The quantification of fucoxanthin from selected South African marine brown algae (Phaeophyta) using HPLC-UV/Vis

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List of Abbreviations

ax	Axial
Caco-2	Human epithelial colorectal adenocarcinoma cells
CAM	Complimentary Alternative Medicine
CAS	Chemical Abstract Service
DEPT	Distortionless Enhancement by Polarized Transfer spectroscopy
DLD	Human adenocarcinoma cells
DPPH	1-diphenyl-2-picrylhydrazyl radical
EC_{50}	Half maximal effective concentration
EMEA	European Medicines Agency
ESR	Electron Spin Resonance
EtOAc	Ethyl acetate
FDA	Food and Drug Administration
G0/G1	Gap 0/ Gap 1 (resting phase in the cell cycle)
GC	Glencairn, Cape Town
HepG2	Liver hepatocellular carcinoma
HL-60	Human Promyelocytic Leukemia cells
HMBC	Heteronuclear Multiple Bond Correlation spectroscopy
HPLC	High Performance Liquid Chromatography
HPLC-UV/Vis	High Performance Liquid Chromatograph – Ultraviolet/Visible
HSQC	Heteronuclear Single-Quantum Correlation spectroscopy
HT-29	Human adenocarcinoma cells
ICH	International Conference on Harmonization
KB	Kommetjie Bay, Cape Town
KOS	Kenton On Sea, Kenton
LOD	Limits of Detection
LOQ	Limits of Quantitation
MAE	Microwave Assisted Extraction
MCC	Medicine Control Council

MeOH	Methanol
MHz	Megahertz
MTBE	Methyl tert-butyl ether
NDK	Noordhoek, Port Elizabeth
NOESY	Nuclear Overhauser Effect spectroscopy
PA	Port Alfred
PC-3	Human Prostate Cancer cells
PLE	Pressurized Liquid Extraction
PTFE	Polytetrafluoroethylene
RI	Refractive Index
ROS	Reactive Oxygen Species
RSD	Relative Standard Deviation
SFE	Supercritical Fluid Extraction
SLE	Solid Liquid Extraction
SOX	Soxhlet
spp.	Species
TLC	Thin Layer Chromatography
TS	Three Sisters, Port Alfred
UAE	Ultrasound Assisted Extraction
UCP	Uncoupling protein
UV	Ultra violet
WAT	White Adipose Tissue
WHO	World Health Organization

Abstract

Marine brown algae (seaweeds) are a rich source of fucoxanthin, a xanthophyll carotenoid that is naturally, an accessory pigment in the process of photosynthesis of sea vegetation such as *Sargassum incisifolium*. Fucoxanthin has been exploited by nutraceutical companies for its anti-obesity effects that has resulted in an increase of seaweed slimming preparations such as FucoThin™. The field is getting widespread consumer attention as interest in fucoxanthin has also transcended to its widespread biological potential which include cytotoxicity, anti-diabetic, anti-oxidant, anti-inflammatory and anti-plasmodium effects. We therefore wanted to identify a reliable source(s) of fucoxanthin from diverse samples of South African marine brown algae in order to explore our medicinal chemistry interests around the cytotoxicity and anti-malarial potential of fucoxanthin.

A known source, *Sargassum incisifolium*, was used to isolate (maceration in CH_2Cl_2/MeOH at 35 °C followed by a hexane/EtOAc step gradient silica column of the crude extract and reversed phase semi-prep HPLC) and characterize (1D and 2D NMR) fucoxanthin (reference standard) in order to develop an analytical method for its determination in selected diverse brown algae commonly found in South Africa. The HPLC [Column: Phenomenex® Synergi™ (250 x 3.0 mm i.d); Mobile phase: ACN/H2O (95:5)] method developed for this analysis was validated according the guidelines set by the International Conference on Harmonization (ICH). Fifteen species were then assessed for fucoxanthin content (µg/g of dried weight) using the developed method. Stability studies on fucoxanthin were also carried out to assess photo- and pH degradation of fucoxanthin.

Zonaria subarticulata (**KOS130226-18**) from Kenton-On-Sea beach and *Sargassum incisifolium* (**PA130427-1**) from Port Alfred beach were found to be the highest producers of fucoxanthin with 0.50 mg/g and 0.45 mg/g dried weight respectively. Fucoxanthin was found to be both photo-labile and sensitive to both acidic and basic pH environments. However, the pigment was more photostable in pure as opposed to extract form and also showed to be more stable at pH 10.0.

Our findings show that *Z. subarticulata* and *S. incisifolium* could be reliable sources of fucoxanthin and can be considered as the algae to use in optimized extraction procedures in further studies. Also, when working with fucoxanthin, it is important to protect it from light. Any consideration

of taking fucoxanthin preparation orally (as a nutraceutical) should consider protecting the active from the harsh conditions of the gastrointestinal tract. Any upscale production of fucoxanthin from seaweed should consider variations such as geographical, seasonal, lifecycle stage, *etc*. of identified algae as these may be important factors in obtaining effective concentrations of fucoxanthin.

Chapter 1

General Introduction

1.1. Natural Products

A natural product may simply be defined as a molecule that is produced by an organism. Natural products have been divided into primary and secondary metabolites. Secondary metabolites are the class of natural products that has been significant in drug discovery. They have been defined to be any substance derived from nature and is produced from metabolic pathways that are not directly responsible for the organism's growth and reproduction (Fraenkel, 1959). Their occurrence is sporadic but may be specific for a certain genus or family *e.g.* tannins, alkaloids, essential oils and carotenoids. It is difficult to separate the organisms' metabolic pathways that produce secondary metabolites (natural products) and those that produce the primary metabolites responsible for growth and development of the organism *e.g.* sugar and proteins as both co-exist to enhance the organism hence the term "metabolome" which encompasses both (Hadacek, 2002).

Nonetheless, humans over the ages have made use of their environment to sustain their existence. The reliance on nature for their basic needs is evident *i.e.* shelter, clothing, transportation and, equally important, medicine (Newman *et al*., 2000). Different cultures, based on their unique surrounding, developed traditional medicinal systems which have contributed to a rich folklore. The origins of the use of plants for medicinal purposes have been reported to belong to pre-Hellenic civilizations dating back to as early as 2900 *BC* (Newman *et al.*, 2000). To touch on this briefly, Mesopotamia is one of the earliest civilizations to make use of over 1000 plant derived substances *e.g.* oils which include *Cedrus* spp. (cedar) and *Papaver somniferum* (poppy juice) while the Egyptian "*Ebers Papyrus*" dating back to 1500 *BC* contain over 700 drugs of plant origin according to Newman *et al*. (2000). In addition, China, India and the Arab world had remarkable contributions to medical folklore (Sneader, 2005). But what was probably a crucial turning point was the Greek's rational development of the use of herbal medicines in the early centuries (*AD*) *e.g.* the writings of Galen and Ibn Sina which was progressively developed, challenged and bettered to give rise to the idea of single entity compounds characterized by the isolation of morphine in the 1800s (Newman *et al.*, 2000). Pharmacologically active compounds from plants

became readily available in the beginning of the 19th century. The challenges of sustaining the production of natural products, overcoming the toxic side-effects, advances in organic and combinatorial chemistry *etc*. were some of the main reasons for the decreased reliance on nature for therapy. A number of research organizations maintained a presence in this field and as a result there is a resurgent interest in the use of nature to develop pharmaceuticals including new and better ways to manipulate the production of natural products (Ganesan, 2004).

1.2. Seaweeds

1.2.1. Classification

Seaweed is a colloquial term given to a type of vegetation found in the sea. Vegetation in the sea is mainly divided biologically into two main groups, benthos (attached form and mainly multicellular) and phytoplankton (free living and mainly unicellular). There is some crossover but that will not be discussed in this text. Algae is another term given to sea vegetation as it is mainly found in this form and are subsequently divided into macroalgae (seaweeds) and microalgae (phytoplankton). Both types are photosynthetic organisms (Levring, 1979). What is mainly important however to our current study is the classification of seaweeds, "in order analyses may bear fruit, it is necessary that the right type of alga should be recognizable and recognized, its course of life understood and its reproductive periods utilized to the full" (Delf, 1943).

In the old systematic classification of algae (early 19th century and prior), color was not allowed as a feature of distinction. Only in 1836, were algae classified into three main groups of green, brown and red algae. Today seaweeds fall into three main phyla *i.e.* Chlorophyta, Phaeophyta and Rhodophyta. Chlorophyta (first class Chlorophyceae) which comprises of the green algae, contains pigments that resemble those of the higher terrestrial plants. Phaeophyta (first class Phaeophyceae) comprises of the brown algae which are complicated multicellular bodies and are often of considerable size. This group is the focus of this study and does contain chlorophyll as the main photosynthetic pigment however; it is masked by high levels of yellow and brown pigments (Boney, 1966; Tilden, 1933). As a result of dominating carotenoids, the algae take a more brownish color. One such interesting dominating pigment is fucoxanthin, the primary focus of this study.

Rhodophyta (subclasses Bangioideae and Floriceae) have dominating red pigments and they are the most abundant of the three divisions (Bolton and Stegenga, 2002). There have been multiple sub-divisions, but the above mentioned divisions are accepted as the main divisions of seaweeds (Levring, 1979; Tilden, 1933). Pigmentation therefore plays a primary role in imparting color to algae and hence their classification. The varying quantity of pigments in algae as needed by the plant, has been described as heritable and can be traced taxonomically (Tilden, 1933). The photosynthetic products (controlled by pigments) are in turn a way of classification. Green algae produce starch, brown algae, laminarin and red algae, Rhodophycean starch. Other methods of classification *i.e.* ultrastructure, morphology, DNA homology, chromosome numbers *etc*. are also used by phycologists (Levring, 1979).

1.2.2. Uses of seaweeds

The extent of use of seaweeds in human society is remarkable and includes its use as food (*e.g.* "Kombu" in East Asian *Laminaria* spp., "Wakame" of *Undaria* spp., and the "Kelp meal" of *Laminaria* spp.), fodder, manure, industrial raw material (*e.g.* agar, carageenans, alginic acid, fucoidan, mannitol and iodine) and probably most relevantly its use for medicinal purposes. Brown algae have been part of medical folklore in many ways (Hoppe, 1979).

Table 1.1: Selected seaweeds and their uses

Seaweed	Medicinal Purposes	Reference
Ascophyllum nodosum:	A drug constituent in obesity preparations.	Hoppe, 1979
Eisenia bicyclis:	The crude extract possesses anti-inflammatory and anticurare activities.	
Laminaria spp.:	Used in Japanese folklore for lowering blood pressure and as "Hai-tai", "Kai-wan" by the Chinese for menstrual difficulties.	
Laminaria japonica:	used in South China for producing a cooling and blood cleaning effect for treatment of glandular weakness and normalizing blood pressure.	
Sargassum natans:	Used in South America for medicinal preparations to cure goiter and renal disorders whilst *Sargassum liniifolium* in India is used for urinary and calculous diseases.	

The use of seaweeds in medicine is therefore not new, neither is the discovery of the bioactives in seaweed responsible for their use in medical folklore. It is the continued discovery of bioactives, despite the abandonment of programs relating to drug discovery from natural sources (mid-20th century) that has opened new, perhaps important and potentially sustainable possibilities in the field. Not only did that resurgence bring back research in drugs from natural products, a new face emerged, the nutraceutical industry. It can be thought of as a merger, the use of plants (and seaweed) as food that not only provides nutrition but added health benefits (*see* section 1.3). Edible seaweeds that provide added health benefits in addition to their inherent nutritional value have sparked an enormous interest in the discovery and exploitation of what is now termed as nutraceuticals.

1.3. Nutraceuticals

From the time of Hippocrates (460 – 377 *BC*) through to the coining of the term "natural product" in scientific research, medicine consisted of an informed choice of natural food products (Andlauer and Furst, 2002). The use of seaweeds as food has been carried on over generations and has since developed a new face from the vantage point of taking seaweed meals for their health benefits. That is basically the concept of nutraceuticals defined. It was formerly defined in 1989 by Defelice and the foundation of innovative medicine as "any substance that may be considered a food or part of a food which provides medical or health benefits, including the prevention and treatment of disease" (DeFelice, 1992). Nutraceuticals may be seen as either an alternative or adjuvant to modern medicine and the market zeal has propagated resurgence of natural product programs from several companies (Ganesan, 2004). They comprise mainly of nutrients, herbal preparations and fortified foods.

Today, India (Ayurveda medicine) is one of the largest homes of medicinal herbs and spices which support their large rural families from where they depend on this form of medicine for their wellbeing. China, another highly populous country, also use their traditional medicine to treat common ailments and for prevention and protective purposes (Newman *et al.*, 2000). The use of traditional medicine is really without a doubt a global concept including here in South Africa but it is in countries like Canada and the USA where that concept has been modernized and

transformed into a booming nutraceutical industry estimated to be worth US $ 30 billion and potentially US $50 billion at a 5% growth per annum (Andlauer and Furst, 2002). The carotenoid market is expected to reach US $ 1.4 billion in 2018[1]. The largest carotenoid market is that of β-carotene worth US $ 261 million in 2010[1]. Carotenoids have been recognized for their use as food, feed and in supplement industries (antioxidant and nutrition enhancing properties).[1]

The industry has expanded its way of delivering these nutraceuticals *i.e.* extracts are now being formulated like their synthetic counterparts. We have seen on the current market, an increase in extracts being delivered in soft gels, capsules, patches, *etc*. something that has sparked not only more interest and excitement but also scrutiny from regulatory bodies. With the advent of the concept of nutraceuticals in the 1990s and the popularity they have gained on an industrial scale, questions and concerns have been raised regarding the safety, quality, efficacy and the regulation of their use by the public.

1.4. Regulations of nutraceuticals

It becomes a great concern for governments and regulatory bodies when consumers avoid allopathic medicine in favor of nutraceuticals to maintain and improve their health. Nutraceuticals are acquiring increasing global attention and currently, these products are not strictly monitored and do not undergo stringent testing and/or clinical trials as synthetic drugs do. The challenges of nutraceutical and natural product industries as a whole is that active constituents may vary as a result of a variety of factors such as climate, soil, season, humidity, *etc*. impacting significantly on collection, identification, quantification and standardization methods. The manufacturing process and storage may be a source of contaminants, heavy metals, solvent residues, *etc*. (Bagchi, 2006). Bagchi (2006) therefore highlighted the need to develop rigorous, standardized manufacturing procedures, quality control and assurance techniques.

Clinical trials are a regulatory requirement for allopathic medicines but not for herbal medicines, a category within which algal preparations fall. But the need to establish the quality, safety and

[1] Carotenoid Market Forecast: http://newhope360.com/supply-news-amp-analysis/carotenoid-market-forecast-grow-14-billion-2018/ [Accessed 17 January 2014].

efficacy profiles for these preparations is ever increasing. Multiple governments and relevant organizations all over the world have been revising and evaluating their approach in the population's use of complimentary alternative medicines (CAMs) as they are known in South Africa. Several hurdles have been placed mainly in the development of nutraceuticals. In South Africa for instance, the Medicine Control Council (MCC) does not require the registration of CAMs in the same way they require for allopathic medicines. CAMs are not approved as safe and/or efficacious by the MCC, they are merely listed in a registry. This has been the position of the organization since 2011 when they passed guidelines that were asking for comments and contributions on the matter from the public and experts in the field[2]. It has since been updated in December 2013. According to their proposal they strictly recommend that the contents of all CAMs be categorically stated and allude to the introduction of stringent tests that allopathic medicines undergo including authentication and validation tests if the components are not in an MCC approved monograph.[2]

The regulations of the MCC are similar to that of either foreign or international regulatory bodies such as Food and Drug Administration (FDA), European Medicines Agency (EMEA), World Health Organization (WHO) and notably guidelines provided by the International Conference on Harmonization (ICH). The guidelines for herbal preparations *i.e.* in consumer use, is still a work in progress. According to the aforementioned regulatory bodies, nutraceuticals are not required to go through clinical trials before being allowed for human consumption but manufacturers of such products are required to carry out basic analysis and categorically define their products according to the standard expected for pharmaceuticals[2].

1.5. Analysis and efficacy of nutraceuticals

Several analytical procedures exist to explicitly define a compound. Most of these have been adopted in order to characterize nutraceuticals. From a regulatory point of view, using the MCC's position on CAMs, a brief mention shall be made on some of these recommended procedures.

[2] MCC Complimentary Medicines – Quality, Safety and Efficacy: http://www.mccza.com/genericDocuments/7.01_CAMs_QSE_Dec13_v2.pdf [Accessed 17 January 2014]

The MCC requires quality information where substance name, composition, structure and general properties, manufacturing details, characteristics and analytical test methods including; validation data, stability data and a proposed compositional guideline are to be included for the active and the finished product. Additional tests include the presence of isomers, optical rotation, microbial contamination, *etc*.[3] What does this all mean for naturally derived products?

The extent to which nutraceuticals are being analyzed has opened several opportunities for the resurgence of drug discovery from natural products; a concept once deemed a thing of the past and less favored in recent years. Some pharmaceutical companies albeit channeling most of their resources to synthetic and combinatorial chemistry routes for drug discovery and development, preserved their interest in natural product chemistry (Ganesan, 2004; Newman *et al*., 2000).

1.6. Future prospects of nutraceuticals

Nutraceuticals are gaining momentum in natural product research whilst grabbing consumer attention. All the techniques of the past and present industries, in particular seaweed harvesting and cultivation, may at a sustained level be expanded into a much more formidable industry around the world including South Africa. This sector of natural products could again become more attractive at commercial level like the community-based harvesting of *Gelidium* spp. (Rhodophyceae, red algae) in the Eastern Cape, South Africa for agar production (Lubke, 1998). This thesis has therefore drawn inspiration from the current activities around marine nutraceuticals and drug discovery.

[3] MCC Complimentary Medicines – Quality, Safety and Efficacy: http://www.mccza.com/genericDocuments/7.01_CAMs_QSE_Dec13_v2.pdf [Accessed 17 January 2014]

1.7. Overview of thesis

1.7.1. Rationale

The research described in this thesis is focused on fucoxanthin, a natural pigment derived from brown seaweed. The bioactive potential of fucoxanthin and the interest generated in its use as a potent nutraceutical, has consistently been reported (*see* section 2.5.2 and section 2.6). The biological activity of the compound has been investigated and there are several suggestions regarding its combined and sole use in the treatment of identified conditions such as cancer and obesity (Maeda *et al.*, 2008). Brown seaweeds and diatoms as previously mentioned, have been identified as the main producers of fucoxanthin and several techniques have been developed and are still being developed to isolate fucoxanthin from these sources and develop them into reliable raw materials.

There are however so many unknowns and clarity is needed in several aspects of the interest that fucoxanthin has so far gathered. The aspects of the chemistry of fucoxanthin, optimized techniques to isolate the pigment and commercial use in nutraceutical formulations (slimming preparations) has been the mainstay of fucoxanthin research in the past decade. The focus could potentially be on the possibility of commercially maximizing sourcing of the raw material fucoxanthin from the known reservoirs (micro and macroalgae). The production of carotenoids has been recognized as one of the most successful activities in algal biotechnology and the demand for natural sources is increasing (Christaki *et al.*, 2013). South African algae have not yet been exhausted in this regard *i.e*. we do not know which algae produces the most fucoxanthin. Therefore, a study that can potentially place emphasis on the prospects of the commercial production of fucoxanthin from seaweed in South Africa, as a functional ingredient, is worthwhile. An opportunity was thus presented, to clarify critical aspects such as, a reliable local source of fucoxanthin and understanding potential factors that could influence effective harnessing of fucoxanthin from algae *e.g.* stability.

