MASTER THESIS IN ANALYTICAL ORGANIC CHEMISTRY

Natural Product Chemistry

Carotenoid content in algal epiphytes: A qualitative and quantitative DAD-HPLC analytical study

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Table of content

Acknowledgments

I want to thank my supervisor Associate Professor Monica Jordheim for the guidance and support throughout this project and for giving me the opportunity to get a project and take part in the research group. I want to thank Ph.D. student Angeliki Barouti for the assistance and for giving me a helping-hand when I struggled with the project. I want to thank all the members of the research group for all the help, support, and laughs, making my time there easier and more enjoyable. I want to thank my family for supporting me all these years. Lastly, I want to thank all my real life and online friends for giving me the strength to continue.

Abstract

The research presented in this thesis involves the qualitative and quantitative analysis of carotenoids found in algal epiphytes on the surface of *Laminaria hyperborea*. In addition, a stability study was conducted by quantifying carotenoid amounts over time using different storage conditions for several sample preparation methods. Both qualitative and quantitative analyses were performed using DAD-HPLC and reversed-phase column.

With an increasing world population and focus on sustainability in the food industry, the interest in utilizing algae as a resource has increased in the West over the past decades. With algae processing comes a lot of waste that is not used and extracted for potentially valuable resources. One type of waste is algal epiphytes growing on brown seaweeds called *Laminaria hyperborea* found off the coast of Norway. This biomass can potentially contain many beneficial compounds which have yet to be explored. One such group is the natural products called carotenoids, pigments aiding algae in photosynthesis and working as antioxidants. This group of natural products might be part of untapped resources that are not exploited.

This project aims to give insight into which carotenoids are present in the algal epiphytes, further quantify the number of carotenoids in samples of different sample preparation methods and lastly, perform a 3-week stability study to see how carotenoids of varying sample preparation methods are affected other storage conditions like temperature and light. Hopefully, this will be useful to determine if epiphytic algae from *L. hyperborea* is of commercial interest to process.

In the qualitative analysis, air-dried (AD), freeze-dried (FD), and powdered freeze-dried (Pow) extracts of epiphytic algae were analyzed using DAD-HPLC to characterize carotenoid compounds. Using the four analytical carotenoid standards, fucoxanthin, lutein, zeaxanthin, and *β*-carotene, the pigments fucoxanthin, lutein, and *β*-carotene were characterized in the extracts using retention times and UV-Visible spectra. Four other peaks were detected as carotenoid-like and tentative identifications were discussed based on retention time, fine UV-vis structure, and λ_{max} values. The carotenoid-like compound named u2 was suggested to be an isomer of fucoxanthin due to matching data with other studies. The three other carotenoid-like unknown compounds were given multiple characterization possibilities.

In the quantitative analysis, the carotenoid contents of samples of the four sample preparation methods air-drying (AD), freeze-drying (FD), N2-freezing (N_2), and the last method – with the thawing of frozen fresh materials were quantified using calibration curves of the standards fucoxanthin, lutein, zeaxanthin, and *β*-carotene. Freeze-drying in sample preparation resulted in the best extraction of carotenoids, and N2-freezing gave the most negligible yield. It was found that fresh biomass retains lutein well, but wet and dry samples are difficult to compare due to the water content difference in the samples. Despite the epiphyte biomass mainly containing red algae, fucoxanthin, a pigment associated with brown algae, was found in the highest quantities. Zeaxanthin, a pigment found in red algae, was not found in the extracts. As *Laminaria hyperborea* was found to contain high fucoxanthin content, there is reason to believe that the fucoxanthin content is coming from *L. hyperborea* parts as a result of the harvest methods used – peeling off epiphytes biomass from the stipe of brown kelp. Only the freeze-drying method managed to extract a quantifiable amount of *β*-carotene. Freeze-drying is, therefore, the preferred method for carotenoid quantification. The amount of unknown carotenoid-like u2 compound was found to be positively correlated with the amounts of fucoxanthin in the samples. This correlation increased confidence that u2 is an isomer of fucoxanthin.

A three-week stability study (T_0-T_3) of fucoxanthin, lutein, and *β*-carotene in extracts of freeze-dried, air-dried, N2-frozen, and thawed biomass showed little difference being stored in a freezer at -20℃, refrigerator at 4℃, and on a bench with no light exposure at room temperature. Exposing samples to sunlight/daylight and constant lamp light on a bench at room temperature had a significant effect, leading to noticeable degradation of all three carotenoids, with lutein and *β*-carotene falling below the limit of quantification after one week and three days, respectively. Statistical analysis found that freeze-dried materials tended to be more affected by different storage conditions but had no significant differing mean values compared to being stored in a freezer or a refrigerator. For fucoxanthin, the air-dried samples had significant differences in stability when being stored in a refrigerator compared to in a freezer. For lutein degradation in N_2-frozen samples, only a freezer compared to a bench with no light exposure had no significant difference.

As epiphytic algae biomass on the surface of Laminaria hyperborea is found to have carotenoid amounts comparable to other species of algae. Therefore, this biomass can be of commercial interest and should be studied further to determine if it’s a resource worth utilizing.

Chapter 1. Introduction

1.1 Motivation and purpose

As society moves towards a more sustainable future, the need for resources with less environmental impact is continually growing. In recent decades, green and carbon-neutral energy systems have evolved exponentially to combat the increased emissions worldwide. The food industry has also affected this evolution, as it is another significant contributor to greenhouse emissions and is facing a problem relating to a growing world population [1]. One part of the solution to sustainable food production is the cultivation of algae [2].

Kelp is a large brown macroalga that grows in shallow oceans, forming extensive forests. Until recently, kelp outside the coast of Norway has not been cultivated and instead harvested using trawlers [2]. The commercial utilization of kelp has also been limited, with the current commercial utilization being the processing of *Laminaria hyperborea*, a type of macroalgae, into alginate, a binding agent used in feed [2, 3]. As stated in a report published by the Institute of Marine Research, "For kelp cultivation to be profitable in Norway, new products and applications must be developed" [2]. It is, therefore, paramount that research is being conducted on species of kelp for the industry to succeed and expand.

One area in underutilized kelp processing is the discarded epiphyte biomass after harvesting [4, 5]. Epiphytes are typical species of algae, bryozoa, mollusks, crustaceans, bacteria, and more that reside on the kelp's thallus (the stipe) [6, 7]. Epiphytes pose many problems for cultivators, hampering growth and damaging the produce [4, 8]. To remove epiphytes after harvesting, chemical treatment and mechanical brushing processes target the problematic species [4, 5]. Epiphytes are therefore considered waste. To maintain sustainability in industry, looking at ways to utilize the waste produced to aim for zero-waste processes is essential. Epiphytic algae should therefore be studied to assess its potential as a resource.

This thesis aims to provide information about carotenoid contents in algal epiphytes, focusing on quantification and characterization based on purchased standards. After applying a previously established extraction method, different sample preparation and storage methods are also explored to map the stability of carotenoids under different environments. This will indicate how samples should be treated to reduce the risk of degradation. All analysis was performed with DAD-HPLC.

1.2 Seaweeds

Seaweeds are ocean-based, multicellular organisms characterized as algae species on a macroscopic level [9]. As the ocean covers 71% of the Earth's surface, algae have space to grow in large quantities worldwide, resulting in many species, with 170,581 recorded as of 2023, accounting for 80 % of the world's oxygen production [10-12]. Macroalgae (macroscopic algae) grow on shallow ocean floors on coasts worldwide, creating extensive forests for marine fauna to seek refuge in. Seaweeds are organized into three different groups: Rhodophyta (red algae), Phaeophyceae (brown algae), and Chlorophyta (green algae) [12]. How a species of seaweed is sorted into a group is based on the coloring of the thallus (stipe) and the pigment content [12]. As algae, seaweeds perform photosynthesis to survive and rely on sunlight and carbon dioxide access to grow [13].

As a resource, seaweeds are primarily used for human consumption in Eastern Asia, which makes up 99% of the global algae industry [4]. In addition to being a food product, seaweeds are also processed into alginates used as thickening and gelling agents in food and feed [2]. Most of the algae industry in Norway involves alginate production by processing wild brown macroalgae called *Laminaria hyperborea* [2]. As previously mentioned, the processing of *L. hyperborea* produces a lot of waste that is not utilized today. The waste, consisting of epiphytes, is the subject of this project's research.

It is well established that algae contain various macro and micronutrients. Some examples are polysaccharides, minerals, proteins, and polyphenols [9, 14]. Algae can therefore be an alternative source of these nutrients, making them interesting for the nutraceutical industry. Pigments are also categorized as micronutrients found in all algae types [14]. Among the pigments are nature's most widespread pigments called carotenoids. These molecules act as antioxidants and light harvesters for plants and algae, offering many health-promoting effects to the human body [15].

1.3 Epiphytes

Epiphytes are, in biology, defined as plants that grow on the surface of other plants in a non-symbiotic way and without acting as a parasite toward the host [16]. Common examples of these are moss species and orchids growing on trees in rainforests. As these epiphytes do not rely on the host for sustenance, like other plants, epiphytes rely on water, carbon dioxide, sunlight, and minerals to survive [16].

As well as terrestrial epiphytes, marine epiphytes also exist. These oceanic epiphytes live on the surfaces of macroalgae and can be grouped into two, namely epiphytic algae and epifauna [4]. The epiphytic algae are either micro or macroalgae and function like other algae do to survive. These functions include photosynthesis and gathering CO_2 and minerals from surrounding seawater. Epifauna can consist of bryozoa, mollusks, crustaceans, bacteria, and isopods [4]. These reside on the kelp surface in the biomes created by the algal epiphytes and can vary depending on factors like sunlight, epiphyte density, predators, and temperature [4]. These will not be interesting in the project but will be part of the biomass analyzed, as removing all epifauna from the algae is difficult.

1.3.1 Epiphytes on *Laminaria hyperborea*

The algal epiphytes studied in this project are found on the stipe of *Laminaria hyperborea*, brown kelp harvested off the coast of Haugesund, Rogaland (table 1). A quantitative study on the epiphytes of *Laminaria hyperborea* was done on kelp harvested off the coast of Skipsholmen island, close to Finnøy, where 50 species were identified and categorized into red, brown and green algae[17]. There, it was shown that the epiphyte content on the stipe consisted mostly of red algae species, followed by green and brown species.

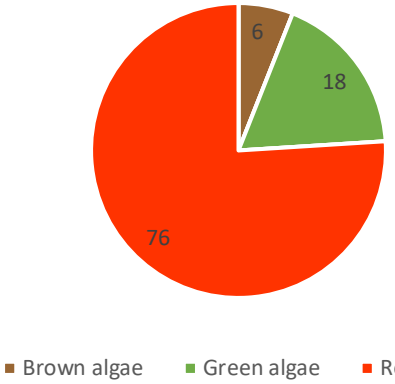
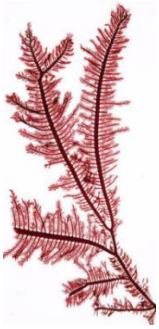


Figure 1: Sector diagram displaying the distribution (in percent) of epiphytic algae species grouped into three groups based on the color of the stipes. The epiphytes were collected from L. hyperborea on the coast of Skipsholmen Island [17].

On the epiphyte biomass used in this project, a qualitative study was done by Ph.D. Angiliki Barouti in the Jordheim research group to identify the different species found on the *L. hyperborea* stipe. Algae specialist Prof. Inga Kjersti Sjøtun supervised this work at the Department of Biological Sciences (UiB). Seven algal epiphytes, five Bryozoa, one sponge, and two Hydrozoa were identified. Seven identified epiphytes were red algae, and one was brown (table 1) [18, 19]. In conjunction with the study done at Skipsholmen, it appears that red algae dominate the epiphytic flora on *L. hyperborea.* Identified red algae were: *Ptilota gunneri* (I), *Palmaria palmata* (II), *Membranoptera alata* (III), *Phycodrys rubens* (IV), *Rhodomela lycopodiodes* (V), and *Polysiphonia stricta* (IV). The brown alga was identified as *Desmarestia aculeata* (VII) (table 1).

Table 1: Identified species of algal epiphytes on the surface of *Laminaria hyperborea*. Identification was performed by Ph.D. student Angeliki Barouti in the Jordheim research group. Species I-VI are red algae, and species VII is a brown alga.

I: *Ptilota gunneri*	II: *Palmaria palmata*	III: *Membranoptera alata*
		
IV: *Phycodrys rubens*	V: *Rhodomela lycopodiodes*	VI: *Polysiphonia stricta*
		
VII: *Desmarestia aculeata*		
		

1.4 Carotenoids

Carotenoids comprise a group of natural products mainly found in marine and terrestrial plants and fungi. So far, over 750 different compounds have been identified, making this an extensive group [20, 21]. As pigments, these compounds have characteristic colors and are responsible for many of the vibrant colors one finds in nature [15]. Carotenoids have been shown to exhibit health-promoting qualities because of their antioxidative properties potentially and as a provitamin [22]. It has therefore been of interest in the nutraceutical industry as a dietary supplement to consumers [23]. Therefore, analysis of potential sources of carotenoids can prove to be of commercial interest.

1.4.1 Structure

All compounds of carotenoids contain a characteristic repeating isoprene structure of alternating carbon double bonds. These are formed when two C20 compounds are linked together by geranylgeranyl diphosphate as the isoprenoid precursor to carotenoids in biosynthesis [20]. This results in most carotenoids containing a C40 carbon chain of eight isoprene monomers [24]. This compound is called lycopene and is the parent molecule from which many carotenoids are derived [25]. Most carotenoids have a linear structure of repeating double bonds in trans-configuration, as trans-configuration is often more thermodynamically stable than cis due to steric hindrance (figure 2) [20]. Still, a cis configuration can occur, resulting in many different stereoisomers as substituents like hydrogen do not pose as a hindrance (figure 4).

Carotenoids are categorized into two groups called carotenes and xanthophylls [15]. The difference is that xanthophylls contain oxygen substituents, while carotenes are strictly hydrocarbons[15]. This difference affects the polarity of the two groups, making it possible to separate them based on both polarity and functional groups, allowing for easy separation concerning analysis (figure 2).

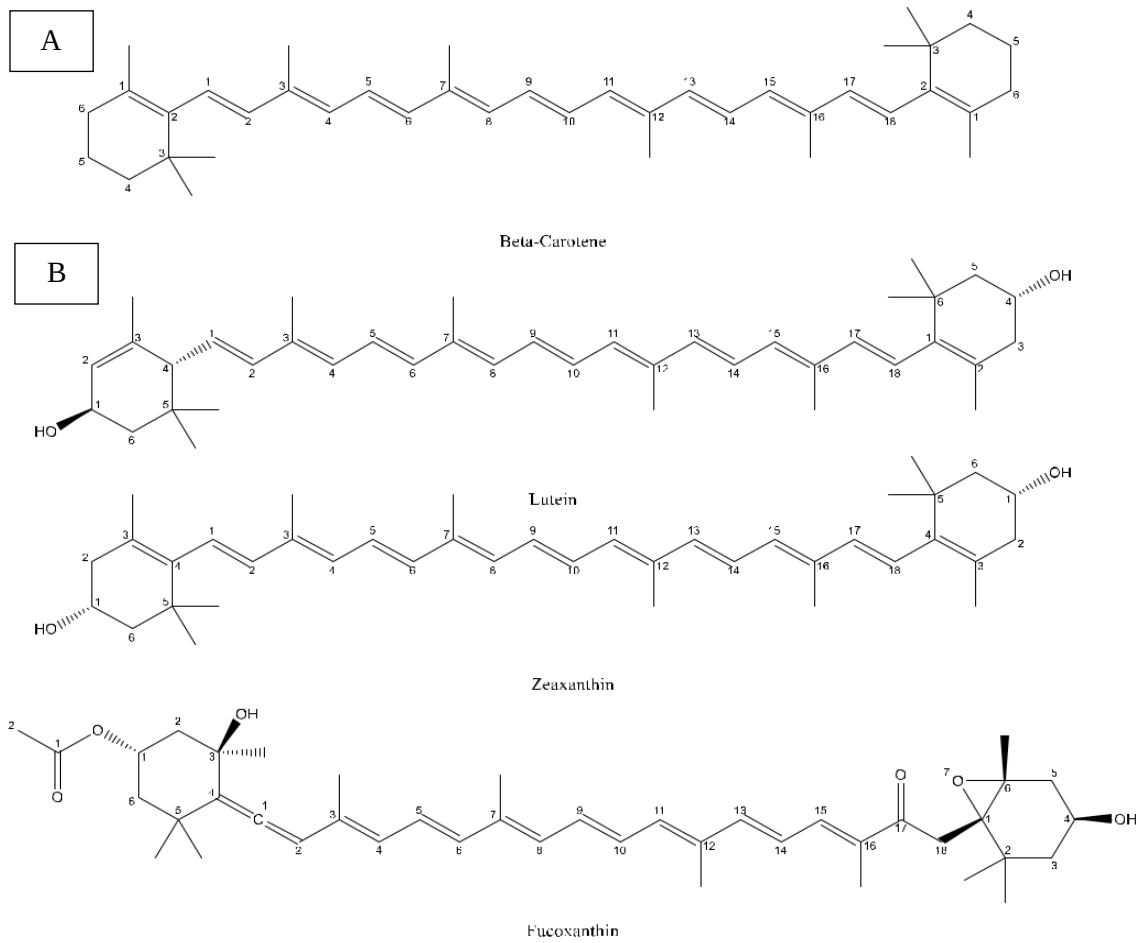


Figure 2: Molecules of carotenoids that were used as analytical standards. A: example of a carotene compound. B: Examples of xanthophyll compounds.

1.4.2 Chemical properties

As pigments, carotenoids display a range of yellow, orange, and red colors (figure 3) [15]. These colors are because carotenoids contain a long chain of double bonds, resulting in conjugated π-bonds. A chain of conjugated π-bonds lowers the energy levels of electrons, making a chromophore in which EMR in the visible spectrum can be absorbed [20]. Therefore, UV-Vis is a preferred detection method, as carotenoids absorb EMR in this wavelength interval [15]. Carotenoids have been shown to possess antioxidative properties of significant interest in carotenoid research [15]. The polyene system of carotenoids makes the compounds susceptible to reactions containing free radicals and oxidation. This happens as unsaturated systems initiate a chain reaction with peroxy-radicals [24]. These radicals can then be quenched by other antioxidative compounds such as vitamin C or E [24]. The potential for carotenoids to prevent reactive oxygen species (ROS) from propagating and causing oxidative stress is why carotenoids have been of interest in the nutraceutical industry [24].

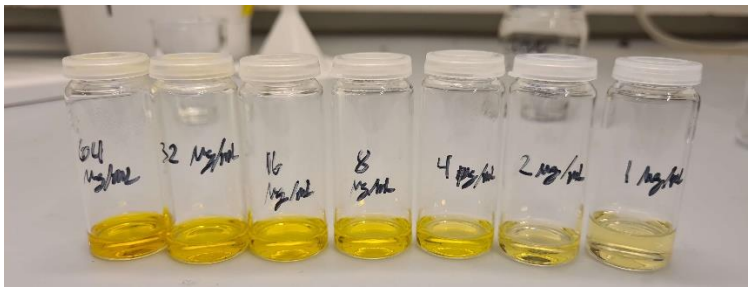


Figure 3: Seven dissolved β-carotene standards at 1 to 64 µg/mL concentrations displaying a reduction in color with lower concentrations.

