KAUNO TECHNOLOGIJOS UNIVERSITETAS

CHEMINĖS TECHNOLOGIJOS FAKULTETAS

MAISTO MOKSLO IR TECHNOLOGIJOS KATEDRA

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Apskritagalvio bandrenio (*Echinops sphaerocephalus*) ir *Chrysochromulina polylepis* dumblių frakcionavimas, analizė ir įvertinimas

Baigiamasis magistro darbas

Vadovas
Prof. Dr. Petras Rimantas Venskutonis

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Final Master Thesis

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SUMMARY

This master thesis consists of two parts. The aim of the first part of the thesis was to determine fatty acid composition, total phenolic content and antioxidant activity of *Echinops sphaerocephalus* extracts made with three different polarity solvents – hexane, acetone and methanol (70:30 %), as well as for extraction residue. Also to perform its supercritical fluid extraction optimization.

Fatty acid composition was determined using gas chromatography. Total phenolic content was determined using Folin-Ciocalteu method. The antioxidant activity of extracts was determined using $DPPH^{\bullet}$, $ABTS^{+\bullet}$ and ORAC assays. The results showed that mostly unsaturated fatty acids compose the oil, the phenolic content of the extracts were dependent on the extraction solvent namely that methanol extracts possessed most of phenolic content and antioxidant activity compared to other two. The residue tests showed opposite results that hexane residue contained more phenols and had more antioxidant activity compared to other extracts. Along those tests optimal supercritical fluid extraction parameters were determined with which to obtain best oil yield.

The aim of the second part of the project was to determine best extraction mode of unidentified target compound of *Chrysochromulina polylepis* using solid phase extraction columns of different polarity and ionization and different solvents of different polarity and then to determine whether that compound had toxic effects on sea life or not using fish gill assay.

Small scale extractions showed that the best target compound yield was using either acetone or methanol solvents with strong anion exchange column however due to large salt content when upscaled to large scale it was proven ineffective. C18 column was then chosen which provided nearly as good yield as SAX column on small scale tests. The fraction containing the target compound was then tested for toxicity but was proven to be non-toxic. However other toxic fractions were isolated and possible toxic compounds detected.

Key words: *Echinops sphaerocephalus, Chrysochromulina polylepis,* antioxidants, supercritical fluid extraction, solid phase extraction, toxicity.

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SANTRAUKA

Šis magistro studijų baigiamasis darbas susideda iš dviejų dalių. Pirmosios dalies tikslas buvo nustatyti *Echinops sphaerocephalus,* Apskritagalvio bandrenio, riebalų rūgščių sudėtį, bendrąjį fenolinių junginių kiekį ir antioksidacinį aktyvumą jo ekstraktuose paruoštuose naudojant tris skirtingo poliškumo tirpiklius – heksaną, acetoną bei metanolį (70:30 %), bei tą patį atlikti su ekstraktų liekana. Taip pat buvo nuspręsta atlikti apskritagalvio bandrenio aliejaus superkrizinio skysčio ekstrakcijos optimizavimą.

Riebalų rūgščių sudėtis buvo nustatyta naudojant dujų chromatografiją, bendrasis fenolių kiekis buvo nustatytas naudojant Folin-Ciocalteu metodą, o antioksidacinis aktyvumas ekstraktuose buvo nustatytas naudojant $DPPH^{\bullet}$, $ABTS^{+\bullet}$ ir ORAC tyrimus. Rezultatai atskleidė, jog bandrenio aliejus daugiausiai sudarytas iš nesočiųjų riebalų rūgščių. Fenolinių junginių kiekis ekstraktuose labai priklausė nuo naudojamo tirpiklio – metanoliniuose ekstraktuose buvo rasta daugiausiai fenolinių junginių bei šio ekstrakto antioksidacinis aktyvumas buvo didžiausias lyginant su kitais ekstraktais. Ekstrakcijų liekanų tyrimų rezultatai parodė, jog liekana, kuri buvo ekstrahuojama tik heksanu turėjo savyje daugiau fenolinių junginių bei demonstravo didesnį antioksidacinį aktyvumą, nei liekanos, su kuriomis buvo atliktos dviejų ar trijų pakopų ekstrakcijos. Taip pat buvo atliktas superkritinio skysčio ekstrakcijos bandrenio aliejui optimizavimas ir nustatyti parametrai išgauti maksimalią aliejaus išeigą.

Antrosios darbo dalies tikslai buvo nustatyti efektyviausią nežinomojo tiriamojo junginio, išskiriamo *Chrysochromulina polylepis* dumblių, ekstrakcijos metodą naudojant skirtingas kietosios fazės ekstrakcijos kolonėles su besiskiriančiais poliškumais ir jonizacija, bei skirtingų poliškumų tirpiklius. Taip nustatyti ar tiriamasis junginys turi toksišką poveikį vandens gyvūnijai atliekant žuvies žiaunų ląstelių toksiškumo tyrimus.

Pirminiai tyrimai parodė, jog geriausia išeiga gaunama ekstrahuojant acetonu arba metanoliu naudojant stiprių anijoninių mainų kalonėlę. Tačiau ji, dėl didelio druskų kiekio didelėje ekstrakcijoje pasirodė esanti neveiksminga, todėl buvo pasirinkta C18 kalonėlė, kuri šiek tiek mažesnę išeigą pirminių bandymų metu. Atliekant toksiškumo tyrimus, ekstraktų frakcija su tiriamuoju junginiu buvo netoksiška, vadinasi ir tiriamasis junginys yra netoksiškas. Tačiau buvo nustatyos toksiškos frakcijos ir identifikuoti pora galimai toksiškų junginių.

Raktažodžiai: *Echinops sphaerocephalus, Chrysochromulina polylepis,* antioxidantai, superkritinio skysčio ekstrakcija, kietosios fazes ekstrakcija, toksiškumas.

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Figure 30. Cell culture survivability when exposed to fractions from C18 column of acetone biomass extract. 70% fraction showed a small bioactivity at highest concentrations. 80% fraction was toxic and first half of 90% fraction was even more toxic, however second half of 90% fraction and a 100% fraction showed no toxicity.

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Figure 33. Comparison between 100% methanol C18 fractions. Methanol extract is toxic and acetone extract is not in these fractions.

ABBREVIATIONS

AAPH	2,2'-azobis(2-amidinopropane) dihydrochloride
$ABTS^{\bullet+}$	2,2′-azino-di-3-ethyl benzothiazoline-6-sulfonic acid
AE	Acetone extract
APCI/MS	Atmospheric pressure chemical ionization mass spectroscopy
AUC	Area under the curve
B	Bush
$DPPH^{\bullet}$	2,2-diphenyl-1-picrylhydrazyl
EE	Ethanol extract
EC_{50}	Effective concentration
ESI	Electrospray ionization
GA	Gallic acid
GAE	Gallic acid equivalents
HPLC	High performance liquid chromatography
LC-MS	Liquid chromatography-mass spectrometry
MS	Mass spectrum
m/z	Mass to charge ratio
ORAC	Oxygen radical absorption capacity
PBS	Phosphate buffered saline
QTOF-MS	Quadrupole time of flight mass spectrometry
ROS	Reactive oxygen species
RSC	Radical scavenging capacity
RT	Retention time

SCFE	Supercritical fluid extraction
SPE	Solid phase extraction
TE	Trolox equivalent
TEAC	Trolox equivalent antioxidant capacity
TPC	Total phenolic content
TPTZ	2,4,6-tri-(2-pyridyl)-s-triazine
UPLC	Ultra performance liquid chromatography
UV	Ultraviolet
$X^{\bullet}$	Free radicals

INTRODUCTION

The variety of natural chemical compounds is near endless. Different life forms biosynthesize hundreds to thousands different compounds that greatly vary in their purpose. Some of those compounds are vital to their producer and some are mortal to their surroundings. This thesis focuses on two different types of compounds. One that increases resilience against negative effects of oxidation in organisms and the other that is potentially toxic to the environment.

The growing food consumption demands for ever growing and adapting market to adapt new sources of food products as well as maximize the use of existing resources. One of such resources might be *Echinops sphaerocephalus* – a plant that has been long known as a bee farm crop to provide bees with reliable source of nectar. More recently some studies were performed that investigated the oil content to see if it would be suitable to use as a food oil source. It was reported, that its seeds contain up to 25% oil of its mass and is consisted with primarily highly valuable unsaturated linoleic fatty acid. This project looks deeper into this oil by investigating the total phenolic content and antioxidant activity of *E. sphaerocephalus* extracts prepared with solvent of different polarity and extract residue.

As well as testing the oil's parameters it is also important to optimize extraction methods and parameters to ensure maximum content yield from raw materials. For this reason it was decided to perform supercritical carbon dioxide extraction optimization. This method is an alternative technique to traditional extraction methods that use organic solvents. It is faster, inexpensive, provides high yields of high purity oil. Response surface methodology was used to evaluate effects of different extraction parameters to establish optimal conditions for the extraction.

In 1988 spring there was an enormous scale of blooming of the unialgal culture of *Chrysochromulina polylepis* algae and seven other *Chrysochromulina* species. This toxic bloom has covered huge areas of Norwegian, Swedish and Danish coasts and killed a huge part of marine population, but especially it has affected the domestic fish farms along those coasts killing nearly all of the farmed fish and causing millions in monetary damages. This was very unusual, because this algae has never been reported forming blooms and especially not so overwhelmingly large ones. There were couple more incidents involving this particular algae, but not as large in scale as that time. (Hanne Kaas, 1989)

Even though a lot of years has passed since the blooming and it has been monitored regularly there is still very little knowledge about the chemistry of *C. polylepis* and what compounds produced by this algae have actually caused that disaster. This research focuses on one unidentified compound with an unusual structure for algal compounds. The aim was to fractionate it and test the toxicity of this compound to see if it is the toxic one.

The combined goals of this thesis are:

- To determine total phenolic content in *E. sphaerocephalus* extracts prepared using three different polarity solvents (hexane, acetone and methanol:water (70:30 %)) and extract residue;
- To determine antioxidant activity of extracts using $DPPH^{\bullet}$ and $ABTS^{+\bullet}$ assays;
- To determine antioxidant activity of extract residue using $ABTS^{+\bullet}$ QUENCHER and ORAC assays;
- To determine the optimal parameters of supercritical fluid extraction for *E. sphaerocephalus* extraction
- To determine the best solid phase extraction mode to fractionate traget compound from *C.polylepis* by differentiating between different SPE columns and solvents;
- To perform bioguided analysis to determine whether or not the target compound is toxic.

1. Literature overview

1.1 Echinops sphaerocephalus

E. sphaerocephalus (Fig. 1) is a perennial plant which belongs to *Asteraceae* plant family and originates from Mediterranean region and South-West Asia. It prefers calcareous soil and grows up to 1.5 m tall. It blooms from July to August producing blue or white blooms which form round racemes 3 to 8 cm in diameter giving species its name (lat. *Echinos* – hedgehog, *ops* – looks). Seeds ripe in early autumn from September to October and contain approximately 25% oil content (A.J. Patel *et al*, 2011). *E. sphaerocephalus* spreads very slowly and usually from places where it was artificially planted before.

Figure 1. *Echinops sphaerocephalus* plant. On the right – blooms on the seed.

1.1.1 Plant industrial uses

E. sphaerocephalus is very rich with pollen and nectar so it is valued as a good culture for honey farms. Its tube like blooms produce 2 to 6 mg of nectar each and easily attracts bees to its scent. It is widely used as a honey giving crop in central and southern Europe (Gert Horn *et al*, 2008). It is easy to grow and can grow in soil that is otherwise unusable for agriculture.

E. sphaerocephalus is also often used as a decorative plant or flower bouquets for its beautiful blooms. Due to high oil content in seeds research programs are currently being performed to see if the *E. sphaerocephalus* seed oil is suitable for cooking oil production which would greatly expand the use of this plant allowing to harvest seeds for oil after blooming and honey season is over.

1.1.2 Chemical composition

E. sphaerocephalus is known to contain carotenoids, tocopherols, sterols, flavonoids, proteins, carbohydrates, aminoacids, chinols and sesqiterpenes as well as some other bioactive compounds such as tiophenes which have insecticidal and fungicidal properties and polyphenols which are important natural antioxidants (Gert Horn *et al*, 2008) and (Nikolas Fokialakis *et al*, 2006). Some of the compounds that are known to be found in *E. sphaerocephalus*:

Apigenin (4,5,7 – trihydroxyflavone) – a typical bioflavonoid which is non-toxic compound which has anticancer properties (Jing Fang, 2005) and (Jing Fang*et al*, 2006). Also found in red wine, tomatoes, beets, parsley.

Chlorogenic acid – a water soluble phenolic acid which is stable in a room temperature. It is known to be a strong natural antioxidant which inhibits glucose intake into the blood flow after food consumption (Margreet R. Olthof *et al*, 2001). Besides antioxidant activity it is also known for its antibacterial and antiviral properties (Jan Oszmianski *et al*, 1990). It is very widespread phenolic acid in plants and fruits including apples, plums, cherries, pears and coffee beans.

Catechin – a flavonoid class phenolic compound which has strong natural antioxidant properties. This compound is known to increase organism resistance to develop prostate and lung cancer because it react with various oxygen forms, nitrogen oxides and hydroxyl radicals (Karrie Heneman *et al*, 2008). It also improves blood circulatory system (Jane V. Higdona *et al*, 2003). It is found in large quantities in green tea, coffee, cocoa, red wine, olives and strawberries.

Quinic acids –crystal acids that have strong antioxidant properties (Wang GF *et al*, 2009). Quinic acids reduce the risk of chronic diseases, heart diseases and cancer tumors. This compound is most common in coffee beans and quinintree peel.

Quinoline alkaloid achinorin and its fission byproducts equinopsin and equinopsidine (Fig. 2) (Gert Horn *et al*, 2008). These compounds are widely used in pharmacy producing substances that have quite wide range of effects such as sensorial enhancer, especially of hearing and smelling, antibacterial, antifungal and anticancer agents, corrosion inhibitors and preservatives (Sudharshan Madapa *et al*, 2008) and (Getachew Belay *et al*, 2011).

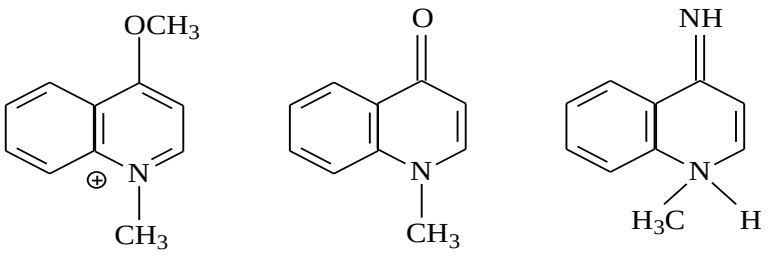


Figure 2. Quinolin alkaloids found in *Echinops* species plants. From left to right Equinorin, Equinopsin, Equinopsidine.

1.2 Lipid oxidation

One of the reasons of food deterioration is lipid oxidation. Products containing fats, especially unsaturated fatty acids, during heating or storing them over the time via oxidation process gain specific bitter taste and rancid off-flavour, reduce their nutritional value and become inconsumable. Oxidation is a process of electron transfer from reductant ions – electron donors – to oxidant ions – electron acceptors. This transfer is powered by a higher affinity of an oxidant. Some of the electron transfers create ions with odd numbered valence electrons which are very reactive and then in turn starts interacting in further oxidation reactions that produce thermodynamically stable oxidation products (Kommineni *et al*, 2000).

There are three stages of lipid autoxidation of lipid oxidation – initiation, propagation and termination. Initial phase produces a variety of peroxides and hydro peroxides. These compounds are nonvolatile and odorless, however they are relatively unstable and may spontaneously decompose forming volatile compounds. The composition of these compounds depends on the fatty acid composition of the oil (Eman *et al*, 2012).

Secondary oxidation phase produces alcohols, aldehydes, ketones and polymeric compounds. These byproducts not only have negative impact on products sensory properties but also negatively affect human health when consumed. When free radical levels reach excess point and organism is unable to neutralize them it creates oxidative stress which incites chronic and degenerative diseases like autoimmune disorders, heart diseases, cancer tumor development, diabetes and others (Eman *et al*, 2012).

1.3 Mechanism of a lipid oxidation

There are four known lipid oxidation mechanisms under which oxidation can occur. Most of them involve some kind of free radicals and some type of oxygen. These four mechanisms are:

Autoxidation – the most common of the four and is defined as a spontaneous reactions between lipids and atmospheric oxygen (Fig. 3) (Gordon M. *et al*, 2001). This process is greatly intensified at high temperatures and is also called thermal oxidation. The mechanism of autoxidation involves free radical initiation, propagation and termination between oxygen and unsaturated lipids. During the initiation lipids loose hydrogen radical in the presence of trace metals, light or heat. Then those free radicals (X^*) react with oxygen and form peroxy radicals (ROO^*). During propagation process previously formed proxy radicals (ROO^*) react with more lipids (RH^*) and produces lipid hydroperoxides ($ROOH^*$) that are primary products of autoxidation (Erickson M. C. *et al*, 2002).

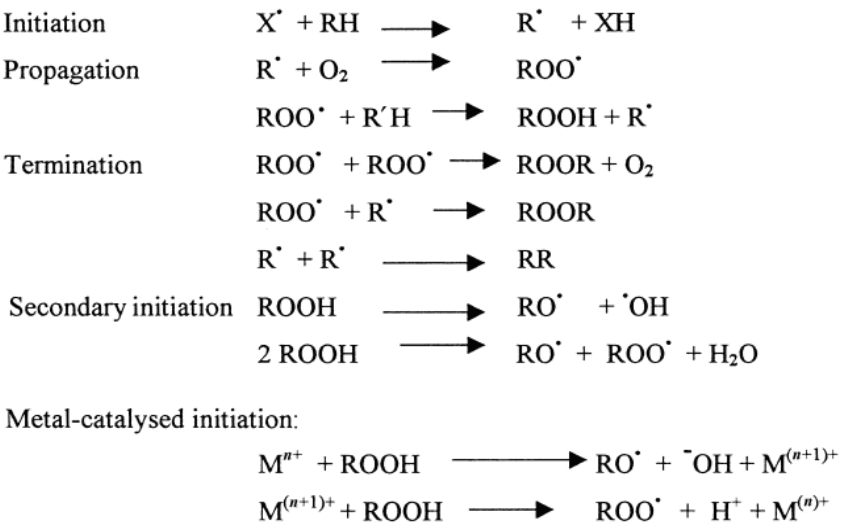


Figure 3. Mechanism of lipid autoxidation.

Decomposition of lipid hydroperoxides. A complicated mechanism which produces multiple of compounds that have bioactive properties and cause flavor deterioration in products containing fats. During decomposition process lipid hydroperoxides (RO-OH) undergo hemolytic fission and form alkoxy radicals (RO^*) which then in turn undergo carbon-carbon fission forming aldehydes, alcohols, esters, furans, hydrocarbons, ketones and lactones (Erickson M. C. *et al*, 2002).

Third mechanism is a reaction between lipid hydroperoxides and oxygen forming bicyclic endoperoxides, cyclic peroxides, dihydroperoxides, epoxyhydroperoxides and ketohydroperoxides. These compounds then fragment like monohydroperoxides forming volatile fission products. Lipid hydroperoxides may also undergo merging into dimers and polymers that in turn can break down producing volatile compounds.

Last mechanisms consists of lipid hydroperoxides and their bifunctional breakdown products interacting with proteins, enzymes and cell membranes. This mechanism is most harmful to human organism, because it affects vital cell functions. Free radicals deteriorate cell membranes, disrupt biological cell functions and creates aging pigments accelerating cell aging process. These age pigments and other oxidation products however, may be neutralized by introducing antioxidant agents such as vitamin E (Perkins E. G. *et al*, 1992).