The main aim of the current study was therefore to find out which of the brown algae common to South Africa was the best source of fucoxanthin. We wanted to achieve this using a simple, reproducible and effective analytical method to quantify the pigment from diverse South African marine brown algae. This knowledge could be a first step in establishing reliable sources of

fucoxanthin to support our medicinal chemistry interests especially antiplasmodial and cytotoxicity activities (Afolayan *et al*., 2008).

Research objectives:

- Isolation and characterization of fucoxanthin from *Sargassum incisifolium* as a reference standard.
- Development of a simple and rapid HPLC method for the quantification of fucoxanthin from diverse samples of marine brown algae.
- Analysis of brown marine algae for fucoxanthin content.
- pH and photostability studies on fucoxanthin.

1.8. References

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Chapter 2

Fucoxanthin: A review

2.1. Introduction

Fucoxanthin is a naturally occurring pigment found more consistently in marine brown algae (macroalgae) and diatoms (microalgae). It is associated with a primary role of contributing to the process of photosynthesis in algae where it acts as an accessory pigment. Fucoxanthin is therefore found in the chloroplasts of relevant algae in close association with chlorophyll, the primary photosynthetic pigment in these organisms (Papagiannakis, 2005).

Fucoxanthin is structurally classified as a carotenoid. Carotenoids are divided into two major categories based on their chemical composition. Carotenes contain predominantly carbon and hydrogen *e.g.* β-carotene whilst xanthophylls are oxygenated carotenes *e.g*. fucoxanthin. There are now between 600 and 800 known carotenoids that have been isolated and chemically characterized, making them one of the most diverse naturally occurring metabolites (Rodriguez-Amaya and Kimura 2004; Guaratini *et al.*, 2009). The carotenoids have adopted some unique structures relative to the long chained parent structure of lycopene. One such group is known as the allenic carotenoids. They bear a unique allene bond and fucoxanthin belongs to this particular class.

Fucoxanthin is also a nutraceutical. It is purported to be a potent multifunctional biomolecule based on the numerous biological activities that have thus far been reported in literature (*see* section 2.5.2). Based on the scientific evidence that is currently available, fucoxanthin is classified as an herbal preparation which greatly exerts its reputed health benefits in a crude form. The commercial value of fucoxanthin has been explored in western countries where fucoxanthin is currently being marketed as algal based crude extracts (rich in the pigment) found in slimming preparations *e.g.* FucoThin™, Fucoxanthin-Slim™ and Brown Seaweed Plus™ (*see* section 2.6).

2.2. Fucoxanthin's most natural role in nature

The brown algae have predominately been associated with the characteristic abundance of carotenoids. In a classification of algae based on pigmentation, Tilden (1933), discussed the relative quantitative dominance of yellow pigments that masked chlorophyll; something that was evident in all Phaeophycean algae. Fucoxanthin as an accessory pigment is understood to function by absorbing blue-green light and passing it on to chlorophyll for the process of photosynthesis (Papagiannakis, 2005; Zigmantas, 2004). This is additional harnessed energy (blue-green wavelength region) from the electromagnetic spectrum that would have otherwise not been used by the plant/algae had it depended on chlorophyll (main photosynthetic pigment) alone during the food production process. In a marine environment, the further the light travels through water, the more filtered it becomes leaving blue light penetrating to larger depths. Brown algae can therefore keep on producing food at relatively deep levels of the sea away from the surface unlike green algae which lack brown pigments or red ones as in the case of some deep red algae that are able to also absorb blue light (Branch, 2000). The value of the pigment to brown algae was therefore investigated. Were the pigment's light absorbing properties vital to the algae as much as chlorophyll? Did it insubordinate chlorophyll in the process of photosynthesis at some stage in the algal lifecycle or did it merely filter off light that would be otherwise harmful to the composition of the algae? (Blinks, 1954).

2.3. The structure and physicochemical properties of fucoxanthin

Fucoxanthin is a typical C_{40} tetraterpenoid, formed from the condensation of eight C_5 isoprenoid units, where tail to tail linkage at the center of the molecule reverses the order resulting in a symmetrical molecule. One of the most important features resulting from this arrangement is an extended conjugated double bond system which forms the chromophore of the molecule (the basis for quantification) whilst imparting an attractive orange color (Rodriguez-Amaya and Kimura, 2004). Fucoxanthin carries cyclic β-ionone end groups, one that is substituted by an epoxy group on positions 5 and 6 and the other coupled to an allenic bond at position 6', 7' and 8'. The two central methyl groups on the polyene chain are in a 1, 6 position relationship *i.e.* C-20 and C-20'

whilst the terminal methyl groups are in a 1, 5 position relationship with the respective central methyl *i.e.* C-19 and C-19' (Miyashita and Hosokawa, 2009). The systematic name is *3'-acetoxy-5,6-epoxy-3,5'-dihydroxy-6',7'-didehydro-5,6,7,8,5',6'-hexahydro-β,β-caroten-8-ol.*[4]

The complete structure of fucoxanthin is shown in (Fig 2.1). The molecular formula is $C_{42}H_{58}O_6$ and the molecular weight is 658.91 Da. Highly conjugated systems such as fucoxanthin are prone to isomerization. The pigment occurs naturally as all-*trans* fucoxanthin and the *cis*-isomers are known to occur as well. Common *cis* isomers are 9', 13, 13', 15 and 15' isomers. Fucoxanthin in this study was found to occur as an orange amorphous powder depending on the quantity and extent of drying, it may otherwise occur as an orange paste and has been reported as orange-colored pigment (Peng *et al*., 2011). The solubility of fucoxanthin in dimethyl sulfoxide, methanol and acetone has been reported[5]. The molecule is freely soluble in these solvents forming a bright orange solution. Fucoxanthin has also been found, in this study, to dissolve readily in acetonitrile, dichloromethane and chloroform. It is insoluble in water. The CAS number is 3351-86 8 and it is marketed as a reference standard of 95 % purity by Sigma Aldrich.[6]

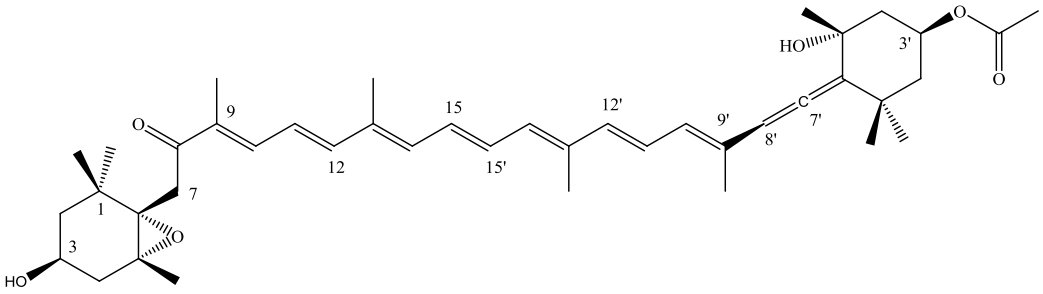


Figure 2.1: The structure of fucoxanthin

[4] Fucoxanthin MSDS (Sigma Aldrich): http://www.sigmaaldrich.com/catalog/product/sigma/f6932?lang=en&region=ZA/ [Accessed 19 January 2014]
[5] Fucoxanthin MSDS (Cayman chemicals): https://www.caymanchem.com/pdfs/13068.pdf/ [Accessed 19 January 2014]
[6] Fucoxanthin MSDS (Sigma Aldrich): http://www.sigmaaldrich.com/catalog/product/sigma/f6932?lang=en&region=ZA/ [Accessed 19 January 2014]

2.4. Biosynthesis of fucoxanthin

Fucoxanthin is regarded as one of the four major carotenoids occurring in nature alongside lutein, violaxanthin and neoxanthin. It has been estimated to account for >10% of total natural production of carotenoids. The carotenoid is largely present in chloroplasts of brown seaweeds but has been isolated in sometimes relatively larger quantities in diatoms (Peng *et al*., 2011). The biosynthesis of fucoxanthin is postulated to be based on the structure of carotenoids (Fig 2.2). The carotenogenesis pathways in phototrophs, like seaweeds, have also been the basis for the chemical synthesis of fucoxanthin which has shown to be very difficult (Yamano *et al.*, 1995; Kanazawa *et al*., 2008).

Based on the structures of known carotenoids occurring in phototrophs (ranging from all types of seaweeds, diatoms and dinoflagellates to higher terrestrial plants) and on the genome sequence data for fucoxanthin producing algae, the biosynthetic pathway of fucoxanthin in brown seaweed has been postulated (Mikami and Hosokawa, 2013). Briefly, the absence of fucoxanthin in red and green algae suggests a unique pathway for brown algae which results in fucoxanthin becoming its major carotenoid. The distribution of xanthophylls, in essence is class-specific, for example, the lack of the xanthophyll cycle (violaxanthin cycle) in red algae explains why there is only the accumulation of a certain type of xanthophyll carotenoids *i.e.* zeaxanthin and lutein (Fig 2.2).

The xanthophyll cycle involves the reversible conversion of zeaxanthin, antheraxanthin and violaxanthin by epoxidation and de-epoxidation reactions under various stress conditions. Brown seaweeds however seemed to have evolved further to have a diadinoxanthin cycle to produce novel xanthophylls including fucoxanthin. Diadinoxanthin is hypothesized to be the precursor to fucoxanthin although another competing hypothesis postulates the formation of fucoxanthin to be via neoxanthin (the allenic carotenoid found in terrestrial plants) albeit the enzymes of that process remain unknown (Mikami and Hosokawa, 2013). The biosynthetic pathway for fucoxanthin remains complicated and inconclusive.

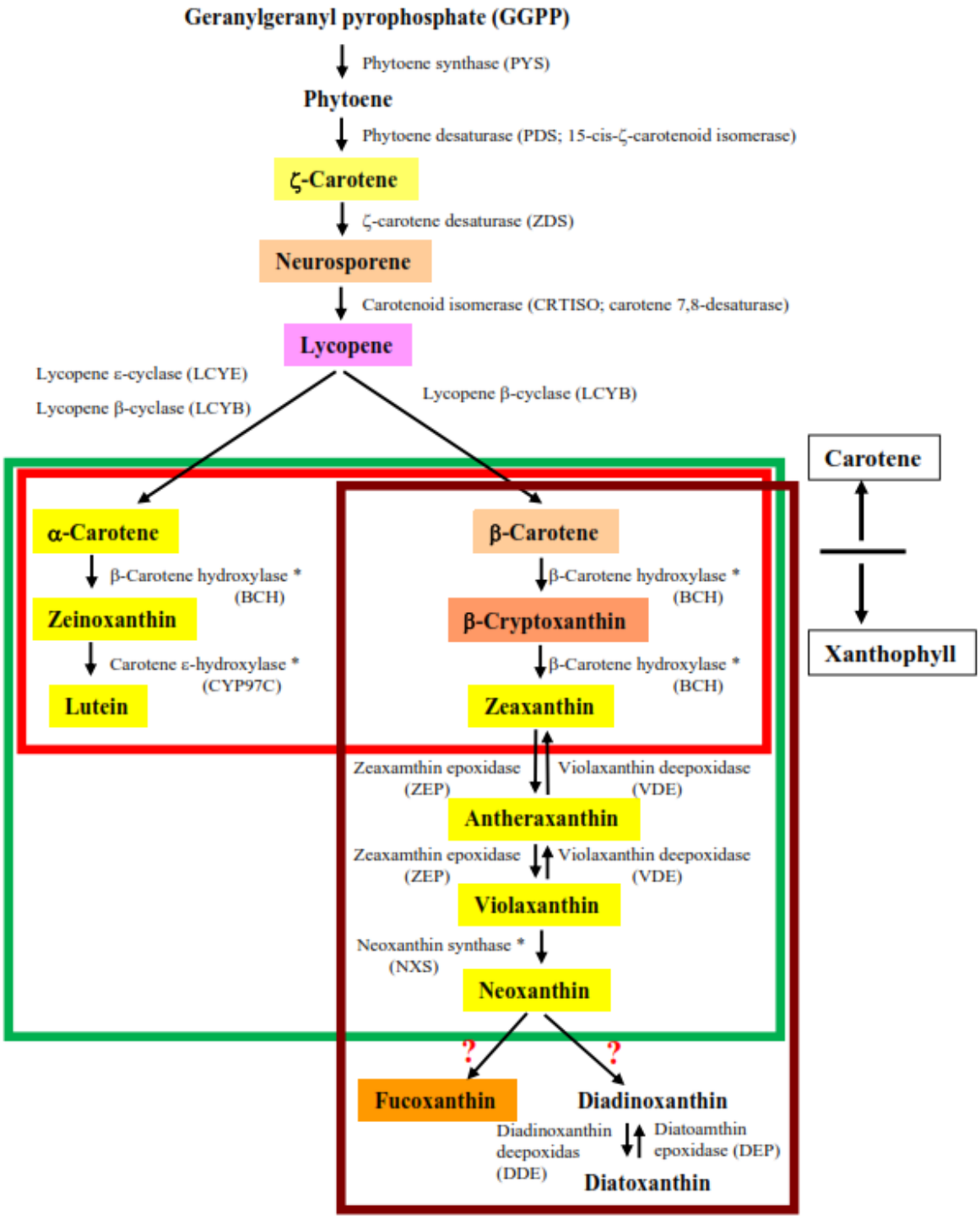
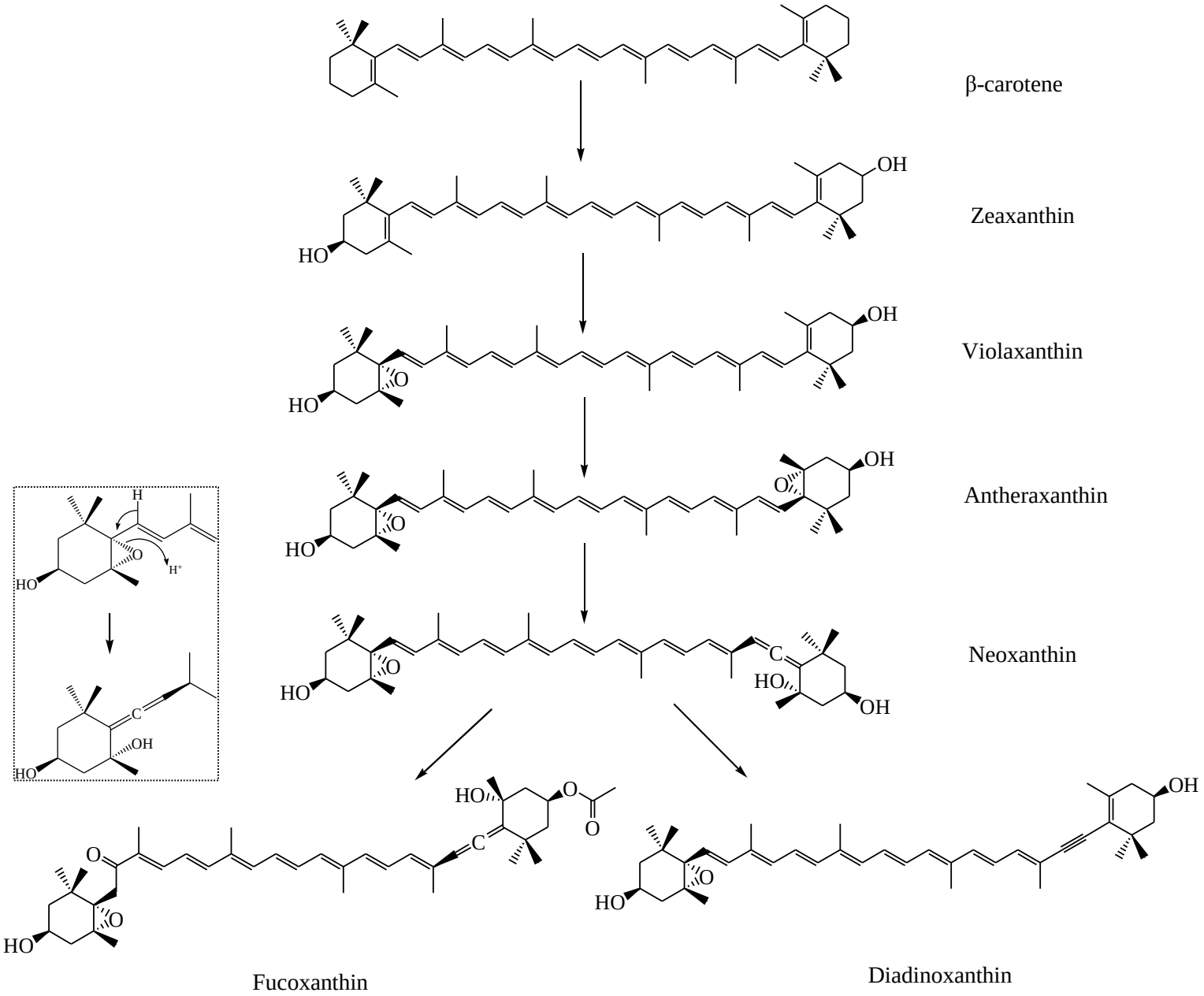


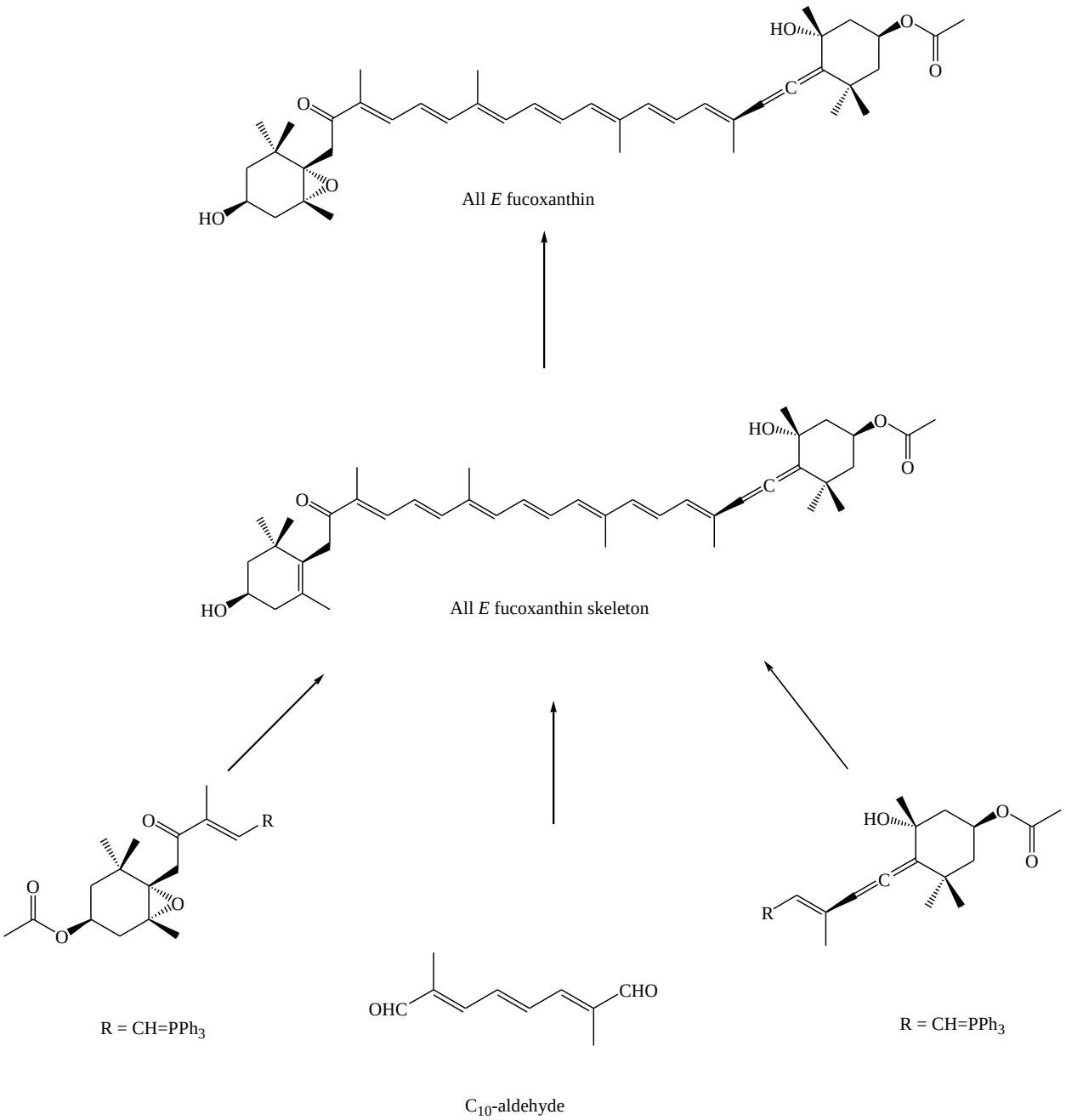
Figure 2.2: Proposed biosynthetic pathway for fucoxanthin: Adapted from Mikami and Hosokawa (2013).