1.2.3 Carotenoid stability

When studying carotenoids' stability in a sample, it is important to understand what parameters affect the alteration or degradation of compounds. One of these is the presence of oxidizing agents. As stated in 1.2.2, carotenoids are susceptible to oxidization as the electron-rich conjugated system is an easy target for electrophilic reagents. Therefore, having oxidizing species present will cause the pigments to react and form short-lived radical compounds [26]. Oxidation reaction with an oxidizing radical:

$$CAR + R_*^+ \rightarrow CAR_*^+ + R \text{ [26] (1.1)}$$

This degradation can result in the formation of apocarotenals and epoxy carotenoids, compounds with lesser favored bioactivity [20]. Added heat and light will speed up oxidation reactions, resulting in rapid degradation [22]. The antioxidant BHT (butylated hydroxytoluene) was used in the extraction process and in the samples to account for the possibility of oxidation during the extraction steps.

Aside from oxidized carotenoid degradation, alteration also poses a potential problem when trying to keep a sample stable. As carotenoids contain an isoprene structure with repeating double bonds, the bonds can rotate and form compounds with a different stereoisomerism, namely isomers [15]. Turning trans double bonds to cis will alter the spatial orientation of the molecule, resulting in changes in properties (figure 4) [20]. The antioxidative properties can change, which is the case for β-carotene, where the 9-cis conformation has been shown to have greater antioxidative properties than all-trans [27]. Alternating stereochemistry influences the samples' ability to absorb different wavelengths. This can be observed without analytical instruments as the pigments will change color over time[20]. The color change is

because an all-trans carotenoid will have a coplanar conjugated system that absorbs longer wavelengths and exhibits a higher absorption coefficient [15] [24]. With cis configuration, the fine structure of the UV-Vis spectrum will be shifted hypsochromically, meaning that the wavelength is decreased.

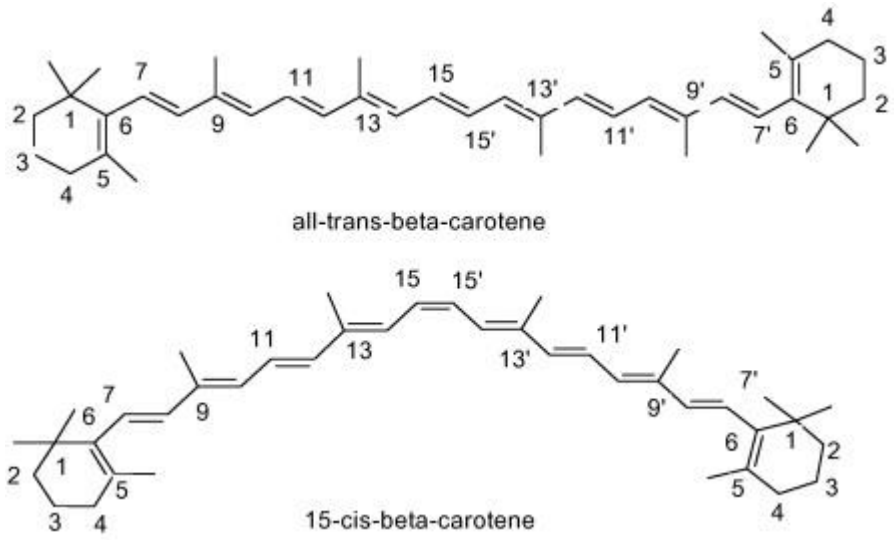


Figure 4: Two examples of stereoisomers of β-carotene found in nature. Above: molecule containing all-trans configuration of double bonds. Below: molecule containing a cis configuration at the center of the double bond chain.

1.2.4 Market value of carotenoids

As a product, carotenoids are widely used in the food- and nutraceutical industries as naturally occurring bioactive pigments and as non-essential dietary supplements for the groups reported antioxidant, anti-inflammatory, and antitumor activities [28]. In 2018, the market value of carotenoids worldwide was 1.5 billion USD, and according to a study conducted by Meticulous Research®, the value is expected to reach 2.26 billion USD in 2030 [29, 30]. Therefore, the market for carotenoids is continually growing, and more are expected to be needed in the future (figure 5). One source of pigments is marine algae, containing bioactive pigments such as chlorophylls, phycobilins, and carotenoids [31]. For pigments in algae, the market value is expected to reach 452.4 million USD in 2025, with a compound annual growth rate of 4% from 2019 [29].

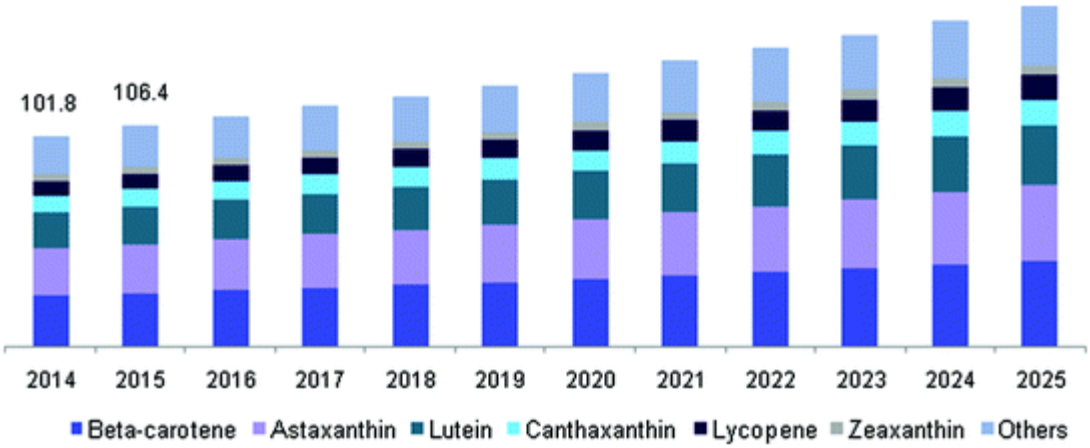


Figure 5: Histogram showcasing the rise in marked demands valued in millions of USD for different carotenoids and the total market size with projections until 2025 [32].

Chapter 2. Materials and methods

2.1 Samples

The epiphytes biomasses in the project were provided by the company Alginor ASA and was harvested in the vicinity of Haugesund. Laminaria *hyperborea* was harvested, and epiphytes were removed from the stem mechanically. The fresh biomass was harvested on the 18th of February 2022, and the powdered biomass was harvested and processed in the spring of 2021.

2.2 Sample preparation

For qualitative and quantitative analysis, the sample preparation methods were freeze-drying (lyophilization), air-drying, and freeze-drying with industrial milling. For the stability study, freeze-drying, air-drying, N2-freezing, and thawing were performed.

2.2.1 Lyophilization

Lyophilization is a technique used to remove water or solvent from a sample. Water removal is done by freezing the water/solvent and placing it in a vacuum chamber [33, 34]. This causes the water/solvent to sublimate from the sample, as the shift in pressure results in a phase transition from solid to gas [33, 34]. Afterward, secondary drying occurs where the temperature is increased under vacuum, resulting in the desorption of water/solvent.

Before lyophilization, epiphyte biomass was weighed in and placed in the freezer until frozen. The freeze-dried biomass was ground using a pestle and mortar, weighed, and placed in a zip lock bag in a freezer at -20°C for storage (figure 6).

Figure 6: Freeze-dried epiphyte biomass. Left: processed by cutting, chopping, and grinding. Right: unprocessed freeze-dried material.

2.2.2 Air drying

Air drying is a method where drying is performed at room temperature, where the water content is evaporated over several days. Frozen epiphyte biomass was thawed and spread out on a plastic sheet left to dry over three days (figure 7). The dried epiphytes were then cut with scissors and chopped in a food processor. The air-dried samples were stored in a sealed beaker at room temperature and removed from light.

Figure 7: Algal epiphytes air-drying on a bench at room temperature.

2.2.3 Thawing

Frozen biomass was placed into a beaker and thawed at room temperature for 30 minutes. The beaker is covered with parafilm and placed in a refrigerator for storage.

2.2.4 Liquid nitrogen-freezing

Liquid nitrogen freezing is when biomass is frozen by submerging it in liquid nitrogen. Algae were submerged in liquid nitrogen and ground with a pestle and mortar (figure 8). The ground biomass was placed in a sealed beaker and the freezer at -20°C for storage.

Figure 8: Equipment used for liquid nitrogen drying algal epiphytes, including liquid nitrogen Dewar flask and processed epiphyte biomass in a beaker.

2.3 Carotenoid extraction

The extraction procedure for carotenoids is taken from a previous master's thesis with the original method designed by a master's student's collaboration with the University of Wageningen, where a method was made to extract carotenoids and chlorophyll [35, 36]. The technique has been slightly altered to cater to the extraction of macroalgae and equipment availability.

Epiphyte biomass (~400 mg) was added to a plastic tube (15 mL). A Sub-zero temperature solution of MeOH and chloroform [5:4] + 0.1% BTH (4.5 mL) was added to the test tube. The plastic test tube was capped and shaken (60 sec) and then placed on ice (10 min). A buffer solution of MeCN and water [1:4] (2.5 mL) was added, creating two phases. The tube was placed on ice (10 min) and centrifuged (1800 rpm, 10 min). The organic green phase was transferred to a sample vial (20 mL). The remaining aqueous phase was extracted twice with chloroform + 0.1% BHT (1 mL) and shaken (60 sec). The tube was placed in an ice bath (10 min) and centrifuged (1800 rpm, 10 min). All three sample vials containing green chloroform phases were transferred and combined into one sample vial and dried under nitrogen gas. The dried sample was dissolved by adding EtOH + 0.1% BHT (1 mL). The supernatant was transferred to a plastic tube (15 mL) and shaken (60 sec). Lastly, the supernatant was centrifuged (3000 rpm, 10 min) and afterward transferred to an amber HPLC vial (2 mL) for analysis.

2.3.1 Chemicals

Table 2: Chemicals used for carotenoid extraction and DAD-HPLC analysis of sample extracts.

Chemical	Formula	Purity [%]	Branding
Acetonitrile	CH_3CN	>99.8	Sigma-Aldrich
Ammonium Acetate	$NH_4CH_3CO_2$		
Butylated Hydroxytoluene (BHT)	$C_{15}H_{24}O$	≥99.0	Sigma-Aldrich
Chloroform	$CHCl_3$	99.0-99.4	Sigma-Aldrich
Ethanol	C_2H_5OH	>99.9	VWR Chemicals
Ethyl Acetate	$CH_3CH_2OCCH_3$	≥99.5	Sigma-Aldrich
Methanol	CH_3OH	>99.9	Sigma-Aldrich
Methyl tert-Butyl Ether	$(CH_3)_3COCH_3$	≥99.8	Sigma-Aldrich

2.3.2 Standards

Table 3: Purchased carotenoid standards used for qualitative and quantitative analysis of carotenoid contents in extract samples.

Chemical	Formula	Purity [%]	Branding
β-Carotene	$C_{40}H_{56}$	≥95% (HPLC)	Sigma-Aldrich
Fucoxanthin	$C_{42}H_{58}O_6$	≥95.0% (HPLC)	Sigma-Aldrich
Lutein	$C_{40}H_{56}O_2$	≥96.0% (HPLC)	Sigma-Aldrich
Zeaxanthin	$C_{40}H_{56}O_2$	≥95.0% (HPLC)	Sigma-Aldrich

2.4 Methods of analysis

2.4.1 High-Performance Liquid Chromatography

High-Performance Liquid Chromatography (HPLC) is an analytical method with high pressure, changing chemical environment, fine particles, and detectors to separate and analyze compounds in a mixture of compounds (figure 9) [37]. This method utilizes high pressure to push the sample through separation columns containing a stationary phase where compounds are separated based on the compounds' molecular makeup and the chemical environment of both the stationary and the mobile phase. The separated compounds are then analyzed with a designated detector [37].

The principles of HPLC are like that of basic extraction, where two phases separate compounds from a mixture based on the molecule's affinity for the two phases [37]. HPLC uses a stationary phase and a mobile phase, where the separation depends on the diffusion of compounds between these phases. Reversed-phase is the most common type of stationary phase used in HPLC, and it consists of a non-polar stationary phase of carbon chains (most commonly C18 or C30) bonded to silica. This type of mode in HPLC separates compounds based on the polarity of the entire molecule. Another common type of stationary phase is the normal phase which uses polar silica particles for separation [37]. This mode of HPLC is best suited to separate based on functional groups, and reversed-phase is used in this project.

The mobile phase is also a crucial part of the separation in HPLC. The strength of the eluent depends on its polarity compared to the stationary phase. A strongly non-polar mobile phase will more easily attract non-polar molecules retained in the non-polar column. One can either do isocratic or gradient elution when eluting the mobile phase. Isocratic elution is when the eluent is kept at a constant concentration throughout the run. Gradient elution is when the concentration changes based on a designed gradient, often containing more than one eluent. As gradient elution gradually changes the polarity of the mobile phase, it is generally preferred as it helps increase separation. A gradient was used in the project (table 4).

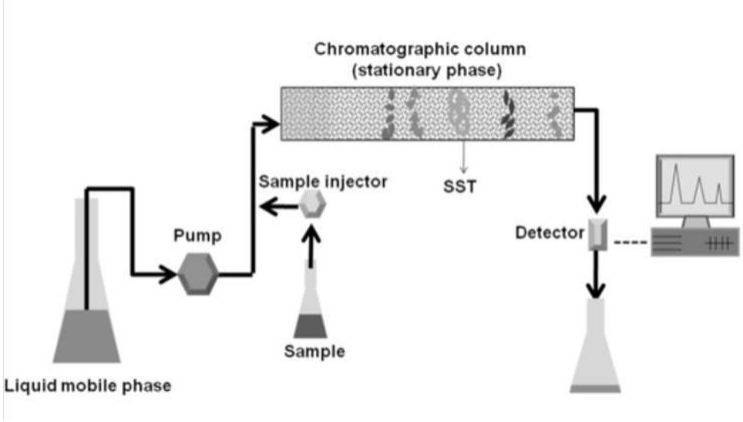


Figure 9: Simplified model containing the essential parts of an HPLC system, including mobile phase, pump, sample injector, stationary phase, detector, and computer [38].

2.4.1.1 Chromatographic separation of carotenoid groups

HPLC is a powerful technique to use for analyzing carotenoids. Many carotenoids give similar UV-Vis spectra but differ in polarity, making reversed phase a suitable method for separation [15]. Carotenoids are, as previously mentioned, divided into two groups: carotenes and xanthophylls, with carotenes being less polar than the xanthophylls. Using a reversed-phase system, one would expect the least polar compounds to elute last and polar first. Therefore, it is safe to assume that carotenes elute after xanthophylls. As there are many different carotenoids, one must be able to separate the individual compounds and not just a collective non-polar/polar group. A gradient is therefore used to alter the chemical properties of the mobile phase concerning polarity [37].

2.4.1.2 Gradient and analytical conditions

The gradient shown in figure 10 was used when analyzing the samples. It describes the percentage of MTBE present in the mobile phase from the start of the analysis to the end. The mobile phase mainly consists of polar and non-polar compounds, methanol and methyl tert-buthyl ether (MTBE). Increasing the MTBE amount over time decreases polarity, making non-polar molecules have less affinity for the stationary phase, resulting in a shorter elution time. To enable good separation, the gradient in table 4 was used. This is an altered version of the gradient used in previous projects, where the gradient has been extended from 45 minutes to 52 minutes to flush the column between each sample analyzed [35].

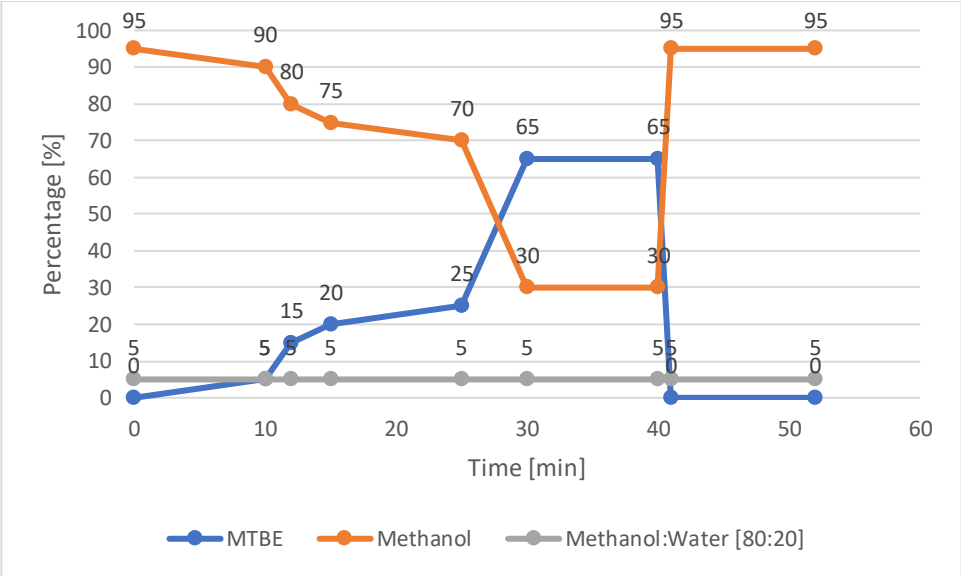


Figure 10: Gradients showing the percentage of eluents pumped at the allocated minutes (table 4). Eluent A is shown in blue, B is shown in orange, and C is shown in grey.

Table 4: Overview of mobile phase composition over 52 minutes of runtime. Each column represents the percentage of an eluent pumped in at the give minutes. A is methyl tert-butyl ether, B is methanol, and C is methanol and water with ammonium acetate.

Time[min]	A [%]	B [%]	C [%]
0	0	95	5
10	5	90	5
12	15	80	5
15	20	75	5
25	25	70	5
30	65	30	5
40	65	30	5
41	0	95	5
52	0	95	5

As reversed-phase is best to separate compounds based on polarity, the reversed-phased column YMC carotenoid C30 was chosen (table 5) with Agilent 1100 as the HPLC system. The rest of the specifications are shown in table 5.

Table 5: Conditions and specifications used when performing HPLC analysis.

Condition	Specification
Instrument	Agilent 1100
Method	CAROC_2
Column	YMC carotenoid C30, µm 4.6 x 250 mm
Injection volume	10 µL/min
Flow rate	1.0 mL/min
UV-Vis range	190-900 nm [2 nm increment]
Eluent A	Methanol
Eluent B	Methyl tert-butyl ether (MTBE)
Eluent C	MeOH + H2O + 0.2% NH4Ac

2.4.2 Diode Array Detector

A Diode Array Detector (DAD) is a detector that utilizes ultraviolet and visible electromagnetic radiation (UV-Vis, EMR) to detect compounds in a matrix. It is commonly used in natural product chemistry as many organic compounds can absorb EMR in the UV-Vis range. Unlike standard UV-Vis detectors, the DAD consists of an array of lamps that allow capturing of a complete spectrum instead of only looking at a few selected wavelengths [37]. Scanning over a range of wavelengths is important as it enables the analysis of UV-Vis peaks across the spectrum from a single compound, revealing characteristic peaks significant in natural product chemistry [39]. Since DAD is a type of UV-Vis-detector, the principles of the detection method are based on the Beer-Lambert law, where absorption of EMR is proportional to the concentration of the sample:

$$A = \varepsilon l c = \log\left(\frac{I_0}{I}\right)$$

A = absorbance; l = length of cuvette; c = sample concentration; I_0 = intensity of UV-Vis EMR sent towards sample; I = intensity of UV-Vis EMR passing through a sample[39].

2.5 Qualitative determination

For qualitative determination, chromatography and DAD spectroscopy are central for identification. The chromatography will help separate compounds and indicate the polarity as the reverse phase is used. Retention time is also important as it helps identify similar peaks from spectrum to spectrum. DAD helps identify compounds based on their characteristic fine structure in the spectra and gives quantized wavelengths specifically to the compounds (figure 11).