The quality of food and its effect on human organism when consumed is significantly influenced by a development of fat rancidity and the interactions of lipid oxidation products and other food components like proteins and amino acids. However the possible damage these compounds may cause can be greatly reduced by understanding the proper ways of food preparation and the importance of addition of antioxidants like vitamin E to the food products to minimize free radical formation and improve their shelf life (Erickson M. C. *et al*, 2002).

1.4 Antioxidants

Antioxidants are a group of compounds that can be referred to as compounds that inhibits oxidation reactions and terminates free radicals. Antioxidant can be added in various stages of food processing to reduce and delay the development of food rancidity.

Antioxidants inhibit oxidation process in two ways. One inhibitory mechanism is free radical scavenging where antioxidant compounds like phenolic compounds directly reacts to free radicals and are consumed in the process. These antioxidants are called primary antioxidants.

Second inhibitory mechanism is a process where antioxidants do not directly interact with free radicals, but instead shuts oxidation reactions by binding metal ions, scavenging or disable oxygen, converting hydroperoxides to non-radical forms or absorbs UV radiation. These antioxidants are called secondary antioxidants (Pokorny, J. *et al*, 2001).

1.5 Antioxidant types

All antioxidants are generally divided into two groups: natural and synthetic antioxidants. Natural antioxidants are a natural oxidation inhibitors that are naturally developed by plants, to protect its fruits from oxidative degradation. Synthetic antioxidants are synthesized or extracted from food products for industrial use.

1.5.1 Natural antioxidants

Plants under natural conditions constantly endure oxidative stress from free radicals, reactive oxygen, prooxidants that are generated from both exogenous sources like heat, light and endogenous sources like H_2O_2 and transition metals. To protect themselves and fruits they bare they have developed natural protection from oxidative harm in form of antioxidants to manage free radicals, lipid oxidation catalysts, oxidation intermediates and oxidation breakdown products. Largest group of natural antioxidants consist of phenolic compounds and most important natural antioxidants are ascorbic acid, carotenoids and tocopherols (Brewer M. S. *et al*, 2011).

L-ascorbic acid – Vitamin C – and its salts like sodium ascorbate and calcium ascorbate are very commonly produced by various plants in high abundance (Fig. 4). It is also one of the most commonly synthesized synthetic antioxidant. Ascorbic acid is a white or slightly yellow crystalline powder. It is commonly used to stabilize beverages, fruits and vegetables. The main antioxidant mechanisms of ascorbic acid are reduction of free radicals, quenching of various forms of oxygen and regeneration of primary antioxidants (Reische D. W. *et al*, 1998). Ascorbic acid has a very strong synergistic interactions with other antioxidant compounds like citric acid, BHA, BHT, α-tocopherol and metal chelators.

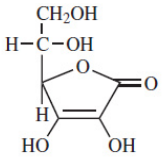


Figure 4. Structure of ascorbic acid.

Carotenoids are soluble in lipids yellow, orange or red pigments common in many plants, fruits and vegetables. Carotenoids are part of tetraterpenoid group. They contain 40 carbon atoms and are produced from 8 isoprene molecules (Fig. 5). Some of the carotenoids are also considered as pro-vitamins like β-carotene, α-caretone and β-cryptoxanthin. Carotenoids are mostly secondary antioxidants who quench singlet oxygen but can also act as primary antioxidants by inhibiting free radicals in the absence of singlet oxygen. They also synergize well with tocopherols. β-carotene, lutein, lycopene and isozeaxanthin are good examples of carotenoids that effectively inhibit oxidation processes (Reische D. W. *et al*, 1998).

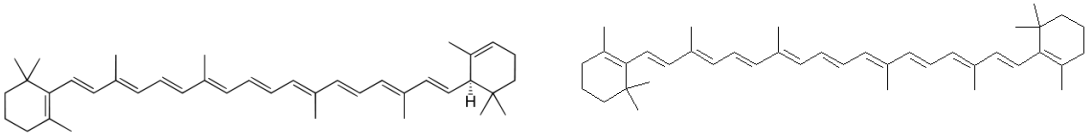


Figure 5. α-Carotene and β-Carotene

Tocols - tocopherols and tocotrienols – are monophenolic and lipophilic compounds that are found in all plant tissues (Fig. 6) (Shahidi F. *et al*, 2000). They are golden to brown color slightly viscous liquids that have specific odour. Tocols are soluble in oil but not in water. Depending on their chemical structure tocopherols and tocotrienols are classified as α-, β-, γ-, δ-. The antioxidant capabilities of tocopherols depending on temperature are of this order δ- > γ- > β- > α-. Tocols act as a free radical inhibitors and terminators. Tocopherols have good synergy with ascorbic acid, citric acid and phospholipids (Shahidi F. *et al*, 2003). They are also used a supplementary ingredients in products that lack resistance to oxidative effects such as animal fats, waxes and butterfats.

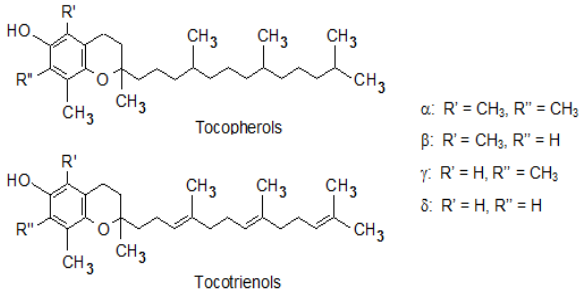
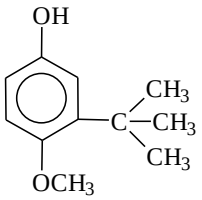


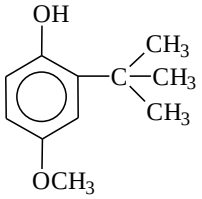
Figure 6. The structure of tocopherols and tocotrienols

1.5.2 Synthetic antioxidants

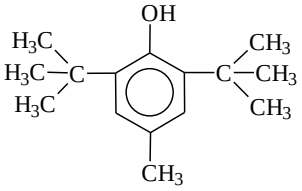
To increase food product resistance to oxidation damage, increase their quality and shelf life it was decided to complement them with additional antioxidants. Since the introduction of synthetic antioxidants there have been synthesized many different antioxidants with different properties (Fig. 7). Not all of these antioxidants share similar capability to inhibit oxidation and terminate free radicals or stability. Only few of them were proven to be suitable for industrial use. Some of the most commonly used synthetic antioxidants in food industry are butylated hydroxyanisole, butylated hydroxytoluene, propyl gallate, tertbutylhydroquinone and ascorbyl palmitate. These antioxidants are mostly used to supplement products with fats and can be directly added to those products to enhance their qualities and shelf life (Lorenzo J. M. *et al*, 2013).



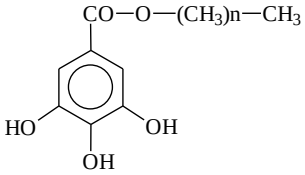
2-*tert*-butyl-4-hydroxyanisole (BHA)



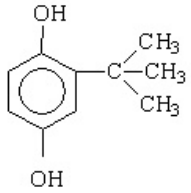
3-*tert*-butyl-4-hydroxyanisole (BHA)



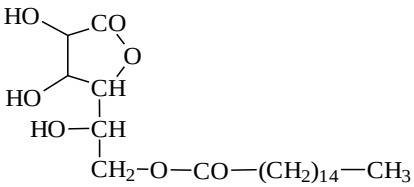
2,6-di-tert-butil-p-hydrokxytoluene (BHT)



Propyl (n=2), octyl (n=7), dodecyl (n=11) gallate



tert-butylhydroquinone (TBHQ)



Ascorbyl palmitate

Figure 7. Synthetic antioxidant structures.

1.6 Supercritical fluid extraction

Supercritical fluid extraction is a separation technique to extract chemical compounds from a raw material using supercritical fluids instead of organic solvents as an extraction solvents (Kraujalis Paulius *et al*, 2014).

Supercritical fluids are referred to as any substance at temperature and pressure above its critical phase at which there is no distinction between liquid or gas phases. Main advantage points of extraction using supercritical fluids is that they can pass solid phase as a gas and at the same time dissolve components like liquid. When a supercritical fluid is near its critical point even slight changes to temperature and pressure leads in major changes on density and many other properties allowing to modify the extraction process to fit many different specific extractions. Carbon dioxide and a little less water are most commonly used supercritical fluids and they are widely applied as substitutes for organic solvents in industrial or laboratory use (Joseph L. Hendrick *et al*, 1992).

Supercritical fluid extraction can be used on small scale for sample preparation or analytical purposes as well as for large scale production of products like oil or removing some unwanted compounds from materials (Fig. 8). In industry this type of extraction often used as a mean of extracting essential oils with carbon dioxide as a supercritical fluid. CO_2 reaches critical phase at 31^0C and 74 bar pressure. This method is highly selective and allows to modify the process to be able

specific compounds from raw materials. If only liquid extraction would be used all of the oil content would be extracted at once, but using supercritical fluid extraction it is possible to first extract volatile oils using low pressure, then at increased pressure lipids and finally phospholipids by adding ethanol to the solvent mixture. This selectivity not only allows to produce products of higher quality but it also allows to create whole new products from sub fractions without the need to further separate the oil (Joseph L. Hendrick *et al*, 1992).

Figure 8. Laboratory (left) and industrial (right) supercritical fluid extractors.

Another advantage to classical diffusion-based extraction methods is that it is much faster. In the classic method the solvent has to diffuse into the matrix of the raw material and then the compounds that are being extracted have to diffuse from the matrix into the solvent. Supercritical fluids have much lower viscosities and have no surface tension which allows them to penetrate even small pores within the matrix that are out of reach for usual liquid solvents. Supercritical fluids also have higher diffusivities then liquid solvents and that along with lower viscosities significantly increase extraction rates. Depending on the quantity of raw material that is being extracted the supercritical fluid extraction may be completed within 10 to 60 minutes, whereas same extraction using liquid solvents may last several hours (Joseph L. Hendrick *et al*, 1992).

Supercritical fluid extraction however has some disadvantages as well. The need to create the pressure in the system increases the cost of the process. The main supercritical fluid solvent – CO_2 – is non- polar so it cannot perform well when extracting polar compounds. There are however solvent modifiers like ethanol that change the properties of the solving allowing to extract wider range of materials at the cost of some benefits of using solvent which is a gas in a room temperature (Joseph L. Hendrick *et al*, 1992).

1.6.1 Optimization of extraction processess

Optimization is a preoperational supercritical fluid extraction tests to determine the operation parameters with which desired goals of the supercritical extraction like highest yield, shortest extraction time or least resource consumption are achieved before upscaling the process to large scale extractions. The optimal parameters depend on the goals that are set to achieve. If the goal is to produce samples in laboratory the optimum parameters will most likely be complete extraction in shortest period of time where as if you need to extract oil for industrial production the optimum is when extraction yields highest amount of extract for lowest resource cost in other words to yield most profit from extraction even though you don't extract all of the compounds. Optimization can be performed by adjusting one or all of these parameters – temperature, pressure and flowrate and in some cases by adding modifiers like ethanol to the solvent (Kraujalis Paulius *et al*, 2014).

Increasing temperature leads to increased diffusion allowing solvents to reach all pores more freely. This effect can also be amplified by reducing the particle size or by swelling the matrix by adding solvent modifiers. Changes in pressure lead to solubility changes. At a very high pressures above critical point it gains synergy with increasing temperature increasing solubility even more. Also there are modifiers that may greatly increase solubility especially when trying to extract more polar compounds. Depending on how you want to optimize your extraction flow rate as an optimization tool can be used differently. To make a cost efficient process flow rate should be matched to solubility sacrificing the speed of the process whereas if speed is the requirement then the flowrate must be set to match diffusion (Joseph L. Hendrick *et al*, 1992).

1.7 *Crysochromulina polylepis*

The algae that is being investigated in this paper is *C. polylepis*. This algae is part of a large algae group called Haptophytes which holds as many as 280 species. The distinctive feature all belonging species hold are their organic or mineralized scales. Although it is hard to establish exact number of species it contains, because some genus like *Chrysochromulina*, that are a part of this group and contains 60 different species on its own that have several different ancestors. The further subgroup of *Chrysochromulina* within haptophytes is called prymnesiales (Linda E. Graham *et al*, 2008).

Prymnesium is a single celled flagellate which has an extremely wide range of of salinity tolerance and also produces toxins that are lethal to fish. They have a short haptonema which does not coil. Species are determines by small ultrastructural differences in their body scales or by molecular methods (Linda E. Graham *et al*, 2008).

Chrysochromulina is a group of approximately 60 species that primarily live in marine environments although there are several freshwater forms. This genus is spread in worldwide regions from arctic to warm climates. They typically have a very long haptonema (Fig. 9) which they use for capturing prey. All known Chysochromulina species are able to perform photosynthesis, they have golden-brown plastids, but phagotrophy is very common among the genus. It is suspected that bacterivory is their way of obtaining phosphate to compensate for low cell-phosphate content. Many of the *Chrysochromulina* species are also photoheterotrophs and are able to take up dissolved organic carbon in the presence of light which helps them survive low-light polar environments. Species are differentiated by molecular methodsor by the structure of their organic fibrillary scales. One cell may have several types of scales in more than one layers (Linda E. Graham *et al*, 2008).

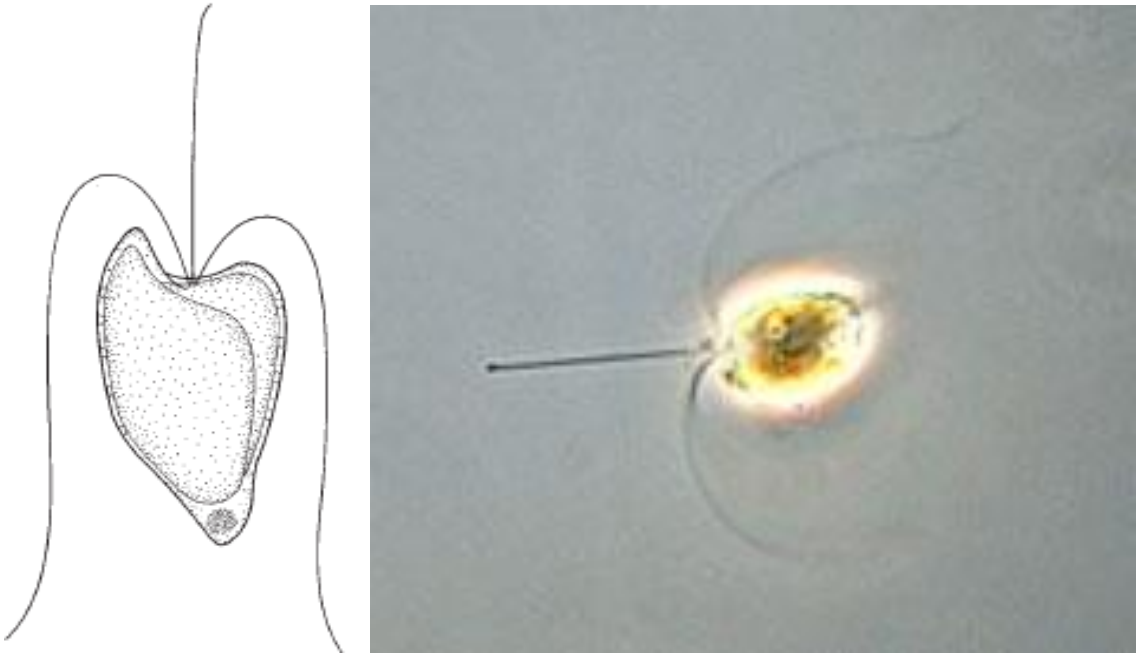


Figure 9. *Chrysochromulina polylepis*.

Some of the marine species of *Chrysochromulina* produce toxins that are lethal to fish. Freshwater species are known to produce odor and compounds that are lethal to tadpoles. There are studies, which have also proved that *C. polylepis* also shows toxic effects towards human erythrocytes and synaptosomes and also synaptic vesicles of a rat brain. However not much is known about the bio products produced by *Chrysochromulina* genus (Anne-Sophie Meldal *et al*, 2009)

1.8 Solid Phase Extraction

Solid phase extraction is an efficient and reliable way to perform sample separations, fractionation and concentration. Wanted goals of sample preparation are achieved through testing a variation of different sorbents – stationary phases – and solvents mobile phases.

Sorbents and solvents for separation are chosen according to what is known about the given sample, dominant compounds, polarity and other factors, to obtain the best results. For that there are different types of mechanisms on which separations are based.

Solid phase extraction is performed in cartridges, on well plates, flat discs or MEPS devices that come in many different sizes. Most commonly used are syringe shaped cartridges that are packed with stationary phase material. There is a wide variety of stationary phase material selection. It is usually a silica based material bonded with specific functional groups designed to perform different extractions. Reverse phase stationary phase is usually bonded with hydrocarbon chains of variable

lengths, anion exchangers have quaternary ammonium or amino groups and cation exchangers have sulfonic acid or carboxyl groups. Sample compounds through the cartridge are being carried via a mobile phase solvents such as methanol and water mixtures, acetonitrile, isopropanol and others using gravitational force or vacuum pumps if the flow must be kept precise and constant (Joseph C. Arsentault, 2012) and (David Harvey *et al*, 2008)

1.8.1 Separations based on polarity

A very important factor defining separations is polarity. Based on polarity there are two kinds of extraction modes: normal-phase and reverse-phase.

In molecular structures of natural products there are a lot of different functional groups that define chemical properties of molecules and chemical reactions. Those properties determines whether that molecule is polar or non-polar. And in chemistry chemical attractions are positive and strong within molecules of same polarity and repulsive and negative between opposite polarities, which means that two polar or two non-polar molecules will attract one another and polar molecule will push away the non-polar (Joseph C. Arsentault, 2012).

The stationary phase and mobile phase during extraction have opposite polarities. Because of that we can differentiate the sample compounds, because molecules of similar polarity are much stronger attracted to the stationary phase, meaning that they will elute slowest and molecules of similar polarity to solvent are going to elute first.

In normal phase extraction the sorbent inside of the column is polar and the solvent is non-polar. So first if we load a sample, which contains many different compounds, into a column those compounds will stick to the column. Then using different elution solvents we can split the sample into different fractions. This happens because solvent's molecules can compete with analyte molecules and replace them in the stationary phase, most non-polar molecules will elute into solvent first, and then, after changing different solvent, we can elute more polar molecules until we fractionate all the solvent (Joseph C. Arsentault, 2012).

Reverse-phase solid phase extraction is opposite to normal one, meaning that the sorbent is non-polar and the solvent is polar. Because of this in reverse phase extraction non-polar molecules move through the column and elute first.

In reverse phase extraction sorbent is very porous, to achieve the largest chromatographic surface, and very non-polar. So in order for sample to sticks between the pores they have to be conditioned. Conditioning is the process, when all the pores of the sorbent's surface are wetted, in order for analyte

to get inside of those pores. If the analyte doesn't get into the pores, it will all elute within the first fraction despite the polarity. More so poor conditioning can lead to analyte loses. If sorbent pores are de-wetted sample doesn't stay in load phase before applying solvents, and passes through column and is lost

Another important aspect of solid phase extraction is the control of the pH. With using only the same solvent it is possible to achieve cardinally different results by only changing pH of the solvent. There are three types of compounds in the samples – neutral, bases and acids. Neutral compounds cannot be ionized and bases and acids can be ionized or de-ionized depending on pH of the solvent.

