Y.; Bao, L.; Nie, B.; Ren, G.; Zheng, C.; Amin, R.; Arnold, R.D.; Jeganathan, R.B.; Huggins, K.W.: Suppression of adipocyte differentiation and lipid accumulation by stearidonic acid (SDA) in 3T3-L1 cells. In: *Lipids in Health and Disease* (2017), Nr. 16, S. 181

92. Lin, J.; Huang, L.; Yu, J.; Xiang, S.; Wang, J.; Zhang, J.; Yan, X.; Cui, W.; He, S.; Wang, Q.: Fucoxanthin, a marine carotenoid, reverses scopolamine-Induced cognitive impairments in mice and inhibits acetylcholinesterase in vitro. In: *Marine Drugs* Bd. 14 (2016), Nr. 67

93. Links, Related: Lipid Mediators : Resolvins , Protectins , and Neuroprotectins (2013), Nr. Vldl, S. 20–23

94. M. E. Cuvelier and C. Berset: *Use of a Free Radical Method to Evaluate Antioxidant Activity*, 1995

95. M, L. Qingyong and W. Chien: Supercritical fluid extraction in herbal and natural product. In: *Talanta* Bd. 53 (2001), S. 771–782

96. Maadane A, Merghoub N, Ainane T, Arrousi H, Benhima R, Amzazi S, et al.: Antioxidant activity of some Moroccan marine microalgae: pufa profiles, carotenoids and phenolic content. In: *Journal of Biotechnology* (2015), Nr. 215, S. 13–19

97. Maadane A, Merghoub N, Ainane T, Arrousi H, Benhima R ; Amzazi S, et al.: Antioxidant activity of some Moroccan marine microalgae: pufa profiles, carotenoids and phenolic content. In: *Journal of Biotechnology* (2015), Nr. 215, S. 13–19

98. Maadane, Amal ; Merghoub, Nawal ; El Mernissi, Najib ; Ainane, Tarik ; Amzazi, Saaïd ; Wahby, Imane ; Bakri, Youssef: Antimicrobial activity of marine microalgae isolated from Moroccan coastlines. In: *Journal of Microbiology, Biotechnology and Food Sciences* Bd. 6 (2017), Nr. 6, S. 1257–1260

99. Machmudah S, Shotipruk A, Goto M, Sasaki M and Hirose T: Extraction of astaxanthin fromhaematococcus pluvialis using supercritical CO2 and ethanol as entrainer. In: *Industrial & Engineering Chemistry Research* (2006), Nr. 45, S. 3652–3657

100. Machmudah, Siti ; Wahyudiono ; Kanda, Hideki ; Goto, Motonobu: Supercritical fluids extraction of valuable compounds from algae: Future perspectives and challenges. In: *Engineering Journal* Bd. 22 (2018), Nr. 5, S. 13–30

101. Macias-Sanchez MD, Mantell C, Rodriguez M, Martinez De La Ossa E, Lubian LM, Montero O: Supercritical fluid extraction ofcarotenoids and chlorophyll a from Nannochloropsis gaditana. In: *Journal of Food Engineering* (2005), Nr. 66, S. 245– 51

102. Mäki-Arvela, Päivi ; Hachemi, Imane ; Murzin, Dmitry Yu: Comparative study of the extraction methods for recovery of carotenoids from algae: Extraction kinetics and effect of different extraction parameters. In: *Journal of Chemical Technology and Biotechnology* Bd. 89 (2014), Nr. 11, S. 1607–1626 — ISBN 1097-4660

103. Martino, Alessandra De ; Meichenin, Agnès ; Shi, Juan ; Pan, Kehou ; Bowler, Chris: Genetic and phenotypic characterization of Phaeodactylum tricornutum (Bacillariophyceae) accessions. In: *Journal of Phycology* Bd. 43 (2007), Nr. 5, S. 992–1009

104. MARTINS, PEDRO F ; DE MELO, MARCELO M R ; SARMENTO, PEDRO ; SILVA, CARLOS M: Supercritical fluid extraction of sterols from Eichhornia crassipes biomass using pure and modified carbon dioxide. Enhancement of stigmasterol yield and extract concentration. In: *The Journal of Supercritical Fluids* Bd. 107 (2016), S. 441–449