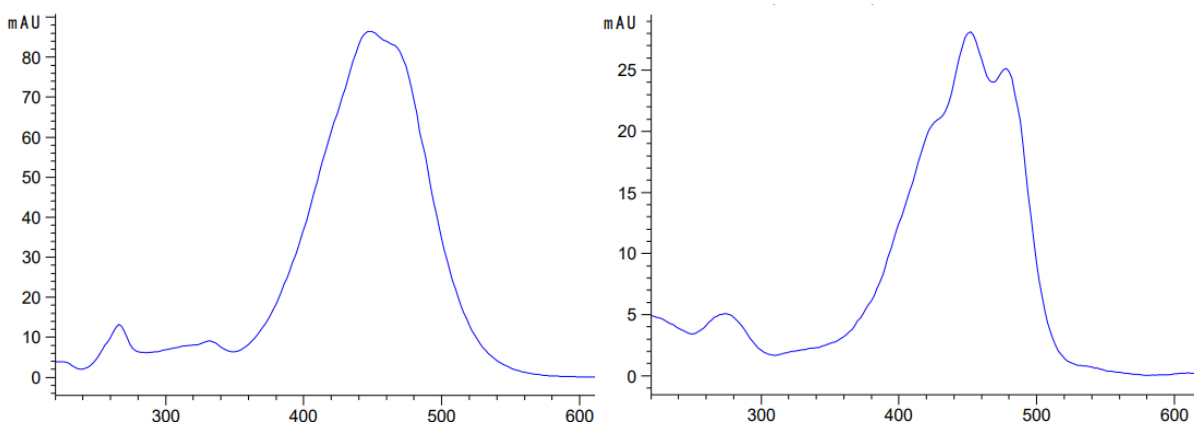


Figure 11: UV-Vis spectra of carotenoid compounds taken from analysis of a freeze-dried sample. Left: fucoxanthin in the sample. Right: b-carotene in the sample.

Standards of fucoxanthin, lutein, zeaxanthin, and b-carotene were used to identify carotenoids that are expected to be present in algae. Mixing the four pigments into one vial and analyzing it with other samples allows one to compare chromatograms to perform tentative identification (figure 12).

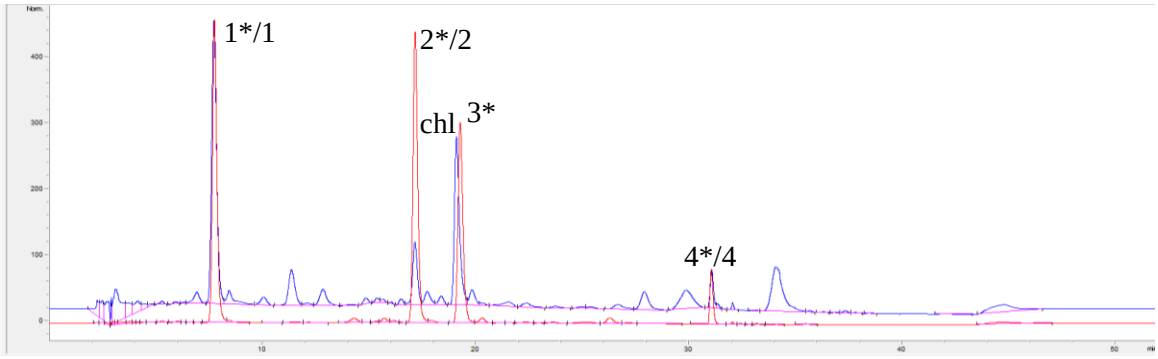


Figure 12: Two chromatograms of the standard mixture (red) and freeze-dried sample (blue) overlayed to identify fucoxanthin, lutein, zeaxanthin, and *β*-carotene. Standards are marked with an asterisk.

The Carotenoid handbook, together with the previous was used to identify carotenoids, suggesting carotenoids based on fine structure and λ-max values [21]. Overlapping of extracts and standard chromatograms aided in identifying expected carotenoid peaks using retention times, fine structures, and λ-max values (figure 12).

2.5.1 Application of ultraviolet-visible light detection for carotenoids

The chromophore in carotenoids makes the compounds able to absorb light in the visible spectrum between 400-500 nm [24]. The length of the chromophore determines the maximum wavelength (λ_{max}), as a more extensive conjugated system of double bonds lowers the energy level required for absorption [39]. Extra isoprene monomers, carbonyls, or rings on terminal sites help extend the ring, resulting in a bathochromic shift to a higher wavelength. A change in wavelength means that it is possible to distinguish carotenoids based on the displayed in the spectrum.

2.5.2 Ultraviolet-visible light absorption and fine structure alteration

Another important factor when it comes to distinguishing the different carotenoids is stereochemistry. As previously mentioned, the chromophore is affected by the size of the conjugated π-bonds and spatial arrangements. If the substituents are not coplanar with the chromophore, the bathochromic shift is less, and the spectrum's shape (fine structure) is changed. This is especially apparent when comparing the carotenoid β-carotene with canthaxanthin, where the characteristic three peaked forms are reduced to a single peak (figure 13).

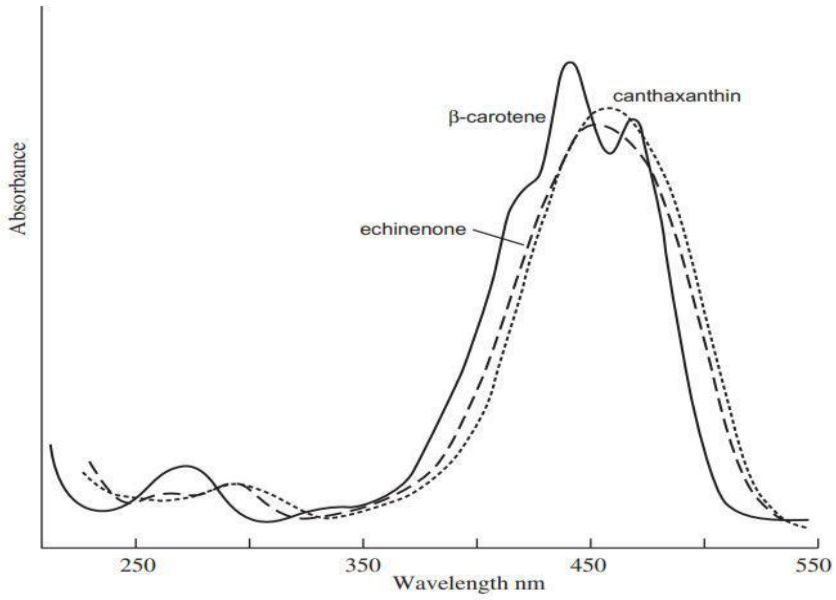


Figure 13: Example of fine structure reduction due to a less coplanar chromophore resulting from altered stereochemistry [15].

Z-isomers can be distinguished from *E*-isomers in two different ways. Compounds with Z-isomerism have a hypsochromic shift of 1-5 nm and have a reduced fine structure. The most noticeable difference is an extra absorption peak that appears 142 nm lower than the λ_{max} [21]. The intensity of this *Z*-peak/cis-peak explains the position of such bonding, as the closer to the middle it is, the stronger the intensity is.

2.6 Quantitative determination

A Calibration curve is a tool based on linear regression that can be used to quantify a mass or property based on limited knowledge with the assistance of a predictive model. The equation can explain the simplest linear regression:

$$y = mx + b$$

y = variable of the y-axis; a = slope of the curve; x = variable of the x-axis.

Different methods are used to ensure the line is closest to every point plotted to draw the line most representative of the linear relationship between the two variables. This ensures that the total standard error is as small as possible. In the least square method, the vertical distance between the data points and the curves is squared and added to a standard error sum. The standard curve is then corrected to have the smallest standard error sum [37]. Assuming that heteroscedasticity does not affect the dataset, the weighted least squares method (WLS) can be used to adjust and refine the calibration curve [40]. This is done by setting the inverse covariance as the weight in the regression. WLS was the method chosen for calibration curve correction as the null hypothesis stating that homoscedasticity is present was not rejected for any calibration curves. These conclusions were results from performing a Breuch-Pagan test, a test where the chi-square test is utilized to conclude whether homoscedasticity is present or not [41]. RStudio was used with the packages: lmtest, zoo, base, datasets, graphics, grDevices, methods, stats, and utils. Five different concentrations of each standard were analyzed with three degrees of freedom.

For quantitative determination, the methods of the standard curve were used. Four standard stock solutions were weighed and dissolved in ethyl acetate with 0,1% butylated hydroxytoluene (BHT). Afterward, six different concentrations of each standard were made to cover a concentration range used to determine the concentration of carotenoids.

Table 6: Overview of standard stock solutions made with concentration and range of calibration curves.

Standard	**$M_{standard}$ [mg]**	**$V_{solvent}$ [mL]**	**Conc. [µg/mL]**	**Conc. Range [µg/mL]**
β-carotene	1550	3.13	203	0.79-12.69
Fucoxanthin	470	2.20	150	0.5-30
Lutein	330	3.10	150	1-150
Zeaxanthin	460	3.07	150	1-150

The different standards were placed in amber HPLC vials (1.5 mL) and placed in increasing order of concentration when the curves were created. The Peak area in the chromatograms was used to plot the curves for each standard, and the peaks were measured at 450±20 nm. The area was found using the automatic integration tool in the software Agilent ChemStation. Baseline correction was set to advanced mode.

When calculating the amount of carotenoids, this equation was used:

$$x\left(\frac{mg}{g}\right) = \left(\frac{A-b}{a}\right) * \frac{V}{m_x}$$

x = concentration; A = peak area (mAU); b = calibration curve intercept; a = slope of the curve; V = solvent volume (mL); mx = mass of sample (mg).

When creating calibration curves, a limit of quantification is needed as models cannot quantify amounts at a certain level due to the presence of standard deviation. To calculate the limit of quantification, a standard deviation of the models' y-intercept and slope is used:

$$LOQ = \frac{10*SD}{S} \quad (1.2)$$

SD is the standard deviation of the y-intercept, and S is the standard deviation of the slope.

Chapter 3. Results and Discussion

3.1 Preliminary extractions and analysis of carotenoid contents of epiphyte

To get a general overview of the pigment contents of the samples, extracts made from the given method were analyzed using DAD-HPLC with the specifications given in table 5. Three samples of equal powdered epiphyte amounts (49.95 mg, 50.09 mg, and 50.10 mg) were weighed in and extracted using the carotenoid extraction method mentioned in section 2.3 (figure 14).

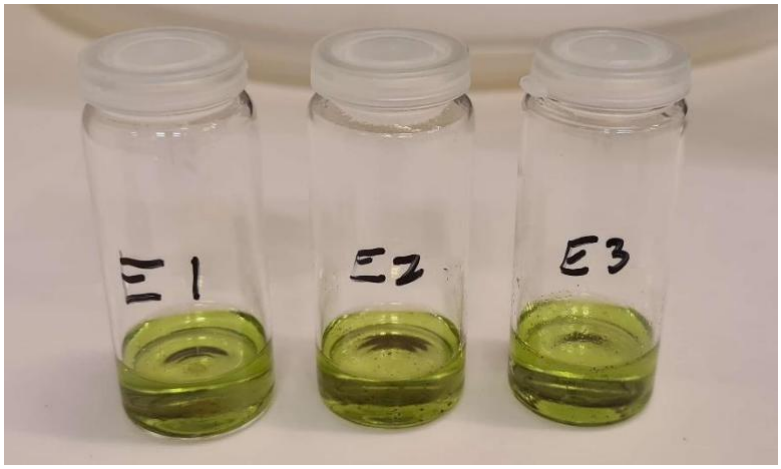


Figure 14: Chloroform diluted pigment extracts of freeze-dried epiphyte material marked E1-3.

Using methanol and chloroform as extraction solvents together with water and acetonitrile, the chloroform phase in the extraction was light green, indicating that pigments were extracted from the biomass, especially chlorophyll (figure 14). It was therefore expected that chlorophyll would be likely to be found in the chromatograms.

Extracts were dried under nitrogen, dissolved in ethanol with added BTH antioxidants and transferred to amber HPLC vials.

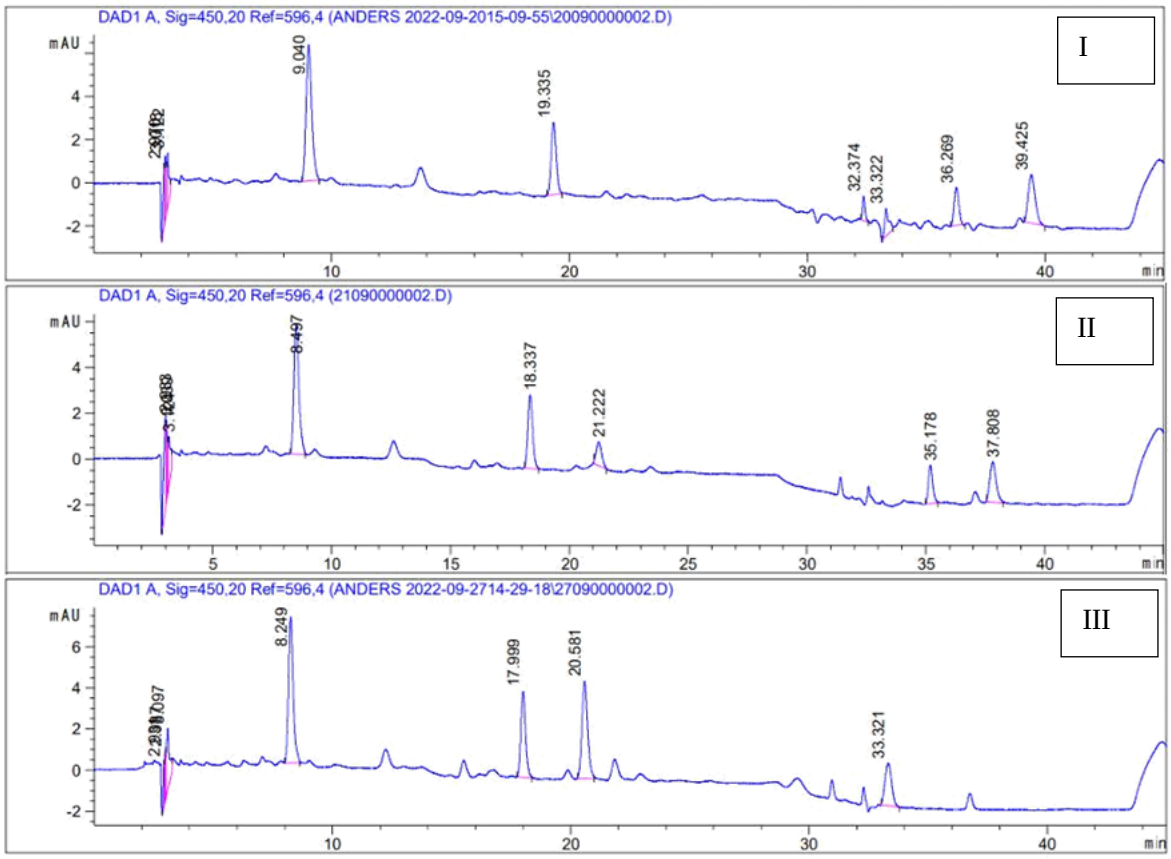


Figure 15: HPLC chromatogram from three parallel extractions (I-III) of powdered epiphytes detected at 450±20 nm.

The three different chromatograms (figure 15) show that all peaks have good separation with few overlaps, suggesting good HPLC conditions, which made us decide to keep the conditions described in table_. The biomass used per sample was increased to 400 mg to get higher concentrations of compounds in the samples. A change in mass was done to ensure that the concentration of carotenoids was enough to quantify the amounts later. Interestingly, the different peaks displayed in chromatograms I-III have different intensities, even though the extracts are from the same powdered biomass. III has a higher intensity than I and II (figure 15). The masses of these samples were 400.6 mg, 399.7 mg, and 399.9 mg for I, II, and III, respectively. The mass alone cannot explain this difference, and a possible inhomogeneity in the powder can explain this. It is also worth noting that the heights of the signals are low, so it is expected to have variations at low intensities.

3.2 Carotenoid profiles of epiphyte biomass

For the characterization of carotenoids, three parallel samples were prepared. The samples were air-dried (AD), freeze-dried (FD), or freeze-dried material with industrial milling, weighing 400 mg (table 7). These samples were analyzed using four analytical carotenoid standards to create co-chromatograms. This mixture contained the carotenoids β-Carotene, fucoxanthin, lutein, and zeaxanthin and was used for characterization.

Table 7: Names of samples analyzed in the profiling with description.

Name	Description
AD	Air-dried epiphytes, chopped, sliced, and ground
FD	Freeze-dried epiphytes, chopped, sliced, and ground
Pow	Freeze-dried and milled to a powder. Provided by Alginor ASA

3.2.1 Selection of standards

The reason why these four compounds have been chosen as standards is that β-carotene, fucoxanthin, lutein, and zeaxanthin are carotenoids that are common in algae, with fucoxanthin, β-carotene, and violaxanthin being present in brown algae, zeaxanthin, α- and β-carotene, and lutein in red algae and β-carotene, violaxanthin, lutein, neoxanthin, and zeaxanthin in green algae[14]. As epiphytes contain an assortment of all three algae groups, these compounds are expected to be present. The epiphytes on *Laminaria hyperborea* have been shown to mostly be red algae, which contains zeaxanthin, β-carotene, and lutein (table 1, figure 1) [17]. It was therefore expected to find these pigments present in the sample types. Zeaxanthin was not found in any of the chromatograms as none of the peaks shared a similar retention time, with the closest signal with partial overlap having a UV-Vis spectrum indicative of chlorophyll. Chlorophyll a was expected to elute in this part of the chromatogram as in a previous project with similar conditions [35].

3.2.2 Retention times of standards

As a C30 reversed-phase column is used, the compound with the highest polarity is expected to be eluted first and least polar at last, as the percentage of polar eluent A is high at the beginning of the gradient (figure 10) [37]. It is shown that the compounds *β*-carotene, lutein, and zeaxanthin elute in this order using a C30 column: lutein < zeaxanthin < *β*-carotene, as the polarity of lutein is higher than zeaxanthin, and zeaxanthin is more polar than *β*-carotene [42]. As a xanthophyll, fucoxanthin is expected to be eluted first, as the compound contains the most oxygen functional groups, resulting in a higher polarity (figure 2). Therefore, the expected order of elution is fucoxanthin, lutein, zeaxanthin, and *β*-carotene, with the longest retention time. This was confirmed when the standards were run separately using the same experimental conditions (figure 16, I-IV). 1*-4* is therefore assigned as the standards in the order previously mentioned.