Ionized compounds become much more polar, so they elute faster but have less retention, which means changing pH can help you improve retention or elution of the compounds. Neutral compounds are not affected by pH changes in the solvent. Note to remember, that pH changes can help you deduce, if the compound has acidic or basic groups (Maria Mansson *et al*, 2014)

1.8.2 Ion Exchange Chromatography

Ion exchange based chromatography works the opposite way as polarity based chromatography, where in polarity based chromatography the rule is that like attracts like and opposites may repel, in Ion Exchange Chromatography it is reversed – likes may repel and opposites are attracted. So for the mechanism to work the analyte and the sorbent must be oppositely charged (Joseph C. Arsentault, 2012).

There are two types of ion exchange mechanism – "strong" and "weak". In the strong anion/cation exchanger either compound or the sorbent will always be charged and in weak ion exchanger either compound or the sorbent may be charged or may not be depending on the pH of the solvent used. Usually the solid phase is charged in ion exchangers.

Strong ion exchange stationary phases has functional groups such as sulfonic acids or quaternary amines which are always ionized. They are used to retain weak ions in the sample which later can be eluted by displacement with ions that are more strongly attached to the stationary phase sites. Also weak ions retained in the column can be eluted by changing the pH of the mobile phase, which un-ionizes them and makes them loose their attraction to the stationary phase and elute. Strong ion exchangers are also often used to purify samples before running them on LC – MS. Strong anion exchangers bind strongly acidic impurities, so that they don't come out with sample targets, as well as strong cation exchanger can bind basic impurities (Joseph C. Arsentault, 2012).

Weak ion exchangers contain such functional groups as secondary amines or carboxylic acids that can be neutralized at some certain pH levels shutting their ion exchange mechanisms. Weak ion exchangers are used to capture strong ions. If these ions later cannot be eluted by displacement then simply stationary phase sites are being neutralized causing strong ions to loose attraction and elute.

It is very important to note never to use strong exchanger with strong compound because they are both charged which makes ion-exchange mechanism impossible to shut off. Because of this the compound might become very difficult to be released from the cartridge requiring very strong acids or bases and high amount of solvent to elute them. This, however, as stated before can be used to capture unwanted compounds to dispose of them (Joseph C. Arsentault, 2012).

1.9 Gill assay

One of the main goals of the project is not only to identify new compounds in algae bio products but especially to identify toxic ones.

In recent years toxicity tests have been developed to avoid using live animals and produce fast, simple and low cost investigation of compound toxicity. It was achieved by using fish-derived cell lines for the tests. Using fish gills it is possible to achieve goals like understanding the mechanisms by which contaminants exert toxicity, to determine the relative toxicity of different contaminants, and to evaluate toxicity of environmental samples. General responses illustrate fundamental cellular activities, which are expressed by all fish cell lines and are affected by a wide range of toxins (Vivian R. Dayeh *et al,* 2013) and (L. E. J. Lee *et al,* 2009).

Fish gill cells are being used for these test. This is due to the high importance of gills for the whole fish organism. Gills are involved in oxygen and other gas exchange, osmoregulation and other vital functions. Because of that damage to the gills usually lead to animal impairment or eventual death and compounds that are highly toxic to the gill tissue is accordingly lethal to the organism as a whole.

There are about 45 fish cell lines available from different fish species to choose from for the test. These cells have epithelioid morphology and form tight mono-cellular layer. Cells can be grown in regular tissue culture surfaces or in trans-well membranes. In Vitro tests with cells also provide some advantages compared to tests with live fish. They allow direct access and investigation of specific functions with higher control of test conditions and allows to bypass some difficulties like response variability due to stress responses, avoiding non targeted tissues, and such (Vivian R. Dayeh *et al*, 2013).

The method used to determine the toxicity in our samples was metabolic activity test using alamar blue reagent. This method allows to detect directly cytotoxic compounds or compounds that require ultraviolet irradiation to become toxic. In this test monolayer fish-derived cells that are present in microwell plates are exposed to toxicants with or without UV irradiation. Cells are then being incubated for set amount of time and at the end of incubation cell viability is measured by assessing metabolic activity by using fluorescent indicator – alamar blue dye. Results can be analyzed fluorometrically or spectrophotometrically (L. E. J. Lee *et al*, 2009).

Experiments are carried out in microwell plates with usually 24 to 96 wells per plate. Cell tissue is grown with media for about 48 hours for cells to strongly attach to the surface of the plate and form confluent monolayer. Before the test, media is removed from plates and media-sample solution is added. Microwell plates are then incubated for set amount of time, usually for 2-3 hours but depending on the specific conditions it can take up to 24-48 hours or even longer. After the incubation media with sample is removed, and dyes are added. Then plates are incubated for another 30 minutes. After the final incubation plates are then read with plate readers. In every plate there are blank wells and wells that are not exposed to toxic compounds – control wells and wells containing varying concentrations of sample done in triplicates for every concentration. Results are expressed as cell viability percentage to the control wells (L. E. J. Lee *et al*, 2009).

Although these are not the only techniques of cell assays and more are being developed but in recent times these methods are one of the most widely applied due to their wide availability, cost-efficiency, low resource and cell tissue requirement and high reproducibility.

2. Materials and methods

2.1 Preparation of E. sphaerocephalus

Before any further experiments the raw material had to be homogenized and made accessible for further research. The *E. sphaerocephalus* was received in packages of 200 grams containing mixed seed and bloom content. This content was milled using Retsch ZM 200 mill (Germany) using 0.5 mm grid.

2.2 Lipid extraction using Soxhlet extractor

Oil samples for the experiments were prepared using soxhlet extractors in three steps. First *E. sphaerocephalus* samples were loaded into sample containers consisting of 10 to 15 g of material in each. Samples are placed in the extractor and collecting vial along with solvent is placed at the bottom of the extractor on a heater. Solvent is then heated at an average 75^0C temperature at which it start boiling. Solvent vapors then travel up the extractor to the container chamber where it is cooled and goes into liquid state. Solvent then dissolves compounds from the container and goes back to collecting vial from where it continues to evaporate more solvent to go to container chamber.

First batch of samples were extracted using 100 mL of hexane for each container into 150 mL volume soxhlet vials. After the extraction samples were dried using vacuum pump.

Second batch of samples were extracted with 100 mL of acetone into 150 mL vials from same *E. sphaerocephalus* that was previous extracted with hexane. Samples were then dried using vacuum pump.

Final samples were extracted with 100 mL of methanol-water (70:30) solution into 150 mL vials from same material that was previously extracted with both hexane and acetone. Samples were then dried using vacuum pump.

2.3 Total phenolic content measurement

The TPC in *E. sphaerocephalus* extracts was determined by a slightly modified Folin–Ciocalteu method (Folin, 1927) using a 96 well microplate reader (Biotek El 808, Vermont, USA). 30 μL of respective dilutions extracts or gallic acid (GA) solutions (0.0625; 0.125; 0.2; 0.25; 0.4 mg/mL) were mixed with 150 μL Folin-Ciocalteu's reagent and with 120 μL 7.5 % sodium carbonate. After 30 minutes the absorbance was measured at 765 nm wave length. The TPC was calculated using (1) equation and the results were expressed as mg of GA equivalents in 1 g of extract.

$$C = \frac{c * V}{m} \quad (1)$$

Where: *C* is TPC; *c* is gallic acid equivalents measured using calibration curve (mg/ml); *V* is sample volume (mL); *m* is the mass of the extract used in analysis.

2.4 DPPH radical scavenging capacity

The RCS of extracts against stable $DPPH^{\bullet}$ was determined by a slightly modified spectrophotometric method (Brand-Williams, 1995) using a 96 well microplate reader (Biotek El 808, Vermont, USA). $DPPH^{\bullet}$ working solution was diluted with methanol to an absorbance of 0.70 ± 0.03 at 515 nm. For each well an aliquot of 7.5 µL extract was mixed with 300 µL of $DPPH^{\bullet}$ solution. The absorbance was taken at 515 nm every minute (overall 41 minutes) until the balance is reached. The inhibition percentage of the absorbance of the $DPPH^{\bullet}$ solution was calculated using the DPPH inhibition equation (2) and the results were expressed as effective concentration (EC_{50}), which denotes the concentration of the sample required to scavenge 50 % DPPH free radicals.

$$\text{Inhibition of } DPPH^{\bullet}, \% = \left(\frac{A_0 - A}{A_0} \right) \times 100 \quad (2)$$

Where: A_0 is absorbance of $DPPH^{\bullet}$ at zero time; *A* is absorbance of $DPPH^{\bullet}$ after 41 min of incubation.

2.5 $ABTS^{+}$ radical scavenging activity

$ABTS^{\bullet+}$ decolourisation test was performed according to an "Improved ABTS radical cation decolourisation assay" (1999). The working $ABTS^{+\bullet}$ solution was made by mixing 7 mM ABTS stock solution with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. The working $ABTS^{+\bullet}$ solution (stable for 2 days) was diluted with PBS to an absorbance of 0.80 ± 0.03 at 734 nm. For reaction 3 µL of extract was mixed with 300 µL of working $ABTS^{\bullet+}$ solution. The absorbance reading was taken every minute until 41 minute using (Biotek El 808, Vermont, USA) microplate reader. The inhibition percentage of the absorbance of the $ABTS^{\bullet+}$ solution was calculated using the (3) equation and the results were expressed as effective concentration (EC_{50}).

$$\text{Inhibition of } ABTS^{\bullet+}, \% = \left(\frac{A_0 - A}{A_0} \right) \times 100 \quad (3)$$

Where: A_0 is absorbance of $ABTS^{\bullet+}$ at zero time; A is absorbance of $ABTS^{\bullet+}$ after 41 min of incubation.

2.6 ORAC assay

The ORAC assay according to Prior et al., (2003) with slight modification was used to study the antioxidant capacity of *E. sphaerocephalus*. The peroxyl radical reacts with a fluorescent probe to form a non-fluorescent product which can be quantified by fluorescence. Samples were prepared by dissolving plant residue in phosphate buffer (PBS); a stock solution of fluorescein was prepared according to this method: 25 µL of samples or trolox reagent were mixed with 150 µl of fluorescein inside 96 wells black opaque microplates. After incubation (15 min at 37 °C) 26 µL of AAPH was added. The microplate was immediately placed in the FLUOstar Omega fluorescent reader (BMG Labtech GmbH, Ortenberg, Germany). Measurements (excitation wavelength 485 nm; emission wavelength 510 nm) were taken every 66 sec (1 cycle), of total 81 cycles. Raw data were analyzed using Mars software (BMG Labtech GmbH, Offenburg, Germany). Trolox dissolved in PBS (6.25 - 200 µM) was used for calibration. Antioxidant curves (fluorescence versus time) were normalized and the area under the fluorescence decay curve (AUC) was calculated using the (4) equation.

$$AUC = 1 + \sum_{i=1}^{i=120} \frac{f_i}{f_0} \quad (4)$$

Where: f_0 is the initial fluorescence reading at 0 min and f_i is the fluorescence reading at time

The final ORAC values were calculated by using a regression equation between the trolox concentration and the net area under the curve (AUC). The antioxidant activity was expressed as µmol trolox equivalent antioxidant capacity in 1 g of extract.

2.7 Supercritical CO_2 extraction

The SCE-CO_2 experiments were carried out using supercritical fluid extractor Helix (Applied Separation, USA). Each extraction was performed using 15 g of *E. sphaerocephalus* sample, which was filled in the vessel between two layers of defatted cotton wool, in both ends, to avoid the particles clogging in the system. The samples were loaded into a 50 mL thick-walled stainless cylindrical extractor vessel with an inner diameter of 14 mm and a length of 320 mm. The temperature of the extraction vessel was controlled by a surrounding heating jacket. The flow rate of CO_2 in the system was controlled manually by the micro-metering valve (back-pressure regulator). The volume of CO_2

consumed was measured by a ball float rotameter and a digital mass flow meter in standard liters per minute (SL/min) at standard state (P_{CO2}=100 kPa, T_{CO2}=20 °C, ρ_{CO2}=0.0018 g/ml). The extracts were collected in glass bottles. The conditions for extraction were set as follows: extraction time 30-120 min, pressure 80-550 bar, extraction temperature 36-60 °C, flow rate of CO_2 2 SL/min. A static time of 10 min was included in to the total extraction time and was constant in all extractions.

2.8 Supercritical fluid extraction experimental design

Response surface methodology (RSM) using central composite design (CCD) was employed to determine the effect of three variables (and a fixed flowrate) on the extract yield and to identify optimum conditions for oil extraction. For data analysis and model establishing the software Design-Expert trial version 8.0.6.1 (Stat-Ease Inc., Minneapolis, MN) was used. Extraction pressure (P), temperature (T) and time (t) were chosen as independent variables. The number of experiments is defined by the formulae:

$$N = (2^f+2f+c) \quad (5)$$

Where f is the number of factors; c is the number of center points.

The complete design consisted of 30 experimental runs with sixteen factorial points, eight axial points and six center points. The data obtained from the CCD design was fitted with a second order polynomial equation, which can be expressed as:

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_{ii} X_i^2 + \sum_{i \neq j=1}^{4} \beta_{ij} X_i X_j \quad (6)$$

Where Y is the predicted response; β_0 is a constant; β_i, β_{ii}, β_{ij} are the coefficients for linearity; X_i and X_j are independent variables. Statistical significance of the model and model variables was determined at the 5% probability level (p<0.05). The adequacy of the model was determined by evaluating the lack of fit coefficient and the Fisher test value (F-value) obtained from the analysis of variance.

2.9 Gas chromatography determination of fatty acid profile

Fatty acid composition of oil obtained by soxhlet extractions was analyzed by gas chromatography (GC). Fatty acid methyl esters (FAME) were prepared by using BF_3 catalyst according to the official AOAC method with slight modifications. FAMEs were analyzed on a HRGC 5300 equipped with a flame ionization detector and 100 m length 0.25 mm (id), 0.20 µm film thickness fused silica capillary column SP™-2560 (Supelco, Bellafonte, PA). Analysis parameters were as follows: injection temperature 220 °C; detector's temperature 240 °C; split ratio 100:1; oven temperature was

programmed in three ramps from 80 °C to 135 °C at 4 °C/min, from 135 °C to 185 °C at 4 °C/min, and from 185 °C to 240 °C at 4 °C/min and held isothermal for 5 min; carrier gas, helium at a flow rate of 20 cm^3/s. The compounds were identified by comparing their retention times with those of a commercial FAME mixture. Duplicate GC runs were performed and the results are presented as a mean. Standard deviations for fatty acids were in the range of 1-5 %.

2.10 Growing algae culture

Chrysochromulina culture was grown in Marine Biological Laboratory, University of Copenhagen, Denmark. The algae was grown in f/2 medium based on autoclaved seawater, salinity 33 PSU, at 15^0C following a light-dark cycle of 16:8 h. The light was provided cool white fluorescent lamps. Starting cell concentration was 5-10.000 cells/ml.

2.11 Biomass extraction.

The biomass was separated from the supernatant using Beckman Coulter AvantiJ-26 XP continuous flow centrifuge equipped with a Beckman Coulter Zonal and Continuous Flow Rotor JCF-Z at an average 40 ml/min flow at 3500g speed 1 liter at a time for small scale tests and 10 liters at a time for large scale tests. Outflowing broth was run through a 30 ml volume C18 column to collect any extracellular metabolites that might be in the supernatant of the algae culture for other future tests. The remaining supernatant from the biomass was separated by centrifuging it at 8500 rpm speed for 15 minutes. After the centrifuging a concentrated broth – supernatant was collected in to a separate vial. Broth, concentrated supernatant and biomass were dried separately.

Biomass was then extracted with acetone and methanol. First extraction were performed with acetone, using 2 ml of solvent to elute the biomass with, then centrifuging it at 4000 rpm speed for 5 minutes. The extract is then collected to a vial. Procedure is repeated for 3 times with both acetone and methanol and collecting extracts separately. Vials were then dried using nitrogen gas.

For large scale tests biomass was eluted with 40 ml of acetone, then centrifuged at 4000 rpm speed for 10 minutes. The extract was then collected into a flask. Procedure was done 4 times with acetone, methanol and ethyl acetate. Extracts of each solvent were collected into separate flasks. Flasks were dried using vacuum drier. Dried flasks were washed with methanol into vials and dried using nitrogen gas.

2.12 SPE column tests

For small scale SPE column tests the biomass pellets were used from the biomass gained from1 liter of culture centrifugate.

For the SAX column tests the pellets were eluted with 450 µl of methanol and mixed with 400 µl of 70% methanol. Evolute AX 1 ml volume columns were used for the analysis. The columns were first equilibrated with 2 ml (2 column volumes) of 100% methanol followed by 70% of methanol in water. Then the 100 µl of the sample solution was loaded into the columns. First fraction was washed with 1 ml of 70% methanol and 1 ml of 100% methanol. The second fraction was eluted with 2 ml of 100% methanol with 1% addition of formic acid (pH2). Fractions were eluted at an average 1 ml per minute flow rate. 100 µl of each fraction were taken for the LC-MS analysis and rest of the fractions were dried with nitrogen gas.

For the C18 column tests the pellets were eluted with 450 µl of methanol. 100 µl of that solution were mixed with 900 µl of molecular water. Strata X 1 ml volume columns were used for the analysis. The columns were first equilibrated with 2 ml of methanol (2 column volumes) and 2 ml of 10% methanol. The 100 µl out of 10% methanol sample solution were loaded into the column. First fraction was washed with 2 ml of 20% methanol, then four more fraction eluted with methanol of increasing concentration in methanol-water solution – 40%, 60%, 80%, and 100%. Each fraction was eluted at an average 1 ml per minute flow rate. 100 µl of each fraction were taken for the LC-MS analysis and the rest of the fractions were dried using nitrogen gas.

2.13 Large scale fractionation

The biomass extracts made with acetone and methanol were used for large scale fractionation.

SAX separation for acetone biomass extract was performed in 15 ml volume column packed with Phenomenex 5 µm Sepra SAX polymer. The sample was dissolved in 5 ml of methanol and mixed with said polymer of column cartridge column then dried and placed into that cartridge. For elution Biotage Isolera One was used which pumped solvents automatically at a constant 10 ml/min flowrate. The column was conditioned with 150 ml of 100% methanol and equilibrated with 150 ml of 70% methanol in water. The first fraction was washed with 150 ml of 70% methanol. The second fraction was washed with 150 ml of 100% methanol, the third fraction was washed with 150 ml of 100% methanol with 1% of formic acid, and the fourth fraction was washed with 350 ml of isopropanol with 1% of formic acid. 100 µl of the fractions were taken for LC-MS analysis.

For SAX column of methanol biomass extract was used 15 ml volume column which was packed with Phenomenex 5µ Sepra SAX polymer. The sample was dissolved in 5 ml of methanol, mixed with the column packing of column cartridge volume, dried, and packed into the cartridge. The column was conditioned with 150 ml of 100% methanol and equilibrated with 150 ml of 70% methanol and the cartridge was placed into the column. After 10 ml of first fraction at 10 ml/min

flowrate column has clogged and it was decided to finish fractionation separately for the cartridge and the column at a 4 ml/mi flowrate. 150ml of each fraction was run separately through cartridge and the column collecting each fraction from both cartridge and the column in to the combined fraction flasks, 300 ml of each fraction total. 100 µl of each fraction were taken for LC-MS analysis.