The biosynthetic pathway can be narrowed down to show how fucoxanthin is synthesized from β-carotene via neoxanthin as postulated by Dambek *et al.* (2012). The formation of the allenic functionality in fucoxanthin is also depicted in (Scheme 2.1).



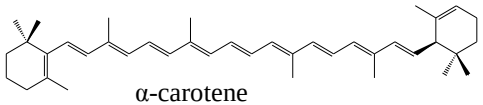
Scheme 2.1: Biosynthetic pathway for the synthesis of fucoxanthin including the formation of the allenic functional group in fucoxanthin. Adapted from Dambek *et al.* (2012).

The total chemical synthesis of fucoxanthin has been reported using oxo-metallic catalysts (Yamano *et al*., 1995). This multistep reaction involved several rearrangement reactions (α-acteylinic alcohols), conversions of α-β-unsaturated ketones, preparation of 8-oxo and allenic Wittig salts and several reduction and isomerization reactions before the preparation of fucoxanthin skeletal compounds. Each step demanded time and varying conditions and the total time of the experiment was in an excess of 85 hours (experimental time). The reported result was a mixture of epoxides (16 mg, 36%) which included an optically active fucoxanthin (Yamano *et al.*, 1995). Fucoxanthin has also been synthesized according to (Scheme 2.2) and the challenges identified were to do with the construction of the β-epoxy ketone coupled with polyene chain, which was shown to be extremely liable to alkali. The synthesis of A-part (C_{15}-8-oxo-compound) and B-part (C_{15}-allenic phosphorium chloride) is shown in (*see* Appendix 2.1).

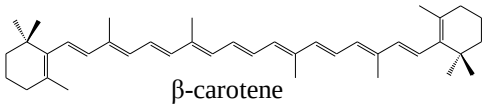


Scheme 2.2: Synthesis of fucoxanthin. Adapted from Ito *et al*. (1994).

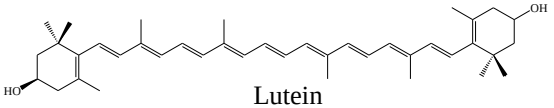
On the other hand, there is the extraction of naturally occurring fucoxanthin which has been reported at length to produce pure fucoxanthin with better yields of as high as 3.7 mg/g (dried weight) of *Sargassum horneri* (Terasaki *et al.*, 2009) when compared to total synthesis of (Yamano *et al*., 1995). Both methods have their fair share of challenges but extraction of seaweeds and other reported sources such as diatoms and dinoflagellates is currently preferred. The extraction of ready-made fucoxanthin from reliable and well known sources using highly efficient techniques may prove to be the more reasonable approach to acquiring fucoxanthin as a raw material (Kanazawa *et al*., 2008). The carotenoids isolated from marine algae in general are shown in (Fig 2.3).



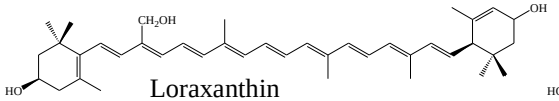
α-carotene



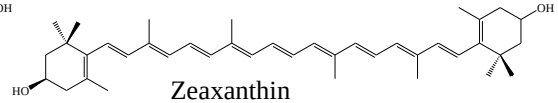
β-carotene



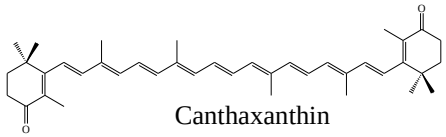
Lutein



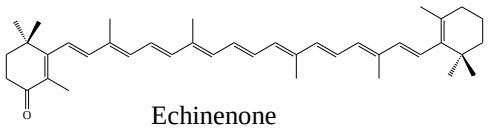
Loraxanthin



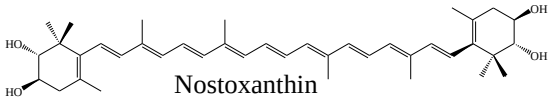
Zeaxanthin



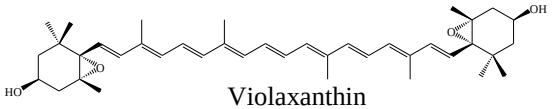
Canthaxanthin



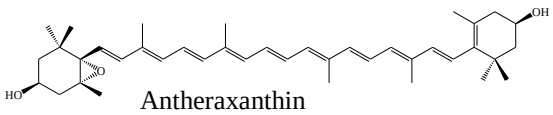
Echinenone



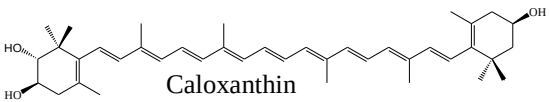
Nostoxanthin



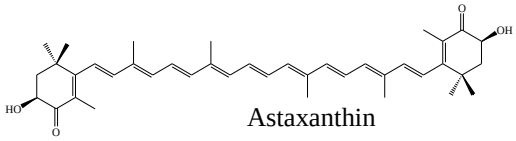
Violaxanthin



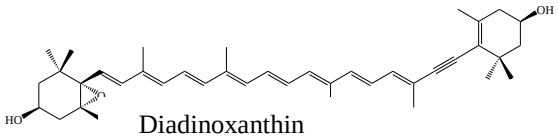
Antheraxanthin



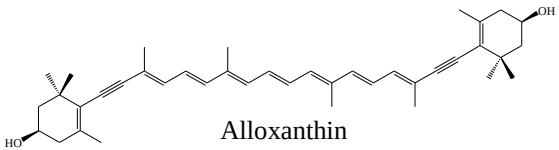
Caloxanthin



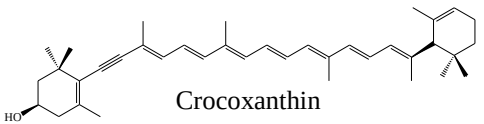
Astaxanthin



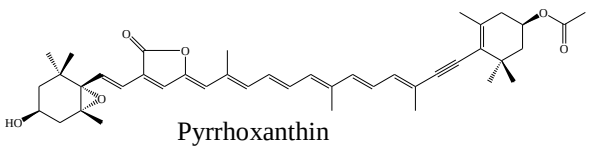
Diadinoxanthin

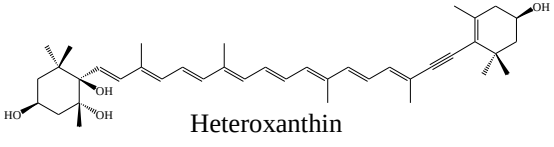


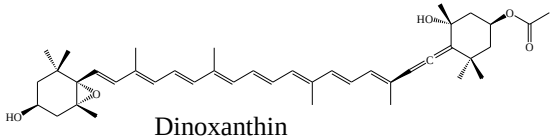
Alloxanthin

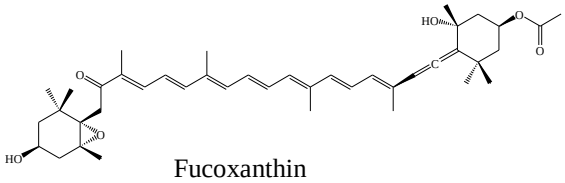


Crocoxanthin









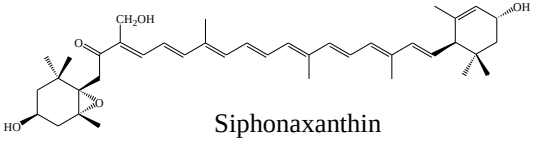
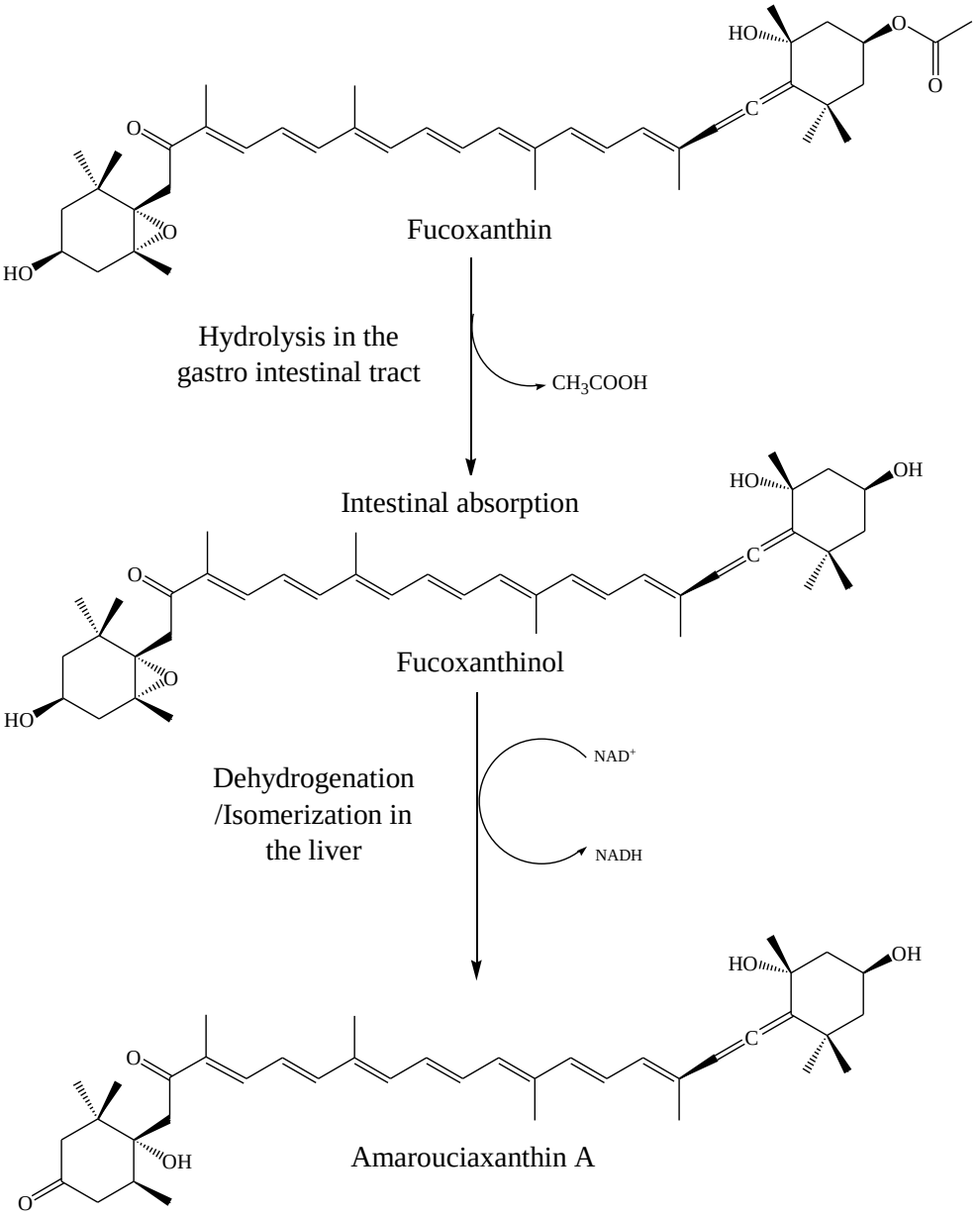


Figure 2.3: Selected carotenoids isolated from marine algae. Adapted from Takaichi (2011).

2.5. Biological activity of fucoxanthin

2.5.1. Bioavailability, metabolism and safety of fucoxanthin

Pharmacokinetic studies carried out in rodents have alluded to the occurrence of metabolites of fucoxanthin rather than the parent molecule itself in either the circulatory system or targeted tissues (Sugawara *et al*. 2002).



Scheme 2.3: Proposed mechanism for the metabolism of fucoxanthin in mice. Adapted from Moghadamtousi *et al.* (2014).

Sugawara *et al.* (2002) administered 40 nmol of fucoxanthin orally to mice and analyzed their blood plasma after 1 hour. No fucoxanthin was found, however, its metabolites were identified *i.e*. fucoxanthinol and amarouciaxanthin A (Sugawara *et al*., 2002; Asai *et al.*, 2004). The distribution of fucoxanthin in mice was also investigated. After a single dose of fucoxanthin (160 nmol), fucoxanthin and metabolites were analyzed in plasma, red blood cells, fat tissue, liver, lung, heart, kidney and spleen (Hashimoto *et al.* 2009). Fucoxanthin was not detected but again fucoxanthinol and amarouciaxanthin A were detected after 1 hour administration. Fucoxanthin was then detected after continuous dosing in erythrocytes, adipose tissue, liver, lung, heart and spleen (Hashimoto *et al*., 2009). The conversion of fucoxanthin to two main metabolites, fucoxanthinol and amarouciaxanthin A is illustrated in (Scheme 2.3).

Fucoxanthin has generally been reported as safe with no abnormal changes observed after larger doses of 10 mg/kg/day were given to rats over 28 days (Kadekaru *et al.*, 2008; Peng *et al*., 2011).

2.5.2. Reported health promoting effects of fucoxanthin

The remarkable biological activities that have been reported have been attributed to the unique structure of fucoxanthin which is different from most common carotenoids such as β-carotene and astaxanthin (Sachindra *et al*., 2007). The ability to scavenge reactive oxygen species (ROS) and free radicals has contributed to its reported antioxidant activity. The MeOH extract of *Hijikia fusiformis* was tested against 1-diphenyl-2-picrylhydrazyl (DPPH) on a TLC plate and was shown to have 65% free radical scavenging activity. After further TLC analysis, the orange spot identified as fucoxanthin, contributed 30% of the total free radical scavenging activity. The other carotenoids β-carotene, β-cryptoxanthin, zeaxanthin and lutein did not show free radical scavenging activity. (Yan *et al*., 1999). Free radical scavenging may prevent potential mutations and in turn prevent the occurrence of heart disease and cancer (Sachindra *et al*., 2007). Extracts of brown seaweeds containing fucoxanthin and phenolics have shown increased antioxidant activities suggesting a synergistic effect (Airanthi *et al*., 2011). Other mechanisms such as the prevention of oxidant formation, scavenging superoxide anions and reduction of active intermediates (*ex vivo*) have been postulated. A comparison (*in vitro*) was made between fucoxanthin, halocynthiaxanthin, fucoxanthinol and α-tocopherol. With regards to scavenging free OH-radicals, generated by the

Fenton reaction system and evaluated by chemiluminescence and electron spin resonance (ESR), fucoxanthin (EC_{50} = 0.25 mg/mL) showed 13 times better activity than α-tocopherol. However in the same study, α-tocopherol (EC_{50} = 63 μM) showed stronger scavenging activity to that of fucoxanthin (EC_{50} = 164 μM) in a DPPH assay (Sachindra *et al*., 2007). Fucoxanthin has been shown to have secondary pathways to exert its antioxidant properties by increasing the activity of catalase which in turn leads to the inhibition of intracellular ROS formation, DNA damage and apoptosis (Ahn *et al*., 2007).

One of the most studied aspects of fucoxanthin is probably its anti-cancer activity. Apoptosis induction, arresting the cell in the G0/G1 phase of the cell cycle and the inhibition of mammalian DNA polymerase has been reported as likely mechanisms (Peng *et al*., 2011). Fucoxanthin induced apoptosis and significantly decreased the cell viability in human promyelocytic leukemia (HL-60) and human prostate cancer cells (PC-3). Other related carotenoids (β-carotene) could not induce the same effect as it lacked the 5,-6-monoepoxide moiety found on fucoxanthin (Hosokawa *et al*., 1999). Fucoxanthin was also found to decrease the viability of human colon cancer cell lines (Caco-2, HT-29 and DLD) in a dose dependent manner (Hosokawa *et al*., 2004). Cytostatic activity, rather than cytocidal, was observed when the G0/G1 phase of the cell cycle of hepatocellular carcinoma cells (HepG2) was targeted by fucoxanthin (Das *et al.*, 2008). The regulative effect of fucoxanthin on biomolecules is therefore related to the cell cycle and apoptosis.

The antiobesity effects of fucoxanthin relative to its cytotoxicity are equally interesting. The accumulation of fat in the body and white adipose tissue causes obesity but it also leads to cytokine disturbance which then increases the risk of many serious diseases such as type 2 diabetes, hyperlipidemia, hypertension and several cardiovascular diseases (Maeda *et al*., 2005). The reduction of excess white adipose tissue masses in rodents (Wistar rats) via adaptive thermogenesis owing to the expression of uncoupling mitochondrial proteins (UCP) has been demonstrated in white adipose tissue (WAT) of fucoxanthin fed mice (Maeda *et al*., 2005). The gene UCP1 results in thermogenesis, a process characterized by fatty acid oxidation, heat production and energy dissipation resulting in a decrease in abdominal fat (WAT). Interestingly enough, after being fed with extracts of *L. japonica* and *L. ochotensis*, lean mice were not affected by fucoxanthin whilst the decrease in visceral fat pad weight, white adipose tissue, and the size of adipocytes was observed in obese mice (Woo *et al.*, 2009). Fucoxanthin was suggested to be the active component

within the extracts (Maeda *et al.*, 2005). The effects of fucoxanthin on the lipid profile of tested mammals included the inhibition of fat absorption and decreased fatty acid synthesis. The carotenoid has therefore been referred to as the regulator of metabolism in fat tissues (Peng *et al.*, 2011).

The issue of human clinical trials is a disputed one. There is little information available. A few reports have emerged and one comprises of a 16 week trial of orally administered xanthigen (fucoxanthin-containing mixture) and pomegranate seed oil (to improve absorption) in obese, non-diabetic and premenopausal women presenting with normal liver fat or non-alcoholic fatty liver disease. The evaluated features included body weight, liver lipids, blood chemistry and resting energy expenditure. Significant losses in body weight, body fat and liver fat content are reported (Abidov *et al*., 2010).