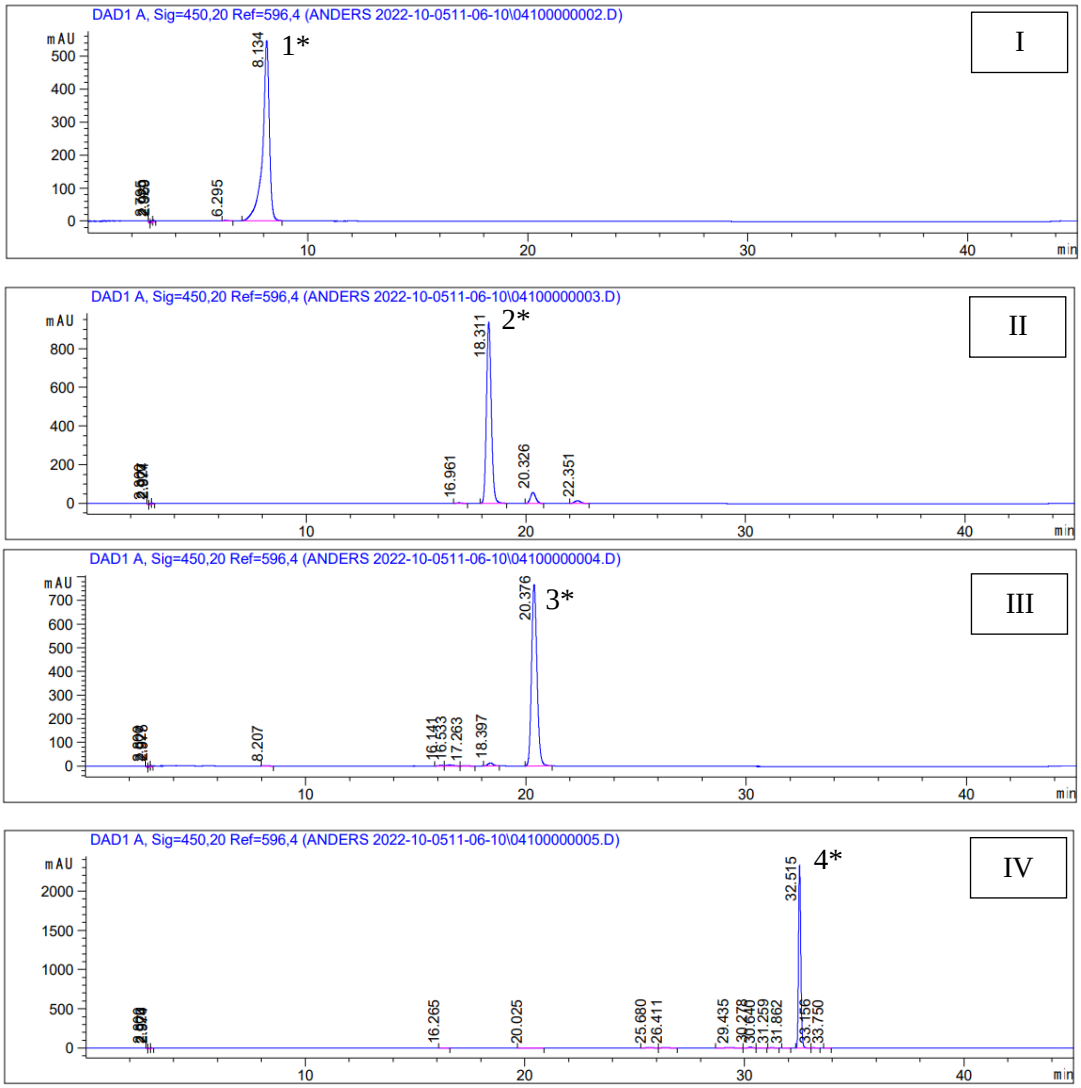


Figure 16: Chromatograms of separate runs of the standards fucoxanthin (I), lutein (II), zeaxanthin (III), and β-carotene (IV). Chromatograms were detected at 450±20 nm.

3.2.2 Identification of compounds using carotenoid standards

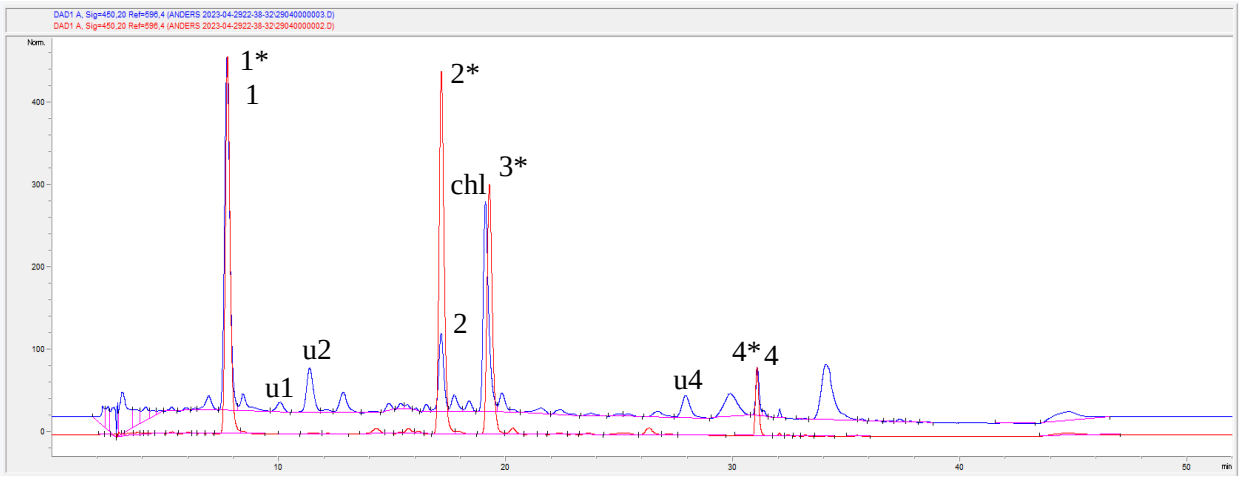


Figure 17: Overlapping chromatograms of freeze-dried sample and standard mixture. Red: standard mixture, blue: freeze-dried sample chromatogram. Data from the chromatograms in appendix_and_. Both chromatograms are detected at 450±20 nm.

Figure 17 shows overlapping chromatograms of freeze-dried and standard mixture with the four standards marked from 1* to 4* and the unknown carotenoid compounds marked as 1 to 4 and u1-4. Aside from a difference in intensity, one can observe that 1*,2*, and 4* overlap with peaks 1, 2, and 3, while 3* partially overlaps with another peak. This indicates that some compounds in the freeze-dried sample have equal retention times to the standard mixture. Table 3 lists the standards used in the project.

For 1*, 2*, and 4*, the retention time (t_R) was 7.74, 17.15, and 31.07 min, respectively (figure 17). The retention times for the unknown compounds (1,3,5) were 7.72, 17.14, and 31.09 min, respectively (figure 17) (appendix 1,2). These retention times have similar values, indicating that three out of four standards overlap well with the unknown peaks. Peaks u1, u2, and u4 do not overlap and cannot be identified using analytical standards.

For a further indication of the four standards, the fine structure of the UV-Vis spectra and the peaks maximums present were compared to give the best possible indication given the methods used.

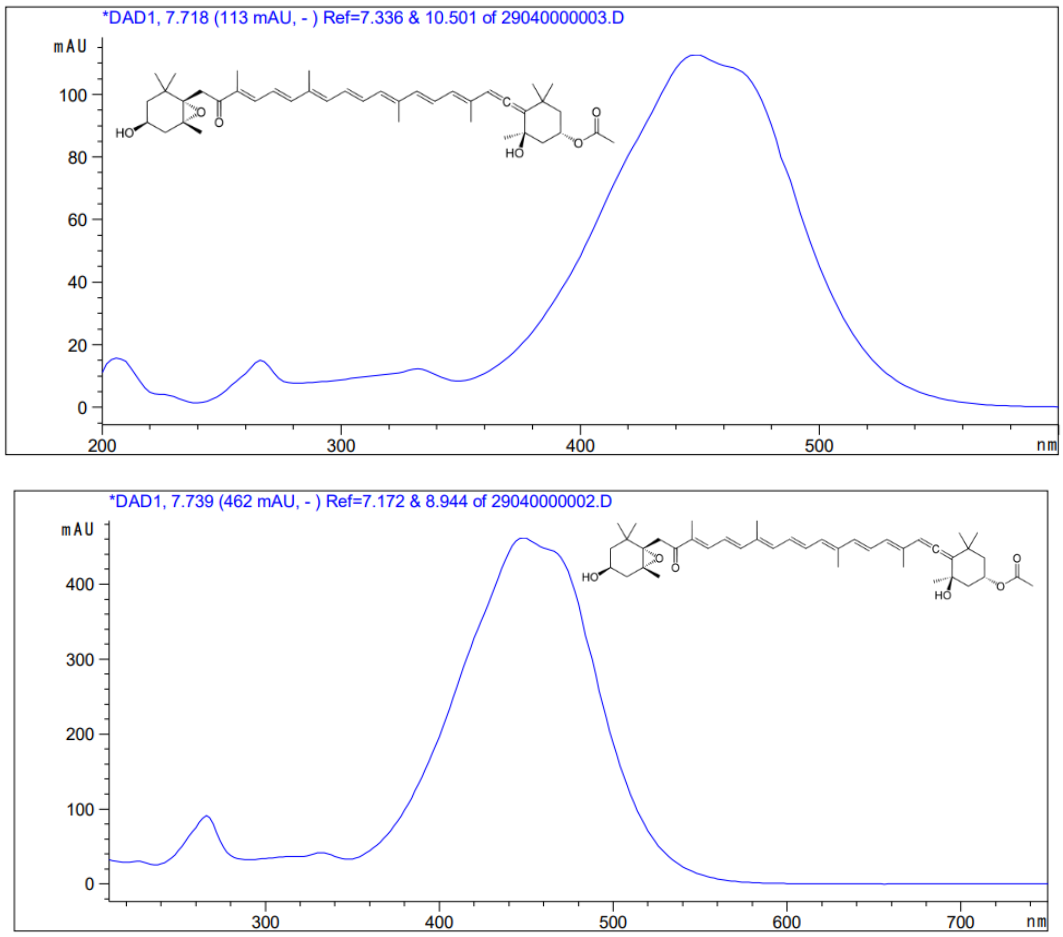


Figure 18: Above: UV-Vis spectrum of peak 1 in freeze-dried (FD) chromatogram; Below: UV-VIS-spectrum of fucoxanthin in the standard mixture. UV-Vis spectra are detected at 450±20 nm.

For compound 1 with a retention of 7.72 (figure 17), the fine spectrum was found to have a UV-VIS λ-max of 446 nm and a local maximum peak of 466, while 1* had a λ-max of 448 with a local λ-max of 466 (appendix 1,2). The two peaks have similar maximums but with a 2 nm difference. Peak 3 in figure 17 has a λ-max of 444 nm with two local λ-max of 422- and 472 nm, respectively (appendix 2). For the standard 2* (figure 17), the UV-Vis spectrum had a local λ-max of 444 with local λ-max of 422- and 470 nm (appendix 1). Peak 3 (figure 17) had a local λ-max of 452 nm with 478 nm as a local maximum (appendix 2). The peak for standard 4* (figure 17, appendix 1) had a λ-max of 452 nm with a local λ-max of 452, meaning 4* and peak 3 have identical values. By comparing the shapes of the UV-Vis spectra of peaks 1, 2, and 3 with the standards 1*, 2*, and 4*, respectively, each unknown compound has quite similar shapes, with nearly equal λ-max values. 3* overlaps partially with peak Chl, but the fine structure of the UV-Vis spectra of these compounds are different, with Chl containing a spectrum like that of chlorophyll α (figure 19).

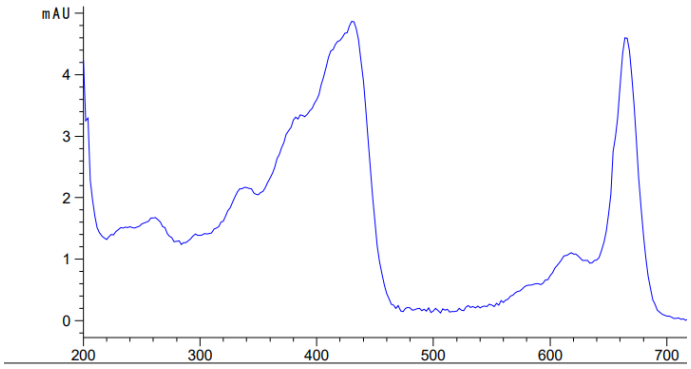


Figure 19: UV-Vis spectrum of chlorophyll peak at 19.84 min in the freeze-dried sample detected at 450±20 nm (figure 17).

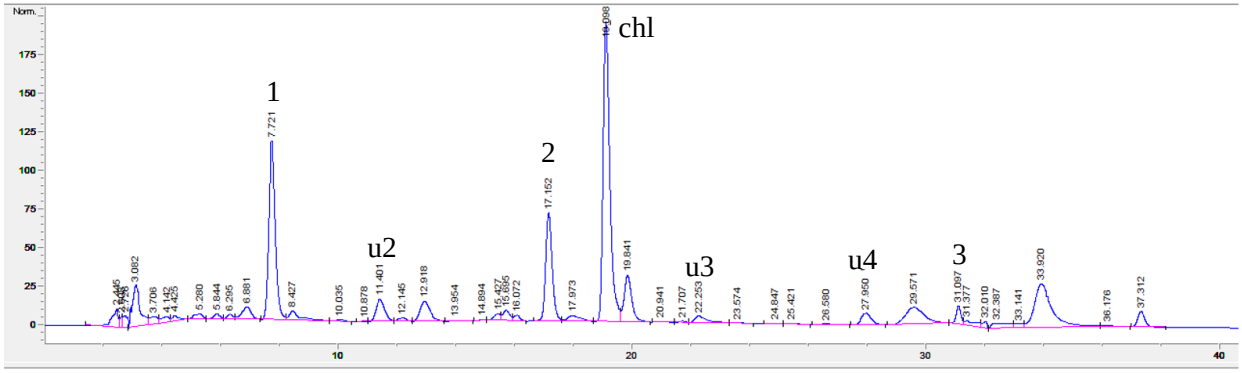


Figure 20: Chromatogram of a powdered sample. Peaks 1-3 are fucoxanthin, lutein, and *β*-carotene. Peak chl is chlorophyll, and u2-4 are unknown carotenoid-like compounds. The chromatogram was detected at 450±20 nm.

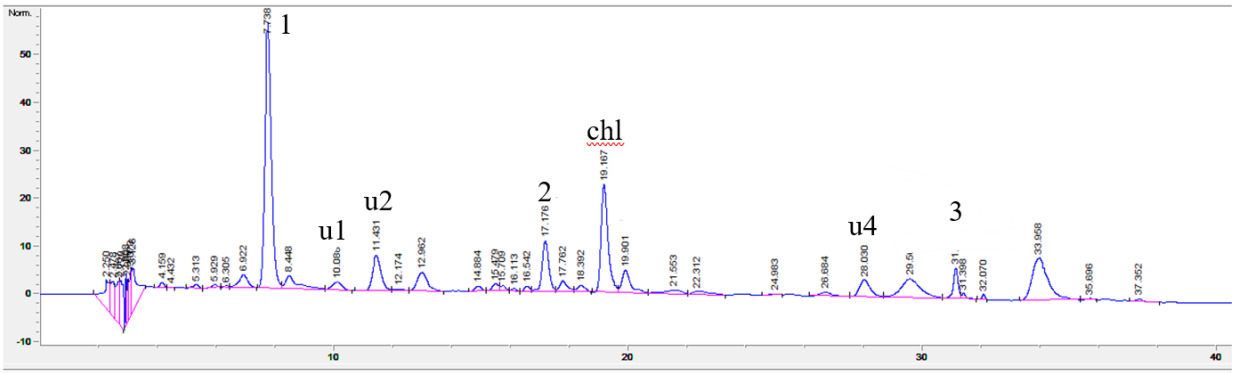


Figure 21: Chromatogram of an air-dried sample. Peaks 1-3 are fucoxanthin, lutein, and *β*-carotene. Peak chl is chlorophyll, and u1-4 are unknown carotenoid-like compounds. The chromatogram was detected at 450±20 nm.

The chromatograms from the powdered and air-dried samples (figure 20 and 21) show three peaks with a similar retention time as standards 1*, 2*, and 4*(appendix 1,3,4). For AD, the peaks elute at 7.74, 17.18, and 31.13 min; for powder, the peaks elute at 7.72, 17.15, and 31.10 min (appendix 3,4). Both samples have a good overlap with the standard mixture sample. Comparing the λ-max values, peak 1 in air-dried (AD) has a λ-max of 448 nm with a local maximum of 466 nm, peak 2 has a λ-max of 444 nm with a local λ-max of 472 nm, and peak 3 has a λ-max of 448 nm with a local λ-max of 478 (figure 21, appendix 4). For powder, peak 1 has a λ-max of 488 nm with a local λ-max of 466 nm, peak 2 has a λ-max of 444 nm and a local λ-max of 472, and peak 3 has a λ-max of 448 with a local λ-max of 478 (figure 22, appendix 3). Compared with the standards. The fine structures of the overlapping compounds have very similar fine structures, but some λ-max have a difference in 2-4 nm. Therefore, the overlapping peaks 1, 2, and 3 in AD, FD, and Pow chromatograms are assigned these compounds. This strengthens the validation that the selected peaks represent the three standards.

3.2.3 Suggestions for unknown carotenoid-like compounds

Excluding the three overlapping compounds validated to be fucoxanthin, lutein, and β-carotene, four other similar signals were detected, perhaps being carotenoids, as the fine structures of these have similar characteristics to that of carotenoids. It is uncertain whether these can be placed into four different compounds, but the signals in the three different chromatograms have similar retention times and fine structures. As only DAD-HPLC and standards have been used to identify specific compounds in the sample, it is impossible to do any characterization of other compounds that might be present in the samples. Only suggestions can be given based on fine structure and literature as a starting point for further analysis in the future.

3.2.4.1 The 1st unknown compound (u1)

The first signal, u1, was found in FD and AD and had a retention time of 10,05 and 10,09 min (figure 17, 21. Appendix 2,4). The λ_{max} of the signals was λ-max of 438 nm with 412/414 nm and 468 nm, and λ-max of 436 with local λ-max of 414 and 468 for FD and AD, respectively (figure 22 A, C). Low intensity makes it difficult to compare the fine structure with other compounds because the shape is poorly defined. As the fine structures contain three peaks, (9'Z), neoxanthin is suggested as it contains a fine structure with three peaks where λ-max is 413 nm with two local λ-max of 437 nm and 466 nm in ethanol (figure 22 B) [21]. Neoxanthin is found in green algae, and its most common form is 9-cis-neoxanthin. It is, therefore, possible that this compound can be present in the sample. Since neoxanthin is a xanthophyll, the elution time makes sense as polar carotenoids elute faster in the reversed-phase system (figure 17, 21).

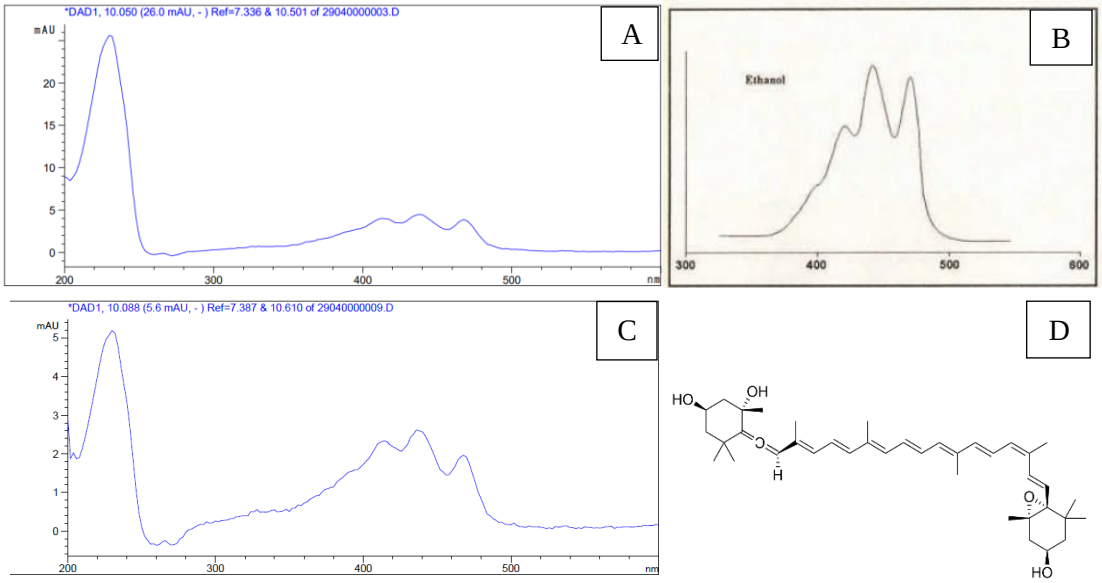


Figure 22: A: UV-Vis spectrum of u1 in freeze-dried extract (figure_). B: Neoxanthin fine structure in ethanol[21] recorded with UV-Vis spectrophotometer. C: UV-Vis spectrum of u1 in air-dried extract (figure_). D: molecule of neoxanthin, the suggested molecule for u1. Figure A and C were detected at 450±20 nm.