For C18 fractionation 30 ml volume columns were used which were packed with C18 polymer. Solvents were pumped using Biotage Isolera One at 10 ml/min flowrate. Samples were dissolved in 5 ml of methanol and mixed with column cartridge volume worth of C18 and dried. Then the sample mixed was C18 and loaded into the cartridge. Column was conditioned with 150 ml of 100% methanol, and equilibrated with 150 ml of 50% methanol in water and 500 ml of 5% methanol in water. Cartridge was then placed into the column. Eleven fraction of methanol-water were washed with 300 ml – 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, and twelfth fraction was washed with 500 ml of isopropanol with 1% formic acid. 100 µl of each fraction were taken for LC-MS.

2.14 LC-MS

All samples were tested on Agilent liquid chromatography-diode array detection-quadruple time of flight mass spectrometry systems as was done in Accurate Dereplication of Bioactive Secondary Metabolites from Marine-Derived Fungi by UHPLC-DAD-QTOFMS and a MS/HRMS Library Sara Kildgaard, Maria Mansson, Ina Dosen, Andreas Klitgaard, Jens C. Frisvad, Thomas O. Larsen and Kristian F. Nielsen. Department of Systems Biology, Technical University of Denmark, Soeltofts Plads 221, Kgs. Lyngby DK-2800, Denmark.

2.15 Gill assay

Fraction toxicity was evaluated using metabolic activity test with alamar blue identicator.

Fraction samples were dissolved in 1.6ml of L-15-minus-FBS media solution with 5% of DMSO. The starting sample concentration in the solvent was 100µg/ml. 96 well plates were used for the test. Plates were planted with gill cells and incubated with media for 48 hours. Before adding the samples incubation media was removed from the plates. Plates were then filled with samples by adding blank and control wells and 10 different concentrations of a sample triplicates. The first triplicate and a control were added with 200µl of sample and media solution. Then 800µl of media were added to the sample diluting it by half. This way plates were filled with 10 different concentration sample solutions from 100µl/ml down to 0.2µl/ml. Plates with sample were then incubated in thermostat for three hours. After three hours fluorescent agent was added to the plates and placed into incubation for 30

minutes. After that time plates were read using colorimeter to see if there was an increase in fluorescence which means that cells were alive and there was no toxic effect caused by the sample. Cells were also inspected with a microscope to look for any visual changes to the gill cells.

3. Results and Discussion

3.1 Antioxidant capabilities of *E. sphaerocephalus* and SCF oil extraction optimization

In today's world of ever expanding consumer market and growing population it is important to explore new possible product ideas and expand the use of existing ones. For this reason the attention was drawn towards *E. sphaerocephalus* as a new potential food processing oil. In order to approve it as a viable source of cooking oil initial tests were performed to test the fatty acid content, antioxidant capabilities, oil content percentage and oil extractability.

E. sphaerocephalus L was received in packages 200 grams each containing a mixture of seeds and blooms from that were collected in 2012, "Wilara", Prienai, Lithuania. The samples were extracted using soxhlet extractor. Initial *E. sphaerocephalus* container load was extracted with hexane to make hexane samples which contain mostly oil content. Then the same material that was previously extracted with hexane was extracted with acetone to make acetone samples. The last batch of samples were prepared using the residue that remained after the first two extraction and extracting it with methanol-water mixture (70:30). The average amount of extract yields were:

1) Hexane extracts – 16.34% of total sample mass;
2) Acetone extracts – 1.12% total sample mass;
3) Methanol extracts – 0.64% total sample mass.

Hexane extract contained mostly lipid fraction as well as some other compounds that gave mildly intense green color. Acetone extract contained a lot of resins and was strongly viscous. Dried methanol extract was sticky dark yellow to brown crystals that still contained some moisture.

These are solid numbers for *E. sphaerocephalus* as a food oil material however numbers are lower than those that are described in other articles found on the oil amount and plant descriptions of this plant where it is reported that ripe seeds contain 25% oil content on average. This is most likely due to described tests were performed using only *E. sphaerocephalus* seed sample whereas the current sample is a mixture of seeds and blooms and seeds usually hold most of the plant oil content. For example other popular oil crops like sunflower seeds contain 28% oil content on average and rape seeds contain 45% oil content.

3.1.1 E. sphaerocephalus fatty acid profiling

One of the most important parts of selecting new plant species for food oil production is determining its fatty acid content which shows the quality, nutrition and stability of the oil. The profile of the fatty acids was analyzed by gas chromatography. Unsaturated fatty acids compose over a 90% of total fatty

acid content. The variety of fatty acids is not diverse (Table 1). Most of the fatty acid content is dominated by linoleic acid (68.32%) along with oleic acid (21.26%), palmitic acid (4.98%) and stearic acid (2.79%). Other fatty acids have less than 1% of abundance. These results show similar fatty acid composition ratio of previously performed research. The oil characteristics of *E. sphaerocephalus* are similar to other unsaturated plant oils like sunflower seed oil, soybean oil or wheat germ oil (Gert Horn *et al*, 2008) and (A.J. Patel *et al*, 2011). The fatty acid composition and the ratio of saturated and unsaturated acids are good indicators that help to evaluate nutritional and functional value of the oil. High unsaturated fatty acid content oil helps to reduce cholesterol content in blood and are generally considered healthier than saturated fats however they are less popular in industrial production because they are less resistant to rancidity and are in a more solid state at room temperature.

Table 1.

The fatty acid composition of *E. sphaerocephalus*.

Fatty acid found in *E. sphaerocephalus*	Fraction of total fatty acids, %	Fraction of total fatty acids as found in literature, %
Linoleic acid	68.32	71.5 – 82.7
Oleic acid	21.26	11.1 – 19.1
Palmitic acid	4.98	3.0 – 7.6
Stearic acid	2.79	1.1 – 3.0
Linolenic acid	0.823	0.7 – 2.0
Behenic acid	0.34	0.3 – 0.6
Lignoceric acid	0.14	
Palmitoleic acid	0.13	
Cis-8.11.14-eicosatrienoic acid	0.12	
Arachidic acid	0.12	
Capric acid	0.1	
Myristic acid	0.07	
Heptadecanoic acid	0.05	
Tricosanoic acid	0.03	
Heptadecanoic acid	0.03	

3.1.2 The assessment of the antioxidant properties of *E. sphaerocephalus* extracts

3.1.2.1 Total phenolic content

Phenolic compounds are referred to as compounds that have the highest potential to neutralize free radicals. The determination of quantity of phenols is a commonly applied in food researches. The total phenolic content within *E. sphaerocephalus* was determined using Folin-Ciocalteu method. Gallic acid was used for calibration curve standard and the results are shown as a gallic acid

equivalents. The amount of total phenolic content value was quite similar between hexane sample – 27.64 mg/g and acetone sample – 28.03 mg/g but methanol extract showed a high phenolic content – 158.69 mg/g.

In comparison to other common food oil crops total phenolic content it shows similarly. Sunflower seed oil has between 21.86 mg/g and 36.14 mg/g of phenolic compounds and rape seed oil is reported to contain 18.76 mg/g to 19.62 mg/g phenolic compounds (Gert Horn *et al*, 2008), (Gert Horn *et al*, 2007) and (Magdalena Karamac *et al*, 2012).

3.1.2.2 DPPH free radical scavenging capacity

The DPPH free radical scavenging capability is a widely used method to determine the strength of antioxidants in the sample. Samples which were prepared in 9 concentrations that ranged within brackets from 0.025% to 1% and were repeated 3 times each were being tested at 515 nm wavelength to check for DPPH scavenging capability. This effect is affected by type of radical, structure and kinetic behavior of phenolic compounds and their concentration. Results are presented as an effective concentration – EC_{50}, mg/mL – which shows the concentration at which 50% of the free radicals are bonded. Out of these three extracts hexane extract showed least DPPH radical scavenging capacity however acetone extract which had similar amount of phenolic compounds had a lot stronger radical scavenging effect. Methanol extract was almost as strong as the acetone extract. Hexane extract – 25.76 EC_{50}, acetone extract – 0.54 EC_{50}, methanol extract – 1.3 EC_{50}. These results show that even though phenolic compound in *E. sphaerocephalus* are spread in all of the extracts hexane extract is considerable weaker when compared to other two extracts. This might indicate that those phenolic compounds with the strongest radical scavenging capacity did not extract with hexane.

In comparison sunflower seed oil DPPH scavenging capacity is reported to range between 27.69 and 45.45 EC_{50} and 11.9 to 51.6 EC_{50} for rapeseed oil (Gert Horn *et al*, 2007), (Muhammad Nadeem *et al*, 2011) and (Hyun-H Jun *et al*, 2014). These results indicate that the *E. sphaerocephalus* is similarly active as other popular oil crops meaning that this plant is suitable for food oil production.

3.1.2.3 $ABTS^+$ radical scavenging capability

Another commonly used method for testing free radical scavenging capability that was being used is $ABTS^+$ assay. Similarly to DPPH assay this method is also based on the ability of antioxidants in the sample to scavenge free radicals which in this case is a synthetic radical $ABTS^+$. Samples which were prepared in 9 concentrations that ranged within brackets from 0.025% to 1% and were repeated 3 times each were being run at 734 nm wavelength. Results are presented as a concentration of antioxidants at which they scavenge 50% of $ABTS^+$ radicals – EC_{50}, mg/mL. Just

like in DPPH assay results are similar. Highest free radical scavenging capability was seen in methanol extract – 0.17 EC_{50} and acetone extract – 0.54 EC_{50} whereas hexane extract had a lot less $ABTS^+$ radical scavenging capacity –18 EC_{50}. This test correlates to the results of DPPH assay that acetone and methanol fractions show a lot stronger antioxidant potential. In comparison rapeseed oil was reported to have $ABTS^+$ radical scavenging capability of 16.7 to 66.1 EC_{50} (Hyun-H Jun, 2014)

Table 2.

The antioxidant activity of the *E. sphaerocephalus* extracts.

Extract	Total phenolic content, mg/g	DPPH radical scavenging capability, mg/mL EC_{50}	$ABTS^+$ radical scavenging capability, mg/mL EC_{50}
Hexane	27.64 ± 0.05	25.76 ± 0.37	18 ± 0.77
Acetone	28.03 ± 0.07	0.54 ± 0.006	0.54 ± 0.003
Methanol	158.69 ± 0.13	1.3 ± 0,009	0.17 ± 0.004

3.1.3 The assessment of antioxidant properties of *E. sphaerocephalus* extraction residue

Today there is a growing need to capitalize as much of raw materials leaving as little waste as possible. Because of this reason there is an interest to perform antioxidant activity test on residue that is left after the extractions to see if they still might be usable tor some purposes after the oil has been extracted out of it. Samples for the experiments were prepared by mixing extraction residue after extractions with microcrystalline cellulose to make samples of appropriate concentrations.

3.1.3.1 Total phenolic content

The phenolic content for the remaining material was evaluated using the same Folin-Ciocalteu method that was used to determine phenolic content of the extracts. The samples of extraction residue mixed with microcrystalline cellulose were tested and results were expressed as milligrams of gallic acid equivalents in one gram of extract and were as following: post hexane extraction residue – 95.03 mg/g, post hexane and acetone extraction residue – 84.60 mg/g, post hexane, acetone and methanol extraction residue – 44.58 mg/g. These results show that there were still a lot of phenolic compounds left in the *E. sphaerocephalus* even after the extractions in fact even more then there were in the extracts themselves.

3.1.3.2 $ABTS^+$ QUENCHER radical scavenging capability

To determine free radical scavenging capabilities for solid sample improved ABTS radical cation decolourisation assay was used. Samples used contained 0.1 µg of the residue mixed with microcrystalline cellulose. Trolox was used for the calibration curve standard. The results are expressed as micromoles of trolox equivalent in one gram of sample and were as follows: post hexane

extraction residue sample – 446.46 μmol/g (111.74 mg/g), post hexane and acetone extraction residue sample – 160.46 μmol/g (40.16 mg/g), post hexane, acetone and methanol extraction residue sample – 37.8 μmol/g (9.46 mg/g).

3.1.3.3 Oxygen radical absorbance capacity assay (ORAC)

Another method used to determine antioxidant activity was quencher ORAC which measures oxidative degradation of fluorescent molecule after being mixed with free radicals. This method is used to determine an antioxidant activities of a wide variety of food products. Samples used contained 0.38 μg of the residue mixed with microcrystalline cellulose. Trolox was used for the calibration curve standard. The results are expressed as micromoles of trolox equivalent in one gram of sample and were as follows: post hexane extraction residue sample – 318.83 μmol/g (79.8mg/g), post hexane and acetone extraction residue sample – 240.42 μmol/g (60.17 mg/g), post hexane, acetone and methanol extraction residue sample – 167.79 μmol/g (41.99 mg/g).

These results correlate with the results of $ABTS^+$ assay experiment confirming that extraction residue still contains antioxidant potential which is reducing in power with each additional extraction.

As the results indicate there is still antioxidant potential remaining in *E. sphaerocephalus* even after the extractions had been performed. There are quite significantly more phenolic compounds compared to the extracts and antioxidant capacity is also relatively high, especially in second and third stage residue samples considering that the acetone and methanol extracts had very low antioxidant properties compared to hexane extract.

Table 3.

Antioxidant activity of extraction residue.

Extract residue	Total phenolic content, mg/g	$ABTS^+$ TEAC, μmol/g	Oxygen radical absorbance capacity TE, μmol/g
Hexane	95.03 ± 2.1	446.46 ± 25.3	318.83 ± 34.3
Hexane, Acetone	84.60 ± 2.9	160.46 ± 8.4	240.42 ± 9.9
Hexane, Acetone. Methanol	44.58 ± 1.7	37.80 ± 6.1	167.79 ± 7.1

3.1.4 Supercritical CO_2 extraction optimization

In order to get conduct further research more of the *E. sphaerocephalus* oil is required. Since soxhlet extraction takes relatively high amount of time and solvents to produce low amount of oil it was decided to use more efficient way in regards of time and resources – supercritical fluid extraction.

This method is widely used in industrial extractions where the size of operations are much greater than laboratory ones and it not only helps to maximize the outcome of the manufacturing but is also much more environment friendly because the solvent used is mostly CO_2 which can be regenerated for multiple uses or just safely released into the atmosphere. Before going to large scale extraction it is recommended to perform small scale optimization to discover the best protocol conditions.

Table 4.

Experimental results of supercritical fluid extraction optimization.

Pressure,	Temperature,	Time,	Yield,
80	36	30	0.077
80	36	120	0.16
80	48	75	0.028
80	60	30	0.03
80	60	120	0.08
315	36	75	14.08
315	48	30	12.64
315	48	30	13.1
315	48	75	13.95
315	48	75	13.96
315	48	75	13.52
315	48	75	12.47
315	48	75	14.42
315	48	75	12.27
315	48	75	14.26
315	48	120	14.08
315	60	75	13.98
550	36	30	8.77
550	36	120	13.88
550	48	75	13.26
550	60	30	12.87
550	60	120	13.25
550	60	120	14.03

Central composite design was used to optimize three independent variables – pressure, temperature, extraction time. The experimental conditions were 36^0C to 60^0C temperature, 80bar to 550 bar pressure and 30 to 120 minutes time. The CO_2 rate was chosen to be a constant variable set to 2 SL/min. flow rate. Experimentally obtained oil yields were ranging between 0.028% and 14.42%. The aim of the optimization was to find the settings with which there would be a largest oil outcome from the material. Based on 23 extraction protocols (Table 4) with different variable settings 30 different protocol solutions (Table 5) that provide the largest oil yield were composed. The optimal conditions to gain the maximum oil yield were 116.97 minutes, 55.49^0C temperature and 42.18 MPa

pressure at a constant 2 SL/min CO_2 flow rate. This generated protocol gave the predicted value of 15.7% oil yield, a considerably higher than the experimentally gained maximum value under the conditions of one of the experiments. However other protocols maybe equally valid if the desired goal is to get as many of the extract in the fastest time period or using as little resources like energy or solvent and the little more gain in yield would be irrelevant. This especially makes this method of extraction attractive to industrial use because of the high flexibility of the method.

Table 5.

Protocol solutions created for supercritical fluid extraction.

Solution number	Pressure, MPa	Temperature, ^{0}C	Time, min	Yield, %
1	40.55	41.77	62.51	14.5
2	39.33	49.07	54.04	14.7
3	44.78	58.97	78.53	15.6
4	33.64	45.70	89.34	14.5
5	43.42	47.94	114.46	15.7
6	43.56	56.78	43.63	14.9
7	41.85	58.43	73.61	15.6
8	33.51	37.41	109.55	14.8
9	46.54	52.04	71.81	14.9
10	42.80	44.45	83.93	15.2
11	45.91	47.39	87.98	15.1
12	35.35	51.57	106.23	15.0
13	40.29	41.00	64.57	14.5
14	42.18	55.49	116.97	15.7
15	37.17	53.01	95.07	15.3
16	41.03	50.90	49.76	14.7
17	34.85	58.64	50.38	14.7
18	37.32	58.33	79.78	15.4
19	43.54	39.98	76.40	14.8
20	39.70	55.04	94.12	15.6
21	44.02	54.15	36.77	14.5
22	37.74	51.73	66.48	15.0
23	47.51	57.45	40.30	14.4
24	38.24	52.35	115.39	15.4
25	40.90	37.59	88.59	15.1
26	43.58	39.10	74.18	14.7
27	44.01	58.48	75.73	15.6
28	38.57	57.03	52.72	15.1
29	44.48	50.99	107.46	15.6
30	33.46	51.06	101.45	14.6

Response surface plots showing the effect of extraction time, temperature and pressure on oil yield are presented in figures 35 - 37. The graphs were obtained by fixing two variables at coded zero level, one of which is a flow rate, while varying the remaining two variables and predicting the response variable. First figure (10) illustrates shows linear effect of the extraction temperature and effects of the pressure on oil yield at a fixed 75 minute timer. In this case the pressure was the main deciding factor of the extraction where increased pressure increases the yield up to a point at around 40 – 45 MPa after which it starts to reduce. The yield starts to decrease because vapor pressure increase after some point leads to reduced density of the solvent and therefore reduced extraction power (D. Westerman, 2006). Increased temperature here provided synergistic effect with increasing pressure.

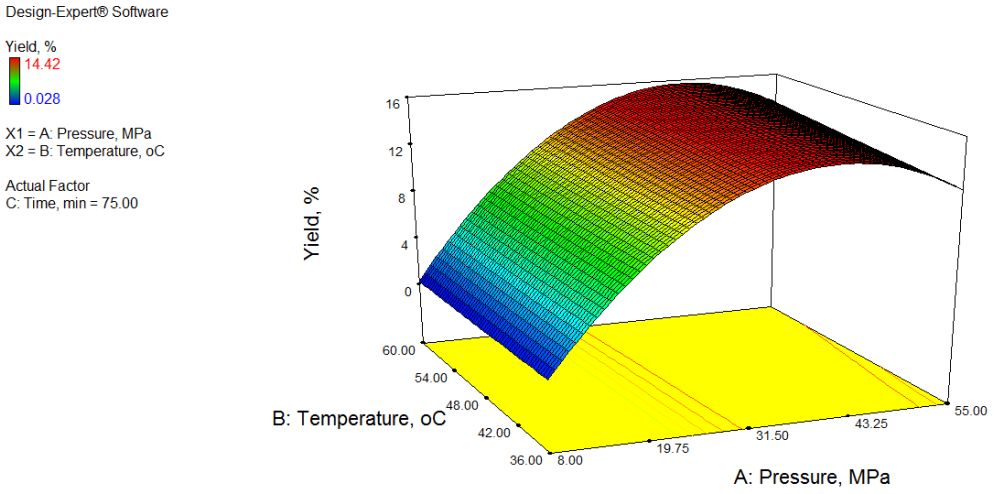


Figure 10. The effects of temperature and pressure on the extraction.