The attraction of large amounts of leucocytes (neutrophils, monocytes-macrophages and mast cells) to an inflamed area is characteristic of the process of inflammation. It is also known to involve the generation of superoxide anion and nitric oxide radicals which is self-damaging. Conventional anti-inflammatory agents suppress cytokine production *e.g*. tumor necrosis factor α, interleukin-1β and inflammatory mediators including nitric oxide and prostaglandin E2 synthesized by nitric oxide synthase and cyclooxygenase which have been reported to be inhibited by fucoxanthin (Kim *et al*., 2010; Heo *et al*., 2010).

A marked decrease in plasma glucose and insulin levels coupled with an improved insulin resistance has led to some suggestions that fucoxanthin possesses antidiabetic activity. KK-A^y mice (obese and diabetic) were used in a study to demonstrate the anti-diabetic effects of fucoxanthin. The mice were fed with 0.1% and 0.2% fucoxanthin and the result revealed a significant decrease in plasma glucose and insulin (Maeda *et al*., 2007). This has been, in part, explained by an up-regulation of glucose transporter 4 in skeletal muscles (Miyashita *et al*., 2011). The lipid metabolism induced by fucoxanthin has also contributed to hepato-protective effects after the hepatic lipid content was lowered and fecal lipids were significantly increased by inhibition of lipid absorption in C57BLN/6N mice that were on a high-fat diet (Park *et al.*, 2011). This decreased the activity of hepatic fatty acid synthesis-related enzymes and normalized hepatic glycogen content which may also contribute, in part, to the antidiabetic properties exhibited by

fucoxanthin. The decrease in lipids may be due to the increase in docosahexaenoic acid (DHA) which increases the hepatic fatty acid β-oxidation in the liver (Maeda *et al*., 2008).

Skin protective effects of fucoxanthin have also been reported in ultraviolet (UV) related damage of cellular constituents and some cutaneous diseases such as pigmentation, laxity, wrinkling, erythema and skin cancer. The antioxidant activity of fucoxanthin has been suggested as the likely mechanism (Urikura *et al.,* 2011). Increased cell survival has also been linked to decreased intracellular ROS in human fibroblasts, inhibition of melanogenesis in melanoma, tyrosinase activity and UV-B-induced skin pigmentation (Urikura *et al*., 2011).

The lesser reported activities of fucoxanthin include anti-angiogenic, cerebrovascular protective, bone-protective, ocular-protective and antimalarial effects. The suppression of the formation of new blood vessels may make fucoxanthin useful in the prevention of angiogenesis-related diseases such as cancer, atherosclerosis, psoriasis and diabetic retinopathy (Sugawara *et al*., 2006). The cerebrovascular protection was investigated in cultured neuronal cells from hypertensive rats and fucoxanthin was found to attenuate neuronal cell injury in hypoxia. It has therefore been suggested that the pigment may have a beneficial effect against ischemic neuronal cell death in stroke-prone, spontaneously hypertensive rats (Ikeda *et al.,* 2003). A macrophage cell line RAW264.7 (able to differentiate into osteoclast-like cells when stimulated) and osteoblast-like cell line MC3T3-E1 were used to investigate the effects of fucoxanthin. It was found to suppress osteoclastogenesis through inhibiting osteoclast differentiation and inducing apoptosis in osteoclasts without antagonizing bone formation. Fucoxanthin may therefore prevent bone diseases such as osteoporosis and rheumatoid arthritis (Das *et al.,* 2010). Fucoxanthin isolated from *Sargassum heterophyllum* exhibited the highest antiplasmodial activity (IC_{50} = 1.5 µM) against a chloroquine-sensitive strain *Plasmodium falciparum* (D10) parasite of all the metabolites isolated and tested (Afolayan *et al*., 2008).

2.6. Recent progress and commercial application of fucoxanthin

The sources and extraction techniques for fucoxanthin are well established. Efforts to commercialize its production using novel techniques such as enzyme assisted and pressurized

liquid extractions are ongoing (Kadam *et al*., 2013). More novel applications include the recently reported *in vitro* encapsulation of fucoxanthin in a microsphere composed of cetyl palmitate-based solid lipid core to protect the compound in simulated gastric conditions (Quan *et al.*, 2012).

To date, the biological activity of fucoxanthin that has been exploited at a commercial scale is its anti-obesity effects where it is reported to be present in several slimming preparations (Li and Liu, 2013). Most products are still only available on the internet (health shops) and are based in the United States of America. Health supplement companies have developed formulations which deliver the brown seaweed crude extract in the form of soft gels, capsules and patches (Li and Liu, 2013) some of the products are shown in (Fig 2.3);

(i) Garden of Life® FucoThin™ consisting of pomegranate oil, *Undaria pinnatifida* (Wakame) and *Laminaria japonica* extracts containing fucoxanthin. The dose is 200 mg and it is recommended to take one soft gel three times a day. Ninety soft gels cost about US $42.[7]

(ii) Only Natural® Brown Seaweed Plus™ consisting of unspecified brown seaweed containing fucoxanthin and green tree extract. The dose is 700 mg and it is recommended to take one capsule daily. Ninety capsules cost US $20.[8]

(iii) Life Extension® Fucoxanthin-Slim™ also consists of xanthigen blend. It is branded as an "*Optimized fucoxanthin extract, non-stimulant thermogenic...*" The dose is 200 mg and 90 soft gels will cost about US $40.[9]

(iv) Smith Sorensen® FucoXanthin Diet Patch-CR™ is an easy adhesive patch that claims to drastically reduce cravings for food and increase metabolism to burn fat. It contains several actives which include concentrated brown seaweed (unspecified), pomegranate and *Hoodia gordonii* (appetite suppressant). It is sold as 10 patches applied daily and these cost about US $40.[10]

[7] FucoThin™: www.fucothin.com/ [Accessed 17 January 2014]
[8] Brown seaweed plus™: http://www.onlynaturalinc.com/best-sellers/brown-seaweed-plus [Accessed 17 January 2014]

[9] Fucoxanthin-Slim™: http://www.lef.org/Vitamins-Supplements/Item00993/Fucoxanthin-Slim.html [Accessed 17 January 2014]

[10] FucoXanthin Diet Patch-CR™: http://www.bettertotalhealth.com/fucoxanthinpatch.php [Accessed 17 January 2014]

Figure 2.4: Commercial slimming products containing fucoxanthin.

These all too familiar statements have been found on the packaging;

"*These statements have not been validated by the Food and Drug Administration. This product is not intended to diagnose, cure or prevent any disease"*.

This winds all the way back to the regulation of herbal preparations discussed earlier (section 1.4). The future prospects of fucoxanthin may thus depend on regulation of naturally derived nutraceuticals, finding the best source of fucoxanthin and technological advancement in cultivation, fucoxanthin production and extraction.

2.7. Future prospects with fucoxanthin

The research on fucoxanthin is indeed leaning towards identifying good sources to sustain commercial production from both diatoms and brown seaweeds. There are reported biotechnological considerations to support this venture *i.e.* marine biotechnology applied to seaweeds and their secondary metabolites (Ibanez and Cifuentes, 2012). Human clinical studies are still pending for fucoxanthin and the future of the compound's usefulness on a larger scale in the pharmaceutical industry will also depend on more reputable clinical studies that demonstrate the efficacy of this compound in humans.

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Chapter 3

The extraction, isolation and characterization of fucoxanthin from the marine brown alga *Sargassum incisifolium* (Turner) C. Agardh.

3.1. Introduction

3.1.1. Fucoxanthin found in *Sargassum incisifolium* (Turner) C. Agardh.

Sargassum incisifolium[11] (Turner) C. Agardh is from a family (Sargassaceae) of brown algae constituting one of the largest and complex seaweeds. The Sargasso Sea, situated in North Atlantic, is believed to have been named as such after the seaweed since the 14th century. It was so abundant as to appear to cover the entire sea as described in the accounts of early explorers, such as Christopher Columbus in 1492, sailing the Atlantic in search of new lands (Deacon, 1942). This is a shear demonstration of survival considering the *Sargassum* spp. are still found in that region which is dominated by *Sargassum natans* (De Széchy *et al*., 2012). Several species of *Sargassum* also occur away from temperate waters. The gullies and intertidal pools of South Africa are one such location where *Sargassum* spp. are one of the many brown seaweeds on the east and overlapping south coast of South Africa (Lubke, 1998). The *Sargassum* spp. in literature, are reported to produce large amounts of fucoxanthin as do several other Phaeophycean algae (Terasaki *et al*., 2009; Miyashita *et al*., 2009). Fucoxanthin has been the subject of study for over a century and it is now nearly 100 years since Willstatter and Page in 1914, isolated the pigment in crystalline state. There has been a mention of the presence of fucoxanthin in *Sargassum flavican* (Jeffrey, 1963, 1968). At the time, the author was attempting to purify chlorophyll *c*. There appeared to be traces of fucoxanthin in stage 1 of the column separation of pigments and reference was made to the earlier work of Strain *et al*. (1944) where fucoxanthin was isolated from *Fucus serratus.* The same feat was also achieved from the same alga in the earliest mentions of isolated fucoxanthin (Willstatter and Page, 1914). The presence of fucoxanthin in the South African brown

[11] *Sargassum heterophyllum* is the heterotypic synonym. *Sargassum incisifolium* was used first in literature. (*Personal Communication,* J.J. Bolton, 3 March 2014)

alga, *Sargassum heterophyllum* (Afolayan *et al*., 2008), has previously been reported making this alga a suitable source of this compound for our studies.

Since the early studies, fucoxanthin has been extracted, isolated and purified from an array of Phaeophycean seaweed with improved yields owing to the technological advancement in isolation techniques (Table 3.1). But what has been equally interesting, is the versatility in terms of the potential biological activity of the pigment (*see* section 2.5.2). The traditional extraction of bioactives from macroalgae is generally characterized by initial techniques such as; soxhlet extraction (SOX), solid liquid extraction (SLE) with subsequent liquid-liquid partitioning.

3.1.2. Previous methods used in the isolation of fucoxanthin from brown algae

The majority of the earlier isolation studies resembles an era with the use of large volumes of organic solvents in time consuming procedures of traditional solid liquid extraction SLE (solvent steeping and maceration), liquid partitioning and soxhlet extraction SOX. Newer research is moving away from these procedures for what is believed to be more commercially viable state of the art techniques such as supercritical fluid extraction (SFE), pressurized fluid extraction (PLE), microwave assisted extraction (MAE) and ultrasound assisted extraction (UAE) among many other innovative procedures. Some of the procedures previously used in the extraction and isolation of fucoxanthin are highlighted in (Table 3.1). The use of organic solvents for the initial extraction of algal pigments (methanol and acetone), isolation and purification of fucoxanthin-rich fractions (hexane, ethyl acetate and acetone) using either solid-liquid or liquid-liquid separation techniques (silica/C18 open and HPLC, separating funnels, counter-current chromatography, *etc*.) are amongst the most common methods used in previous studies. There is a mix of bulk extractions of algal fronds in the order of kilograms (3 kg of *Ecklonia bicyclis*, 10 L acetone) using centrifugal partition chromatography (Kim *et al.,* 2010), medium scale gram quantities (5 g *Undaria. pinnatifida*, 2 mL/min liquefied CO_2) by supercritical fluid extraction at supercritical conditions of 40 MPa at 25 °C producing remarkable yields of ~80 mg/g dry weight (Quitain *et al.,* 2013) and small scale milligram quantities (100 mg freeze dried *U. pinnatifida*, 15 mL methanol) by using hexane initially, then chloroform in a liquid-liquid extraction procedure (Fung, *et al.,* 2013).

Table 3.1*:* Selected methods used previously in the extraction and isolation of fucoxanthin from brown algae.

Sample	Method of extraction/isolation	Comments	Ref.
Sargassum heterophyllum **(wet fronds)**	MeOH; CH_2Cl_2/MeOH (2:1); step gradient (hex/EtOAc).[12]	Bioactive fucoxanthin (antiplasmodial activity)	Afolayan *et al*., 2008
Fucus serratus **(air dried, ground)**	Acetone/methanol (7:3) extraction; silica gel column chromatography; crystallization from acetone-hexane.	0.04 mg/g dried alga	Haugan and Liaane-Jensen, 1989.
Padina australis **(dried and ground)**	Acetone/methanol (7:3) extraction; liquid-liquid partitioning; silica gel column chromatography.	Yield 0.43 mg/g	Jaswir *et al.*, 2011.
Fresh algae of 5 species including *Turbinaria conoides, Sargassum filifpendula.*	Maceration in DMSO then dried in argon gas before silica gel separation using methanol/isopropyl alcohol.	Fucoxanthin content 0.158 – 0.26 mg/g.	Zailanie and Purnomo, 2011.
Freeze–dried *Undaria pinnatifida* from frozen storage.	Solvent soaking in methanol, then hexane/water liquid-liquid extraction and centrifugation.	The extraction was small scale (100 mg of freeze dried material).	Fung *et al.*, 2013.
Wet fronds of *Eisenia bicyclis* previously stored at - 20°C.	Soaked in acetone, partitioned in hexane/ethanol/water then CPC.	Applicable to industrial production.	Kim *et al.*, 2010.
Finely ground powder of *Undaria pinnatifida.*	MAE and HSCCC and UV-guided collection of fractions.	The extraction yield was 0.726 mg/g.	Xiao *et al.*, 2012.
Fresh fronds of *Eisenia bicyclis* from storage at -20°C.	PLE on fresh algae and HPLC analysis	Purity not reported. Quantified as 0.39 mg/g.	Shang *et al*., 2011.
Powdered dried *Undaria pinnatifida* (commercial wakame)	Supercritical carbon dioxide fluid extraction	~80 mg/g of dry weight.	Quitain *et al.*, 2013

[12] This method was chosen for this study as we were more familiar with the technique in our lab.

Sufficient fucoxanthin of high purity was required for subsequent studies on the analysis of fucoxanthin in crude extracts as will be discussed in later chapters. Therefore a procedure that was efficient, with reasonable yields, one that did not cause degradation of the target compound and had relatively few manipulations was required.

3.1.3. Chapter aims

The first aim of the overall study, was to isolate sufficient fucoxanthin to support the analytical method used in the determination of its content, in diverse samples of South African brown algae (*see* chapter 4 and 5). The difficulty to obtain commercial standards of high purity was confirmed by Bidigare (1991) when the author stipulated the recommendations for the analysis of algal carotenoids. This is true even to this day simply because of the difficulty in synthesizing the carotenoid or extracting it in large quantities and high purity.[13] Therefore fucoxanthin isolated and purified from *S. incisifolium* was elected as the reference standard for later studies (*see* Chapters 3, 4 and 5).

Chapter objectives:

1. Isolate fucoxanthin of high purity from fresh alga *S. incisifolium*.
2. Confirm the identity of fucoxanthin using spectroscopic techniques such as 1D and 2D NMR

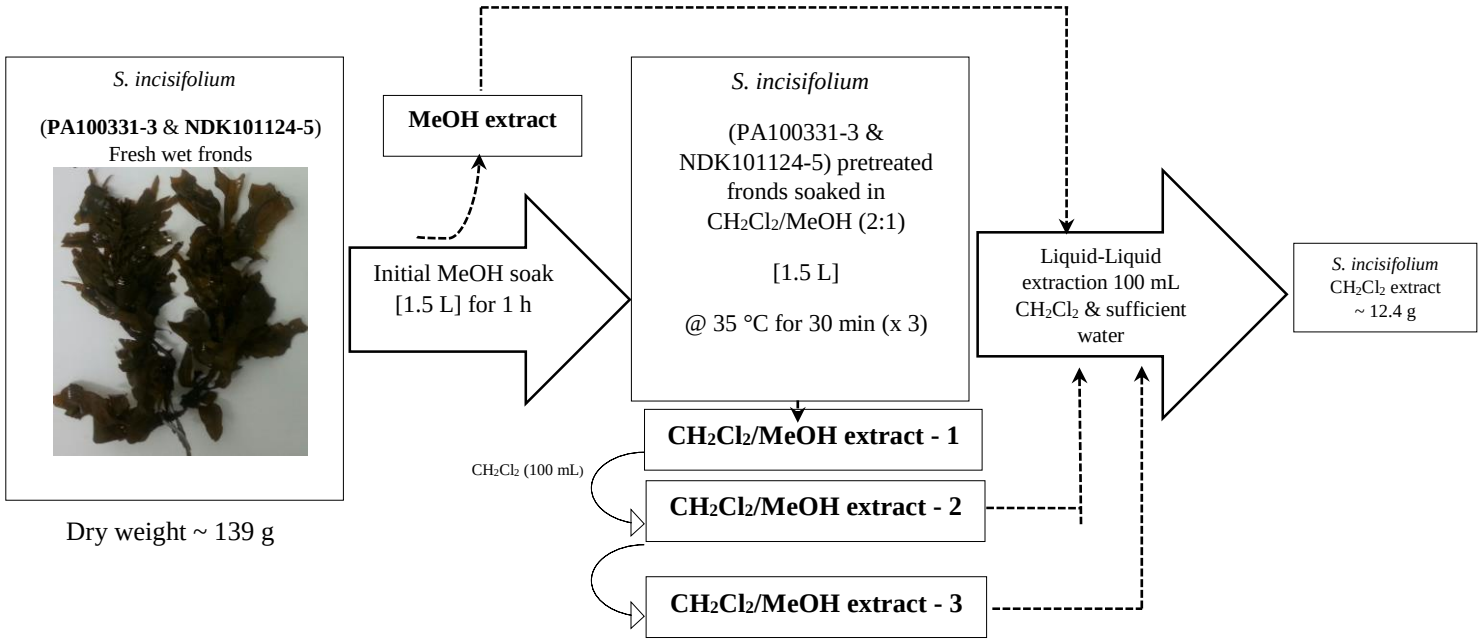
[13] Standards in literature are mainly supplied by Sigma-Aldrich. The purity is reported as 95%. MSDS available online: http://www.sigmaaldrich.com/catalog/product/sigma/f6932?lang=en&region=ZA. [Accessed 19 February /2014]. This compound is relatively expensive (R585/mg) and would require additional purification before use as a reference standard.

3.2. Results and discussions

3.2.1. Extraction and isolation of fucoxanthin

Since the main objective of this part of the study was to isolate fucoxanthin as a reference standard, no attempts were made to optimize the isolation procedure. Instead, we adapted the extraction and isolation procedure from Afolayan *et al*. (2008). Furthermore, we also knew that fucoxanthin was a relatively minor constituent of *S. incisifolium* which could potentially complicate the isolation procedure (Afolayan *et al*., 2008). The algae (**NDK101124-5** and **PA100331-3**) used in the study had been in storage at -18 °C for three years.