3.2.4.2 The 2nd Unknown compound (u2)

The following signal, named u2, was found in the chromatograms of air-dried (AD), freeze-dried (FD), and powdered (Pow) with the retention time of 11,43 min, 11,35 min, and 11,40 min, respectively (Appendix 2, 3, 4). The UV-Vis spectra have a λ-max of 444 nm, 446 nm, and 444 nm, with a local λ-max found on the spectrum for the peak in the chromatogram for powdered extract (Pow). The fine structure of all signals has a similar shape and is reminiscent of the UV-Vis spectrum for fucoxanthin (figure 23). From the handbook, no other carotenoid had a similar fine structure and λ-max. It is therefore proposed that the three signals found could be a derivative of fucoxanthin or an isomer. A study on fucoxanthin isomers in microalgae *Isochrysis sp.* using a similar HPLC setup found a peak with a similar fine structure eluting after fucoxanthin (figure 24, A) [43]. It was reported to have λ-max values of 444 nm and 462 nm which are identical to the λ-max values of u2. 9-cis-fucoxanthin is, therefore, likely to represent u2.

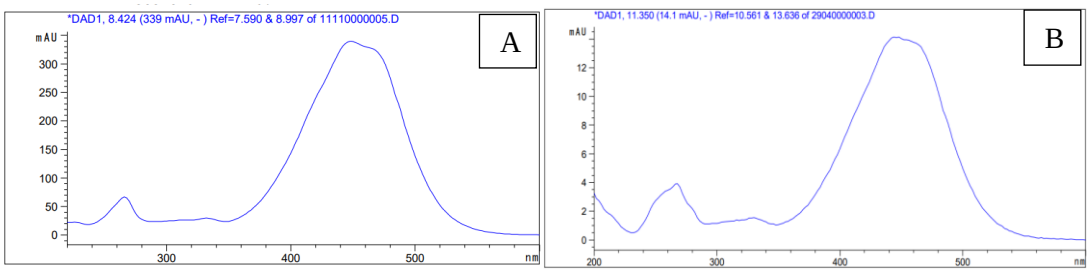


Figure 23: A: DAD spectrum of fucoxanthin from a fucoxanthin standard. B: UV-Vis spectrum of peak (u2) in the FD chromatogram. Spectra were recorded at detected at 450±20 nm.

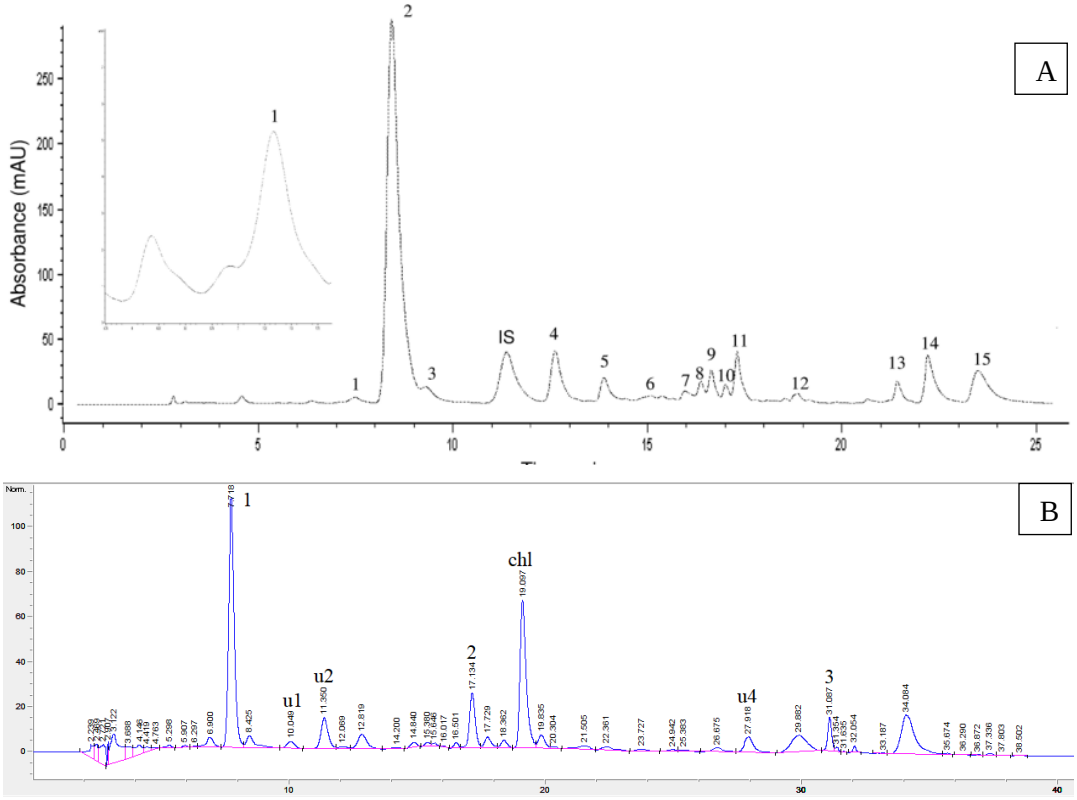


Figure 24: A: Chromatogram from *Isochrysis sp.* analysis in a study on fucoxanthin isomers [43]. Peak 1 is fucoxanthin, and 1 is 9-cis-fucoxanthin. B: Chromatogram of freeze-dried epiphyte sample. Peaks 1-3 are fucoxanthin, lutein, and β-carotene. Peaks u1-4 are unknown carotenoid-like compounds. Chl is chlorophyll. Chromatogram was detected at 450±20 nm.

3.2.4.3 The 3rd Unknown compound

The carotenoid-like signal, named u3, was found in the chromatogram for powdered extract (Pow) and had a retention time of 22.25 min (figure 20. Appendix 3). As this signal had low intensity, the fine structure had low resolution making it difficult to discern which fine structure it resembled by comparing it to other peaks and literature (figure 25, A). The λ_{max} was 440 nm with a local λ_{max} of 468 nm (appendix 3). From these values and the shape of the fine structure, one compound is suggested as a viable candidate for this compound, namely lactucaxanthin, a xanthophyll identified from extracts of the green algae called *Chlorella ellipsoidia* and the common vegetable *Lactuca sativa,* also known as lettuce [44]. It is important to stress that suggestions are only based on shape and λ_{max} values and should not be taken as confident identifications. Further identification and confirmation with NMR spectroscopy and preparative HPLC are needed, possibly assisted by appropriate analytical standards.

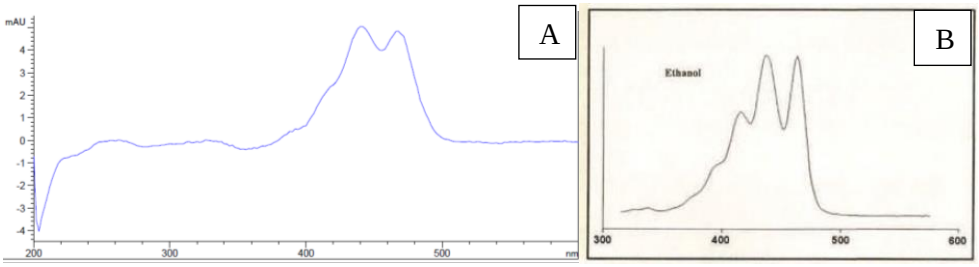


Figure 25: A: UV-Vis spectrum of u3 in the powdered extract detected at 450±20 nm. B: Spectrum of lactucaxanthin in an ethanol sample recorded on UV-Vis spectrophotometer [21].

3.2.4.3 The 4th unknown compound

A third group of signals, named u4, are found at the retention times 27.92, 27.95, and 28.03 min for FD, AD, and Pow and are the latest carotenoids-like signals found, aside from *β*-carotene (Figure 17, 20, 21. Appendix 2, 3, 4). The λ-max on the respective signals is found to be 444 nm, 474 nm, and 446 nm, all with a local maximum of 474 nm (appendix 2, 3, 4). The fine structure located on the signals from freeze-dried (FD) and powdered (Pow) has identical λ-max, while the signal found in air-dried (AD) is negligible due to having a signal intensity close to zero (figure 26 A, B, C). Many carotenoid compounds have been suggested to be present in any of the mentioned chromatograms, as many carotenoids have similar fine structures with similar λ-max and local λ-max found (figure 26, D). The suggested compounds are anhydrolutein 1 and 2, antheraxanthin, zeinoxanthin, epilutein, and cryptoxanthin. Only zeinoxanthin, antheraxanthin, and cryptoxanthin have been found in algae and are viable compounds [45, 46].

As the fine structure is similar to *β*-carotene, the peak may belong to an isomer of this carotenoid. Previous studies on the isomerism of *β*-carotene found that 15-cis-*β*-carotene and 13,15-di-cis-*β*-carotene elute before *β*-carotene in a reversed phased column [47, 48]. It was reported that 13,15-di-cis-*β*-carotene had λ-max values of 436 nm, and 15-cis-*β*-carotene had λ_{max} values of 425 nm, 446 nm, and 474 nm [48]. In comparison, it was found that u4 had λ_{max} values of 444/446 nm and 474 nm, making 15-cis-*β*-carotene a better fit(appendix 2, 3, 4). The UV-Viss spectrum of 15-cis- *β*-carotene has been identified to have a cis-peak at 338.7 nm [49]. This peak is not present in the fine structure of u4, making it unlikely that 15-cis-*β*-carotene is present in the spectrum (figure 26, A, B).

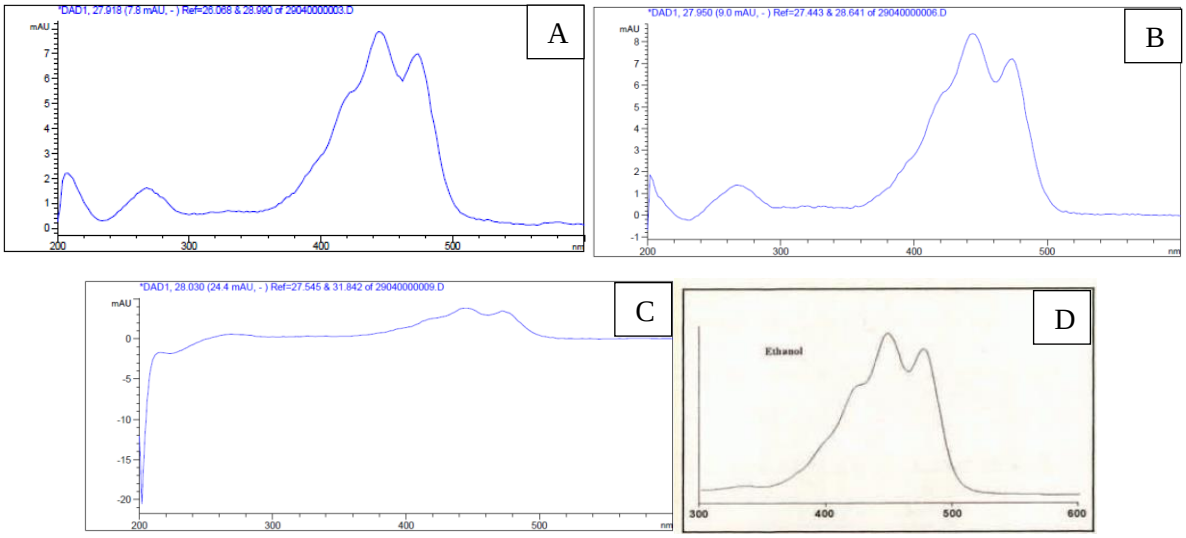


Figure 26: A: UV-Vis spectrum of u4 in the freeze-dried sample. B: UV-Vis spectrum of u4 in the powdered sample. C: UV-Vis spectrum of u4 found in the air-dried sample. D: fine structure of the compounds: anhydrolutein, antheraxanthin, zeinoxanthin, epilutein, and cryptoxanthin detected in a UV-Vis spectrophotometer. Spectrums A, B, and C were detected at 450±20 nm.

It is important to stress that comparing UV-Vis spectra detected with DAD and UV-Vis spectra recorded with UV-Vis spectrophotometer can be dangerous, as the analytes in the sample are affected by different environments. In DAD-HPLC, samples are pumped through a system in a mobile phase containing many different solvents, which can affect λ_{max} and fine structures. In a spectrophotometer, samples are analyzed on one or a few wavelengths in a cuvette dissolved in one solvent under static conditions. This must be taken into account when comparing spectra.

3.3 Quantitative determination

For quantitative determination, three parallels of freeze-dried, air-dried, and powdered samples containing 400 mg of material were analyzed with DAD-HPLC and quantified using calibration curves of four standards. After extraction, the samples were stored in a freezer at -20℃ until analysis.

3.3.1 Calibration curve quantification

Standards of xanthophylls and carotenes were chosen to quantify unknown compounds to cover a wide range of polarities. This was done as one cannot rely on having standards for every compound found in complex samples. *β*-Carotene, fucoxanthin, lutein, and zeaxanthin were chosen as discussed in 3.2.1. For unknown compounds u1 and u2, the calibration curve of fucoxanthin was selected as the signals had a retention time of 10 min and 11 min, closest to the fucoxanthin peak (figure 17). Unknown u3 was quantified with the zeaxanthin calibration curve due to the peaks' proximity to the standard peak (figure 17). U4 was quantified using the *β*-carotene standard curve closest to the carotene retention time (figure 17). These peaks were chosen to be quantified as they had carotenoid-like fine structures and had signals above the limit of quantification.

Table 8: Calibration curves of standards with associated values made in RStudio. St.d[y]: residual standard deviation, St.d[a], standard deviation of the slope, st.d[b]: standard deviation of intercept, LOD: limit of detection, LOQ: limit of quantification. R^2: Coefficient of determination.

Compound	Function	R^2	St.d[y]	St.d[a]	St.d[b]	LOD[b]	LOQ[b]
Fucoxanthin	y=102.1106x-1.4641	0.9997	4.738	0.3971	1.613	0.1531	0.4640
Lutein	y=98.1544x+0.8002	0.9998	11.85	0.4104	6.886	0.3984	1.207
Zeaxanthin	y=82.6087x-10.8341	0.9994	16.05	0.5563	9.332	0.6411	1.943
***β*-Carotene**	y=63.538x+38.509	0.9975	5.971	1.576	5.000	0.3101	0.9398

Each calibration curve was made by preparing five solutions of dissolved carotenoid standard from a standard stock made from dissolving the standard powder in ethyl acetate. Each solution was analyzed three times to establish the standard deviation of each data point. The absorption data and the calculated concentration were plotted in a RStudio script where R2, regression line, and standard deviations for residual, slope, and intercept were calculated using the weighted least square method after applying the Breuch-Pagan test. The coefficient of determination (R^2) of all calibration curves is above 0.995, deeming the curves fit for purpose(table 8) [37]. The limit of quantification (LOQ) was used to determine if the chromatograms signals should be quantified.

3.3.2 Carotenoid quantities in epiphyte samples

Comparing the three sample types, powdered has the highest carotenoid content of 5.38 µg/mg DW, with air-dried containing 2.03 µg/mg DW and freeze-dried 3.70 µg/mg (table 9). The same trend is found when looking at the percentage of how much the DW of the sample contains carotenoids and carotenoid-like compounds. As both freeze-dried and powdered have a higher carotenoid content, freeze-drying is the method of choice when preparing epiphyte samples to be extracted (table 9). In a study done on purple carrots, it was shown that freeze-drying led to the smallest reduction of carotenoids (0.7%) compared to air-drying, which had a significant loss (36.2%) [50]. Another study showed that freeze-drying carrots also retained more carotenoid content (70.37 mg/100g) than air-drying (34.16 mg/100g) [51]. This supports the trend in table 9 that freeze-drying yields a higher extraction of carotenoids.

Table 9: Total carotenoid content of air-dried, freeze-dried, and powdered samples.

	AD	**FD**	**Pow**
Sum [µg/mg]	2.03	3.70	5.38
% in sample	0.203	0.370	0.538

Table 10: Overview of quantified carotenoids in air-dried, freeze-dried, and powdered samples with standard deviation. Blank cells indicate that the peaks were not present in the chromatograms.

	Amount [µg/mg]		
Compound	**Air-dried**	**Freeze-dried**	**Powdered**
Fucoxanthin (1)	1.25±0.0125	2.17±0.0039	2.63±0.0152
Lutein (2)	0.302±0.00247	0.582±0.00287	1.53±0.0111
β-Carotene (3)	0.0705±0.00777	0.238±0.0037	0.216±0.0018
Carotenoid-like (u1)	0.567±0.000734	0.0842±0.000472	
Carotenoid-like (u2)	0.219±0.00834	0.364±0.00667	0.435±0.00329
Carotenoid-like (u3)			0.269±0.0007
Carotenoid-like (u4)	0.137*	0.257±0.0162	0.306±0.0009

*Value does not have standard deviation due to parallels falling under LOQ

In previous projects, the carotenoid content in the microalgae *T. Chuii* was 0.33% of biomass; in P. tricornutum, the contents were 6.71 ± 0.53 µg/mg [35, 36]. Another study done on carotenoid contents of red algae species off the coast of New Zealand found 1.64 µg/mg DW in *Porphyra columbina*, 4.12 µg/mg DW in *Melantlia abscissa*, 8.72 µg/mg DW in *Cladhymenia oblongifolia*, and 9.18 µg/mg DW in *Vidalia colensoi* [52]. Compared to the contents found in different types of microalgae and seaweeds, the carotenoid contents in the epiphyte biomass are of an equal magnitude (table 10). They can be seen as a comparable source of carotenoids, especially microalgae and red algae species.

Comparing the u2 contents in the different samples, one can observe a positive correlation between the amounts of fucoxanthin and the amounts of u2 in the samples (figure 27). More fucoxanthin in a sample can therefore mean that more u2 is found. This further supports that u2 is 9-cis-fucoxanthin as more fucoxanthin allows more isomerization.