Figure (11) shows the effect of time and pressure on the extraction at the set 48^0C. The effect in this case is similar to the first figure. The increased pressure here also contributes most to increased extraction up to a point at around 40 – 45 MPa after which it starts to go down due to reduced solute density and solvent power. Time of the extraction gives synergistic effect of increased extraction the longer it takes.

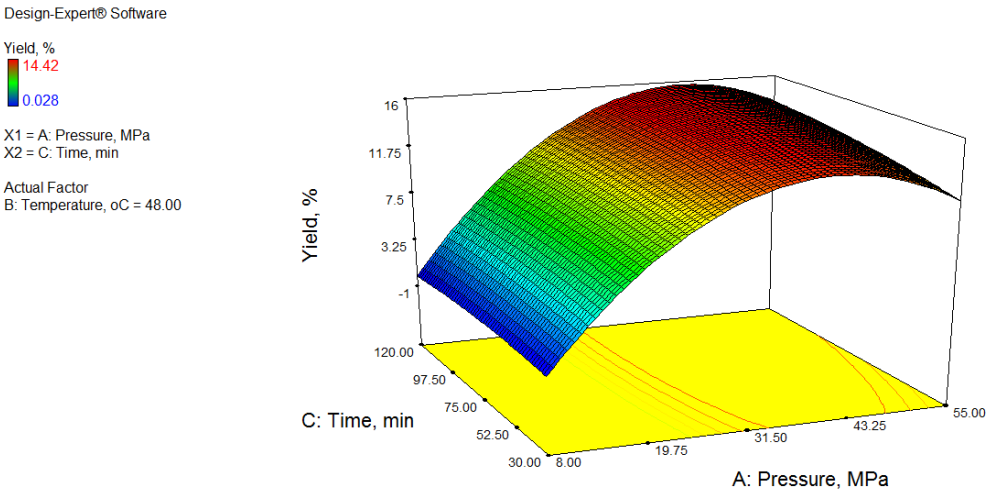


Figure 11. The effects of duration and pressure on the extraction.

The last figure (12) shows the effect of the duration of the extraction and the effect of temperature at a set 31.5 MPa pressure. In this case at low temperatures the longer the duration the more oil is extracted. At low to medium duration the temperature greatly increases the yield however with greatly increasing temperature time stops being a positive factor and extraction yield reduces. This is most likely due to higher density of the solvent which means increased solvent strength at lower temperatures and its reduction at higher. Increased temperature however increases volatility of the solute which increases the yield.

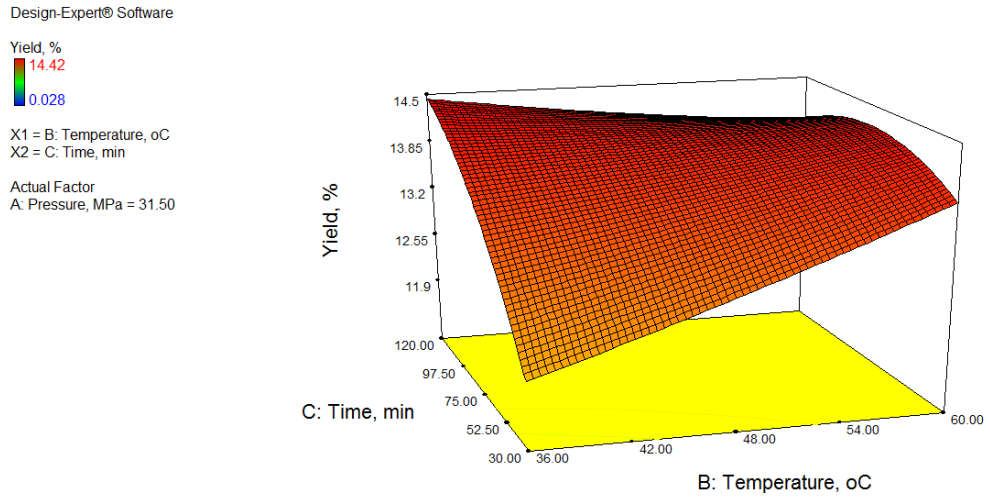


Figure 12. The effects of duration and temperature on the extraction.

Considering all of the figures it is evident that the most influential parameters are the pressure and the extraction time whereas temperature have less important impact on the oil yield. Other studies also report that the extraction time and the pressure have most impact on the supercritical fluid extraction of seed oil (S. Liu *et al*, 2009) and (V. Y. Ixtaina *et al*, 2010).

The remaining residue after the supercritical fluid extractions may be also used as a source to extract other compounds from. As tested with antioxidant methods it still contains a lot of phenolic compounds and shows antioxidant properties. It is also worth taking into consideration that supercritical fluid extraction is performed at lower temperatures then most of other extraction methods and less reactive solvents thus it probably retains more unaltered compounds especially proteins. With more research on the residue it might be successfully used as a source of secondary substances or applied to animal fodder or even human foods such as bakery.

3.1.5 Model analysis

To assess the effect of supercritical CO_2 extraction parameters on the total oil yield the response surface methodology was used (RSM).Second order polynomial regression model which is an empirical relationship between dependent variables and the independent test variables – time, temperature and pressure – is given in the following equation (7).

$$Y = 13.85 + 6.17\times P + 0.32\times T + 0.66\times t + 0.45\times P\times T + 0.67\times P\times t - 0.60\times T\times t - 7.34\times P^2 + 0049\times T^2 - 0.39\times t^2 \quad (7)$$

Predicted values were calculated using a second order polynomial equation (6) and compared to experimental values (Fig. 13). The adequacy of the model was evaluated by the total determination coefficient (R^2) value of 0.99 which indicates a reasonable fit of the model to the experimental data. Adjusted coefficient of determination (R^2) of 0.98 which is a measure of the amount of variation about the mean explained by the model. Even though adjusted coefficient is slightly off from predicted coefficient of determination (R^2) of 0.89 it is still aligns in agreement with it.

Model evaluation is presented in the table of the analysis (Table 7). The Student test (*p*-value) was used to determine significance of each model. The analysis of quadratic regression models for oil yield suggested that the model was significant. With an *F*-value of 119.14 and the "lack of fit" was not significant relative to the pure error with a *p*-value of 0.3217. The model shows that the factor with the largest effect on oil yield was extraction pressure – P ($p<0.0001$), extraction time – t ($p = 0.0254$) and extraction temperature – T ($p = 0.2263$). The interaction between time and pressure – tP ($p = 0.0385$) had the highest effect on oil yield and was significant. The only second-order term that was significant ($p<0.0001$) was pressure – P^2.

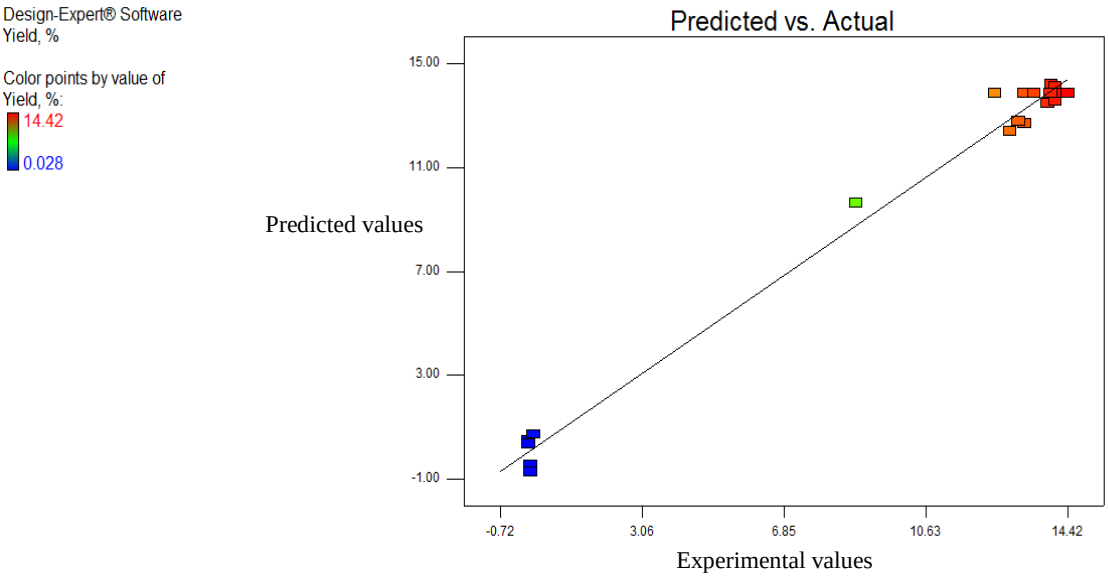


Figure 13. Predicted values versus experimental values.

3.2 Bio-guided Analysis and Fractionation of *C. polylepis*

3.2.1 Small scale chemical profiling

3.2.1.1 Dereplication

There was very little information about the compounds produced by the *C. polylepis* algae. In order to find out more about its chemistry, novel and in particular toxic compounds. The initial extractions were performed and samples were run on LCMS. Chromatograms revealed a lot of novel compounds that were not registered in the Antibase data base compound collection and only compound that was registered in Antibase as a compound found in *C. polylepis* was chlorophyll β.

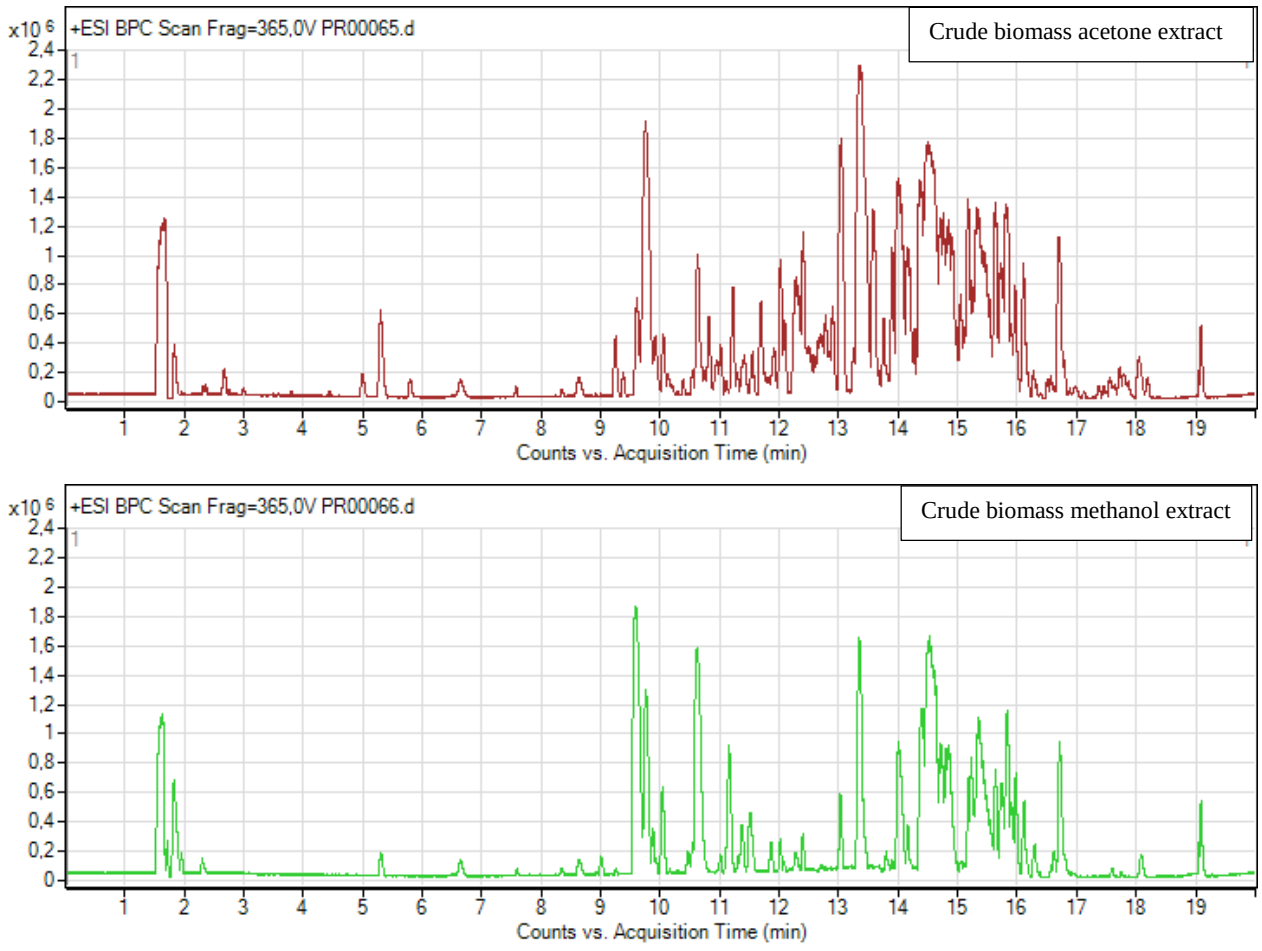


Figure 14. Base peak chromatograms of acetone crude biomass extract and methanol crude biomass extract showing wide range of compounds not registered in databases.

One compound in particular was very interesting. Looking closer into mass spectra a compound which contains Cl, Br and S was discovered. The adduct pattern showed an ion with a loss of m/z 80 hinting that the compound has SO_3^- group (Fig. 15). This compound is very interesting, because the combination of these elements in one compound is very rare. Especially the presence of Br, because

there are very few cases of microalgae containing bromine. Given the rare composition this compound was presumed to be one of the compounds causing the *Chrysochromulina* toxicity and it was decided that it should be purified to closely investigate its structure and toxicity. Upon searching Antibase 2012 database for the mass with no hits it was presumed that this is a unique novel compound.

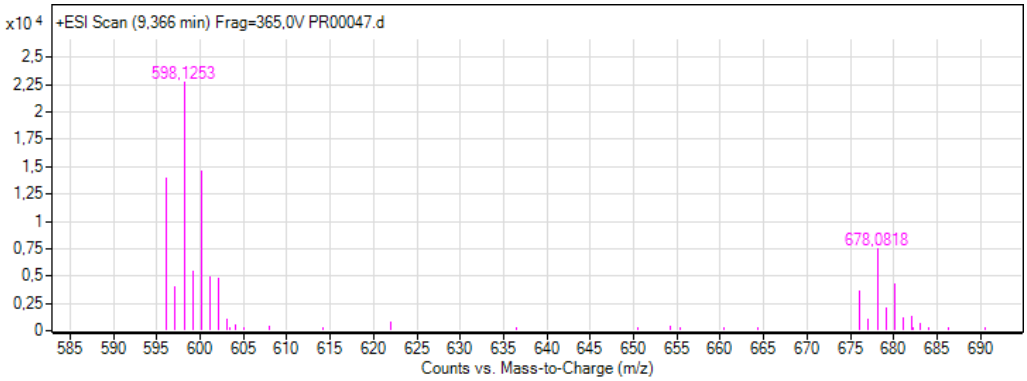


Figure 15. Novel compound isotope and fragment pattern MS. Adduct loss of m/z 80 inclines that the compound has a SO_3^- group.

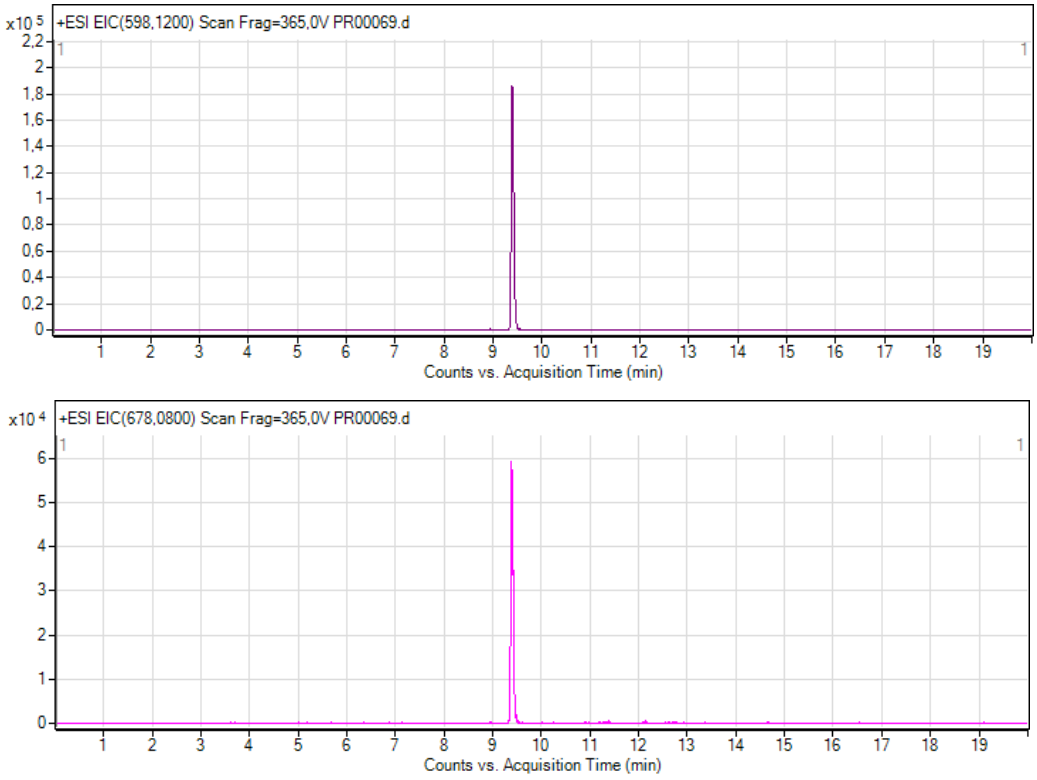


Figure 16. Extracted ion chromatogram of ion fragment with a mass of 598 Da showing greater abundance than ion with a mass 678 Da in *C. polylepis* crude acetone extract.

After a closer inspection of mass spectrum it was discovered that the ion fragment with a loss of SO_3 and mass of 598 Da was more abundant than the molecular ion with a mass of 678 Da so it is better to look for fragment ion or both ions in the chromatograms (Fig. 16).

3.2.1.2 E-SPE column tests for deciding future purification strategy.

In order to begin further experimentations with a novel compound it is necessary to have a sample of purified compound. Since the amounts of compounds produced by algae are very small it is very important to choose a most efficient extraction mode. Before going to large scale extraction methods are first tested in small scale analytical environment.

At first small amounts of sample were produced for initial tests. Biomass was separated from supernatant by centrifuging. Then a few different solvents including acetone, acetonitrile, butanol and methanol were used to extract compounds from the biomass pellets to determine which ones were most efficient.

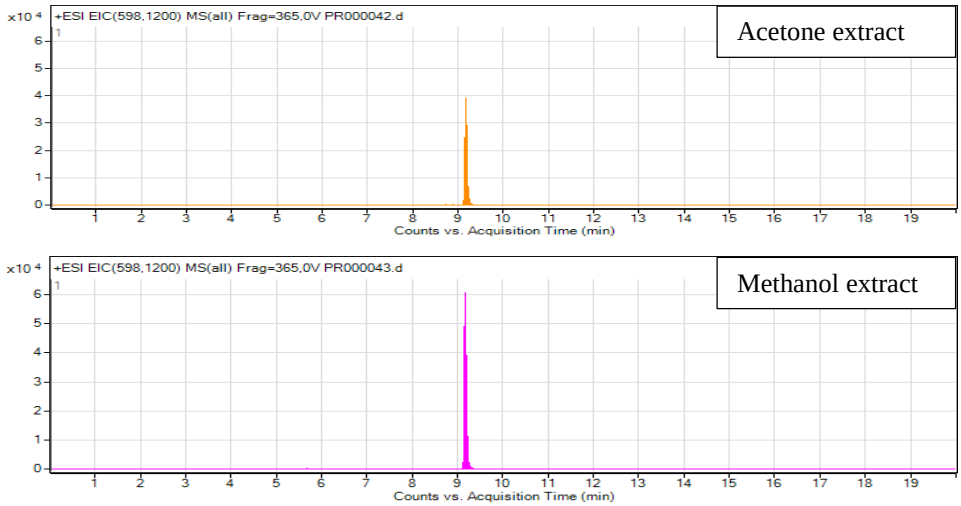


Figure 17. Extracted ion chromatograms showing fragment ion with mass 598 Da abundance within *C. polylepis* strain P039 crude biomass extracts of two most efficient solvents: top chromatogram – acetone extract, bottom chromatogram – methanol extract.