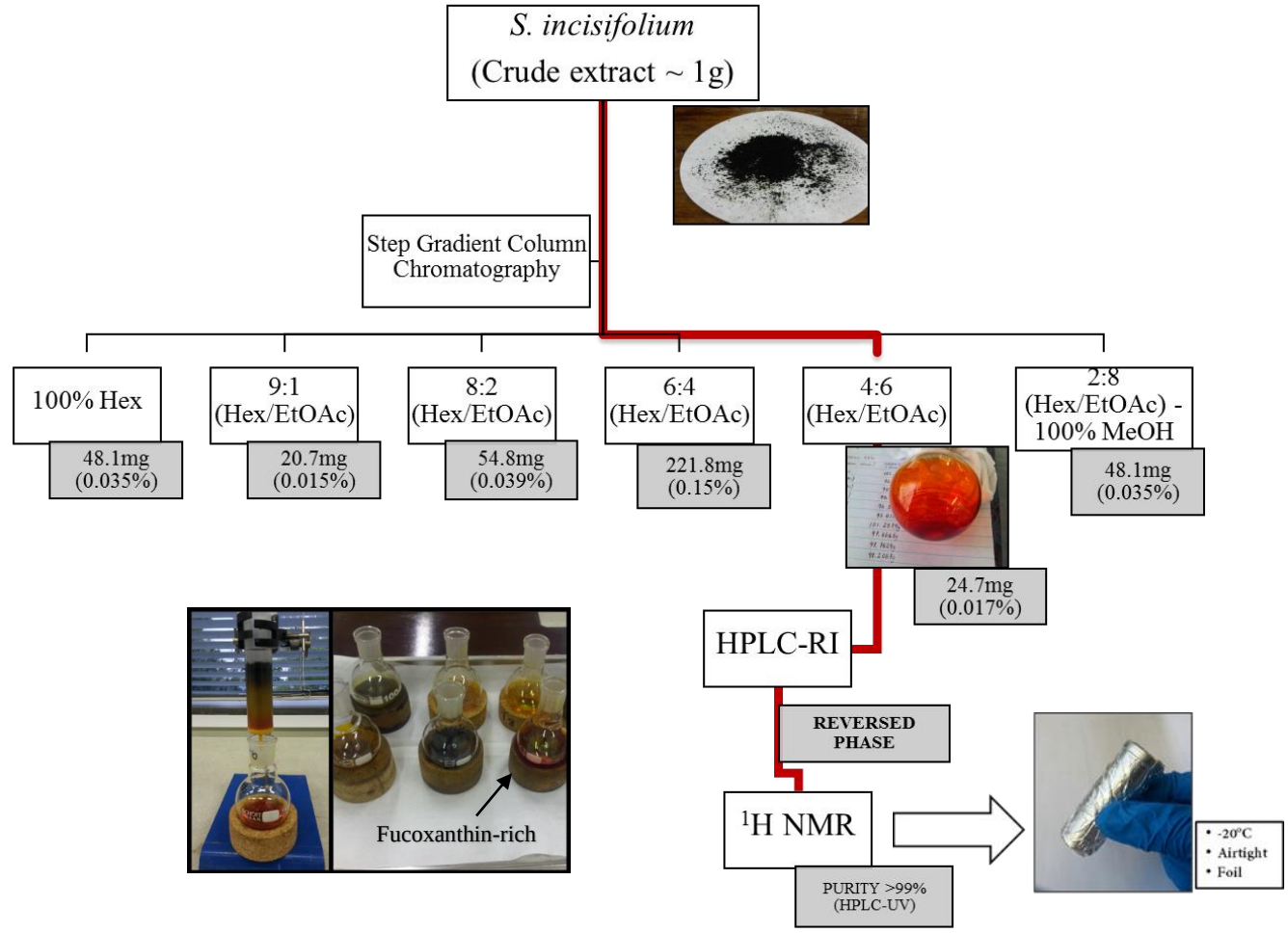
The partially thawed algae were first extracted with MeOH[14] followed by repeated extraction with CH_2Cl_2-MeOH (2:1). The solvent extracts were concentrated and re-extracted with CH_2Cl_2 to give MeOH extract (1.93 g) and CH_2Cl_2 extract (12.4 g). (Scheme 3.1).



Scheme 3.1: The extraction of *Sargassum incisifolium*

[14] The varying amounts of moisture present in the fresh/frozen algae often results in variable extraction yields. The methanol extraction step partially dehydrates the algae and thus facilitates the extraction efficiency and also give rise to more consistent results.

The crude CH_2Cl_2 extract obtained above was further fractionated by column chromatography on silica gel using solvent combinations of increasing polarity. Gratifyingly, fraction 6, **BM13_22 (6)** (hexane-EtOAc, 4:6) eluting as a red band, gave almost pure fucoxanthin (Scheme 3.2).



Scheme 3.2: The isolation of fucoxanthin from *Sargassum incisifolium*.

The effectiveness of the above procedure is well illustrated when the ^{1}H NMR (600 MHz) spectra of the different step-gradient fractions are compared (Fig 3.1). Fucoxanthin is almost completely masked in the crude extract as shown by the dominant green color and the ^{1}H NMR spectra of crude *S. incisifolium*, (**BM13_16 (1)**).

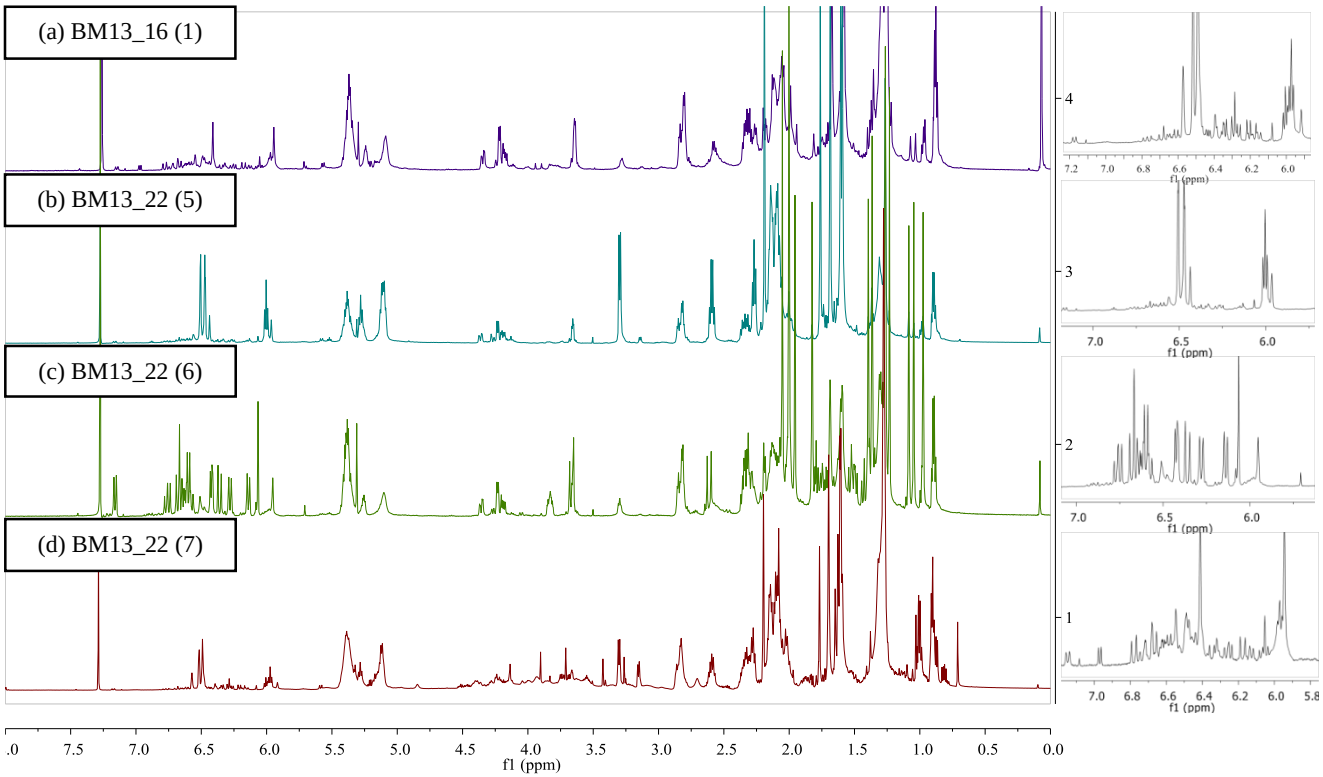


Figure 3.1: Stacked ^{1}H NMR spectra for the crude extract and step gradient fractions. (a) Crude extract, (b) fraction 5, hexane/EtOAc (6:4), (c) fraction 6, hexane/EtOAc (4:6), (d) fraction 7, hexane/EtOAc (2:8) with expansions showing regions between 6.0 and 7.2 ppm.

The fucoxanthin-enriched column fraction obtained above was further purified by semi-preparative HPLC. A comparison of semi-preparative HPLC chromatograms obtained for fucoxanthin under normal- and reversed phase conditions is shown in (Fig 3.2). It appears that the fucoxanthin isolated under reversed phase conditions is more pure (by analytical HPLC[15] and ^{1}H NMR spectroscopy) than when it is purified by normal phase HPLC (Fig 3.3). The yields are also much improved and the run time is reduced significantly.

[15] This part of the work was done after the analytical procedure developed in chapter 4. At this stage we had identified a number of challenges regarding the stability of fucoxanthin under different conditions. In particular, we identified the formation of a fucoxanthin degradant (during the experimental work) which eluted after the main fucoxanthin peak. This degradant was more easily "visualized" by analytical HPLC than ^{1}H NMR spectroscopy. Therefore, particular attention was placed on reducing exposure of samples to light, air and heat.

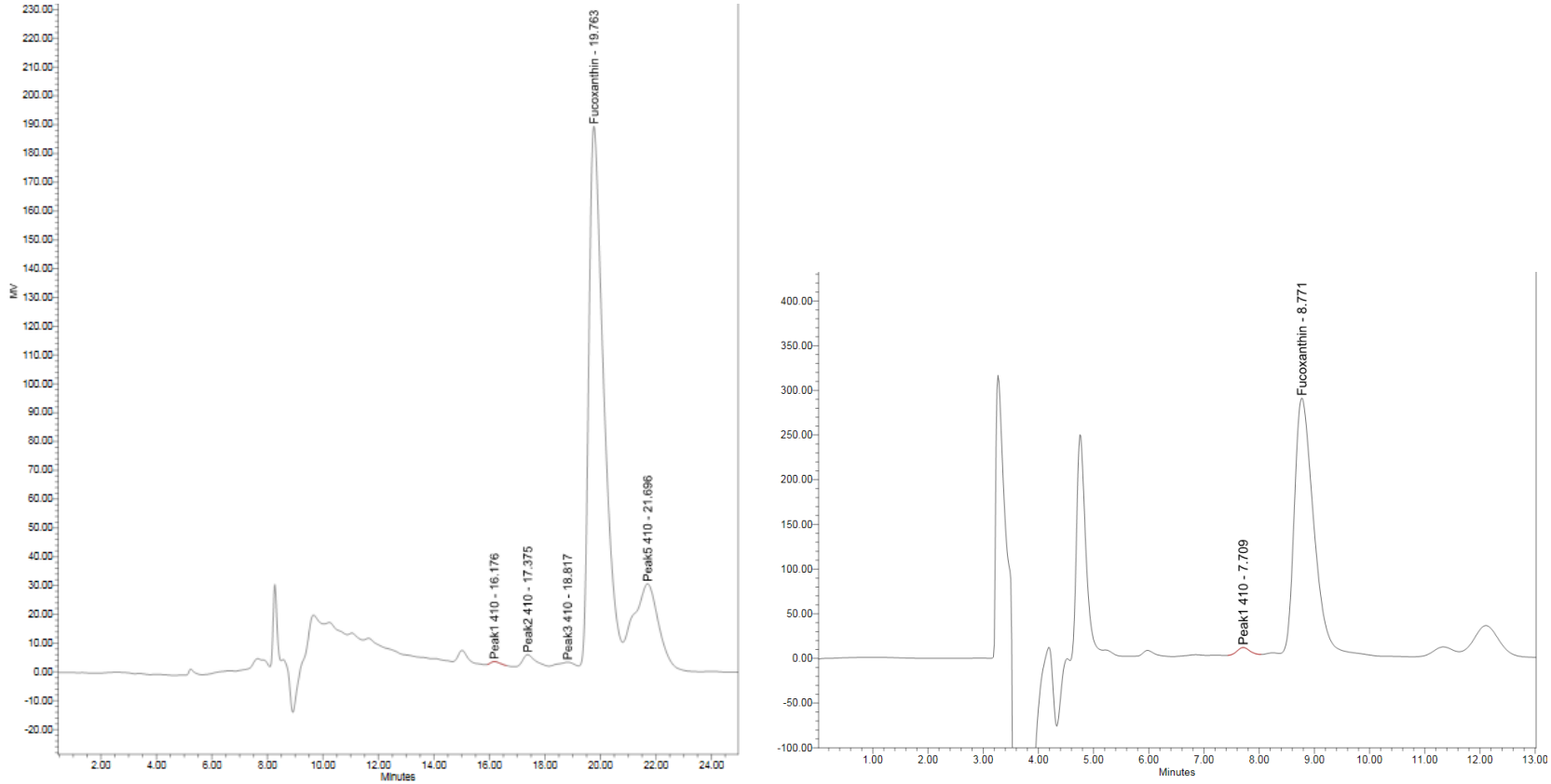


Figure 3.2: Semi-preparative HPLC chromatograms for crude fucoxanthin (**BM13_73 (6)**) on (a) normal phase (b) reversed phase. Conditions. (a) Column: Whatman® Partisil 10 500 x 10 mm i.d., mobile phase: hexane/EtOAc (4:6), flow rate: 3 mL/min, detector: refractive index (b) Phenomenex® Luna™ 250 x 10 mm i.d., mobile phase: ACN/H_2O (95:5).

The final purity of the fucoxanthin (**BM13-73b**) obtained was greater than 97% by HPLC and was used for the HPLC method validation (*see* chapter 4), quantification of fucoxanthin in brown macroalgae (*see* chapter 5) and stability studies (*see* chapter 6). This compares well to the standard obtained from Sigma-Aldrich® which was only 94% pure (*see* Appendix 3.1).

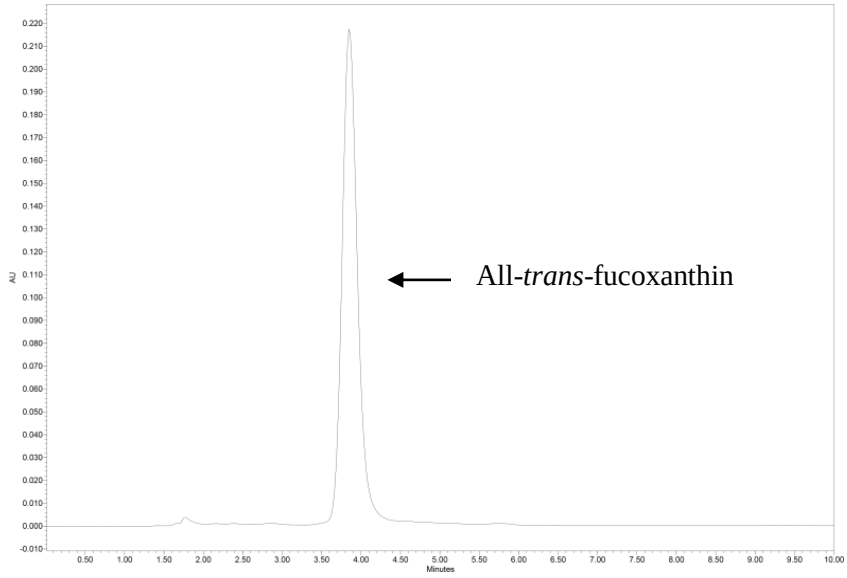


Figure 3.3: HPLC chromatogram of pure fucoxanthin (**BM13-73b**), 99% (HPLC). Conditions: Phenomenex® Synergi™ C-18 250 x 3.0 mm i.d.; mobile phase: ACN/H_2O (95:5).

3.2.2. Characterization of BM13-73b

Because of the number of related carotenoids that occur in marine algae and the possibility of isolating different isomers of fucoxanthin, we deemed a thorough characterization of our sample of fucoxanthin necessary. Furthermore, some discrepancies in the reported data for fucoxanthin have been observed. In the section below we used a number of spectroscopic techniques to identify characteristic functional groups in fucoxanthin and to confirm the geometry of the double bonds as all-*trans*. In addition, we assigned the complete structure using one- and two dimensional NMR experiments and compared chemical shift values to those in the literature (Table 3.2).

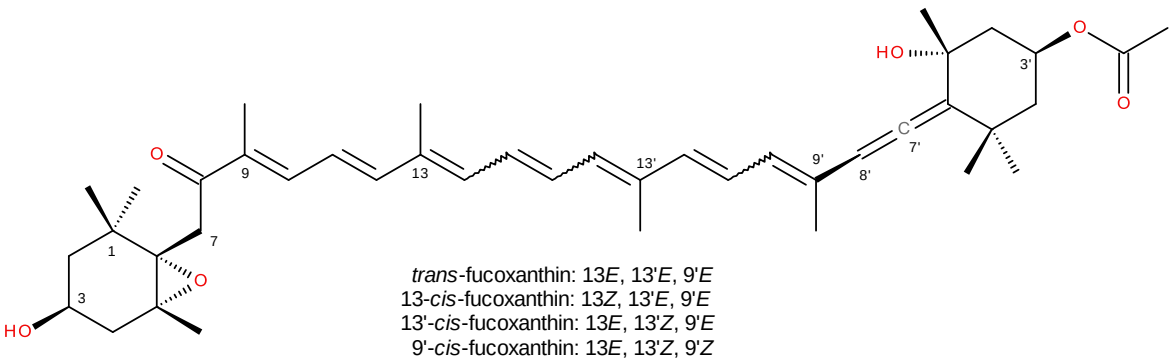


Figure 3.4: The structure of all-*trans*-fucoxanthin and its isomers.

3.2.2.1. One- and two-dimensional NMR studies of BM13-73b

The ^{1}H NMR spectrum of fucoxanthin (**BM13-73b**) (Fig 3.5, Table 3.2) shows several well-resolved and characteristic signals. The methine doublet at δ_H 7.15 (*d, J* = 11.70 Hz, H-10) is consistent with the β-proton of an α,β-unsaturated carbonyl system while the complex overlapping olefinic signals between δ_H 5.0 and 7.0 is characteristic of the unsaturated polyene chain of fucoxanthin. Broad multiplets at δ_H 3.81 (H-3) and δ_H 5.37 (H-3') are characteristic to the hydroxy-methine and the more deshielded acetoxy-methine protons in the molecule. The two mutually coupled doublets (^{2}J = 18.3 Hz) at δ_H 2.59 and δ_H 3.60 were assigned to CH_2-7 and confirmed by the HSQC NMR spectrum (HSQC correlations to δ_C 40.89). The singlet at δ_H 6.05 (H-8', the allenic proton) is an unusual feature amongst carotenoids as it represents one of the unique features of fucoxanthin, the allenic functional group, making it key in identifying the pigment. The presence of ten methyl singlet signals between δ_H 2.05 and δ_H 0.90 is easily distinguished in the ^{1}H NMR spectrum and confirmed by the HSQC data (Fig 3.6). The methyl singlet δ_H 1.98 (Me-20 and Me-20') is shared by two methyl groups and overlaps with the methylene peak for H-2'.

A comparison of the ^{1}H NMR chemical shifts (Table 3.2) of **BM13-73b** with those of the reported isomers of fucoxanthin *i.e.* 9'-*cis*, 13- and 13'-*cis* confirmed that **BM13-73b** is indeed, all-*trans*-fucoxanthin. Table 3.3 summarizes the key chemical shift differences between the various fucoxanthin isomers.