105. MCWILLIAMS, ANDREW: The Global Market for Carotenoids BCC Research Report Overview The Global Market for Carotenoids (2018), Nr. June

106. MEISER, A.; SCHMID-STAIGER, U.; TRÖSCH, W.: Optimization of eicosapentaenoic acid production by Phaeodactylum tricornutum in the flat panel airlift (FPA) reactor. In: *Applied Phycology* (2004), Nr. 16, S. 215–225

107. MIRÓN, A.S.; GARCIA, M.C.C.; GÓMEZ, A.C.; CAMACHO, F.G.; GRIMA, E.M.; CHISTI, Y.: Shear stress tolerance and biochemical characterization of Phaeodactylum tricornutum in quasi steady-state continuous culture in outdoor photobioreactors. In: *Biochemistry Engineering* (2003), Nr. 16, S. 287–297

108. NEUMANN, ULRIKE ; DERWENSKUS, FELIX ; FLISTER, VERENA FLAIZ ; SCHMID-STAIGER, ULRIKE ; HIRTH, THOMAS ; BISCHOFF, STEPHAN C.: Fucoxanthin, a carotenoid derived from Phaeodactylum tricornutum exerts antiproliferative and antioxidant activities in vitro. In: *Antioxidants* Bd. 8 (2019), Nr. 6, S. 1–11

109. NOBREB, MARCELO F, PASSOS R, BEIRAO L, PALAVRA A, GOUVEIA L ANDMENDES R: Supercritical carbon dioxide extraction of astaxanthin and other carotenoids from the microalga Haematococcus pluvialis. In: *Journal of European Food Research and Technology* (2006), Nr. 223, S. 87–790

110. PALOZZA, P.; KRINSKY, N.I.: β-Carotene and α-tocopherol are synergistic antioxidants. In: *Archives of Biochemistry and Biophysics* (1992), Nr. 297, S. 184–187

111. PANE, G., CACCIOLA, G., GIACCO, E., MARIOTTINI, G. L., & COPPO E.: Assessment of the antimicrobial activity of algae extracts on bacteria responsible of external otitis. In: *Marine Drugs* (2015), S. 6440–6452

112. PANGESTUTI, RATIH ; KIM, SE KWON: Biological activities and health benefit effects of natural pigments derived from marine algae. In: *Journal of Functional Foods* Bd. 3, Elsevier Ltd (2011), Nr. 4, S. 255–266 — ISBN 1756-4646

113. PARK, P. W., R. E. GOINS.: In situ preparation of fatty acid methyl esters for analysis of fatty acid composition in foods. In: *Journal of Food Science* (1994), Nr. 59, S. 1262–1266

114. PENG, J., YUAN, J. P., WU, C. F., & WANG, J. H.: Fucoxanthin, a marine carotenoid present in brown seaweeds and diatoms: Metabolism and bioactivities relevant to human health. In: *Marine Drugs* Bd. 9 (2011), Nr. 10, S. 1806–1828

115. PÉREZ, MARÍA JOSÉ ; FALQUÉ, ELENA ; DOMÍNGUEZ, HERMINIA: Antimicrobial action of compounds from marine seaweed. In: *Marine Drugs* Bd. 14 (2016), Nr. 3, S. 1–38

116. PREMVARDHAN L, SANDBERG DJ, FEY H, BIRGE RR, BÜCHEL C, VAN GRONDELLE R: The charge-transfer properties of the S2 state of fucoxanthin in solution and in fucoxanthin chlorophyll-a/c2 protein (FCP) based on stark spectroscopy and molecular-orbital theory. In: *The Journal of Physical Chemistry* Bd. 112 (2008), Nr. 37, S. 11838–53

117. PRONYK C. AND MAZZA G.: Design and scale-up of pressurized fluid extractors for food and bioproducts. In: *Journal of Food Engineering* Bd. 95 (2009), Nr. 2, S. 215–226

118. PUROHIT, RAHUL ; VENUGOPALAN, P: Polymorphism : An Overview (2009), Nr. September, S. 882–893

119. QIN, Y.: Bioactive Seaweeds for Food Applications. Natural Ingredients for Healthy Diets (2018)

120. REBOLLOSO-FUENTES, M. M., VAVARRO-PÉREZ, A., RAMOS-MIRAS, J. J., & GUIL-GUERRERO, J. L.: Biomass nutrient profiles of the microalga Phaeodactylum tricornutum. In: *Food Biochemistry* (2001), Nr. 25, S. 57–76