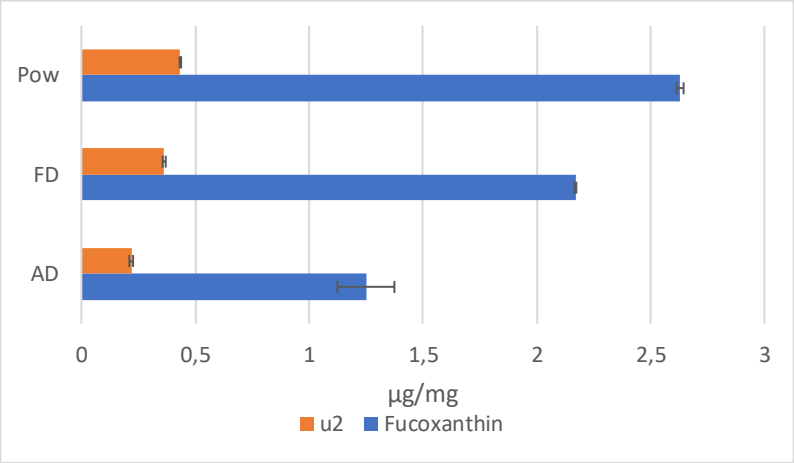


Figure 27: Comparison of fucoxanthin and u2 content in freeze-dried (FD), air-dried (AD), and powdered samples (Pow).

3.3.2.1 Fucoxanthin contents

Table 11: Fucoxanthin content of epiphyte and L. hyperborea samples compared to the total carotenoid contents.

Compound	Air-dried	Freeze-dried	Powdered	*L. Hyperborea*
Fucoxanthin [µg/mg]	1.25	2.17	2.63	7.13
Total content [µg/mg]	2.03	3.70	5.38	8.52
% Fucoxanthin	61.4	58.6	48.9	83.8

As shown in subchapter 1.3, most of the epiphyte species found on the stipe of *L. hyperborea* are part of the red algae group, with brown algae making up the smallest. It is, therefore, interesting to look at the quantified amounts of fucoxanthin in the samples, as this compound is most associated with brown algae, as discussed in subchapter 3.2.1. For all sample types, fucoxanthin makes up most of the carotenoid contents, containing 61.4 % of the contents in the air-dried sample, 58.6% in freeze-dried, and 48.9% in powdered (table 11). As it is not expected that fucoxanthin is naturally occurring in red algae, it is believed that the pigment stems from contamination, as biomass from the L. hyperborea could have ended up in the epiphyte biomass during the removal process. Therefore, a sample of air-dried L. hyperborea was extracted to analyze the carotenoid contents. With a percentage of 83.8, fucoxanthin makes up most of the carotenoid content in L. hyperborea and is deemed a good source of this pigment, as expected from a brown alga (table 11). This has been shown in a previous work where 16.5 mg/g fucoxanthin was quantified in *L. hyperborea* using qNMR spectroscopy [53].

As the powdered samples have a higher carotenoid content than the freeze-dried, there is reason to believe that freeze-drying and industrial milling are more effective ways to extract carotenoids from the epiphytes (table 11). Although this might be true, it is important to note that the powdered epiphyte produced by Alginor ASA was not harvested with the epiphyte material used to make the air-dried and freeze-dried materials. Season harvested and time spent in storage make a difference in the quantified amounts [54, 55]. This might explain why the amount of fucoxanthin found in the powdered sample is around 10 percent points lower than for air-dried and freeze-dried.

3.4 Stability of carotenoids based on different biomass preparation and storage conditions

3.4.1 Aim and design of the three-week stability study (T_0-T_3)

In the stability study, the aim was to observe how different parameters affect the concentration of carotenoids in samples over a period set to three weeks (T_0-T_3). As the established extraction method was used, the stability study focused on how sample preparation (table 12) and the storage of samples affect the analytes (table 13).

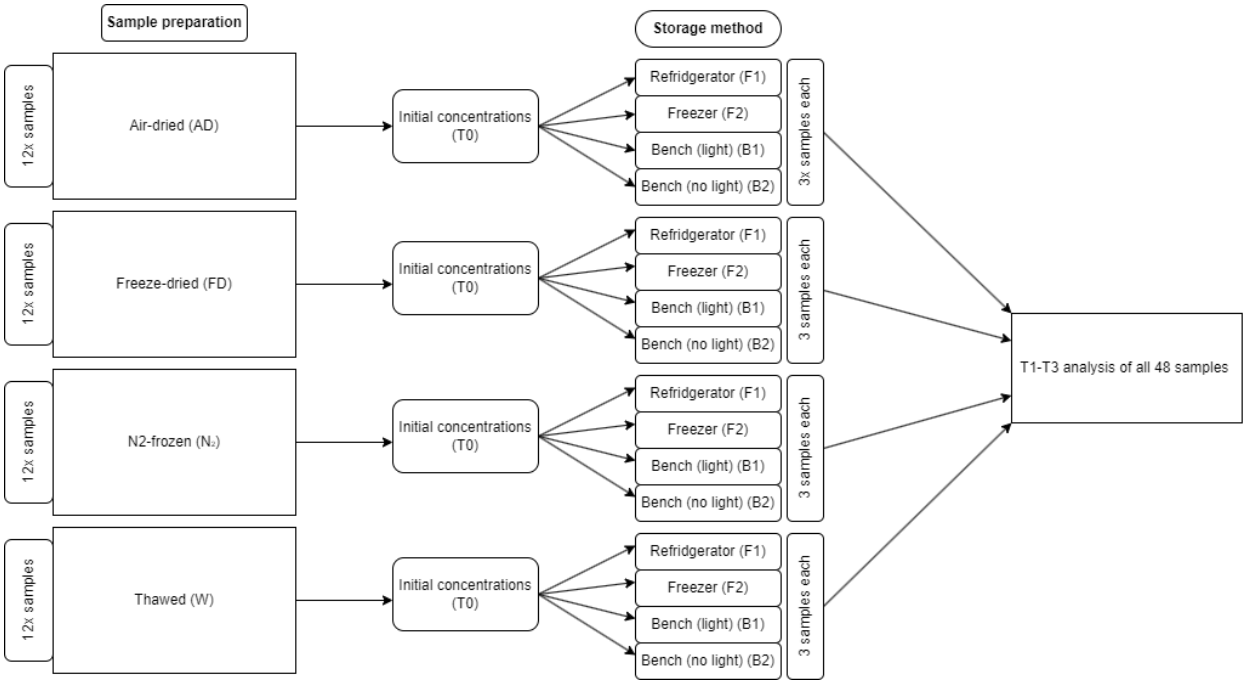


Figure 28: Diagram of the three-week (T_0-T_3) stability project design showcasing how samples of different preparation methods are grouped into different storage method categories. Twelve samples were made per sample preparation method, and initial concentrations of carotenoids were analyzed. The twelve samples were split into four storage methods: refrigerator (F1), freezer (F2), bench with light exposure (B1), and bench without light exposure (B2). Samples were stored for a three-week period with analysis each week (T_1-T_3).

A design (figure 28) was set up to perform different sample preparation methods, testing different parameters known to cause carotenoid degradation. Air-drying, freeze-drying, liquid nitrogen freezing, and thawing were performed for sample preparation (figure 12). Heat and light were chosen for degradation parameters as these affect carotenoids (see subchapter 1.2.3). For storage, three locations were chosen based on temperature and lighting. These were in a refrigerator (F1), in a freezer (F2), on a bench at room temperature with exposure to lamp light and sunlight (B1), and on a bench at room temperature wrapped in aluminium foil (B2) (table

13). This gave in total of three locations with no exposure to light at three different temperatures and two locations of equal temperatures with a difference in light exposure.

Table 12: Shortened labels for sample preparation methods assigned to samples according to the methods stated in subchapter 2.2.

AD	**Air-dried – dried at room temp**
FD	Freeze-dried – dried using lyophilization
N2	Liquid nitrogen [N_2] – dried with liquid nitrogen
W	Wet – thawed -> wet biomass

Table 13: Shortened labels for storage description of samples used for every type of sample preparation listed in table_.

F1	**Stored in a refrigerator at 4°C**
F2	Stored in a freezer at -20°C
B1	Stored in a fume hood with constant exposure to light at room temperature
B2	Stored in a fume hood with no light exposure at room temperature

Each sample was prepared with 400 mg of epiphyte biomass and extracted using the method described in subchapter 2.3. Twelve samples of each preparation method were extracted, totaling 48 samples. These twelve samples were sorted into four storage methods, giving three parallel samples each. Before the first analysis for initial quantification, all samples were kept in a freezer at -20°C to prevent variable loss before the initial quantifications. After the initial quantification, each sample was placed into its assigned storage location, following the design shown in figure 28.

3.4.2 Initial quantifications (T_0)

Before storage, all samples were analyzed with the conditions in table 5, and the compounds fucoxanthin, lutein, and β-carotene were quantified. These compounds were chosen as they have been identified in subchapter 3.2. Initial quantification is interesting as it shows the number of carotenoids present in samples after different preparation.

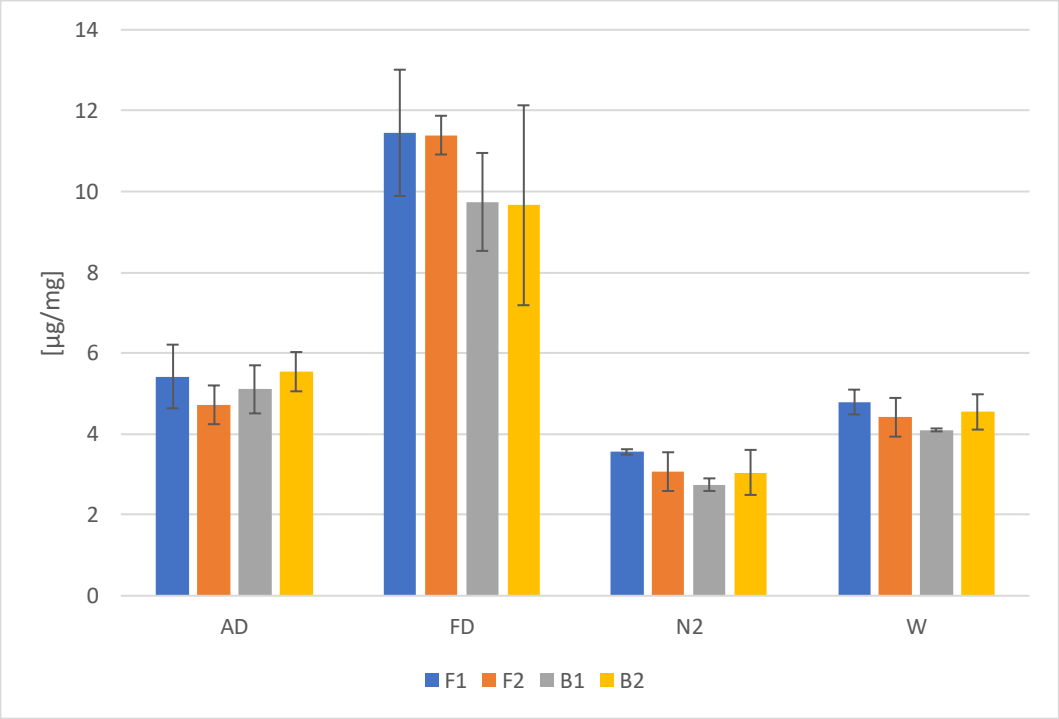


Figure 29: Average initial amounts (µg/mg) of fucoxanthin in air-dried (AD), freeze-dried (FD, N_2-frozen (N_2), and thawed samples (W), labeled with storage methods: in a refrigerator (F1), in a freezer (F2), on a bench in room temperature with exposure to lamp light and sunlight (B1), and on a bench at room temperature wrapped in aluminium foil (B2)

From the initial quantifications (T_0) of fucoxanthin, samples of the freeze-dried extract have the highest amount per mg DW (figure 29). This is followed by air-dried samples, thawed samples, and N_2-frozen. With the highest amounts, freeze-dried samples also have the highest standard deviations, especially freeze-dried on a bench with no light (FD-B2) (figure 29). It is expected that freeze-dried samples have a higher carotenoid amount, as discussed in subchapter 3.3.

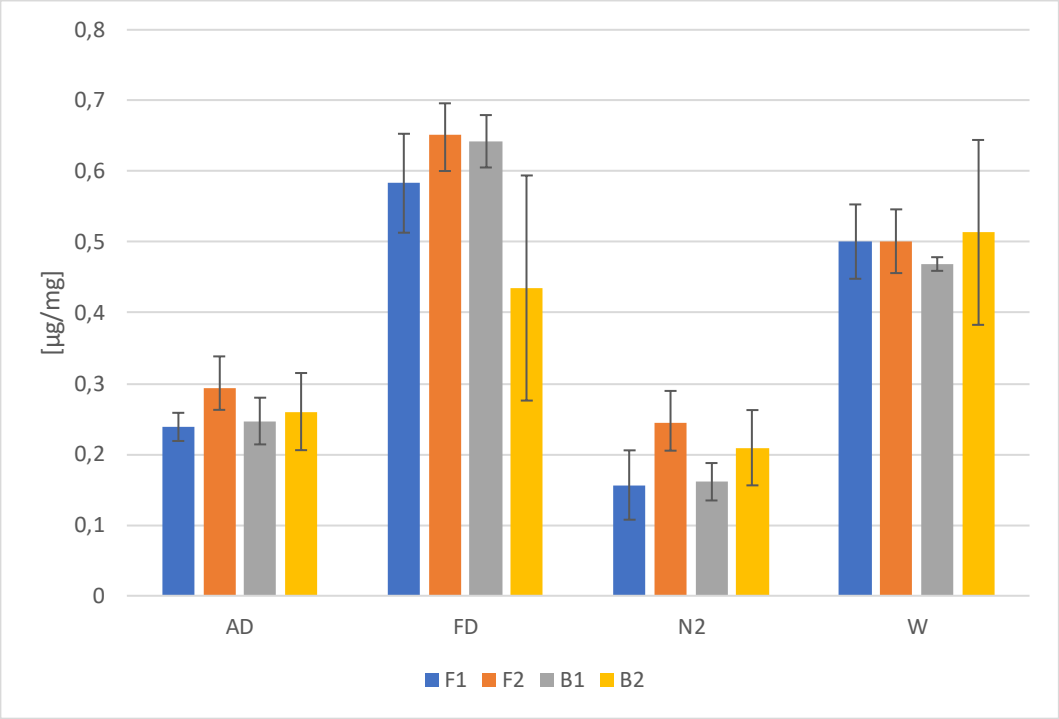


Figure 30: Average initial amounts (µg/mg) of lutein in air-dried, freeze-dried, N_2-frozen, and thawed samples, labeled with storage methods: in a refrigerator (F1), in a freezer (F2), on a bench in room temperature with exposure to lamp light and sunlight (B1), and on a bench at room temperature wrapped in aluminium foil (B2)

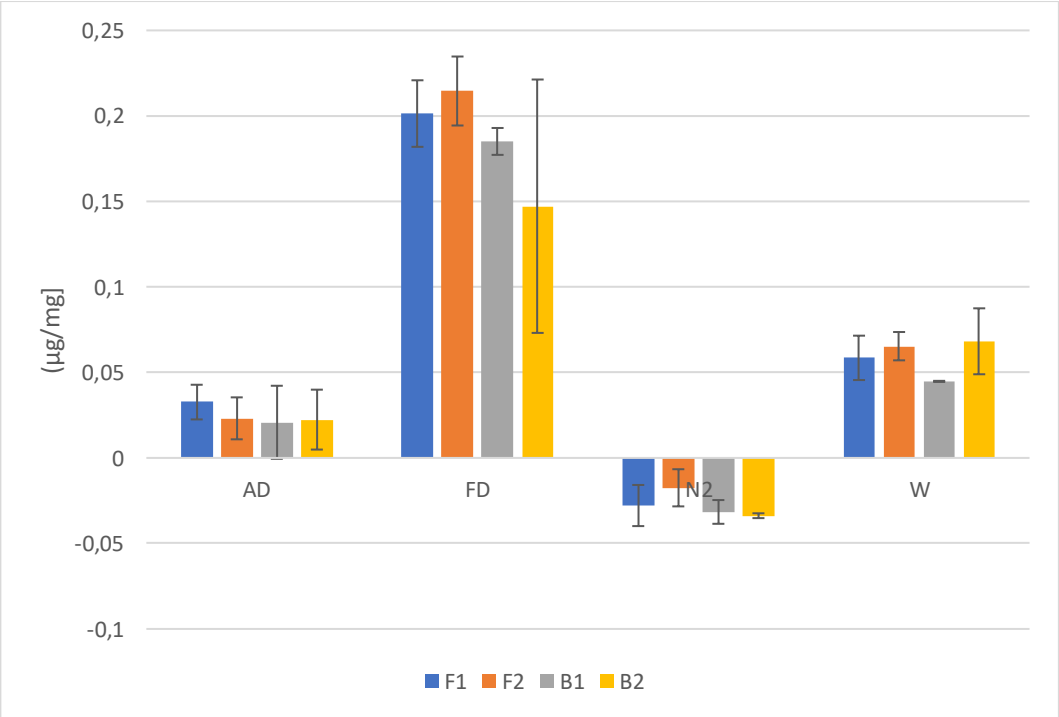


Figure 31: Average initial amounts (µg/mg) of *β*-carotene in air-dried, freeze-dried, N_2-dried, and thawed samples, labeled with storage methods: in a refrigerator (F1), in a freezer (F2), on a bench in room temperature with exposure to lamp light and sunlight (B1), and a bench at room temperature wrapped in aluminium foil (B2)

For initial lutein levels, freeze-dried and thawed extracts have the highest content, followed by air-dried and N_2-frozen with the least amount (figure 30). As the biomasses of FD and AD have the water removed, but N_2 and W have not, one cannot make a direct comparison as most of the mass in the wet samples are from water content. One study showed that algae can have a water content of 75.95-96.03 % [56]. Still, it is not sure why thawed biomass contains as much lutein. Lutein is a xanthophyll and is, therefore, a polar compound. Still, one should expect similar results from the quantified fucoxanthin amounts, as fucoxanthin is more polar than lutein (figure 2). This indicates that lutein and fucoxanthin behave differently in the sample preparation stage.

The initial quantification shows that only freeze-dried had a detectable amount of β-carotene after all sample preparation methods were performed, as all other values fell under the limit of quantification (figure 31). This explains why the values for N_2 drop below zero. The standard deviation for freeze-drying on the bench with light (FD-B2) is much higher than the rest, as seen in figure 29, 30, and 31.

From the three histograms (figure 29, 30, 31), freeze-drying is most effective for extracting carotenoids in the samples, while freezing the biomass with liquid nitrogen yielded the lowest quantities for all three compounds. While air-drying yielded a slightly higher amount of fucoxanthin (figure 29), the thawed samples had higher amounts of lutein and β-carotene (figure 30, 31). A previous study on extractions of carrots showed that fresh material yielded better carotenoid amounts than air-dried, but less for freeze-dried [51]. This indicates that fresh samples help extract more carotenoids than air-dried or N_2-frozen. Another study on different sample preparation methods on the brown algae *Phyllaria reniformis* showed that frozen material retained more carotenoids than dried and fresh material [57]. Using liquid nitrogen (N_2) as a way to freeze biomass for easier grinding proved unnecessary as it did not remove any water from the sample like thawing frozen biomass and retained less.

It is not sure why many of the B2 samples have higher standard deviations than the others (figure 29, 30, 31). As all samples have been stored in the freezer until initial quantification, storage should not be the cause. High standard deviation can result from poor extraction leading to high variation in carotenoid concentrations. As only freeze-drying of epiphyte biomass managed to help extract detectable amounts of β-carotene, only freeze-dried samples were analyzed in the β-carotene part of the storage experiment.

3.4.3 Storage of samples

In a three-week period, carotenoid contents of the known compounds fucoxanthin, lutein, and *β*-carotene were quantified once for each parallel sample. The system and conditions used in the analysis are detailed in table 5. Three parallel samples were made for each sample preparation and storage method, totaling 48 samples. The average signals of three parallel samples were plotted for each storage method to compare how each method impacted the carotenoid quantity of each sample preparation method (figure 32, 33, 34, 35).