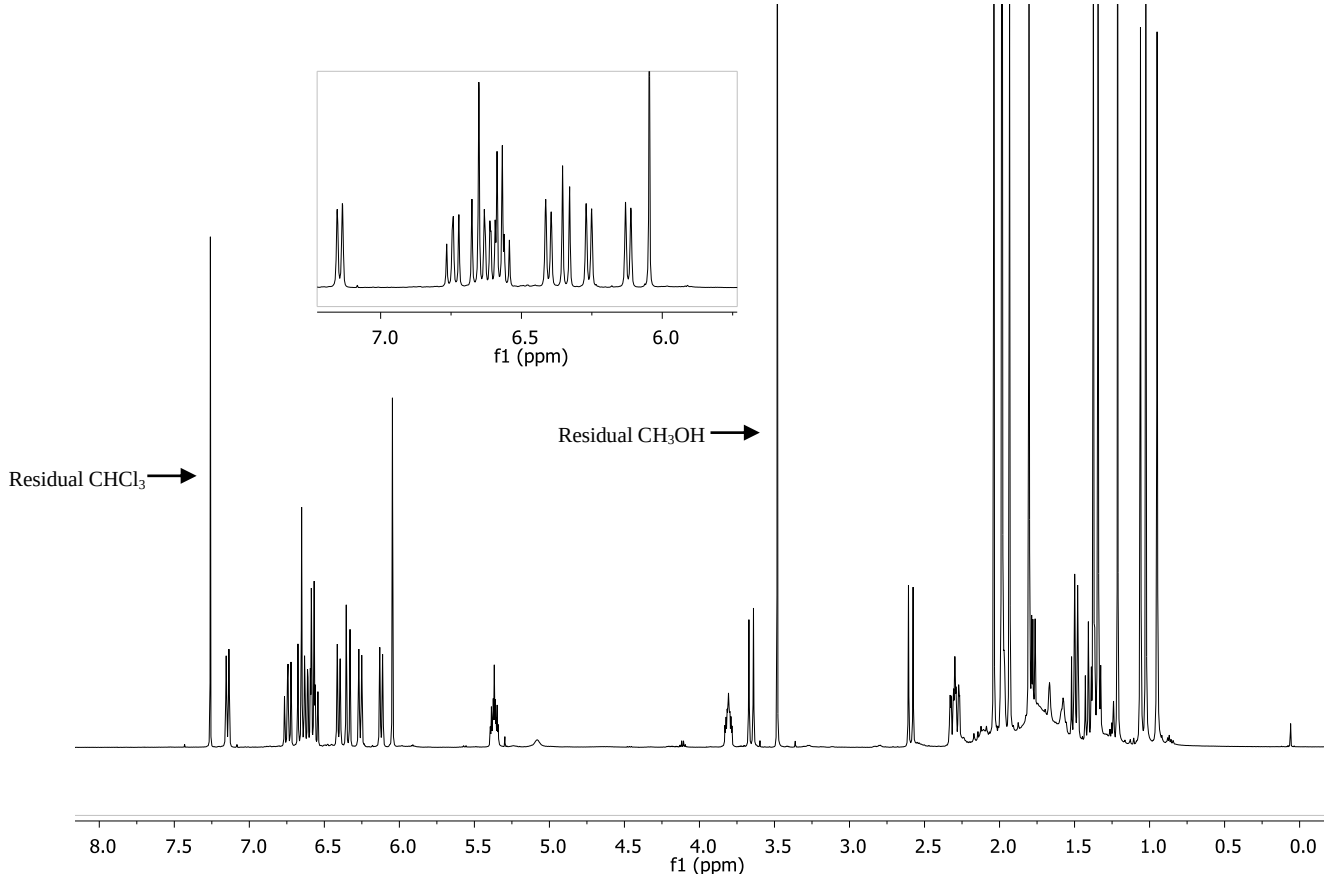


Figure 3.5: ^{1}H NMR spectrum for fucoxanthin (**BM13-73b**) (600 MHz, $CDCl_3$).

Table 3.2: ^{1}H NMR and ^{13}C NMR chemical shift comparisons of **BM13-73b**, all-trans-fucoxanthin and cis-fucoxanthin isomers.

	All *trans* fucoxanthin (Kim *et al*., 2012)		**BM13-73b**		**13'-*cis* [16]**	**13-*cis* [6]**	**9'-*cis* [6]**
C no.	**δ_C, type**, ($CDCl_3$, 100MHz)	**δ_H, mult**, *J* (Hz) ($CDCl_3$, 400 MHz)	**δ_C, type** [17] ($CDCl_3$, 150 MHz)	**δ_H, mult**, *J* (Hz)[18] ($CDCl_3$, 600 MHz)	**δ_H, mult**, *J* (Hz) ($CDCl_3$, 400 MHz)	**δ_H, mult**, *J* (Hz) ($CDCl_3$, 400 MHz)	**δ_H, mult**, *J* (Hz) ($CDCl_3$, 400 MHz)
1	35.60		35.25, **C**				
2	46.90	1.36 (*dd*, *J* = 8.7, 14.2 Hz, H-2ax), 1.49 (*dd*, *J* = 2.9, 14.2 Hz, H-2eq),	46.90, **CH_2**	1.37, *m* [19] 1.50, *m* [3]	1.36 ax 1.50 eq	1.36 ax 1.50 eq	1.35 ax 1.50 eq
3	64.20	3.80 (*m*, H-3),	64.42, **CH**	3.81, br *m*	3.82 ax	3.82 ax	3.82 ax
4	41.50 41.50	1.77 (*dd*, *J* = 8.7, 14.2 Hz, H-4ax), 2.29 (*dd*, *J* = 2.9, 17.8 Hz, H-4eq),	41.71, **CH_2**	1.78 , *m* [3] 2.30, *m* [3]	1.79 ax 2.32 eq	1.79 ax 2.33 eq	1.79 ax 2.32 eq
5	66.00		66.32, **C**				
6	66.90		67.22, **C**				
7	40.60	2.59 (*d*, *J* = 20.4 Hz, H-7), 3.64 (*d*, *J* = 20.4 Hz, H-7),	40.89, **CH_2**	2.59, (*d*, *J* = 18.3 Hz) 3.66, (*d*, *J* = 18.3 Hz)	2.60 3.66	2.61 3.67	2.60 3.66
8	170.40		197.70, **C**				
9	134.30		134.58, **C**				
10	139.00	7.14 (*d*, *J* = 12.8 Hz, H-10).	139.24, **CH**	7.15 (*d*, *J* = 11.70 Hz)	7.15	7.20	7.15
11	123.20	6.58 (*m*, H-11).	123.38, **CH**	6.57, *m*	6.57	6.59	6.57
12	144.90	6.66 (*t*, *J* = 12.8 Hz, H-12).	145.12, **CH**	6.67, *m*	6.70	7.23	6.67
13	135.30		135.54, **C**				
14	136.60	6.40 (*d*, *J* = 11.6 Hz, H-14).	136.67, **CH**	6.40, (*d*, *J* = 11.60 Hz)	6.40	6.30	6.41
15	129.30	6.67 (*m*, H-15).	129.42, **CH**	6.64, *m*	6.90	6.82	6.61
16	24.90	1.02 (*s*, Me-16).	25.01, **CH_3**	1.02, *s*	1.04	1.04	1.04
17	28.00	0.95 (s, Me-17),	28.22, **CH_3**	0.95, *s*	0.99	0.94	0.96
18	21.00	1.21 (*s*, H-18).	21.26, **CH_3**	1.21, *s*	1.23	1.23	1.22
19	11.80	1.93 (*s*, H-19).	11.84, **CH_3**	1.93, *s*	1.94	1.95	1.94
20	12.60	1.98 (*s*, H-20).	12.80, **CH_3**	1.98, *s*	1.99	1.99	1.99

[16] [1] H NMR data for the geometric isomers of fucoxanthin obtained from (Haugan *et al.*, 1992)
[17] Carbons were assigned on the basis of HSQC, DEPT-135 NMR data and comparison to literature values (Kim *et al*., 2012)
[18] There are slight discrepancies in chemical shifts and coupling constants (*e.g*. Kim *et al*., 2012 and Imbs *et al*., 2013).
[19] Coupling constant could not be determined due to overlapping signals.

Table 3.2 *continued*

1'	35.00		35.88, **CH**				
2'	45.20	1.41 (*dd*, *J* = 10.4, 14.9 Hz, H-2'ax), 2.00 (*dd*, *J* = 2.9, 14.9 Hz, H-2'eq).	45.16, **CH_2**	1.41, *m* 1.98, *m*	1.42 ax 1.99 eq	1.41 ax 1.99 eq	1.40 ax 1.99 eq
3'	67.80	5.37 (*tt*, *J* = 8.8, 12.0 Hz, H-3').	68.17, **CH**	5.37, br *m*	5.39	5.39	5.39
4'	45.10	1.53 (*dd*, *J* = 10.4, 14.9 Hz, H-4'ax), 2.29 (*dd*, *J* = 2.9, 17.8 Hz, H-4'eq).	45.37, **CH_2**	1.52, *m* 2.29, *m*	1.52 ax 2.29 eq	1.52 2.29	1.50 ax 2.28 eq
5'	72.60		72.79, **C**				
6'	117.30		117.53, **C**				
7'	202.20		202.48, **C**				
8'	103.20	6.04 (*s*, H-8').	103.46, **CH**	6.05, *s*	6.07	6.06	6.58
9'	132.40		132.65, **C**				
10'	128.40	6.12 (*d*, *J* = 11.6 Hz, H-10').	128.62, **CH**	6.12, (*d*, *J* = 11.3 Hz)	6.18	6.14	6.01
11'	125.50	6.71 (*t*, *J* = 12.0 Hz, H-11').	125.69, **CH**	6.59, *m*	6.60	6.59	6.72
12'	137.00	6.34 (*d*, *J* = 11.6 Hz, H-12'),	137.21, **CH**	6.34, (*d*, *J* = 15.0 Hz)	6.88	6.36	6.30
13'	138.00		138.20, **C**				
14'	132.00	6.26 (*d*, *J* = 11.6 Hz, H-14').	132.29, **CH**	6.26, (*d*, *J* = 11.70 Hz)	6.13	6.26	6.29
15'	132.40	6.71 (*dd*, *J* = 12.0, 14.2 Hz, H-15').	132.62, **CH**	6.74, (*dd*, *J* = 14.0, 12.0 Hz)	6.90	6.66	6.76
16'	29.00	1.37 (*s*, Me-16').	31.31, **CH_3**	1.37, *s*	1.39	1.39	1.39
17'	31.90	1.06 (*s*, Me-17').	32.13, **CH_3**	1.06, *s*	1.08	1.07	1.09
18'	31.10	1.34 (*s*, H-18').	31.39, **CH_3**	1.34, *s*	1.36	1.36	1.38
19'	13.90	1.80 (*s*, H-19').	13.97, **CH_3**	1.80, *s*	1.82	1.81	1.82
20'	12.80	1.98 (*s*, H-20').	12.80, **CH_3**	1.98, *s*	2.00	1.98	1.99
21'	197.70		170. 01, **C**				
22'	21.30	2.03 (*s*, Me, C-3'OAc).	21.51, **CH_3**	2.03, *s*	2.04	2.04	2.04

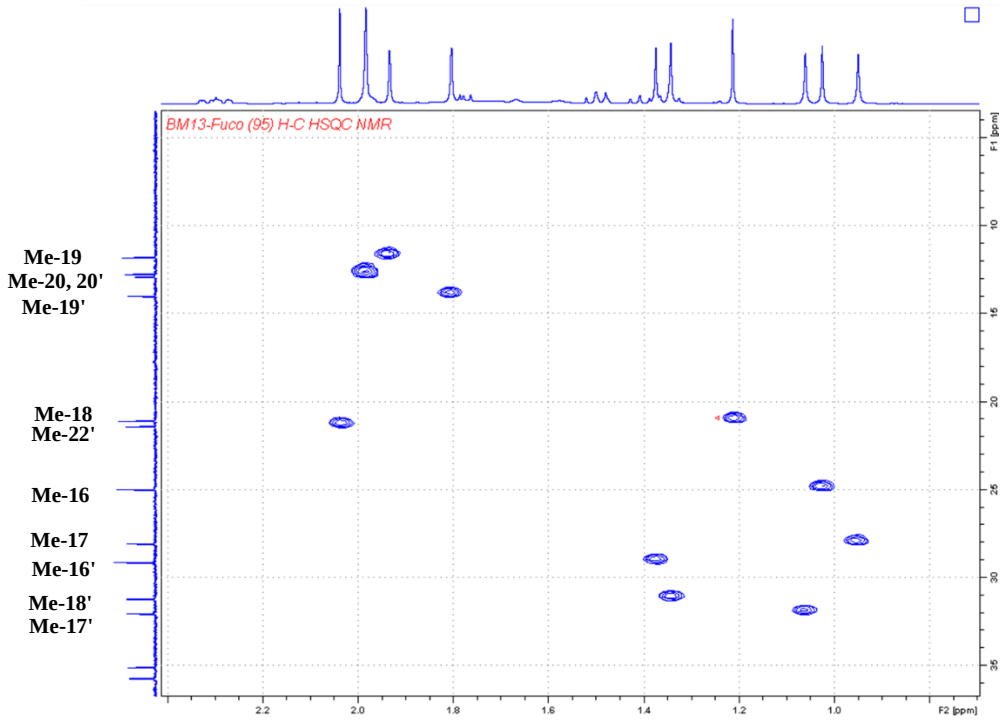


Figure 3.6: HSQC NMR spectrum of **BM13-73b** showing eleven methyl signals.

Table 3.3: Comparisons between chemical shifts of **BM13-73b** and isomers of fucoxanthin: 9', 13' and 13-cis.

Proton	BM13-73b (δ_H)	9'-*cis* (δ_H)	13'-*cis* (δ_H)	13-*cis* (δ_H)
H-8'	6.05	6.58	-	-
H-10'	6.12	6.01	6.18	-
H-11'	6.59	6.72	-	-
H-12'	6.34	6.30	6.88	-
H-14'	6.26	-	6.13	-
H-15'	6.74	-	6.90	6.66
H-10	7.15	-	-	-
H-12	6.67	-	6.70	-
H-14	6.40	-	-	-
H-15	6.64	-	6.90	-
H-17	0.95	-	0.99	-

The ^{13}C NMR spectrum of **BM13-73b** exhibiting forty two carbon signals, confirms yet more key features of fucoxanthin. The presence of the deshielded allenic bond that resonates at δ_C 202.2, the two deshielded carbonyl carbons (C-8-ketone and C-3'-acetate) at δ_C 197.70 and δ 170.01 respectively, sixteen olefinic signals between δ_C 150 and δ_C 115, five oxymethine carbons between δ_C 60 and δ_C 70, ten methylene and eleven methyl signals between δ_C 50 and δ_C 10 are all key features demonstrated by the ^{13}C NMR spectrum (Fig 3.7).

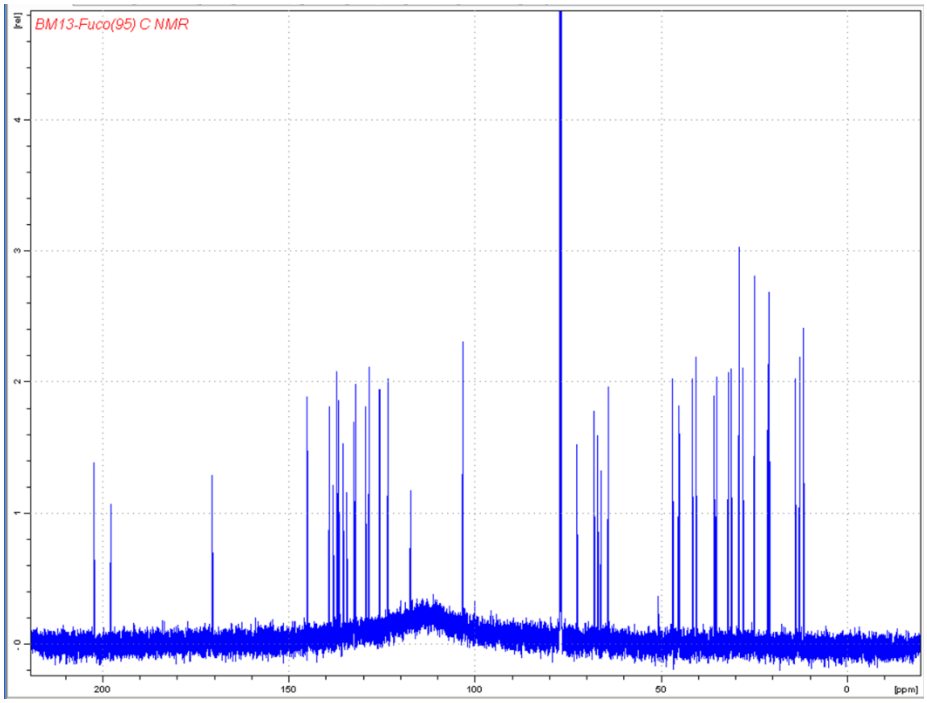


Figure 3.7: ^{13}C NMR spectrum of **BM13-73b** (150 MHz, $CDCl_3$).

Surprisingly, several publications reported the chemical shift for C-8 as δ_C 170 and the acetate carbonyl as δ_C 197.70 (Kim *et al.*, 2012; Imbs *et al.*, 2013). This intuitively appears incorrect. Analysis of the HMBC spectrum of **BM13-73b** (Fig 3.8) confirms this assignment, where strong correlations between the carbonyl δ_C 197.70 and methyl singlet (Me-19) as well as the H-7 methylene and H-10 olefinic protons are observed. Correlations between the carbonyl δ_C 170.01 and H-3' further supported the reassignment of these chemical shifts.

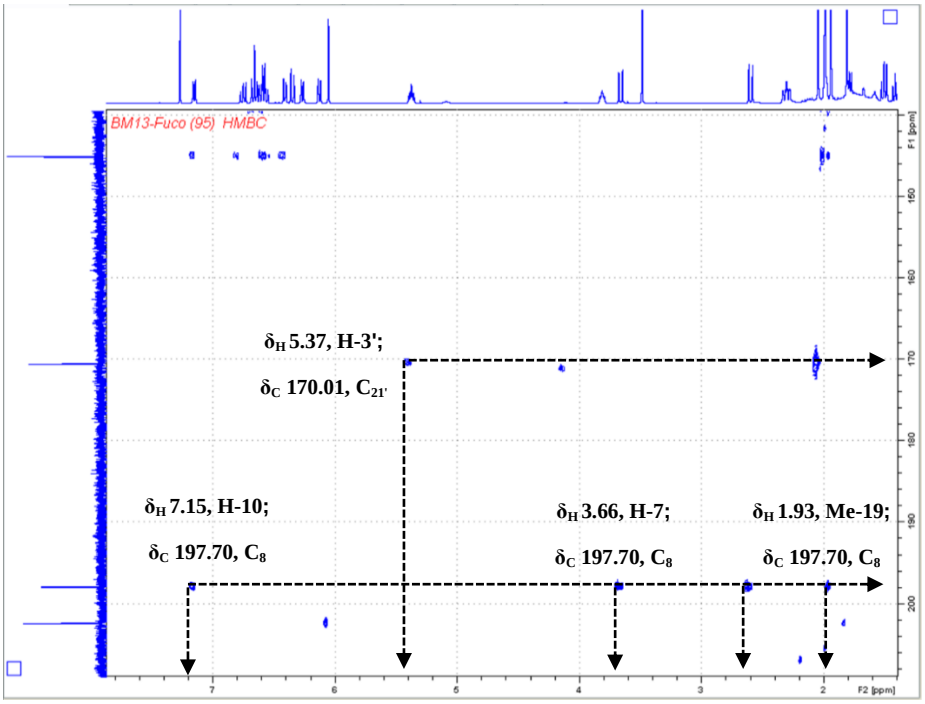


Figure 3.8: HMBC spectrum of **BM13-73b** showing the correlations used in the reassignment of chemical shifts for C-8 and the C-3' acetate.