Methanol extract has slightly better target compound abundance, although some strains showed better target compound extraction with acetone, and has another notable advantage – acetone extract has a lot more unwanted side compounds extracted from the biomass like chlorophylls and other large molecular weight compounds (fig. 17).

Biomass extracts were then used to determine the best separation mode for the target compound. Several small scale solid phase extraction columns of different modes were used. Methods chosen to test first were normal phase C18 column, mixed mode column. And because there is a $-SO_3$ group in the compound it is most likely possible to catch the compound using ion exchange mechanism so weak anion exchange column and strong anion exchange column were also chosen for initial separation tests.

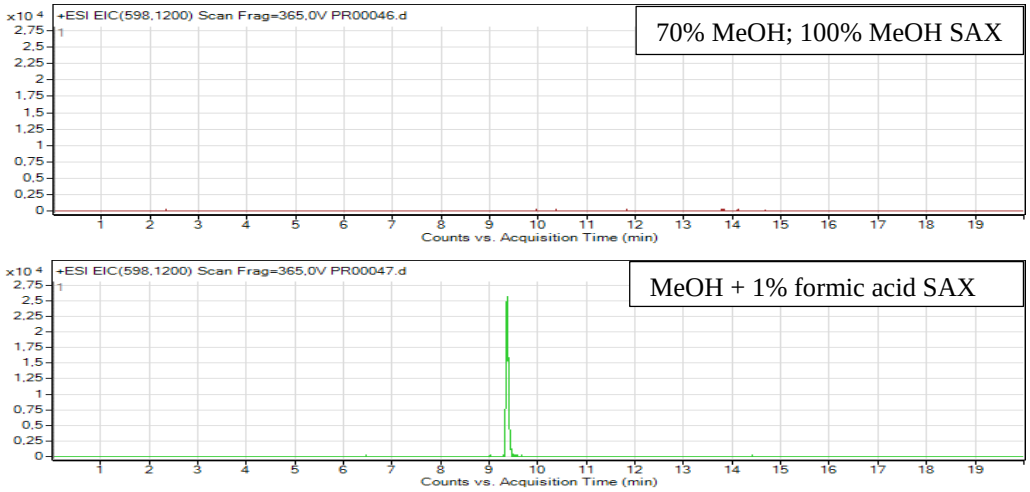


Figure 18. The abundance of compound fragment with mass 598 Da in SAX column fractions.

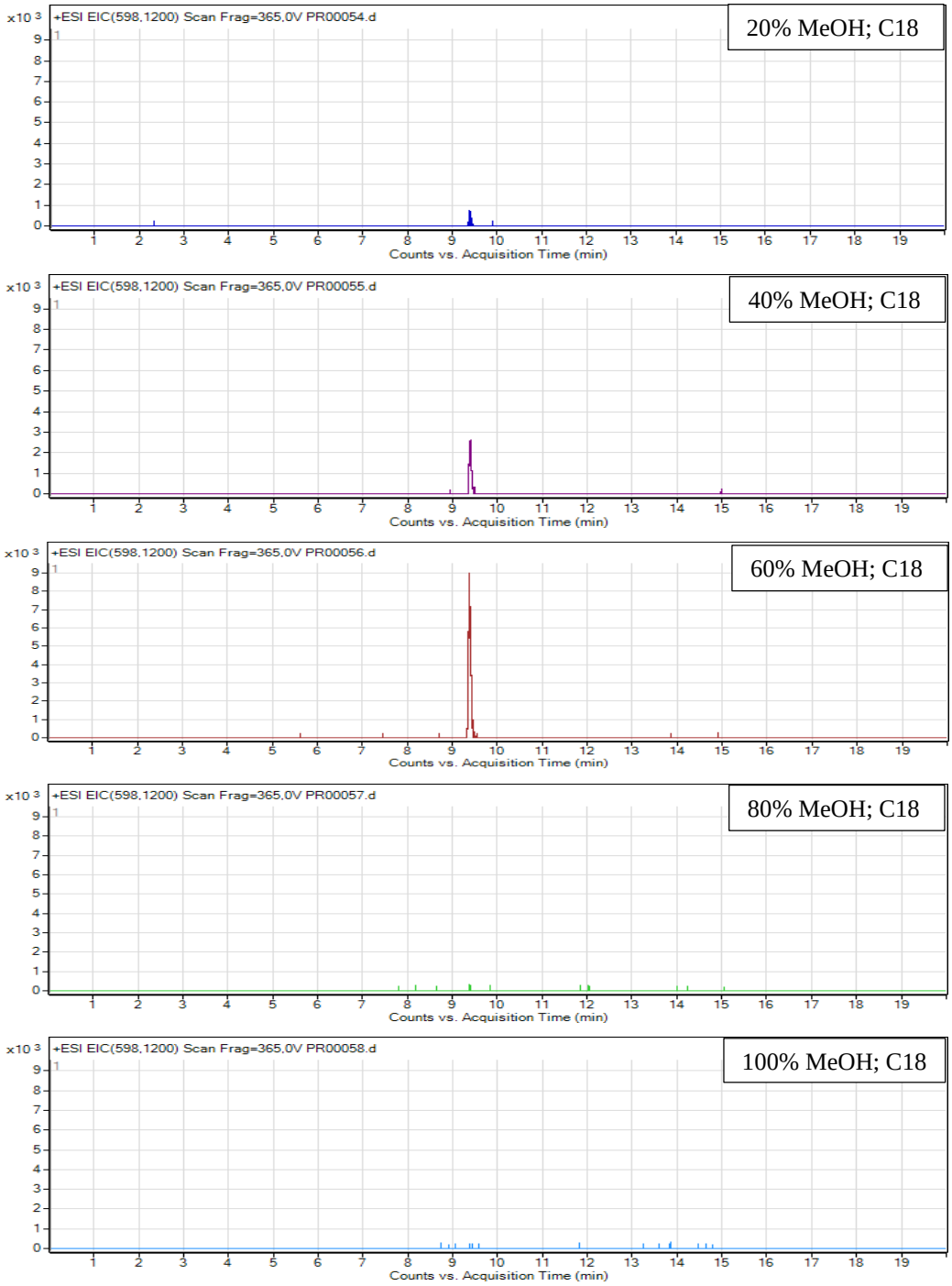


Figure 19. The abundance of compound fragment ion with mass 598 Da in C18 column fractions.

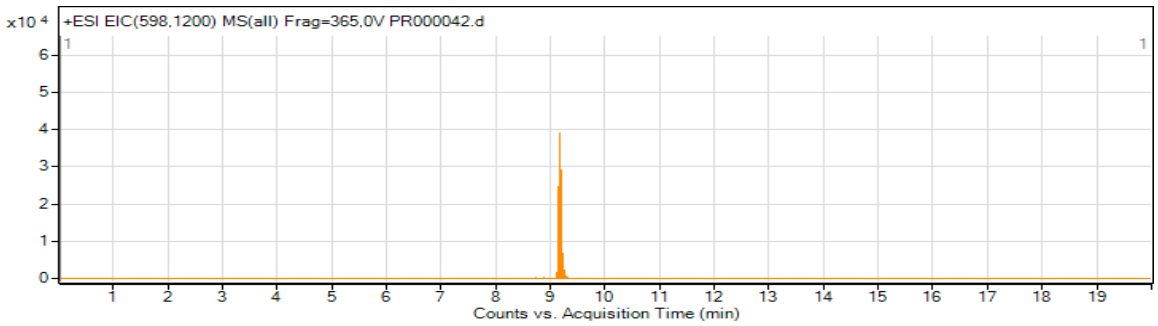
Best target compound extraction was observed using strong anion exchanger in methanol fraction with added 1% of formic acid (pH 2) (Fig. 18). Quite good target compound separation was also observed in C18 60% methanol and 40% water fraction which was the main fraction containing our target compound in C18 mode (Fig. 19). By small scale column results strong anion exchange mode was deemed best suited for large scale compound crude separation.

3.2.1.3 Best compound producing strain

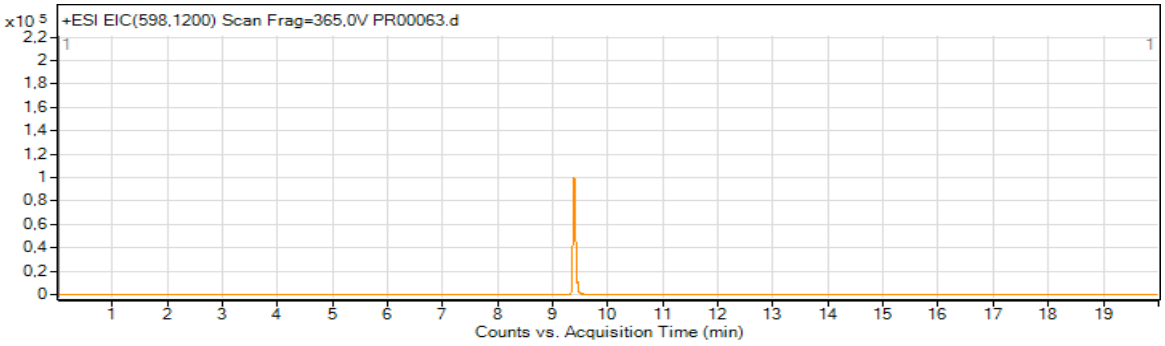
Different strains of same algae sometimes produce different amounts of the same compound. In order to grow batch of strain which produces most of the target compound it is important to see which one of available strains produces most of it.

Five strains of *C. polylepis* were analyzed during this project, two strains from Norway – K0259 and K0617, and three strains from Copenhagen University – P038, P039 and P041.

Strain P039



Strain K0617



Strain K0259

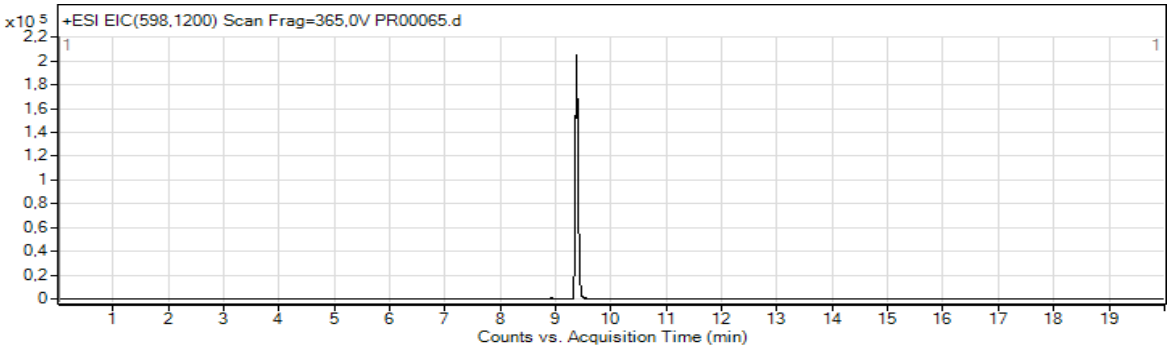


Figure 20. Extracted ion chromatogram showing an abundance of compound with mass 598 in strains that the compound is present in *C. polylepis* and its different strains.

Out of 5 investigated strains two strains, P038 and P041, did not produce our target compound. Out of remaining three the most productive strain was K0259 second K0617 and least productive P039 (Fig. 20), although all of the strains produced relatively similar amount of the target compound. Even though other two strains produced higher amount of the compound, the strain P039 was chosen for further research because it was easier to obtain and was cultivated in a local University and its relatively similar compound production compared to other strains.

3.2.2 Large scale chemical profiling

3.2.2.1 Culture cultivation

After optimal small scale parameters and the producing strain was chosen and the cultivation of strain P039 began. Four batches of 10 liters of culture were grown divided in two separate harvestings of 20 liters per harvest with a two week gap between harvests.

Each harvesting of 20 liters of P039 culture was centrifuged at 3500 × g in a continuous average flowrate of 40 ml min^{-1}. Centrifuge model was Beckman Coulter, AvantiJ-26 XP equipped with a Beckman Coulter Zonal and ContinuousFlow Rotor JCF-Z. While centrifuging the outflowing broth was run through a 30 ml volume C18 column to see if there could be collected any extracellular metabolites. The collected biomass was slimy and sticky indicating it might contain quite a lot of polysaccharides. After the biomass was collected it still had some supernatant with it. The biomass was then placed in centrifuge where it was separated from the remaining supernatant spinning it 15 minutes at 4000 rounds per minute. After the centrifuging a concentrated broth – supernatant was collected in to a separate vial. Broth, concentrated supernatant and biomass were dried separately.

After the separation the biomass was eluted with solvents to extract the chemical compounds. First it was eluted with acetone, then methanol and finally ethyl acetate. Extractions were performed by mixing the biomass with 40ml of solvent then it was shaken with whirlpool for two minutes and centrifuged for fifteen minutes at 4000 rounds per minute speed. The procedure was repeated for four times for each solvent used. Out of each fraction a 100µl sample were taken for initial LC-DAD-MS analysis. After that fractions were dried using nitrogen. Dried samples seemed to be very salty even after some additional washing with methanol. Sample fractions from two separate harvests at first were prepared separately to see if there were any differences in chemistry between harvests but it was proved incorrect by LCMS. Combined dried biomass extract yields were:

Acetone fraction – 0.5232 g;

Methanol fraction – 0.198 g;

Ethyl acetate fraction – 0.0391 g.

Figure 21. Base peak chromatograms of three biomass crude extracts.

LCMS showed that ethyl the acetate fraction did not contain any of the target compound, however the first two fractions had a similar amount target compound so tests were decided to be applied for both fractions simultaneously.

3.2.2.2 Strong anion exchange column

As seen in small scale tests that the SAX column was the best way to extract the compound it was then proceeded to upscale it.

First two 15 ml volume columns were filled with Phenomenex 04G-4414 5µm Sepra SAX polymer for both acetone and methanol biomass fractions. Sample was re-dissolved in methanol and mixed with column cartridge volume of that polymer and the cartridge was loaded into the column.

For solvent pumping Biotage Isolera One was used. At first the column was conditioned by 150 ml of methanol and equilibrated with 150ml of 70% methanol. The first fraction was taken by eluting column with 150 ml of 70% methanol. The second fraction was eluted with 100% methanol, which was divided into two parts, because after first 120ml, which were collected to one vial, a very bright orange solution was coming out of column which, 30ml of it, was decided to collect separately. The third fraction was eluted with 150ml of methanol with an addition of 1% formic acid. The fourth fraction was eluted with 350ml of isopropanol with 1% of formic acid. 100µl of fractions were taken for LCMS analysis.

The same procedure was started for the methanol fraction of the biomass however after the first fraction started running after 10ml of solvent had run column started clogging increasing pressure

above 8 bars causing Isolera to shut off. Salts were suspected to be clogging the column so the flowrate was reduced to 4 ml/min and an additional 300ml of water were run through the column in order to wash away the salts, but as soon as 70% methanol was tried to run the column started clogging again. It appeared that running column separate without the cartridge did not cause the pressure problems so not knowing how much of the compounds from the cartridge eluted into the column already it was decided to run double the volume of solvent, 300ml, for each fraction, half through cartridge and half through column into same respective fractions. 100µl of fractions were taken for LCMS analysis.

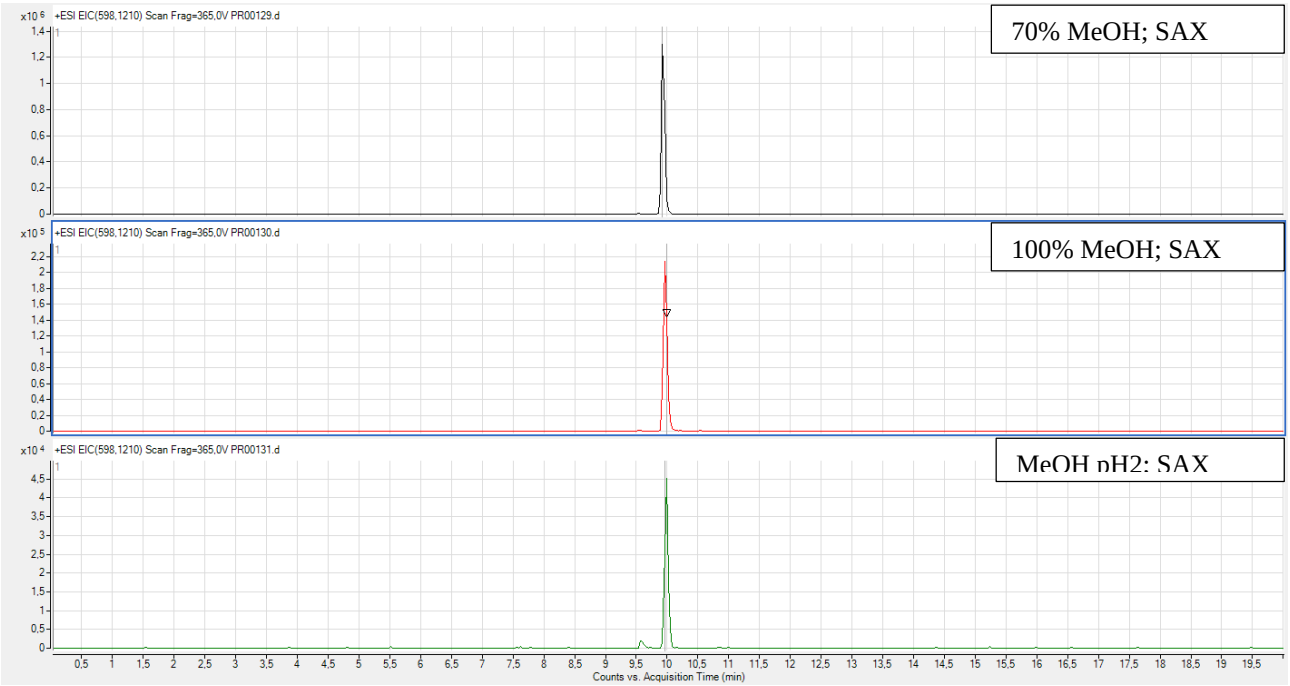


Figure 22. Target compound retention in SAX column fractions in 70%MeOH, 100%MeOH and MeOH pH2 fractions of acetone biomass extract from top to bottom respectively

Samples taken from the fractions were run in LCMS. The chromatograms unfortunately showed almost no retention of our target compound (Fig. 22). Apparently the overwhelming amount of salts in the sample over competed sample compounds for ion exchange spots shutting down ion exchange mechanism thus causing our compound not to fractionate in the same way as it did during the small scale SPE tests.

It was decided to pool fractions together and try running a C18 column in order to separate the salts that were interfering ion exchange. Acetone and methanol fractions were re-dissolved and pooled accordingly.

3.2.2.3 C18 column separation

As in SAX separation columns were once again prepared, however this time, since methanol fraction cleared a little more and had less mass smaller column was chosen. For the acetone fraction a 30 ml volume column was packed with C18 polymer. The sample was dissolved in methanol mixed with cartridge volume of polymer and loaded into the cartridge.