3.4.3.1 Impact on fucoxanthin/Fucoxanthin stability

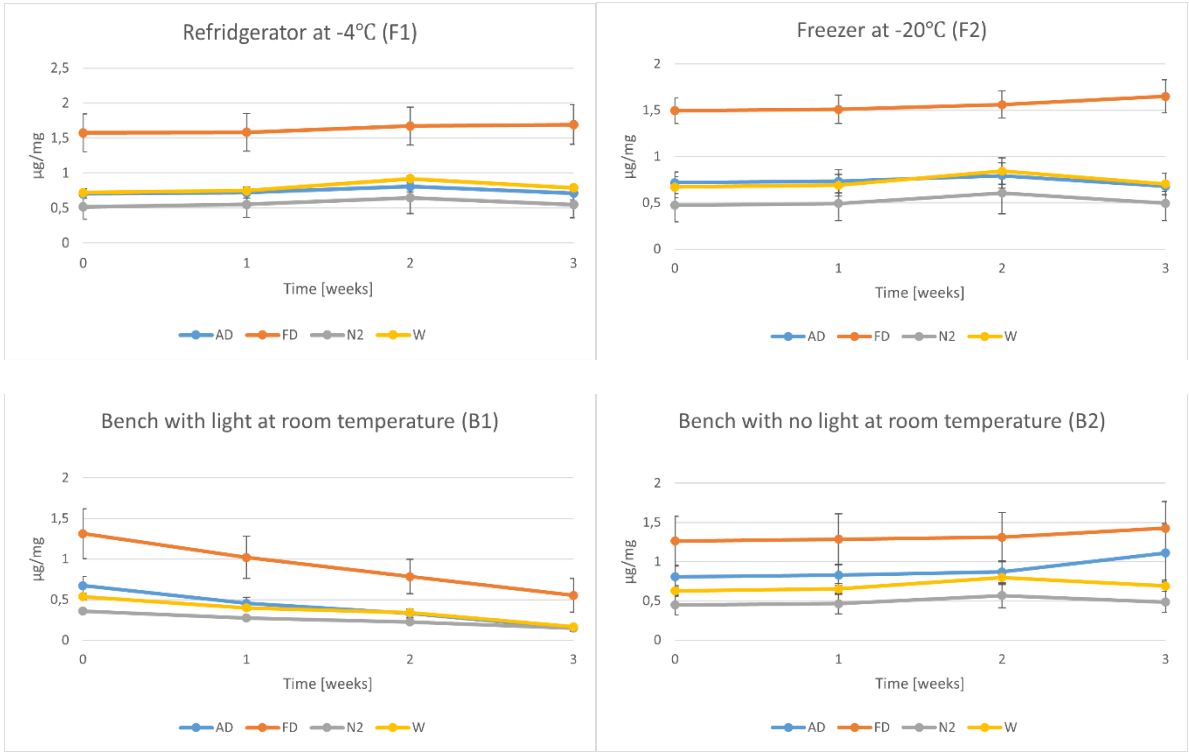


Figure 32: Stability plots showing the quantified amounts of fucoxanthin (µg/mg) measured each week for three weeks, including initial concentrations (T_0-T_3). Samples were stored in a refrigerator (F1), in a freezer (F2), on a bench exposed to light (B1), and on a bench with no light exposure (B2).

From the plotted results (figure 32), one can observe that F1, F2, and B2 have similar plots, with all sample preparation methods having stable amounts throughout the three-week storage period. Most comparable are plots for the refrigerator (F1) and freezer (F2), but F2 has much smaller standard deviations for freeze-dried (FD) samples, indicating that storing carotenoids under colder temperatures leads to lower variability. Room temperature with no light (B2) appears to retain fucoxanthin well but has the highest standard deviation comparing freeze-dried values (FD) with F1 and F2. The fucoxanthin amount in plot B2 air-dried samples (AD) rose in the last week, deviating from the thawed samples (W). Due to the high standard deviation in the last data point (T_3) for AD, this sudden increase might not be significant (figure 32). The stability plot for the bench with room temperature and light (B1) is significantly different from the other storage methods, and all samples decrease in fucoxanthin content over time (figure 32). Constant lamp light and daylight significantly affected the degradation compared to the bench with no light. All quantified values in stability plot B1 were above the level of quantification (LOQ) (see Appendix 6). This was also true for stability plots F1, F2, and B2 (figure 32).

3.4.3.2 Impact on Lutein/Lutein Stability

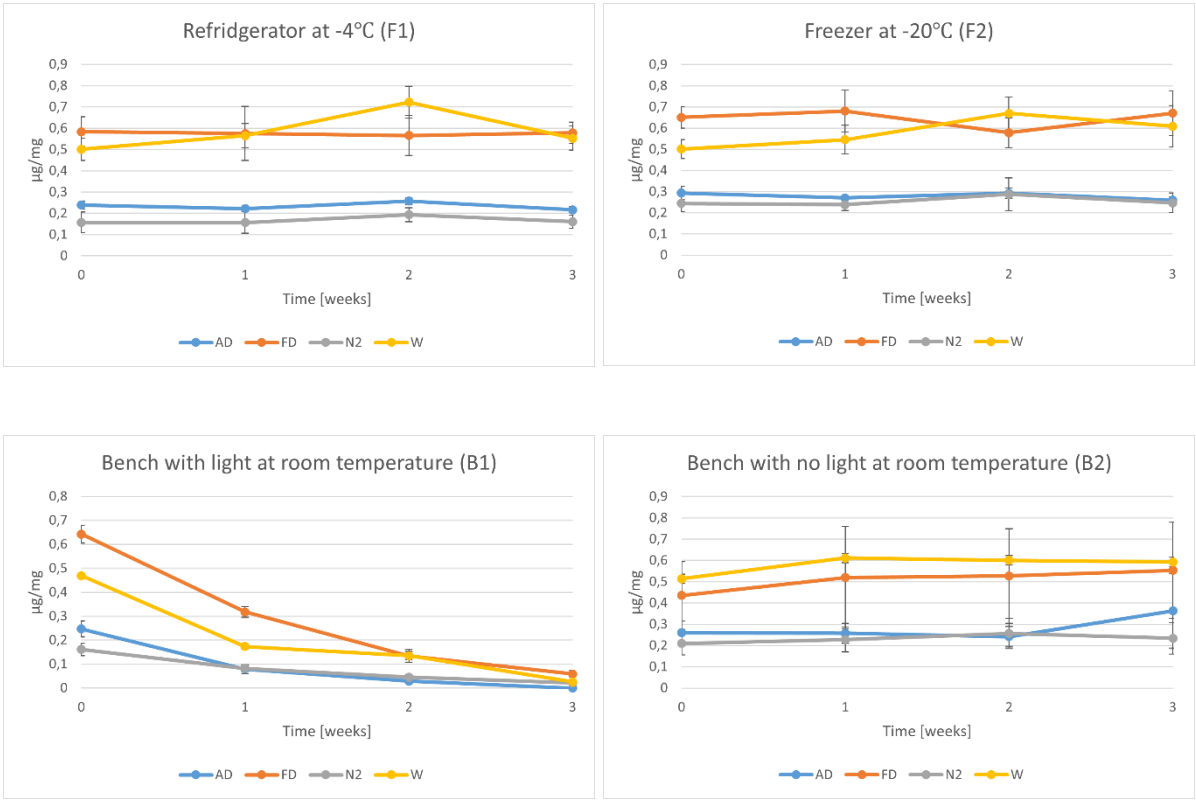


Figure 33: Stability plots showing the quantified amounts of fucoxanthin (µg/mg) measured for a three-week period, including initial concentrations (T_0-T_3). A: Samples stored in a refrigerator (F1). B: Samples stored in a freezer (F2). C: Samples stored on a bench exposed to light (B1). D: Samples stored on a bench with no light exposure.

As for the stability plots in fucoxanthin, the lutein plots F1, F2, and B2 have similar stability plots showing horizontal trends with different variability (figure 32, 33). As discussed in subchapter 3.4.2, the lutein amount is much higher in the thawed biomass (W) than fucoxanthin amounts in W (figure 33), resulting in freeze-dried and thawed biomass having similar amounts in the plots. Light also significantly impacts lutein, as shown when comparing the bench with (B1) and without light exposure (B2) (figure 33). B1 and B2 were at room temperature, but B1 was also exposed to sunlight and constant lamp light. Looking at concentrations, the samples for air-dried (AD) and N2-frozen (N2) fell under the limit of quantification after week 1 (T_1), while freeze-dried (FD) and thawed (W) fell under LOQ after two weeks (see appendix 7). All other signals after week two were rendered non-quantifiable. For plots F1, F2, and B2, all signals recorded were above the LOQ of lutein (see Appendix 7).

3.4.3.3 Impact on *β*-carotene/ *β*-Carotene stability

For the stability study of *β*-carotene, a three-day separate study was performed on the bench with light (B1). The change in time interval was due to *β*-carotene degrading too quickly and did not need a three-week study (see Appendix 8). The initial concentration study showed that only freeze-drying led to extracting a detectable amount of *β*-carotene(See Appendix 8). Therefore, only freeze-dried extracts were stored and analyzed for *β*-carotene.

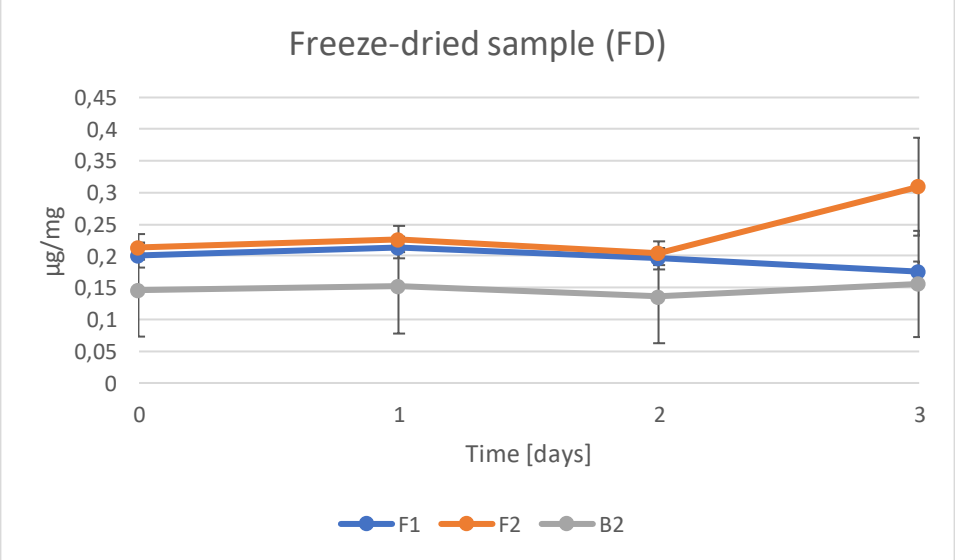


Figure 34: Stability plot of *β*-carotene in freeze-dried samples (FD) at different storage conditions. Initial conditions and analysis every week in a three-day. Storage conditions: in a refrigerator (F1), in a freezer (F2), and on a bench with no light exposure (B2).

In the three-week stability study for β-carotene in the storage conditions F1, F2, and B2, the stability plots show three stable storage conditions from initial concentration to week two (T_2) (figure 34). After week two, the β-carotene amount in the freezer-stored sample increased while the sample on the bench with no light exposure slightly declined. The small reduction is significant as the standard deviations of these samples are smaller than the decline and can therefore be explained as degradation. The increase of β-carotene in F2 has a high standard deviation but is insufficient to explain the significant increase in week 3. It is unclear why this incline happens, as a previous study shows that β-carotene is expected to degrade over time [58].

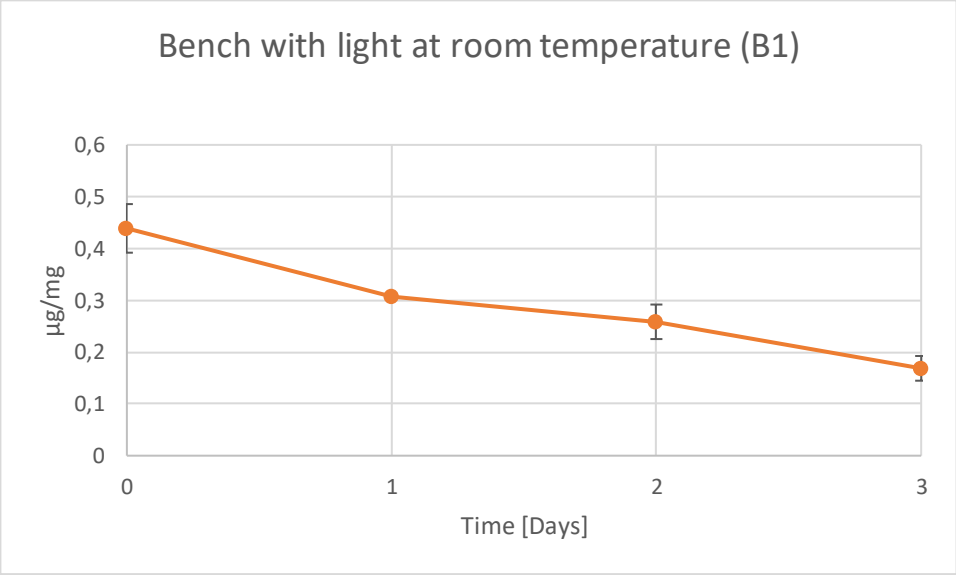


Figure 35: Stability plot for a three-day stability study of β-carotene content in freeze-dried extracts (FD). All samples were stored at room temperature and exposed to sunlight and constant lamp light (B1). Day 1 does not contain a standard deviation due to data loss.

The stability plot for β-carotene in freeze-dried extract (FD) stored on a bench at room temperature exposed to light (B1) (figure 35) shows a steady decrease of β-carotene over three days. The amount of β-carotene is reaching the limit of quantification on day three and is expected to go below LOQ on day 4, projected from the trend made by the data points from concentrations (see appendix 9). Compared to the plots in the 3-week study, there is a clear difference between exposing β-carotene to light (figure 34, 35). Comparing fucoxanthin, lutein, and β-carotene, β-carotene is the pigment that reached the LOQ first, but this does not have to mean that β-carotene degrades quicker as the samples contain more fucoxanthin than β-carotene, and each calibration curve has different levels of LOQ (table 8). A study on the stability of carotenoid powders found that the β-carotene does have a faster degradation rate than lutein [58]. This supports the findings in the study, as lutein and β-carotene have comparable amounts

in freeze-dried samples. A kinetics study is needed to create models showing how each carotenoid degrades over time.

3.4.3.5 Statistical analysis

From the stability plots in subsection 3.4.3, it was observed that storage methods refrigerator (F1), freezer (F2), and bench with no light exposure (B2) had similar stability. It is, therefore, unclear if the different storage methods have a significantly different impact on the degradation of the three carotenoids. To determine if a different storage method impacts the contents of carotenoids in samples, statistical analysis has been used to look at the variance in multiple groups to determine whether there is a significant difference between these. The groups in question are samples with the same sample preparation method but with different storage conditions.

To analyze if there is a significant difference between the variances of storage conditions, ANOVA (analysis of variance) was used on three groups of data, namely T_0-T_3 data from the storage conditions refrigerator (F1), freezer (F2), and bench with no light exposure (B2) for freeze-dried (FD), air-dried (AD), N_2-frozen (N2) and thawed samples (W). The null hypothesis used in the ANOVA test was that none of the storage conditions are significantly different. After performing the ANOVA test, it is determined if there is a statistical difference between some of the three groups, but not which of the three groups are (see appendix 10, 11, 12). Therefore, the post hoc test, called the Bonferroni correction, and a t-test were used to figure which groups are significantly different based on the comparison of mean values.

For a test to reject the null hypothesis, the F-score must be higher than the F-critical value, and the P-value must also be under 0.05 for there to be a significant difference. These values have been considered when deciding whether the groups reject the hypothesis or not. Values for calculated F and P values can be found in appendix 10, 11, and 12.

Table 14: Post Hoc (Bonferroni) test performed on samples under F1, F2, and B2 storage conditions. The sample preparation methods were shown in the ANOVA test to have a significant difference in sample preparation. The answers "yes" or "no" signifies if the Post Hoc test found a significant difference between the storage conditions. "no" means the null hypothesis is not rejected, meaning there is no significant difference between the groups. "yes" rejects the hypothesis and concludes that there is a difference.

	Fucoxanthin		**Lutein**		**β-Carotene**
Storage conditions	**AD**	**FD**	**N2**	**FD**	**FD**
F1 vs. F2 [Significant?]	Yes	No	Yes	No	No
F1 vs. B2 [Significant?]	No	Yes	Yes	No	Yes
F2 vs. B2 [Significant?]	No	Yes	No	Yes	Yes

ANOVA and post hoc were performed on groups for fucoxanthin, lutein, and β-carotene plots. From table 14, it is shown which sample preparations had groups where there was a significant difference between storage conditions. For *β*-carotene, only freeze-dried samples had any significant differences in variation between being stored in a refrigerator (F1), freezer (F2), or on a bench exposed to light (B2). There was a difference in variance for lutein for N2-frozen (N2) and freeze-dried (FD). Air-dried (AD) and freeze-dried (FD) fucoxanthin had significant differences.

For β-carotene, there was a significant difference between storing freeze-dried samples in the refrigerator and on a bench with no light and between a refrigerator and bench with no light (table 14). Therefore, no significant difference is found between storing *β*-carotene in a refrigerator (F1) and a freezer(F2). For lutein, a significance was found between storage conditions for N2-frozen (N2) samples and for freeze-dried (FD). Freeze-dried samples significantly differed when comparing freezer (F2) and bench with now light (B2). This means there was no significant difference in the degradation of lutein when freeze-dried extracts were stored in a refrigerator vs. freezer and refrigerator vs. bench with no light. For N2-frozen samples, it was found a significant difference between the storage conditions freezer (F2) vs. refrigerator (F1) and refrigerator (F1) vs. bench with no light exposure (B2). There was no significant difference in lutein degradation between storing N2-frozen samples in the freezer (F2) and storing samples on the bench with no light. Fucoxanthin found a significant difference in variance for freeze-dried (FD) and air-dried (AD) groups under different storage conditions. For freeze-dried samples, it was found a significant difference in mean values between refrigerator (F1) and bench with no light exposure (B2) and freezer (F2) and bench with no light exposure. There is, therefore, no significant difference between storing freeze-dried samples in a freezer vs. refrigeration for fucoxanthin degradation. For air-dried samples (AD), it was found

that only refrigerated samples (F1) had a significant difference compared to samples stored in the freezer (F2)

The statistical analysis shows that freeze-dried samples are affected mainly by different storage conditions, as there was a significant difference for freeze-dried samples in all three carotenoids. Except for N2-frozen samples (N2) for lutein and air-dried for fucoxanthin (AD) finding, a significant difference between storing in a freezer (F2) and a refrigerator (F1), no other sample preparation extracts for lutein, fucoxanthin, and β-carotene found a difference between these conditions. Therefore, storing in a freezer or a refrigerator has no discernible difference in a 3-week period for freeze-dried. A stability study over a more extended period should be conducted to understand the degradation process better.