Table 3.4: NMR spectroscopic data for **BM13-73b** showing HMBC, NOESY correlations.

C no.	δ_C, type ($CDCl_3$, 150 MHz)	δ_H, **mult**, *J* (Hz) ($CDCl_3$, 600 MHz)	δ_C, **type**, ($CDCl_3$, 100MHz)	**HMBC (^{1}H-^{13}C)**	**NOESY**
1	35.25, **C**		35.60		
2	46.90, **CH_2**	1.37, *m* 1.50, *m*	46.90	**C-16**, **17**, **C-1**, **7**	
3	64.42, **CH**	3.81, br *m*	64.20		
4	41.71, **CH_2**	1.78 , *m* 2.30 , *m*	41.50 41.50	**C-3**, **5** 18 **C-3, 5, 6**	
5	66.32, **C**		66.00		
6	67.22, **C**		66.90		
7	40.89, **CH_2**	2.59, (*d*, *J* = 18.3 Hz) 3.66, (*d*, *J* = 18.3 Hz)	40.60	**C**-1, **6**, **8**, 18 **6, 8**	**Me-16**
8	197.70, **C**		170.40		
9	134.58, **C**		134.30		
10	139.24, **CH**	7.15 (*d*, *J* = 11.70 Hz)	139.00	**C-8**, **11**, **12**, **19**	
11	123.38, **CH**	6.57, *m*	123.20	**C-10, 12, 13**	
12	145.12, **CH**	6.67, *m*	144.90	**C-10, 14, 20**	
13	135.54, **C**		135.30		

14	136.67, **CH**	6.40, (*d, J* = 11.60 Hz)	136.60	**C-12**, **20**, **15'**	
15	129.42, **CH**	6.64, *m*	129.30	**C-11, 14'**, **20**	
16	25.01, **CH_3**	1.02, *s*	24.90	**C-1**, **2**, **17**,	**H-7**
17	28.22, **CH_3**	0.95, *s*	28.00	**C-1**, **2**, **16**,	
18	21.26, **CH_3**	1.21, *s*	21.00	**C-4**, **5**	
19	11.84, **CH_3**	1.93, *s*	11.80	**C-8**, **10**, **12**	
20	12.60, **CH_3**	1.98, *s*	12.60	**C-12**, **14**, **15**	
1'	35.88, **C**		35.00		
2'	45.16, **CH_2**	1.41, *m* 1.98, *m*	45.20	**C-1'**,**3'**, **16', 17'** **C-3'**	
3'	68.17, **CH**	5.37, br *m*	67.80	**C-2'**, **4'**, **21'**	**Me-16'**
4'	45.37, **CH_2**	1.52, *m* 2.29, *m*	45.10	**C-2'**, **3'**, **5'**, **6'** **C-5'**	
5'	72.79, **C**		72.60		
6'	117.53, **C**		117.30		
7'	202.48, **C**		202.20		
8'	103.46, **CH**	6.05, *s*	103.20	**C-1', 5'**, **6'**, **7'**, **10', 9'**, **19'**	
9'	132.65, **C**				
10'	128.62, **CH**	6.12, (*d, J* = 11.3 Hz)		**C-8'**, **11'**, **12'**, **13'**, **19'**	
11'	125.69, **CH**	6.59, *m*		**C-9'**, **10', 12'**, **13'**	
12'	137.21, **CH**	6.34, (*d, J* = 15.0 Hz)		**C-10'**, **11'**, **14' 13'**, **20'**	
13'	138.20, **C**				
14'	132.29, **CH**	6.26, (*d, J* = 11.70 Hz)		**C-12'**, **13', 20'**	
15'	132.62, **CH**	6.74, (*dd*, *J* = 14.0, 12.0 Hz)		**C-12'**, **13'**, **14**, **15**	
16'	31.31, **CH_3**	1.37, *s*		**C-17'**, **1', 4', 6'**,	**H-3'**
17'	32.13, **CH_3**	1.06, *s*		**C-3'**, **6'**, **16'**	
18'	31.39, **CH_3**	1.34, *s*		**C-3'**, **5'**	
19'	13.97, **CH_3**	1.80, *s*		**C-8'**, **10'**, **11'**, **12'**, **14'**, **15'**	
20'	12.80, **CH_3**	1.98, *s*		**C-12'**,**14'**, **15'**	
21'	170. 01, **C**				
22'	21.51, **CH_3**	2.03, *s*		**C-21'**	

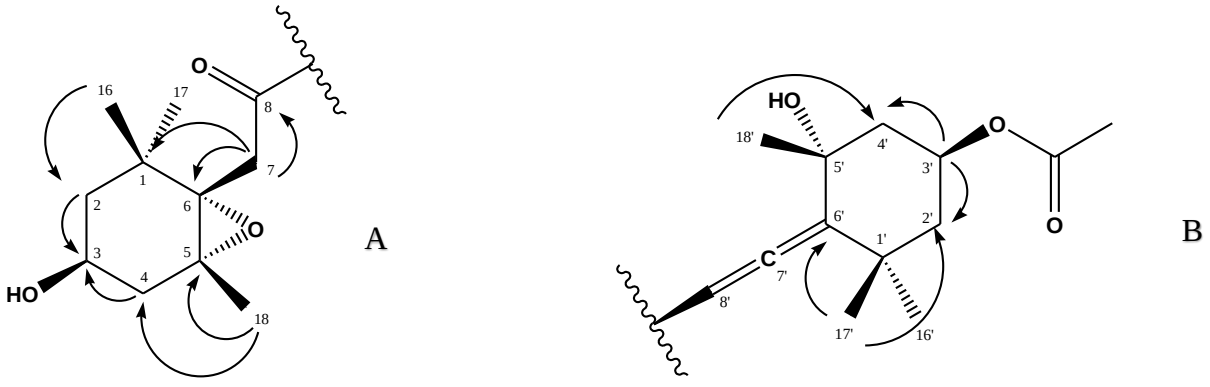


Figure 3.9: HMBC correlations about ring A and B characteristic to the molecule of fucoxanthin.

Key HMBC correlations were observed characteristic to the A and B ring of fucoxanthin. The strong correlations observed between H-7 and the α,β-unsaturated ketone at position 8 further substantiate the aforementioned correction we made to the assignment of δ_C 197.70 previously assigned to C-3'-acetate instead of C-8 as reported by Kim *et al*. (2012). More correlations also confirm the assignments of Me-16 to be on the same ring as the methylene protons of C-2 at δ_H 1.37 and δ_H 1.50. The correlations observed between δ_H 5.37 and C-2' and C-4' further confirm that the more deshielded broad multiplet oxymethine is indeed on ring B and coupled to the acetate (Table 3.4; Fig 3.9).

The assignment of the complex overlapping signals of the olefinic region were further substantiated by more key HMBC correlations shown in (Fig 3.10). Long range correlations between H-8' and C-11' *i.e.* (δ_H 6.05, δ_C 125.50) confirmed the proper assignments for close chemical shifts δ_H 6.57 and δ_H 6.59 to be H-11 and H-11' respectively. The methyl, methylene (δ_C 11 – 50) and oxymethine signals (δ_C 60 – 70) confirmed the position of rings A, B and the allenic functional group (Fig 3.11; Fig 3.12). The methyl groups (Me-19' and Me-20') are confirmed by HMBC correlations between H-12' and H-10' and C-19' and C-20' (δ_H 6.26, δ_C 13.97 and δ_H 6.12, δ_C 12.80) as well as methyl group (Me-19) with correlations between H-10 and C-19 (δ_H 7.15 and δ_C 11.84) all shown in (Fig 3.12).

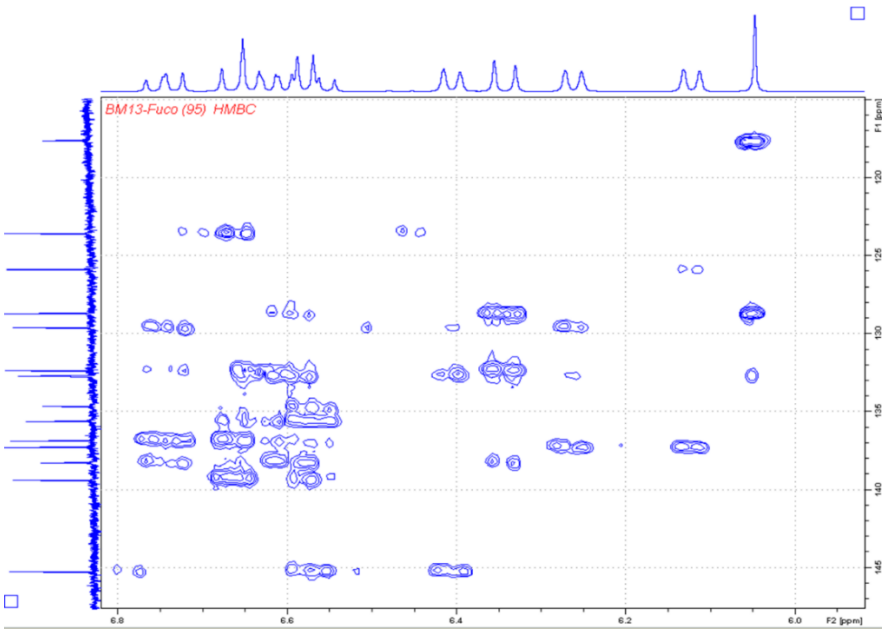


Figure 3.10: HMBC spectrum for **BM13-73b** showing correlations from the olefinic region characteristic to the molecule of fucoxanthin.

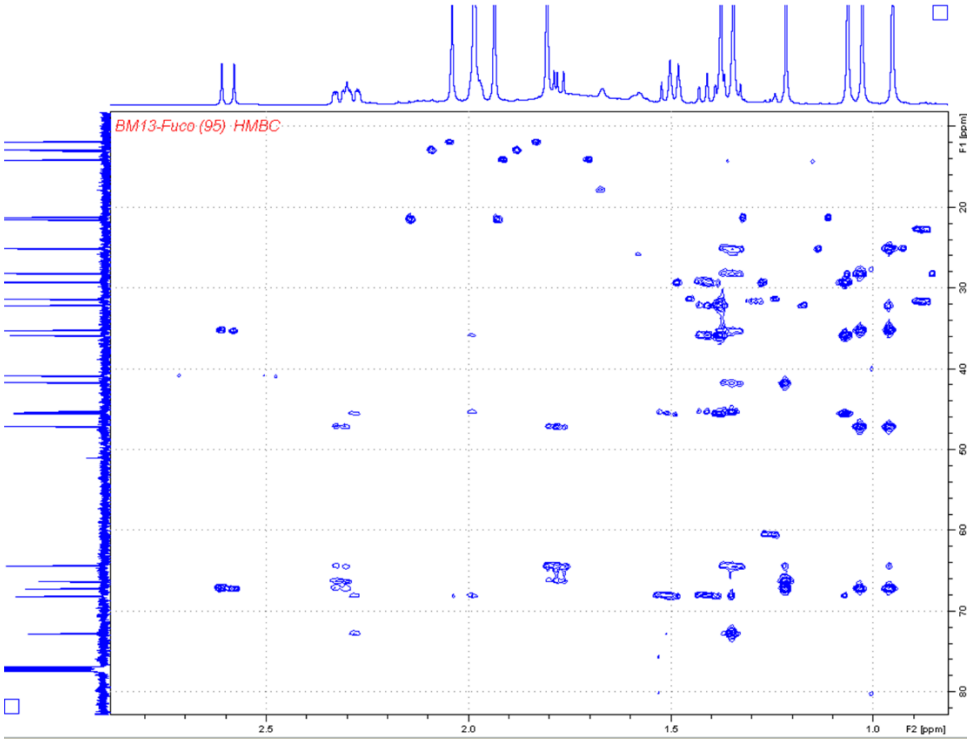


Figure 3.11: HMBC spectrum for **BM13-73b** showing correlations from the methylene and methyl regions characteristic to the molecule of fucoxanthin.

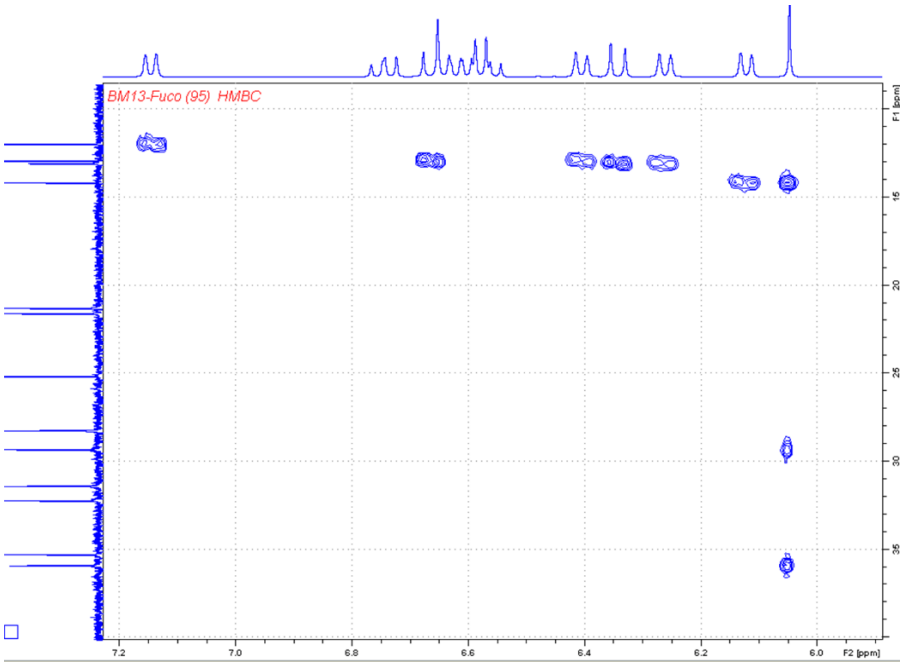


Figure 3.12: HMBC spectrum for **BM13-73b** showing correlations from the oxymethine signals and the allenic functional group on the molecule of fucoxanthin.

3.2.2.2. UV-Vis

The UV-Vis profile of **BM13-73b** (Fig 3.13) was compared to that of Rajauria and Abu-Ghannam (2013) to further authenticate the isolated reference standard as fucoxanthin. The absorption maximum (λ_{max}) of 446 nm agrees with reported values for fucoxanthin isolated from the brown seaweed *Himanthalia elongata* (Rajauria and Abu-Ghannam, 2013). It was also important to determine the λ_{max} as it was going to be pivotal in the method development and validation experiments (*see* chapter 4) as well as subsequent studies in the quantification of fucoxanthin from several brown seaweed (*see* chapter 5) and stability studies (*see* chapter 6).

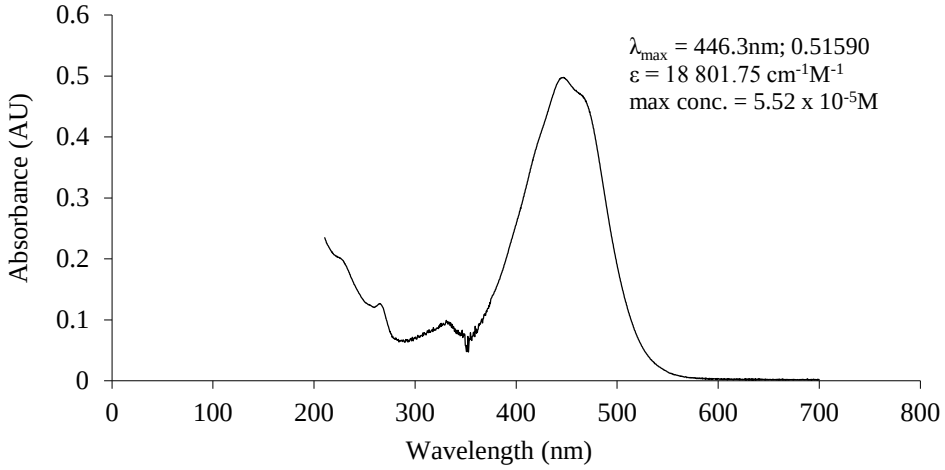


Figure 3.13: UV-Vis spectrum for **BM13-73b** showing wavelength of maximum absorption (446 nm)

3.2.2.3. IR

Using IR (Fig 3.14), we were able to attribute the absorption bands that are characteristic to the functional groups present in **BM13-73b** to further authenticate the identity of the target compound, fucoxanthin. The absorption bands characteristic to fucoxanthin were identified in the FT-IR spectra of **BM13-73b** and the wave numbers listed (*see* section 3.3.4.2) compared well with those reported in literature (Rajauria and Abu-Ghannam, 2013; Haugan and Liaane-Jensen, 1992).

Rajauria and Abu-Ghannam (2013), showed a comparison of their purified fucoxanthin and standard fucoxanthin (*see* Appendix 3.2).

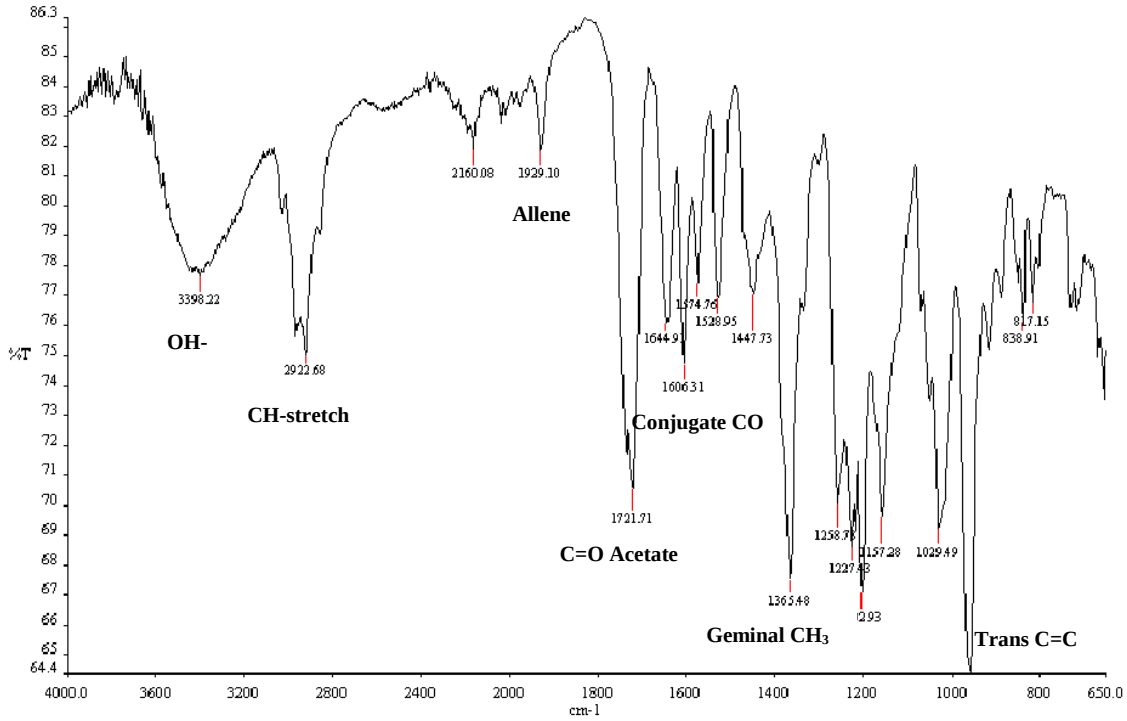


Figure 3.14: FT-IR spectrum for **BM13-73b** showing key wave numbers characteristic to fucoxanthin.