121. REYES, FABIÁN A. ; MENDIOLA, JOSÉ A. ; IBAÑEZ, ELENA ; DEL VALLE, JOSÉ M.: Astaxanthin extraction from Haematococcus pluvialis using CO 2-expanded ethanol. In: *Journal of Supercritical Fluids* Bd. 92 (2014), Nr. August, S. 75–83

122. De Riso, V; Raniello, R.; Maumus, F.; Rogato, A.; Bowler, C.; Falciatore, A.: Gene silencing in the marine diatom Phaeodactylum tricornutum. In: *Nucleic Acids* Bd. Res. 37 (1 (2009), S. 96

123. Rodríguez-Meizoso I, Jaime L, Santoyo S, Cifuentes A, Garcia-Blairsy Reina G, Senorans FJ and Ibanez E: Pressurized fluid extraction of bioactive compounds from Phormidium species. In: *Journal of Agricultural and Food Chemistry* (2008), Nr. 56, S. 3517–3523

124. Round FE, Crawford RM, Mann DG: The Diatoms: biology and morphology of the genera. In: *Cambridge University Press, Cambridge, UK* (1990)

125. Ruenngam D, Shotipruk A, Pavasant P, Machmudah S and Goto M: Selective extraction of lutein from alcohol treated chlrella vulgaris by supercritical CO2. In: *Chemical Engineering & Technology* (2012), Nr. 35, S. 255–60

126. Ruzickova, J.; Rossmeisl, M.; Prazak, T.; Flachs, P.; Sponarova, J.; Veck, M.; Tvrzicka, E.; Bryhn, M.; Kopecky, J.: Omega-3 PUFA of marine origin limit diet-induced obesity in mice by reducing cellularity of adipose tissue. In: *Lipids* (2004), Nr. 39, S. 1177–1185

127. Ryckebosch, Eline ; Bruneel, Charlotte ; Termote-verhalle, Romina ; Goiris, Koen ; Muylaert, Koenraad ; Foubert, Imogen: Nutritional evaluation of microalgae oils rich in omega-3 long chain polyunsaturated fatty acids as an alternative for fish oil. In: *FOOD CHEMISTRY* Bd. 160, Elsevier Ltd (2014), S. 393–400

128. Ryckebosch, Eline ; Bruneel, Charlotte ; Termote-Verhalle, Romina ; Muylaert, Koenraad ; Foubert, Imogen: Influence of extraction solvent system on extractability of lipid components from different microalgae species. In: *Algal Research* Bd. 3, Elsevier B.V. (2014), Nr. 1, S. 36–43

129. S. Lucas , E. Alonso, J. Sanz and M. Cocero: Safety Study in a Supercritical Extraction Plant. In: *Chemical Engineering & Technology* (2003), Nr. 26, S. 449–461

130. S. Pastoriza, C. Delgado-Andrade, A. Haro, and J. A. Rufián-Henares: Analytical Methods A physiologic approach to test the global antioxidant response of foods. The GAR method. In: , 2011

131. S.U. Kadam, B.K. Tiwari, C.P. O'Donnell: Application of novel extraction technologies for bioactives from marine algae. In: *Journal of Agricultural and Food Chemistry* (2013), Nr. 61, S. 4667–4675

132. Sahena, F. ; Zaidul, I. S.M. ; Jinap, S. ; Karim, A. A. ; Abbas, K. A. ; Norulaini, N. A.N. ; Omar, A. K.M.: Application of supercritical CO2in lipid extraction - A review. In: *Journal of Food Engineering* Bd. 95, Elsevier Ltd (2009), Nr. 2, S. 240–253 — ISBN 0260-8774

133. Schütz, E.: Supercritical fluids and applications − a patent review. In: *Chemical Engineering & Technology* (2007), Nr. 30, S. 685−688

134. Shang, Y. F.; Kim, S. M.; Lee, W. J.; Um, B. H.: Pressurized liquid method for fucoxanthin extraction from Eisenia bicyclis. In: *Journal of Bioscience and Bioengineering* (2011), Nr. 111, S. 237−241