The solvents were pumped using Biotage Isolera One. At first the column was conditioned with methanol then equilibrated with 150ml 50% methanol and 500ml of 5% methanol at 25 ml/min flowrate. Then the cartridge was placed into the column. For fractionation flowrate was set to 10 ml per minute. First a water phase was eluted with 500 ml of 5% methanol and 95% water and then 10 fractions of 300 ml each with gradually increasing methanol concentration by 10% each fraction from 10% to 100% of methanol and fraction 12 was eluted with isopropanol with 1% addition of formic acid. A 90% methanol fraction was split into two vials because there was a clearly visible fractionation of orange colored compound dividing fraction into two parts of 160 and 140 ml. 100µl of each fraction were taken for LCMS analysis.

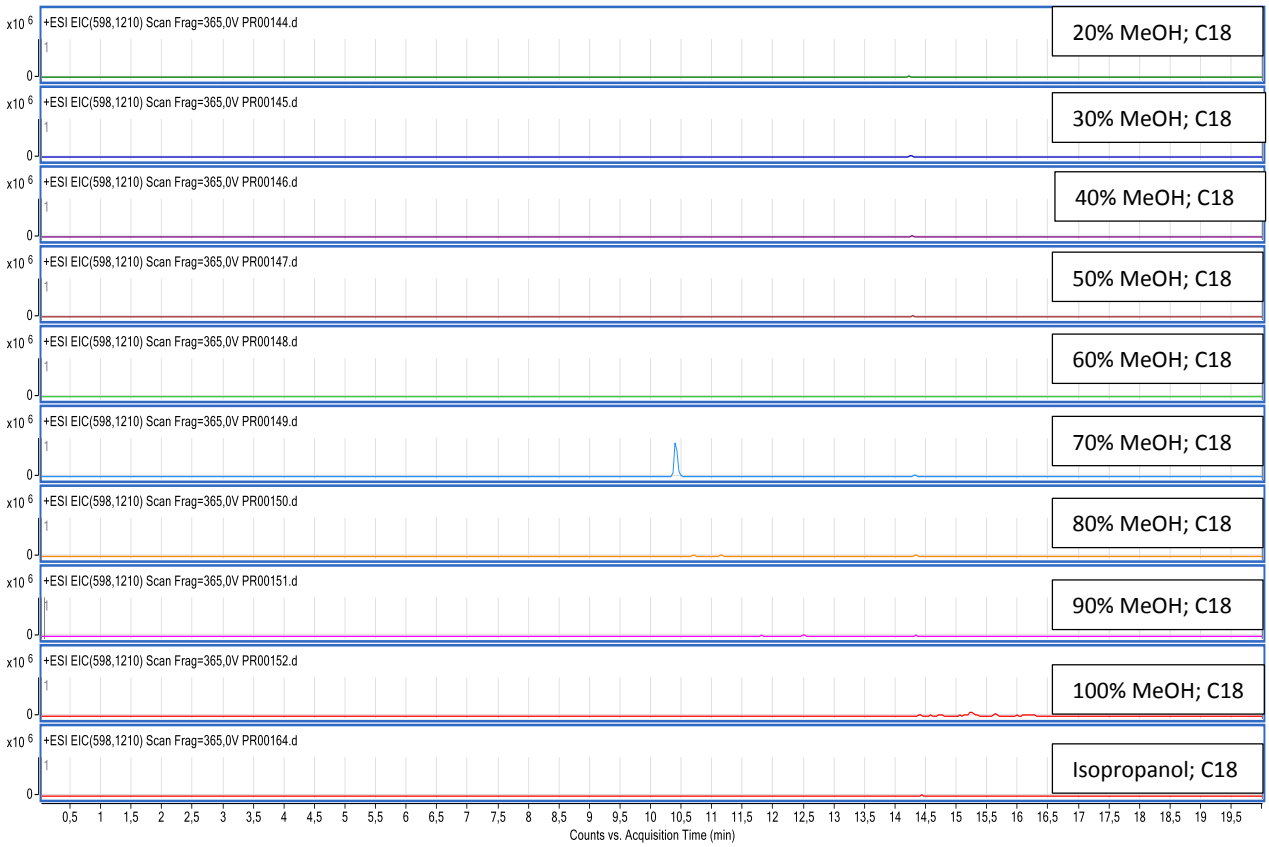


Figure 23. Extracted ion chromatograms of target compound ion fragment in acetone biomass extract C18 fractions from 30% methanol fraction to 100% methanol and an isopropanol fraction.

For the methanol extract 15 ml volume cartridge was chosen which was packed with C18 polymer. Sample was re-dissolved in methanol and mixed with cartridge volume of polymer and loaded into cartridge.

The column was conditioned with 50ml of methanol and equilibrated with 50ml of 50% methanol and 5% methanol and water solution at 25ml/min flowrate. The cartridge was loaded into the column. The flowrate for fractionation was set to 10 ml/min. The first fraction was eluted with 300 ml of 5% methanol and 95% water solution. The next 10 fractions were eluted with 150 ml of water and methanol solutions of gradually increasing methanol concentration by 10% per fraction from 10% to 100%. Fraction 12 was eluted with isopropanol with 1% of formic acid.

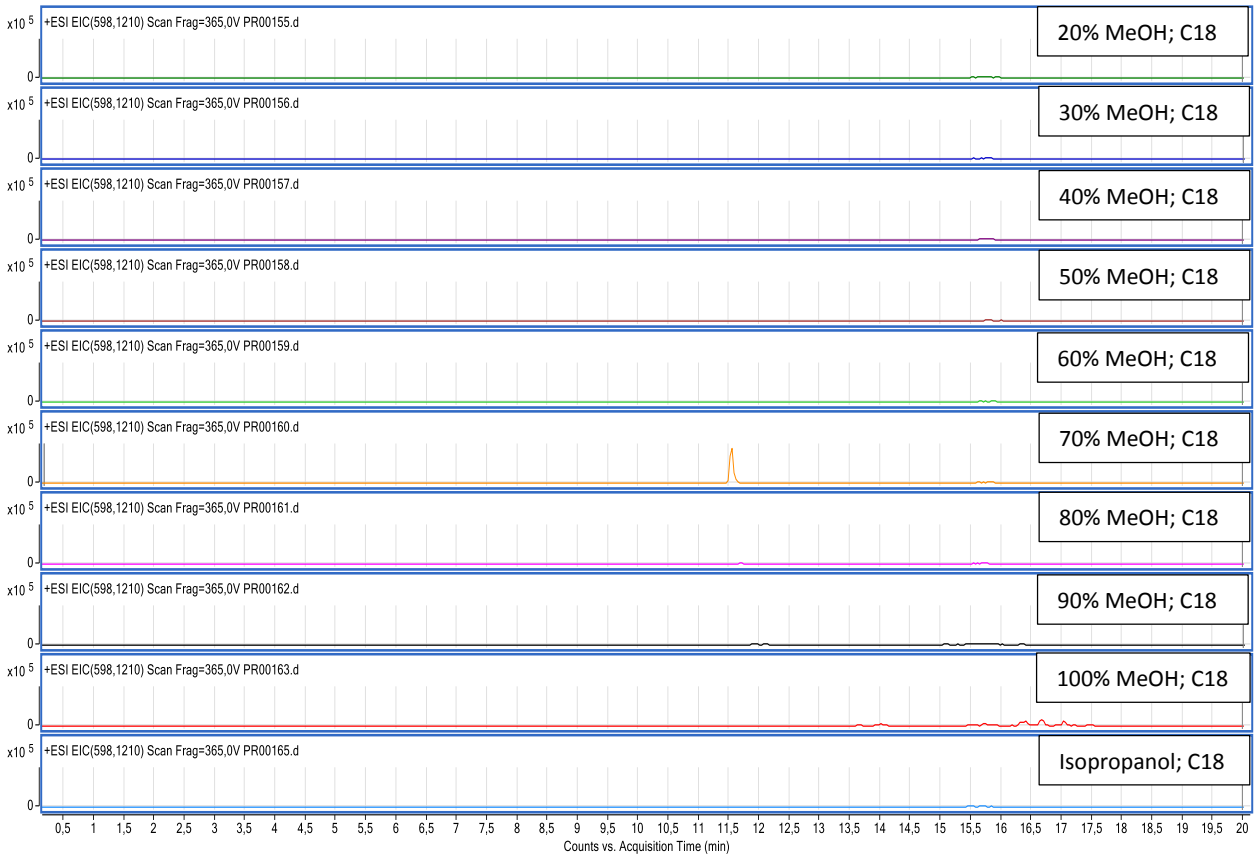


Figure 24. Extracted ions chromatograms of target compound fragment in methanol biomass extract C18 fractions from 30% methanol to 100% methanol and an isopropanol fraction.

Surprisingly the target compound eluted in only 70% methanol fraction unlike the small scale experiment where it was spread in few fractions before (Fig. 23; 24) most of the compound eluting in 80% methanol concentration. The purity of the compound in the fraction was also surprisingly very high, especially in the methanol extract fraction (Fig. 25), with LCMS chromatograms showing only one small extra compound which might also possibly be a novel compound previously unnoticed due

to overlapping compounds in chromatograms. The high purity of the methanol fraction is mainly because many of the organic compounds eluted from the biomass in acetone fraction so unfortunately acetone extraction fraction was not so pure, but the amount of the target compound is relatively similar.

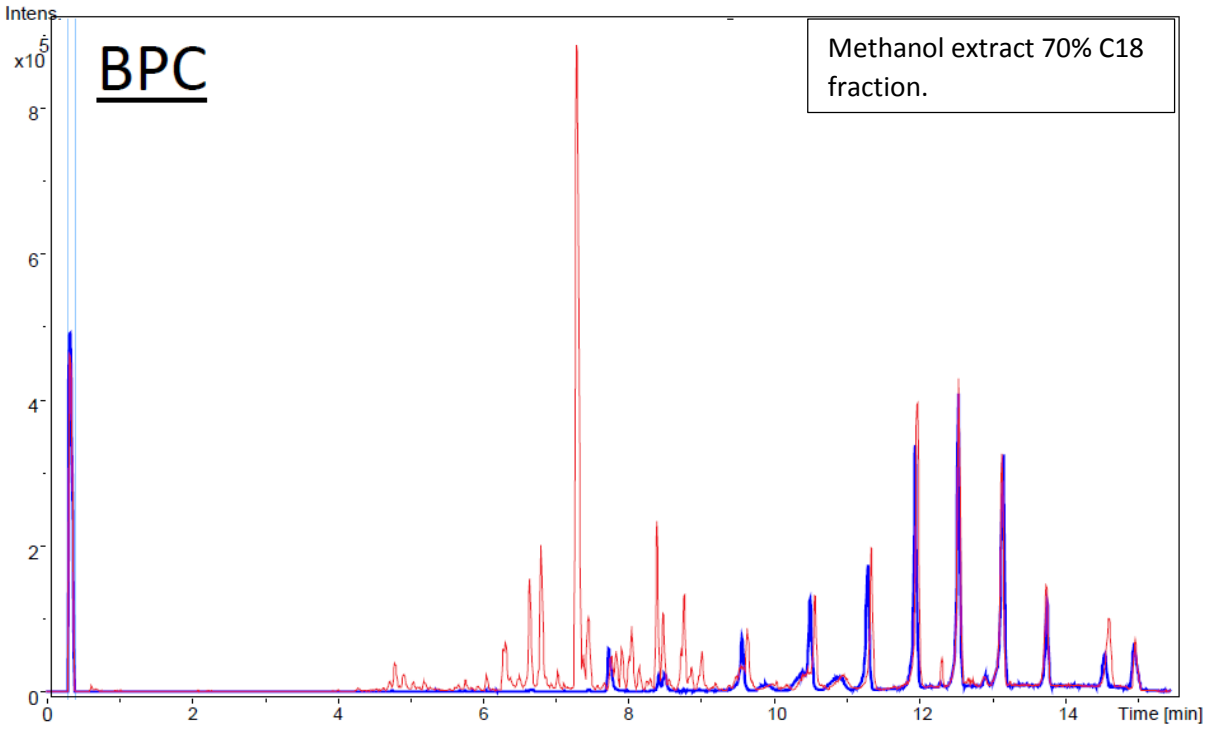


Figure 25. Methanol biomass extract C18 70% methanol fraction. Blue lines show the blank sample.

3.2.3 Compound purification optimization

The next step after the C18 separation which gave the fraction with the target compound was the compound's purification. In order not to lose some of the compound in the HPLC column and to elute with as large gap from other compounds in the fraction it was necessary to do final optimization. At first the previous tests data was scanned for the target compound UV spectrum. Fortunately the target compound has a clearly visible UV which made purification a lot easier (Fig. 26).

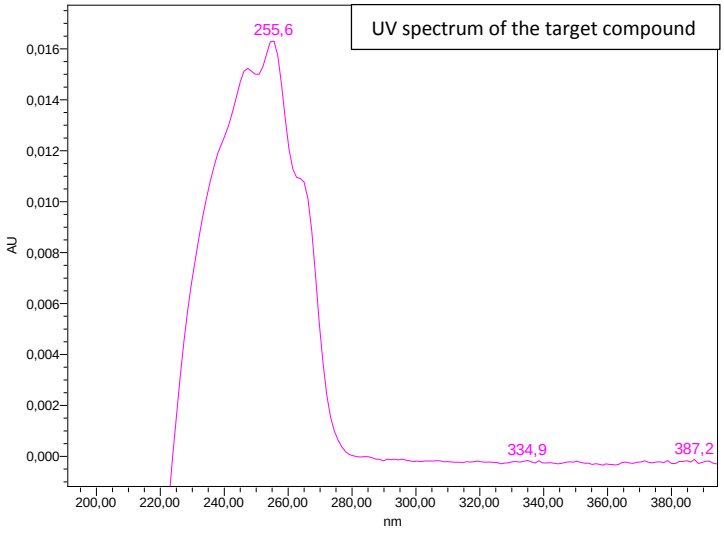


Figure 26. UV spectrum of the target compound.

After the UV was obtained several different elution gradient tests were run on Waters semi-prep HPLC using Luna C18 5micra column to see which acetonitrile-water solution gradient and elution times provide the best separation of the compounds in the HPLC running 70% C18 fraction with the target compound (Fig. 27). 10 different gradient runs were performed with 200 µl injections of 200 µg/ml concentration sample solutions in methanol.

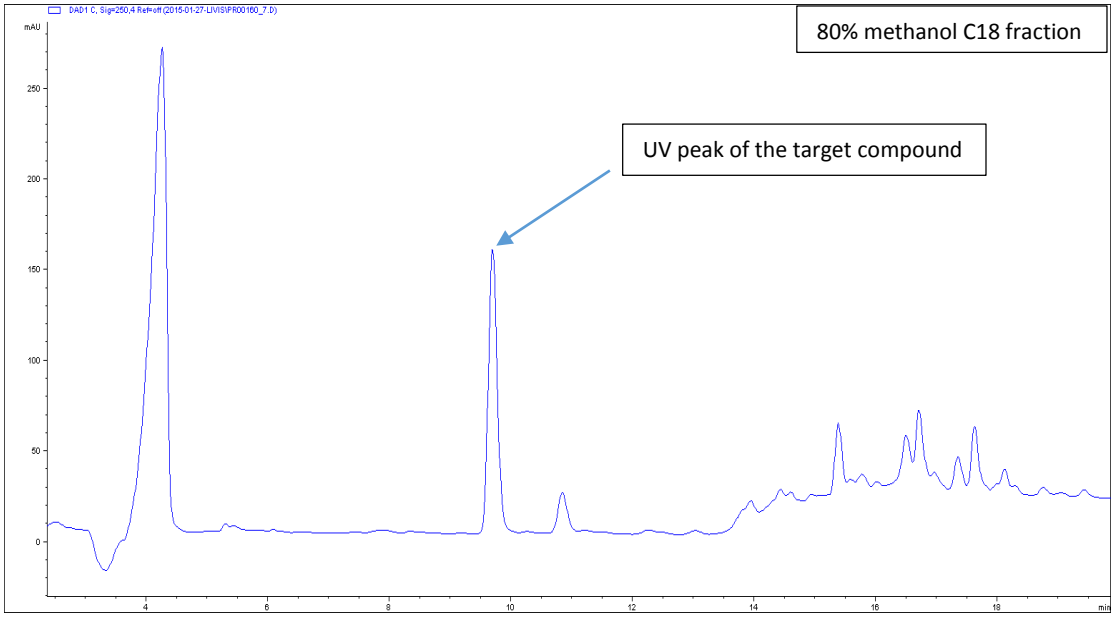


Figure 27. UV spectrum of an acetone biomass extract C18 column 70% methanol fraction using the solvent gradient, which provided best separation of the target compound.

The best compound separation in the UV spectrum was achieved by using this gradient:

Table 6.

Gradient providing best target compound separation within C18 70% methanol fraction HPLC run.

Time, min	Acetonitrile concentration, %	Flow
0	40	0.5
2	50	0.5
10	55	0.5
12	100	0.5
15	100	0.5

This gradient protocol was decided to be used during the final purification of the target compound. Target fraction should be dissolved 450 µl of methanol and injected 3 times 150 µl each into the separation HPLC using 200 µl loop.

3.2.4 Toxicity tests

To see if our target compound or any other compound extracted from the *C. polylepis* was toxic, biomass extract C18 fractions were analyzed with gill assay. 15µg of each fraction were taken for the test. Water fractions (5% methanol/95% water) were not tested for toxicity.

Fraction samples were dissolved in 1.6ml of L-15-minus-FBS media solution with 5% of DMSO. The starting sample concentration in the solvent was 100µg/ml. 96 well plates were used for the test. Plates were planted with gill cells and incubated with media for 48 hours. Before adding the samples incubation media was removed from the plates. Plates were then filled with samples by adding blank and control wells and 10 different concentrations of a sample triplicates. The first triplicate and a control were added with 200µl of sample and media solution. Then 800µl of media were added to the sample diluting it by half. This way plates were filled with 10 different concentration sample solutions from 100µl/ml down to 0.2µl/ml. Plates with sample were then incubated in thermostat for three hours. After three hours fluorescent agent was added to the plates and placed into incubation for 30 minutes. After that time plates were read using colorimeter to see if there was an increase in fluorescence which means that cells were alive and there was no toxic effect caused by the sample. Cells were also inspected with a microscope to look for any visual changes to the gill cells.

After running methanol biomass extract C18 fraction samples first seven fractions (10% methanol fraction – 70% methanol fraction) showed no toxic effect on the gill cells at all. 80% methanol C18 fraction however was very toxic showing lethal dose of 55mg/ml. Watching through a microscope cells were shrunk to a small round shape (Fig. 28). 90% and 100% methanol fractions were even more toxic to the gill cells having LD50 – 25mg/ml and LD50 – 8 mg/ml respectively (Fig. 29). Cells affected with the samples of those two fractions were completely lysed.