From the statistical analysis, differences have been found between different storage methods through statistical significance. Still, as one can observe in the stability plots (figure 32-34), not much has affected the carotenoid amount in the samples stored in a refrigerator (F1), freezer (F2), and on a bench with no light exposure (B2). In a study on carotenoid stability of the microalga *Phaeodactylum tricornutum*, four different storage conditions were used, including storing at -20℃, 4℃, 20℃ and 4℃ with no vacuum packing [59]. After 35 days of storage, it was concluded that no storage conditions had any significant difference. This supports the findings in this project, as the carotenoid amounts in samples have been stable for all samples not exposed to light. A study on the kinetics of carotenoid degradation in einkorn and bread wheat found that the total carotenoid content was affected by a change in temperature after long storage periods [55]. The temperatures -20 ℃, 5℃, 20℃, 30℃ and 38℃ were applied to the samples for 239 days, resulting in a total loss of 2.9% for the sample at -20℃, and a loss of 71% for the sample stored at 38℃ [55]. Temperatures above room temperature and longer periods are therefore needed to observe the degradation of carotenoids in samples better.

Chapter 4. Conclusion and further work

4.1 Conclusion

The compound fucoxanthin, lutein, and β-carotene were identified with four unknown carotenoid-like signals in the qualitative analysis of algal epiphytes. The first unknown was suggested to be the compound neoxanthin, the second was proposed to be 9-cis-fucoxanthin, the third was suggested to be lactucaxanthin, and the fourth was suggested to be zeinoxanthin, antheraxanthin, and cryptoxanthin. It is also possible that the fourth unknown compound is an isomer of β-carotene.

The quantitative analysis found that powdered samples of freeze-dried material gave the best extraction of carotenoids when compared to freeze-drying and air-drying. Fucoxanthin was the most abundant carotenoid in the samples, and because the biomass mainly consists of red algae, it was concluded that fucoxanthin came from parts of L. hyperborea under the removal of epiphytic material from the stipe. A correlation between the fucoxanthin and the second unknown carotenoid-like compound was found, suggesting a correlation between the two compounds.

The three-week stability study found that temperatures -20℃, -4℃, and room temperature had a small effect on the stability of carotenoids. The light significantly impacted stability as exposure to lamp light and daylight caused degradation of fucoxanthin, lutein, and β-carotene. Statistical analysis found that freeze-dried samples were mainly affected by different storage conditions compared to air-dried, thawed, and N_2-frozen biomass. For freeze-dried samples, no significant difference in stability was found between samples stored in the freezer vs. refrigerator. A distinction was found between freezer vs. bench with no light exposure and refrigerator vs. bench with no light exposure. β-Carotene was found to degrade quicker than lutein, but kinetic models are needed to give further evidence on this observation.

4.2 Further work

4.2.1 Qualitative analysis

In the characterization of unknown carotenoids in subchapter 3.2, more work should be done to identify the four selected peaks u1-u4. The λ_{max} values found in the UV-Vis spectrum, comparative λmax values, and fine structure in literature are insufficient to identify a specific compound. Due to some compounds having equal chromophores, but different molecular formulas, a matching fine structure cannot be used to distinguish compounds based on UV-Vis data only. This is why multiple compounds were suggested for peak u4. To discern between the proposed compounds, HPLC with a mass spectrometry detector (MS) can be used as it detects compounds based on mass. Therefore, two compounds with an equal chromophore but different molecular formulas can be distinguished. One can use the mass-to-charge ratio found in literature and utilize Selective Ion Monitoring (SIM) to look for matching signals from the suggested compounds. This method allows you to look for specific masses instead of a wide range, minimizing noise and increasing sensitivity.

For structural identification, NMR spectroscopy can be utilized to analyze the structure of different carotenoids with both 1D and 2D spectra of carbon and hydrogen. Analyzing the structure of the molecules can help distinguish carotenoids containing various functional groups and different constitutional isomers. A problem with NMR spectroscopy for carotenoids is that they all have similar structures, making it challenging to discern compounds in a complex sample. It is, therefore, important that the carotenoids are separated. This can be done using preparative HPLC, separating compounds based on retention times.

As solvents influence the λ_{max} values in a fine spectrum, different solvents should be used in samples and compared to see how the λ_{max} values and overall fine structure change. This can be important as variable λ_{max} values can make it harder to discern compounds from each other.

4.2.2 Quantitative analysis

Seasonal variation was not explored in this project when quantitative analysis was performed. The time of harvest impact the natural product content in algae as the biological activities change with the seasons. Mapping the contents of algal epiphytes throughout the year can give information about when epiphytes are more lucrative to process and extract, which is interesting for algae processing industries.

Different sample preparation methods and epiphyte biomasses were analyzed when performing quantitative analyses, but the same extraction technique was used throughout the project. As the extraction method was designed for microalgae, more can be done to change the extraction method for it to suit the extraction of larger amounts of biomass better, as macroalgae were explored in the project. For health and safety concerns, it might be of interest to chemists to swap some of the solvents in the extraction method with less hazardous chemicals if the extraction method is used extensively. One proposal is to change chloroform with dichloromethane. The calibration curves should be made from a less volatile solvent to reduce a potential increase in residual standard deviation and give a better limit of quantification.

4.2.3 Stability study

The storage conditions refrigerator (F1), freezer (F2), and bench with no light exposure (B2) were effective at preventing the carotenoids from degrading in the three-week period. Therefore, a study with a longer time interval should be performed as a short time interval like T_0-T_3 will keep carotenoids stable, and differences in amount cannot be explained due to standard deviation.

The temperature did not have a noticeable effect on the carotenoid stability in this project. Previous studies it has been shown that increasing heat will affect stability. A study with the introduction of heating samples of different temperatures higher than room temperature would give more insight into how this parameter affects the stability of carotenoids in epiphytic algae.

As carotenoid degradation leads to derivatives, more time should be spent on observing, characterizing, and analyzing potential isomers of carotenoids that might have been under storage. This could have given insight into how the different carotenoids degraded over time.

Chapter 5. References

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Chapter 6 Appendix

Appendix 1: λ_{max} values and retentions times of carotenoid standards found in standard mixture chromatogram.

Assigned number	Compound	Retention Time [min]	LUV-VISmax [nm]	UV-VISmax [nm]	LUV-Vismax [nm]
1*	Fucoxanthin	7.739		448	466
2*	Lutein	17.146	422	444	470
3*	Zeaxanthin	19.		450	476
4*	β-Carotene	31.065		452	478

Appendix 2: λ_{max} values and retentions times for carotenoid and carotenoid like compounds found in the chromatogram of a freeze-dried sample.

Assigned no.	Compound	t_R [min]	LUV-VISmax	UV-VISmax	LUV-VISmax
1	Fucoxanthin	7718		446	466
u1	Unknown carotenoid-like	10.049	412/414	438	468
u2	Unknown carotenoid-like	11.350		444	462
2	Lutein	17.134	422	444	472
u4	Unknown carotenoid-like	27.918		444	474
3	β -carotene	31.087		452	478

Appendix 3: λ_{max} values and retentions times for carotenoid and carotenoid like compounds found in the chromatogram of a powdered sample.

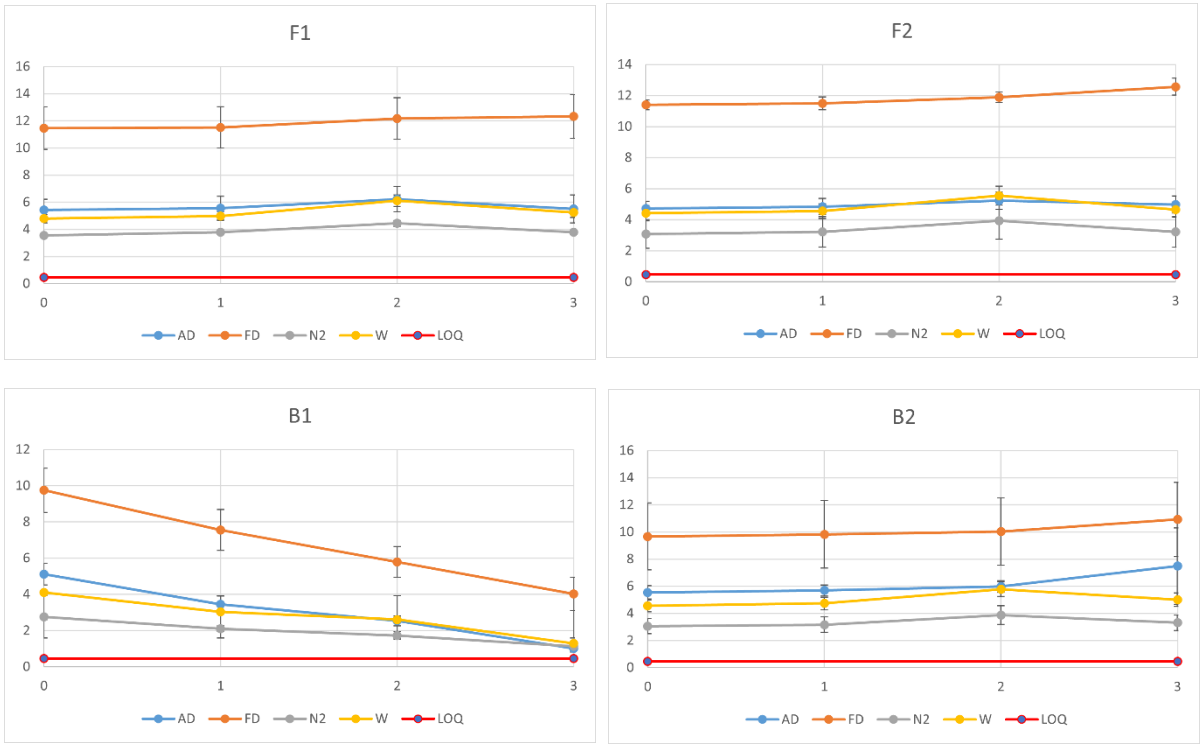
Assigned no.	Compound	t_R [min]	LUV-VISmax	UV-VISmax	LUV-VISmax
1	Fucoxanthin	7.721		448	466
u2	Unknown carotenoid-like	11.401	412/414	444	462
2	Lutein	17.152	422	444	472
u3	Unknown carotenoid-like	22.253		440	468
u4	Unknown carotenoid-like	27.950		444	474
3	β -carotene	31.097		448	478

Appendix 4: λ_{max} values and retentions times for carotenoid and carotenoid like compounds found in the chromatogram of an air-dried samples.

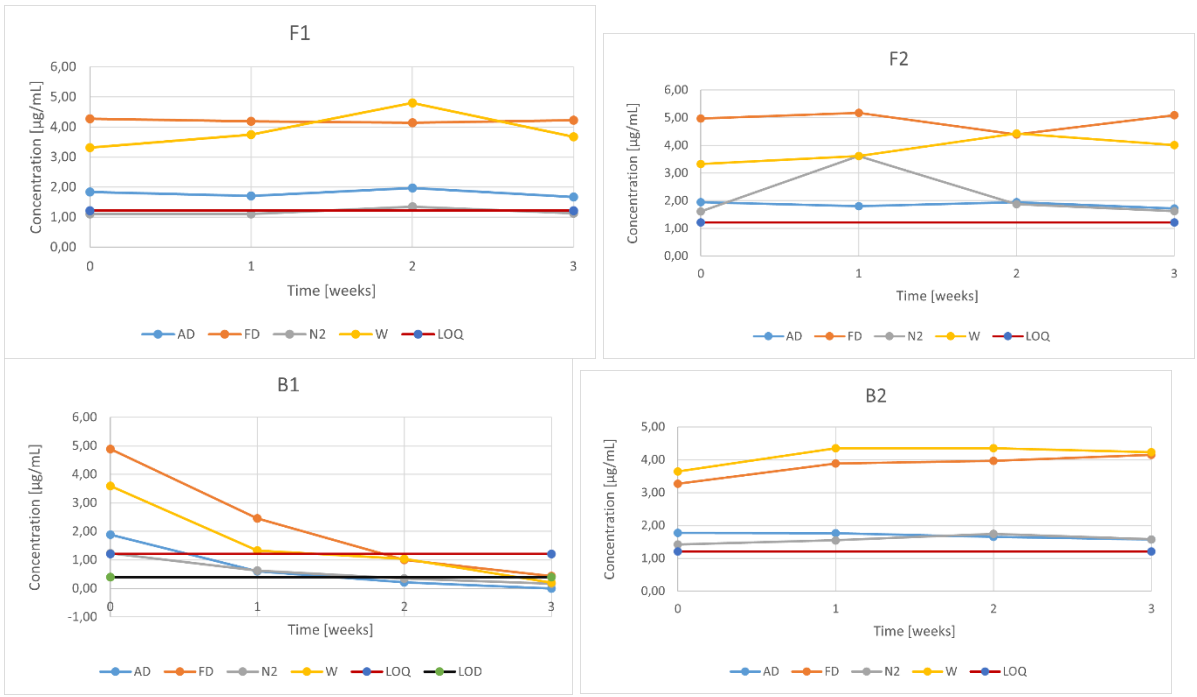
Assigned no.	Compound	t_R [min]	LUV-VISmax	UV-VISmax	LUV-VISmax
1	Fucoxanthin	7.738		448	466
u1	Unknown carotenoid-like	10.088	414	436	468
u2	Unknown carotenoid-like	11.431		446	
2	Lutein	17.431		444	472
u4	Unknown carotenoid-like	28.030		446	474
3	β -carotene	31.125		448	478

Appendix 5: λ_{max} values and retentions times for carotenoid and carotenoid like compounds found in the chromatogram of a *Laminaria hyperborea*.

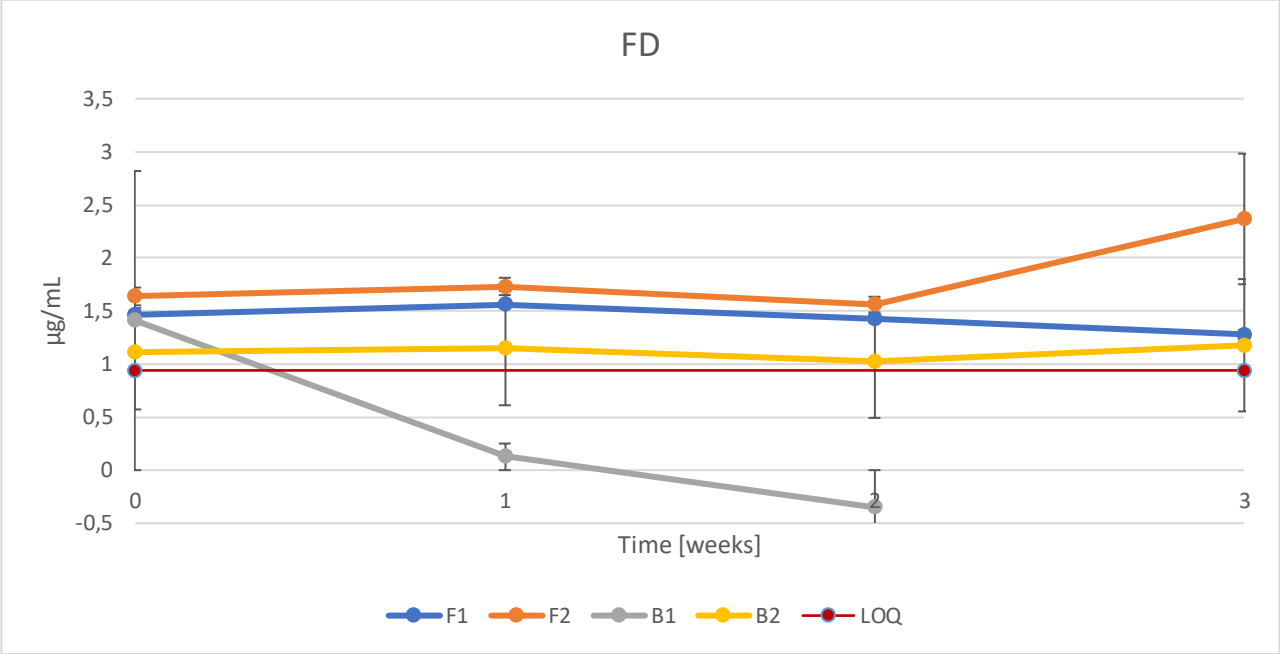
Assigned no.	Compound	t_R [min]	LUV-VISmax	UV-VISmax	LUV-VISmax
1	Fucoxanthin	7.751		448	466
u2	Unknown carotenoid-like	11.463		444	462



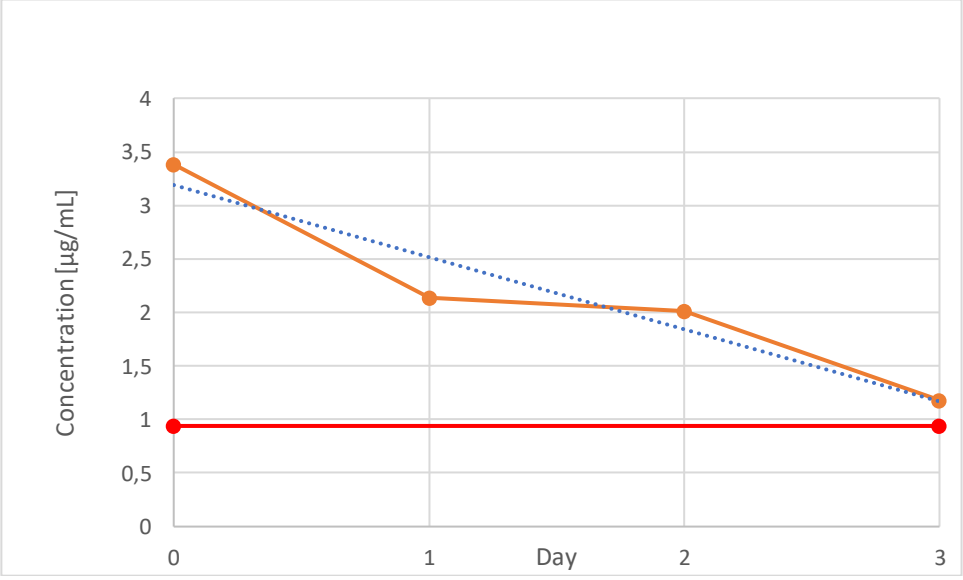
Appendix 6: Measured concentrations of fucoxanthin in samples over a three-week period with four different sample preparation methods.



Appendix 7: Measured concentrations of lutein in samples over a three-week period with four different sample preparation methods.



Appendix 8: Three-week stability study of β-carotene in freeze-dried (FD), air-dried (AD), N2-froze, (N2), and thawed biomass (W) stored in four different storage conditions. Conditions were in the refrigerator (F1), in a freezer (F2), on the bench with light exposure (B1), and on a bench without light exposure (B2).



Appendix 9: Stability plot for a three-day stability study of β-carotene in a freeze-dried extract (FD). Samples were stored at room temperature exposed to sunlight and constant lamp light (B1).

Appendix 10: Calculated values using one tailed ANOVA analysis of amounts of fucoxanthin under the storage conditions F1, F2, and B2 for AD, FD, N2, and W.

Fuco	F-value	P-value	F-critical
AD	4,683324	0,04036052	4,256494729
FD	15,297853	0,00127255	4,256494729
N2	2,70182939	0,12049349	4,256494729
W	0,81166577	0,47415243	4,256494729

Appendix 11: Calculated values using one tailed ANOVA analysis of amounts of lutein under the storage conditions F1, F2, and B2 for AD, FD, N2, and W.

Lutein	F-value	P-value	F-critical
AD	2,27322968	0,15880903	4,256494729
FD	11,5365316	0,00328442	4,256494729
N2	20,8667575	0,00041712	4,256494729
W	0,00612748	0,9938954	4,256494729

Appendix 12: Calculated values using one tailed ANOVA analysis of amounts of β-carotene under the storage conditions F1, F2, and B2 for AD, FD, N2, and W.

Beta	F-value	P-value	F-critical
FD	9,41355497	0,00622279	4,256494729