135. Shang YF, Kim SM, Lee WJ and Um BH: Pressurized liquid method for fucoxanthin extraction from eisenia bicyclis (kjellman) setchell. In: *Journal of Bioscience and Bioengineering* (2011), Nr. 111, S. 237–241

136. Shirsath, S.; Sonawane, S.; Gogate, P.: Intensification of extraction of natural products using ultrasonic irradiations − a review of current status. In: *Chemical Engineering and Processing: Process Intensification* (2012), Nr. 53, S. 10−13

137. Silva Benavides, A.M.; Torzillo, G.; Kopecký, J.; Masojídek, J.: Productivity and biochemical composition of Phaeodactylum tricornutum (Bacillariophyceae) cultures grown outdoors in tubular photobioreactors and open ponds. In: *Biomass Bioenergy* (2013), Nr. 54, S. 115–122

138. Simopoulos, Artemis P: Omega-3 Fatty Acids in Inflammation and Autoimmune Diseases. In: *Journal of the American College of Nutrition* Bd. 21, Taylor & Francis (2002), Nr. 6, S. 495–505

139. Sørensen, M., Berge, G. M., Reitan, K. I., & Ruyter, B.: Microalga Phaeodactylum tricornutum in feed for

Atlantic salmon (Salmo salar) —Effect on nutrient digestibility, growth and utilization of feed. In: *Aquaculture* (2016), Nr. 460, S. 116–123

140. SOSA-HERNÁNDEZ, JUAN EDUARDO ; ESCOBEDO-AVELLANEDA, ZAMANTHA ; IQBAL, HAFIZ M.N. ; WELTI-CHANES, JORGE: State-of-the-art extraction methodologies for bioactive compounds from algal biome to meet bio-economy challenges and opportunities. In: *Molecules* Bd. 23 (2018), Nr. 11

141. STAHL, W.; SIES, H.: Bioactivity and protective effects of natural carotenoids. In: *Biochimica et Biophysica Acta - Molecular Cell Research* (2005), Nr. 1740, S. 101–107

142. STEINRÜCKEN, P.; PRESTEGARD, S.K.; DE VREE, J.H.; STORESUND, J.E.; PREE, B.; MJØS, S.A.; ERGA, S.R.: Comparing EPA production and fatty acid profiles of three Phaeodactylum tricornutum strains under western Norwegian climate conditions. In: *Algal Research* (2018), Nr. 30, S. 11–22

143. STEYTLER, D.: Supercritical fluid extraction and its application in the food industry. In: *Separation Processes in Food and Biotechnology Industries: Principles and Applications* (1996)

144. SUGAWARA, T., KUSHIRO, M., ZHANG, H., NARA, E., ONO, H., & NAGAO, A.: Lysophosphatidylcholine enhances carotenoid uptake from mixed micelles by Caco-2 human intestinal cells. In: *The Journal of Nutrition* Bd. 131 (2001), Nr. 11, S. 2921–2927

145. SURENDHIRAN, D., VIJAY, M., SIRAJUNNISA, A.B., SUBRAMANIYAN, T. & SHANTHALIN, A.: A green synthesis of antimicrobial compounds from marine microalgae Nannochlorpsis oculata. In: *Journal of coastal life medicine* Bd. 2 (2014), Nr. 11, S. 859–863

146. SURESH, A., PRAVEENKUMAR, R., THANGARAJ, R., OSCAR, F.L., BALDEV, E., DHANASEKARAN, D., & THAJUDDIN, N.: Microalgal fatty acid methyl ester a new source of bioactive compounds with antimicrobial activity. In: *Asian Pacific Journal of Tropical Disease* (2014)

147. TANABE, YUKIKO ; SHITARA, TOMOFUMI ; KASHINO, YASUHIRO ; HARA, YOSHIAKI ; KUDOH, SAKAE: Utilizing the Effective Xanthophyll Cycle for Blooming of Ochromonas smithii and O. itoi (Chrysophyceae) on the Snow Surface. In: *PloS one* Bd. 6 (2011), S. e14690

148. TANG S, QIN C, WANG H, LI S AND TIAN S: Study on supercritical extraction of lipids and enrichment of DHA from oil-rich microalgae. In: *Journal of Supercritical Fluids* Bd. 57 (2011), Nr. 1, S. 44–49