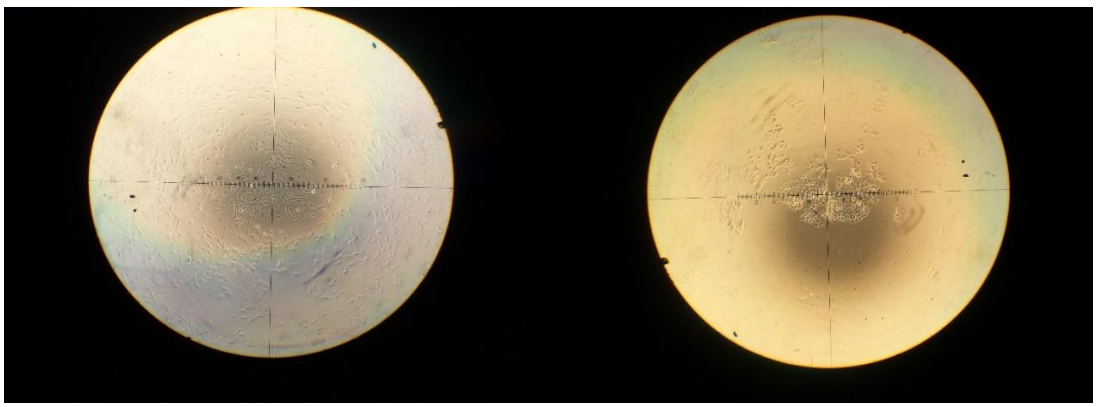


Figure 28. In the left picture it is shown monolayer cell culture in plate wells. Right picture shows dead cells, affected by toxins. If toxins are very strong and lyses the cells they are not visible at all.

Acetone biomass extract C18 fraction samples were even more toxic then the methanol extract fractions. Like with methanol extract C18 samples first seven fractions up to 70% methanol C18 fraction showed no toxic effect on the gill cells. 80% methanol C18 fraction again showed very toxic effects on the gill cells having LD50 – 20mg/ml. Cells were all dead and shrunk and round shaped. First part of 90% methanol C18 fraction again showed even higher toxicity which again resulted in cells being lysed and showing LD50 at only 1,5 mg/ml concentration of the sample (Fig. 30). However second part of 90% C18 fraction showed no effect on the cells at all meaning that the orange colored compound that was separated in the first part of the fraction was most likely the toxic one. Unlike in methanol extract fraction samples, however, 100% methanol C18 fraction showed no toxicity either. Isopropanol fraction was not toxic as well.

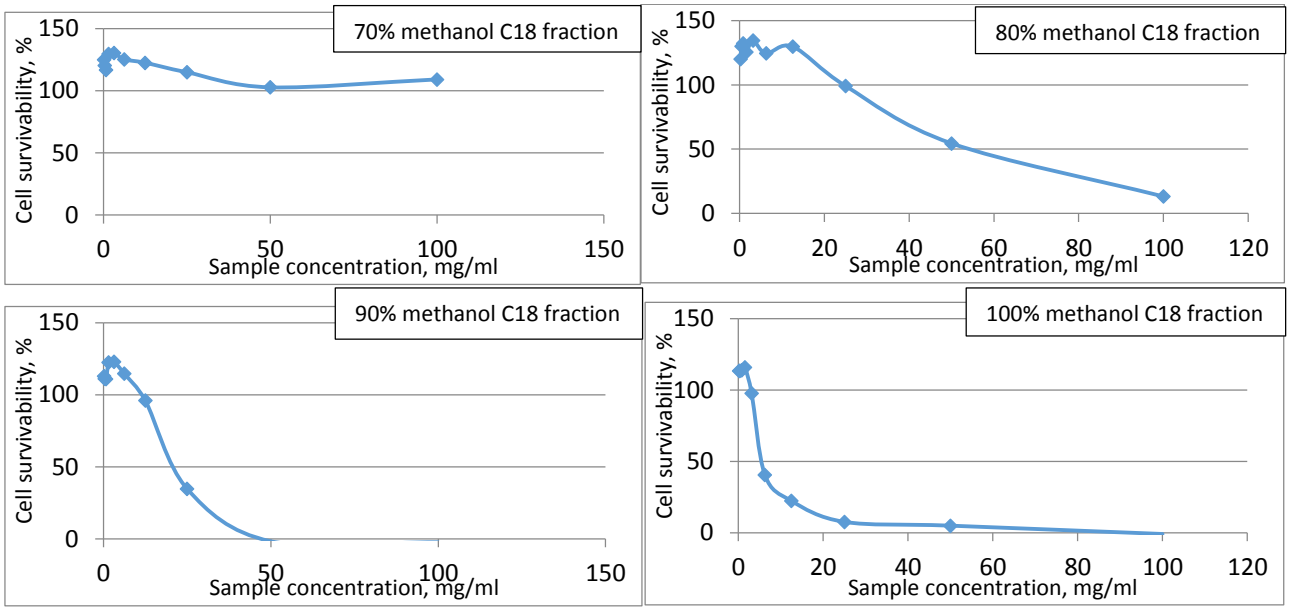


Figure 29. Cell culture survivability when exposed to fractions from C18 column of methanol biomass extract. 70% fraction with the target compound showed no bioactivity towards the gill cells, however fractions of 80% to 100% show increasing toxicity.

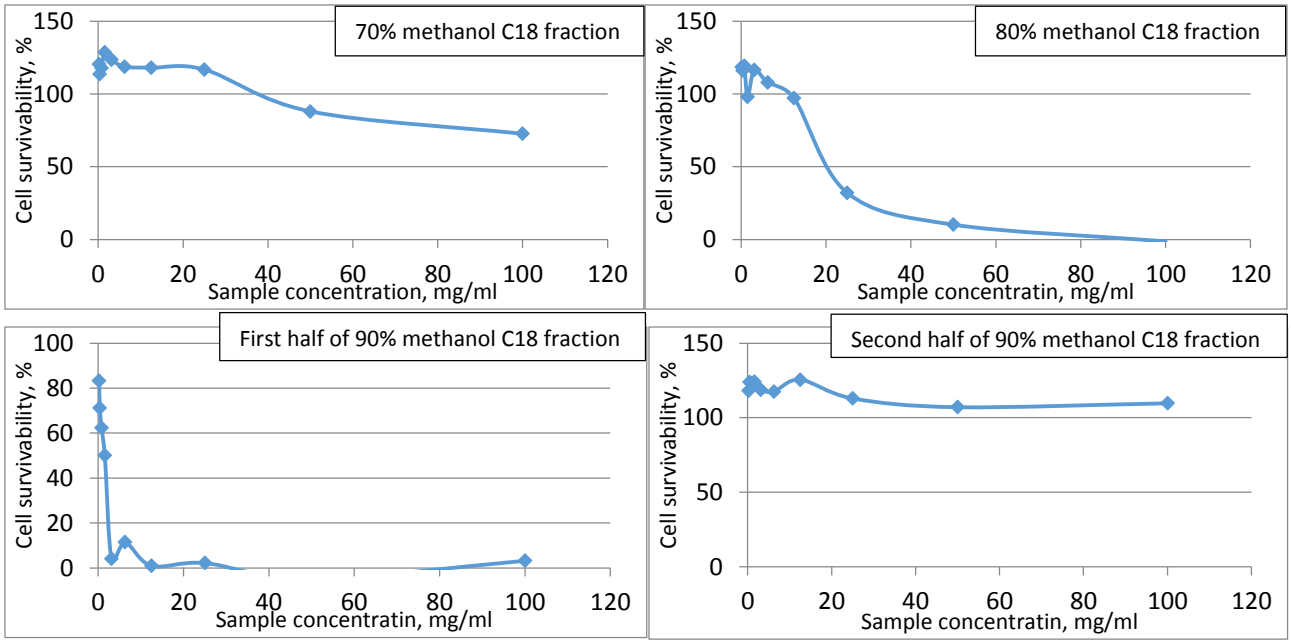


Figure 30. Cell culture survivability when exposed to fractions from C18 column of acetone biomass extract. 70% fraction showed a small bioactivity at highest concentrations. 80% fraction was toxic and first half of 90% fraction was even more toxic, however second half of 90% fraction and a 100% fraction showed no toxicity.

Strangely 20% fraction form acetone biomass extract C18 column showed toxicity with an unusual cell survivability pattern (Fig. 31). However it is not very likely that this fraction is toxic and it might have possibly been a methanol leftover in the sample that bio-actively affected gill cells.

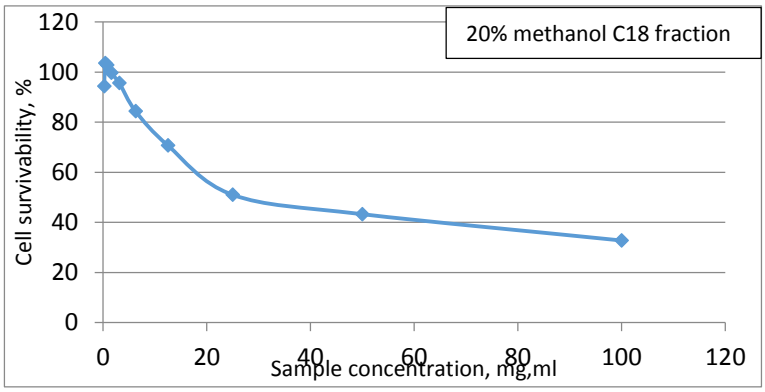


Figure 31. Gill cell survivability when affected with 20% methanol C18 fraction of acetone biomass extract.

Unfortunately our target compound was proven to be non-toxic. However upon inspecting chromatograms of fractions that showed toxic activity and comparing those to those that were non-

toxic few compounds were excluded that might have been those, which were most likely to be the ones that produced toxicity towards the gill cells. From the comparison of the 70% methanol fraction to the 80% methanol fraction a compound m/z 487 mostly stands out in the chromatograms both in acetone biomass extract and methanol extracts. Same pattern is visible in both methanol and acetone extracts and in both cases 70% methanol C18 fraction was non-toxic and 80% was highly toxic (Fig. 32).

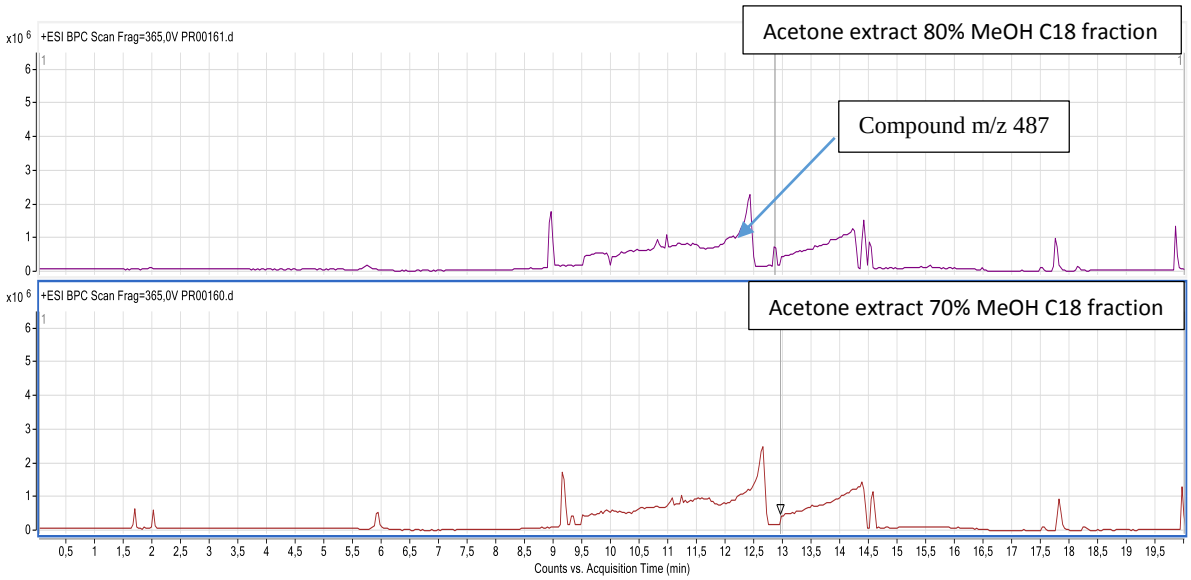


Figure 32. Comparison between Acetone biomass extract C18 70% and 80% methanol fractions. 70% fraction was not toxic and 80% fraction showed high toxicity.

Another compound that caught the eye was compound m/z 568. It was detected in methanol extract 100% methanol C18 fraction which was toxic, but it was not present in its acetone fraction counterpart which might lead to a presumption that it might be toxic compound in that fraction (Fig. 33).

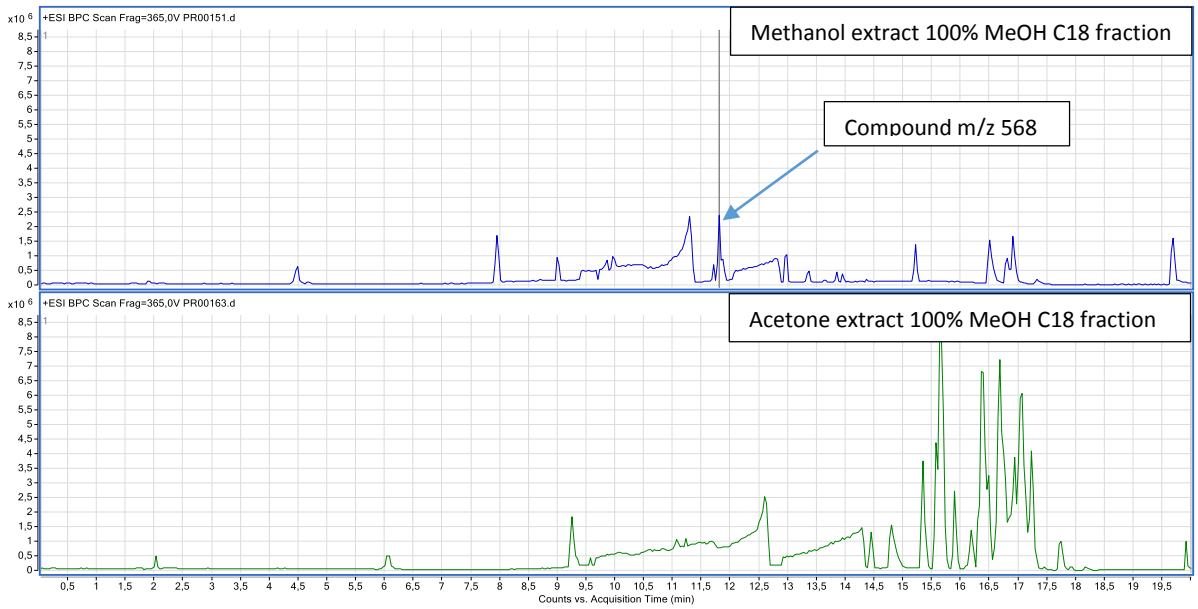


Figure 33. Comparison between 100% methanol C18 fractions. Methanol extract is toxic and acetone extract is not in these fractions.

CONCLUSION

In conclusion this project has provided some information regarding the adaptability of *Echinops sphaerocephalus* oil for food purposes. Analysis showed:

- The total phenolic content of the extracts were 27.64 mg/g for hexane extract, 28.03 mg/g for acetone extract and 158.69 mg/g for methanol TPC for extraction residues were 95.03 mg/g for hexane extraction residue, post hexane and acetone extraction residue – 84.60 mg/g and post hexane, acetone and methanol extraction residue – 44.58 mg/g. Results show that there are still a lot of phenolic content remaining. Most of the phenolic content is extracted with methanol which means that majority of phenols are polar.
- The DPPH radical scavenging capacity of extracts were 25.76 mg/ml EC_{50} for hexane extract, 0.54 mg/ml EC_{50} for acetone extract and 1.3 mg/ml EC_{50} for methanol extract. The $ABTS^{+}$ radical scavenging capacity of extracts were 18 mg/ml EC_{50} for hexane extract, 0.54 mg/ml EC_{50} for acetone extract and 0.17 mg/ml EC_{50} for methanol extract. Results from these both tests correlate and show that oil fraction possesses much weaker radical scavenging capacity then the rest two fractions. More polar phenols extracted with acetone and methanol have much stronger antioxidant power.
- The $ABTS^{+}$ Quencher trolox equivalent antioxidant capacity was 466.46 μmol/g TE for hexane extraction residue, 160.46 μmol/g TE for second stage extraction residue and 37.8 μmol/g TE for third stage extraction residue. The ORAC trolox equivalent antioxidant capacity for all three stages of extraction residues were 318.83 μmol/g TE for first, 240.42 μmol/g TE for second and 167.79 μmol/g for third. These results show that there is still some antioxidant activity left in the residue even after all three extractions took place. Also that there are many polar antioxidants, that are not extracted with oil.
- Supercritical fluid extraction optimization for *E. sphaerocephalus* has been performed and parameters to achieve highest yield were modeled – 117 minutes, 56^{0}C temperature and 42 MPa pressure at a constant 2SL/min CO_2 flowrate. Solutions to optimize other parameters were also modeled.

  When compared to sunflower seed oil and rapeseed oil antioxidant activity is quite similar making it a good candidate for food oil production. Fatty acid profiling showed that *E. sphaerocephalus* is mostly composed of unsaturated fatty acids and mostly of high valued linoleic acid. Extraction residue may also be used for further processing as there are still

antioxidants remaining and further tests could be done to investigate composition of other compounds. Based on these results it can be said that there might be good perspectives to use *E. sphaerocephalus* as a food oil source.

In conclusion of the second part of the project it has given us new insights into the chemistry and bioactivity of natural products produced by *C. polylepis*. Analysis showed:

- The best small scale method to capture target compound was SAX SPE column however when upscaled to a large scale extraction it was inefficient due to a large amount of salt content from a biomass. Because of this reason C18 column was chosen which was almost as good as SAX column in small scale tests.
- The gill assay showed no toxicity in a fraction that contained target compound which means that it was not toxic.

Also a UV spectrum of the target compound was extracted and the best HPLC purification protocol was designed to use when purifying the target compound.

Bio-guided analysis using a gill assay showed that the compound X did not show any toxicity. However the tests revealed that there are indeed compounds highly toxic for the marine environment produced by *C. polylepis*. Results also provided preliminary range in which those compounds elute. After chromatogram comparison of toxic and non-toxic fractions two preliminary suspects for toxic activity were marked – compound m/z 487 and compound m/z 568. These compounds might as well be non-toxic or there might be more toxic compounds that are not so easy to spot in base peak chromatograms due to many other compounds present in the fractions or their abundance might be pretty low for them to clearly be seen in chromatograms. To confirm that those two compounds are toxic or not and whether there are more toxic compound further fractionation and bio-guided gill assay analysis should be carried out.

PADĖKA/ACKNOWLEDGEMENTS

Pagarbiai dėkoju mokslinio darbo vadovui profesoriui dr. Petrui Rimantui Venskutoniui už suteiktą informaciją bei pagalbą tiriamojo darbo metu ir jo metu gautų rezultatų aptarimą. Taip pat dėkoju už sukurtą galimybę išvykti stažuotei į Danijos Technikos Universitetą. Taip pat norėčiau skirti padėką ir likusiam Kauno Technologijos Universiteto akademiniam kolektyvui už suteiktas žinias bei palaikymą.

****

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APPENDIX

Table 7.

Analysis of variance table for response surface quadratic model.

Source	Sum of squares	df	Mean square	*F*-Value	*p*-value
Model	678.41	9	75.38	119.14	< 0.0001significant
A-Pressure, MPa	380.13	1	380.13	600.80	< 0.0001
B-Temperature, oC	1.05	1	1.05	1.66	0.2263
C-Time, min	4.36	1	4.36	6.89	0.0254
AB	1.62	1	1.62	2.56	0.1409
AC	3.59	1	3.59	5.67	0.0385
BC	2.84	1	2.84	4.48	0.0603
A^2	148.02	1	148.02	233.95	< 0.0001
B^2	6.689E-003	1	6.689E-003	0.011	0.9201
C^2	0.42	1	0.42	0.66	0.4343
Residual	6.33	10	0.63		
Lack of Fit	3.84	5	0.77	1.55	0.3217 not significant
Pure Error	2.48	5	0.50		
Corrected Total	684.73	19			