149. THAIPONG K, BOONPRAKOB U, CROSBY K, CISNEROS-ZEVALLOS L AND BYRNE DH.: Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts. In: *Journal of Food Composition and Analysis* Bd. 19 (2006), Nr. 6, S. 669–675

150. TIBBETTS, SEAN M ; MILLEY, JOYCE E ; LALL, SANTOSH P: Chemical composition and nutritional properties of freshwater and marine microalgal biomass cultured in photobioreactors (2015), S. 1109–1119

151. TRIVEDI, J., AILA, M., BANGWAL, D.P., KAUL, S., GARG, M.O.: Algae based biorefinery—How to make sense? In: *Renewable and Sustainable Energy Reviews* (2015), Nr. 47, S. 295–307

152. USHAKUMARI, U. N., & RAMANUJAN, R.: Isolation of astaxanthin from marine yeast and study of its pharmacological activity. In: *International Current Pharmaceutical Journal* Bd. 2 (2013), Nr. 3, S. 67–69

153. VALDERRAMA JO, PERRUT M AND MAJEWSKI W: Extraction of astaxanthin and phycocyanine frommicroalgae with supercritical carbon dioxide. In: *Journal of Chemical & Engineering Data* (2003), Nr. 28, S. 827–830

154. VALDEZ-FLORES M, GERMA BAEZ LJ, GUTIERREZ-DORADO R, MEDINA-GODOY S, NORZAGARAY-VALENZUELA C, HERNANDEZ- VERDUGO S, ET AL.: Improving bioactivities of Jatropha curcas protein hydrolysates by optimizing with response surface methodology the extrusion cooking process. In: *Industrial Crops and Products* Bd. 85 (2016), S. 353–360

155. VEITH, T.; BÜCHEL, C.: The monomeric photosystem I-complex of the diatom Phaeodactylum tricornutum binds specific fucoxanthin chlorophyll proteins (FCPs) as light-harvesting complexes. In: *Biochimica et Biophysica Acta* (2007), Nr. 1767, S. 1428–1435

156. DI VISCONTE, GINO SCHIANO ; SPICER, ANDREW ; CHUCK, CHRISTOPHER J. ; ALLEN, MICHAEL J.: The microalgae biorefinery: A perspective on the current status and future opportunities using genetic modification. In: *Applied Sciences (Switzerland)* Bd. 9 (2019), Nr. 22

157. VAN DER VOORT, MARCEL P.J. ; SPRUIJT, JOANNEKE ; POTTERS, JORIEKE ; DE WOLF, PIETER L. ; ELISSEN, HELLEN J.H.: Socio-economic assessment of Algae-based PUFA production. In: *Public Output report of the PUFAChain project* (2017), Nr. 613303

158. WANG, SONG ; SAID, INAMULLAH HAKEEM ; THORSTENSON, CANDICE ; THOMSEN, CLAUDIA ; ULLRICH, MATTHIAS S. ; KUHNERT, NIKOLAI ; THOMSEN, LAURENZ: Pilot-scale production of antibacterial substances by the marine diatom Phaeodactylum tricornutum Bohlin. In: *Algal Research* Bd. 32, Elsevier (2018), Nr. March, S. 113–120

159. WHYTE, J.; SOUTHCOTT, B.: An extraction procedure for plants: extracts from the red alga Rhodomela larix. In: *Phytochemistry* (1970), Nr. 9, S. 1159−1161

160. WU, HUALIAN ; LI, TAO ; WANG, GUANGHUA ; DAI, SHIKUN ; HE, HUI ; XIANG, WENZHOU: A comparative analysis of fatty acid composition and fucoxanthin content in six Phaeodactylum tricornutum strains from different origins. In: *Chinese Journal of Oceanology and Limnology* Bd. 34 (2016), Nr. 2, S. 391–398

161. YEN HW, CHIANG WC AND SUN CH: Supercritical fluid extraction of lutein from Scenedesmus cultured in an autotrophical photobioreactor. In: *Journal of Taiwan Institute of Chemical Engineering* (2012), Nr. 43, S. 53–57

162. ZHENG, C. J., YOO, J. S., LEE, T. G., CHO, H. Y., KIM, Y. H., & KIM, W. G.: Fatty acid synthesis is a target for antibacterial activity of unsaturated fatty acids. In: *FEBS Letters* Bd. 579 (2005), Nr. 23, S. 5157–5162