3.2.3. Conclusion

Sufficient fucoxanthin of high purity (99% by analytical HPLC), that was evidently of better quality compared to the purchased standard from Sigma-Aldrich® (94%, *see* supplementary chapter), was successfully isolated from *S. incisifolium.* Traditional extraction techniques (maceration) followed by a hexane/EtOAc step-gradient fraction and reversed phase semi-prep HPLC were used to consistently obtain fucoxanthin (~11 mg/g dry weight) with each subsequent extraction. Characterization experiments including one- and two-dimensional NMR, UV-Vis and IR confirmed the authenticity of all-*trans*-fucoxanthin.

3.3. Experimental

3.3.1. General procedures

All NMR experiments to identify and characterize fucoxanthin were recorded on a Bruker® Avance™ 600 MHz spectrometer using $CDCl_3$ as solvent. ^1H NMR spectra were recorded at 600 MHz and ^{13}C NMR experiments at 150 MHz. All step-gradient column chromatography procedures were performed using silica gel 60 (0.040-0.063mm, 230-400 mesh) from Merck KGaA (Darmstadt, Germany). Organic solvents were used for the extraction and isolation of fucoxanthin. Methanol was of HPLC, UV and liquid scintillation grade (Methanol 215, Romil-SpS™) from Romil LTD (Cambridge, UK) distributed by Microsep™ (South Africa). Dichloromethane, hexane and ethyl acetate was of liquid chromatography grade (Lichrosolv®) from Merck KGaA (Darmstadt, Germany). Hexane and ethyl acetate for HPLC were also of liquid chromatography grade from Merck chemicals. Water used in any procedure was distilled (unless otherwise mentioned) and absolute ethanol was sourced from the chemistry stores (Rhodes University, South Africa)

The HPLC procedures to purify fucoxanthin were carried out using a Waters® 1515 isocratic HPLC Pump and Waters® 2414 Refractive Index (RI) detector (Massachusetts, USA) distributed by Microsep™ (South Africa). The system was also fitted with a Rheodyne® injector. The reversed phase semi-prep column Phenomenex® C-18 Luna (2), 250 x 10.0 mm i.d. (California, USA) was used. Waters® Empower2™ computer software was used to assist the operation of the HPLC system and for all the necessary peak identification and peak area calculations. UV spectra were measured with a GBC® 916 Spectrophotometer with Cintra™ software (Connecticut, USA). Standard quartz UV cuvettes were used and the sample (**BM13-73b**, 2.85×10^{-5} M) was dissolved in MeOH. The experiment was done in triplicates, the absorbance recorded and the molar absorptivity estimated by calculation. IR spectra were recorded on a Perkin Elmer® Spectrum 100 FT-IR Spectrometer. The sample (**BM13-73b**, ¼ full spatula) was placed onto the KBr crystal embedded on the machine's sample platform and analyzed. Orange amorphous powder; IR (KBr) ν_{max} cm^{-1}: 3398, 2923, 1929, 1722, 1606, 1365, 1259, 1201-950.

3.3.2. Plant material

S. incisifolium for this study was collected from two different beaches at low tide. **PA100331-3** (Port Alfred, South Africa on the 31st of March 2010) and **NDK101124-5** (Noordhoek, Port Elizabeth, South Africa on the 24th of November 2010) and stored in the deep freezer at -18 °C in room G4 located in the Pharmaceutical Chemistry division (Rhodes University, South Africa). Work on these particular algae was commenced on the morning of the 14th of February, 2013 which translates to approximately 3 years of storage.

3.3.3. Extraction and isolation[20]

Frozen algae **PA100331-3** and **NDK101124-5** (139 g) were thawed at room temperature and soaked in MeOH (1.5 L, 2 L glass conical flask) for 1 hour. The fronds, pretreated in MeOH, were re-extracted (x 3) in a mixture of CH_2Cl_2/MeOH (2:1, 1.5 L) and heated at a maintained temperature of 35 °C for 30 min. The three extracts were filtered (gravitational paper filtration) and then concentrated in reverse order by rotary evaporation. Any traces of water were removed from the organic phase by a phase separation procedure which involved extraction with CH_2Cl_2 (100 mL). The polytop vials were sealed with parafilm, wrapped in aluminum foil and stored in the freezer at -18 °C. The initial procedure for the extraction of brown seaweed, *S. incisifolium* is detailed in (Scheme 3.1). The extracted fronds were left in the fume hood to dry.

Silica gel column fractionation was carried out on a ten step gradient column using hexane, EtOAc and MeOH. The first step was 100 % hexane.[21] The gradient of increasing polarity was initially designed to change by a factor of 10 % *i.e*. the second step had 90 % hexane and 10 % ethyl acetate. The gradient was then altered at step 4 to change by a factor of 20 % *i.e*. from a mixture of hexane/EtOAc (8:2) to (6:4) until step 8 which comprised 100% EtOAc. MeOH was introduced into the gradient in a (1:1) mixture with EtOAc for step 9 and was used as the column wash for

[20] The extraction and isolation of fucoxanthin was as described according to literature (Afolayan *et al*., 2008) with slight modifications.

[21] The CH_2Cl_2 extract was poorly soluble in hexane (first step for the gradient column). Approximately 1 g of extract was therefore dissolved in sufficient CH_2Cl_2 and 2 spatula-full silica gel 60 (mesh 70-230) were added to the round bottom flask containing the extract. The mixture was homogenized by swirling before the solvent was carefully (low pressure) evaporated off by rotary evaporation. The extract adsorbed onto the silica was scraped off in preparation for the gradient column.

step 10 (100 % MeOH). Ten fractions were afforded, **BM13_22 (1-10),** dried under vacuum and analyzed by NMR (Fig 3.1, *see* Appendix 3.1). **Fraction (6)** obtained from a step gradient was further purified using reversed phase HPLC using ACN/H_2O (95:5) to afford pure fucoxanthin, **BM13-73b**.[22]

3.3.4. Characterization of BM13-73b

3.3.4.1. NMR

One-dimensional ^{1}H NMR, ^{13}C NMR and DEPT-135 NMR experiments were carried out on **BM13-73b**. The sample was then further analyzed by another set of two-dimensional NMR experiments *i.e.* Correlation Spectroscopy (COSY), Heteronuclear Single-Quantum Correlation spectroscopy (HSQC), Heteronuclear Multiple Bond Correlation spectroscopy (HMBC) and Nuclear Overhauser Effect spectroscopy (NOESY).

[22] This procedure (Extraction and isolation) was repeated several times as needed. **BM13-73b** is from fraction 6, **BM13_73 (6)**

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Chapter 4

Analysis of fucoxanthin from the brown alga *Sargassum incisifolium* (Turner) C. Agardh by HPLC-UV/Vis: Method development and validation

4.1. Introduction

4.1.1. The analysis of herbal extracts including carotenoids

Analytical procedures in the pharmaceutical industry, are in part useful in the categorical description of an active compound. It is as required by the main pharmaceutical regulatory authorities such as Food and Drug Administration (FDA), International Conference on Harmonization (ICH), European Medicines Agency (EMEA) and the World Health Organization (WHO). The EMEA expects phytochemical characterization of herbal substances and this includes qualitative (chromatographic fingerprinting) and quantitative analysis of active markers. The organization views quality to be independent of traditional use therefore all general guidelines pertaining to quality must apply for herbal preparations.[23] The WHO in their quest to ensure the quality assurance of herbal preparations, recommended the use of HPLC amongst other spectroscopic instruments in a document released in 2007.[24] The versatility and appropriateness of HPLC in the fingerprinting of herbal extracts has been widely reported and its numerous advantages highlighted (Table 4.1). One such advantage is demonstrated by the improvement of the quantification of single entities from crude extracts. In the 1960s, quantification of pigments was developed by spectrophotometric means. It became a standard procedure using equations such as that of Strickland and Parsons albeit being deemed to overestimate or underestimate pigment quantities *e.g.* carotenoids, chlorophylls *a, b, c* and derivatives because some pigments were difficult to differentiate adequately (Jeffrey, 1999). The advent of HPLC in the 1980s, surely for

[23] EMEA guidelines for herbal preparations (2008): http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003211.pdf/ [Accessed 20 January 2014]
[24] WHO. Quality assurance of herbal preparations (2007): http://www.who.int/medicines/areas/quality_safety/quality_assurance/QualityAssurancePharmVol2.pdf/ [Accessed 20 January 2014].

algal chemistry, made it possible to simultaneously determine the concentrations of pigments and acquire information unattainable by other analytical procedures (UV, Fluorometry, *etc*.) in a single run making it ideal and very common (Bidigare *et al.,* 2005). For emerging science, the view held by governments and regulatory authorities placed aside, the identification of actives from natural sources is fundamental and has not stopped the development of procedures to identify, authenticate and quantify secondary metabolites of herbs and algae alike. HPLC is an established, relevant and recommended analytical technique for the analysis of herbal preparations and this text will focus on fucoxanthin analysis (Table 4.1).

Routine analyses of algal extracts and pigments is dependent on the characteristics of the compound(s) of interest. Ultraviolet/Visible (UV/Vis) HPLC for example, is predominately useful for compounds with a strong chromophore(s) and inherent delocalized electron systems. Fucoxanthin's dominating carbon-carbon conjugated double bond system is responsible for the orange to brick-red color imparted to the molecule (Maoka *et al.,* 2002). The color of a compound is somewhat indicative of the wavelength at which it absorbs energy. Orange/red colored substances for instance, absorb blue/violet light *i.e*. this is the color missing when the substance reflects light. Routinely so, fucoxanthin and related carotenoids have been previously analyzed by UV/Vis at wavelengths ranging from about 400 – 500 nm (blue/violet region). Selected methods used to analyze fucoxanthin (Table 4.1).

4.1.2. Method development and validation characteristics for HPLC

Developing an analytical procedure takes inspiration from previous attempts, what has been done before whether successful or not. It largely depends on the objective of the analysis. Manufacturers of analytical equipment however, usually spearhead the recommendations when they highlight the capability and operating parameters of their equipment. Agilent technologies® devised a general protocol for method development using their HPLC designs;

- Describe the sample
- Establish goals
- Consider sample preparation

- Choose detector
- Choose chromatographic mode
- First isocratic run
- Optimize separations
- Predict problems
- Validate and release method[25]

The sample has to be adequately characterized in order to assess the plausibility of even considering the use of HPLC. The non-volatile and thermo-labile nature of most natural products warrants the use of HPLC in the both qualitative and quantitative experiments. Once established, the sample and goals of the analytical procedure in turn determine the detector and chromatographic mode to run the analysis under. The first isocratic and subsequent runs are monitored using factors that monitor the performance of the stationary phase specifically with respect to the both the sample and carrier (mobile phase). These include selectivity and capacity factors, peak resolution and peak symmetry.

Selectivity factor:

Selectivity is a measure of peak separation. It describes how differently two components behave in the same chromatographic mode. The larger the selectivity factor, the further apart the two peaks are from each other. It is a ratio of the capacity factors of two peaks. Selectivity must be greater than 1.0 (Ornaf and Dong, 2005).

Capacity factor/Retention factor (k'):

Retention depends on the different polarities of the mobile phase and the column stationary phase. It describes how far a peak is from the void volume as well as measure how many times an analyte is retained relative to an unretained compound. The capacity/retention factor is proportional to retention. An acceptable range is between 1 and 20 (Ornaf and Dong, 2005).

[25] The LC Handbook – Agilent technologies: http://www.chem.agilent.com/Library/primers/Public/LC-Handbook-Complete-2.pdf/ [Accessed 21 January 2014]

Peak resolution (R_s):

Resolution describes how far apart two peaks are relative to how broad they are. A resolution of 1.0 shows there is clear baseline differentiation. A resolution of 1.5 or greater is often required as it allows for a more robust separation and quantitation (Ornaf and Dong, 2005).

The main aim for an analytical procedure is nothing more complicated than to analyze an expected or unknown response. However, an analytical method that is not reproducible is of not much use. One needs to be confident about scientific results obtained. The suitability for the intended purpose of any analytical procedure must therefore be demonstrated. This process is termed validation.

There are several, necessary validation characteristics. The ICH provides a standard to follow when carrying out various tests on an analytical procedure. The characteristics often include; linearity, accuracy, precision, specificity, detection limits, quantitation limits, range and robustness. All these tests contribute to provide an overall sound knowledge of the capabilities of the analytical procedure. The following characteristics will be of relevance to this text;

Specificity:

The ICH defines specificity as "the ability to assess unequivocally the analyte in the presence of components which may be expected to be present". In the case of HPLC, the resolution calculations of closely eluting peaks may be used to demonstrate the specificity of the analytical procedure, so does the visual inspection of the homogeneity of the peaks of interest (Huber, 2007)

Linearity:

The ICH defines linearity as "the ability (within a given range) to obtain results which are directly proportional to the concentration (amount) of the analyte in the sample". A standard stock solution and serial dilutions may be used to demonstrate linearity and appropriate statistical methods are employed *e.g*. plot of the data, calculations for correlation and a calibration equation. A minimum of five concentrations is required (Huber, 2007).

Accuracy:

"The closeness of agreement between the value which is accepted either as the true conventional value or an accepted reference value and the value found." Accuracy may be reported as % recovery of known analyte applied to the analytical procedure (Huber, 2007).

Precision:

"The closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogenous sample under prescribed conditions". There are three levels of precision; repeatability (intra-assay), intermediate precision (different days, different analyst and different equipment) and reproducibility (different labs). Precision may be demonstrated by standard deviation and coefficient variation *i.e*. the relative standard deviation (RSD) (Huber, 2007).

Recovery:

"An assay of a known amount of analyte added to the sample". It assesses the extraction efficiency of the designed method (Huber, 2007).

Limits of detection:

"The lowest amount of analyte that can be reliably detected without necessarily being quantified as an exact value". This may be determined by visual assessment, signal to noise ratio and standard deviation of the response and the slope (Huber, 2007).

Limits of quantitation:

"The lowest amount of an analyte in a sample that can be quantitatively determined with suitable accuracy and precision". As is with limits for detection, visual assessment and/or estimation by calculation using the standard deviation of the response and the slope is sufficient to demonstrate this characteristic (Huber, 2007).

4.1.3. Methods used previously in the quantitative analysis of fucoxanthin

Selected methods used in the analysis of fucoxanthin are summarized in Table 4.1.

Table 4.1: HPLC methods used previously in the analysis of fucoxanthin/fucoxanthin containing algal extracts.

Matrix analyzed	Sample preparation	Mobile Phase/conditions	Columns	Detection	Reference
Dried powder of Wakame *Undaria pinnatifida*	Extraction with acetone followed by silica column (hexane/acetone)	MeOH/ACN (70:30) Flow rate: 1 mL/min	Develosil® ODS-UG-5; C-18 250 x 4.6 mm i.d. 5 µm particle size 10 x 4.0 mm i.d. guard; C-18	UV-Vis 450 nm	Maeda *et al.*, 2005
Algal cultures *Pavlova lutheri*	Acetone extraction under centrifugation	Linear gradient ACN/H_2O (90:10) to EtOAc 100%, 2 mL/min for 20 min	Rad-Pak® A Octadecyl silica x 2 in series 5 µm particle size	UV-Vis 405-436 nm	Wright and Jeffrey, 1987
Lyophilized, milled *Hijika fusiformis*	Acetone extraction followed by a silica gel column (hexane/ethyl acetate) then dark orange band separated by flash chromatography chloroform/acetone (10:1)	$CHCl_3$/Me_2CO (9:1) Flow rate 1 mL/min	Spherisorb® Silica gel column 5 µm particle size 250 x 4.6 mm i.d. 40^{0}C	UV-Vis 450 nm	Yan *et al.*, 1998
Dried, ground seaweed *Sargassum binderi* & *Sargassum duplicatum*	Cold acetone/methanol (7:3) extraction followed by partitioning in hexane/methanol (1:9) then a silica gel column (100% hexane - hexane/acetone (6:4)	MeOH/ACN (7:3) Flow rate 1 mL/min	HPLC prep double Develosil® (ODS) RP column (250 x 4.6mm i.d.) Guard column 10 x 4.0 mm i.d. under dim yellow light	PAD 450 nm	Jaswir *et al.*, 2012
Dried, milled *Fucus serratus*	Silica gel column stepwise gradient hexane/ethyl acetate (10:0) - (4:6) flash chromatography for fuco-rich fraction LiChroprep® RP-18 (40-63 µm; 240 x 11 mm) acetonitrile/methanol/water (75:15:10) plus 0.1 % ammonium acetate	ACN/MeOH/H_2O (75:15:10) 1 g/L ammonium acetate Flow rate 1 mL/min	TSK gel ODS 80Ts (Tosoh) 250 x 4.6 mm i.d. Pelliguard® LC-C18 20 x 2 mm i.d.	UV-Vis 450 nm	Sugawara *et al.*, 2002
Purchased dry *Undaria pinnatifida* and *Sargassum fusiforme* and fresh *Laminaria japonica*	2 g soaked in water (5min); Irradiated with microwaves; Concentrated extract was dissolved in hexane/ethyl acetate/ethanol/water (5:5:6:4) and introduced into the HSCCC system	ACN (A)/H_2O (B) gradient system. 90 -100 % A (10 min) 100 -100 % A (10 -12 min) 90 - 90 % A (12 -18 min) Flow rate: 1 mL/min	Kromasil C-18 250 x 4.6 mm i.d. 5 µm Guard: 4.0 x 3.0 mm 5 µm	UV-Vis 450nm	Xiao *et al.*, 2012

The common feature in the studies reviewed show the extensive use of reversed phase HPLC with UV-Vis detection. Although the ICH guidelines for the validation of analytical procedures were chiefly designed for pharmaceutical substances these principles may also be applied to herbal products. To the best of our knowledge, fucoxanthin analysis in previous studies have not included validation. Only reports on carotenoid analysis have included validation of their methods (Kao *et al*., 2012).

4.1.4. Chapter Aims

The aim of the research in this chapter was to develop an HPLC method and validate its suitability for the quantification of fucoxanthin from crude extracts of diverse samples of South African marine brown algae (*see* chapter 5). The ability of HPLC to adequately produce separated pigments of higher purity in a continuous and repetitive manner, makes it an ideal analytical procedure to quantify fucoxanthin when compared to thin layer chromatography which was reported to be in turn, much better than column chromatography in this respect (Abaychi, 1979). Therefore HPLC was selected as a reliable analytical option over its almost sure alternative, a stand-alone UV-Vis procedure which is unable to distinguish between compounds in a continuous manner.

Chapter objectives:

1. Develop an HPLC analytical procedure (qualitative and quantitative) for the determination of fucoxanthin (isolated from marine brown algae *S. incisifolium*) and the quantification of the pigment from brown algal crude extracts (*see* chapter 5).
2. Validate (external calibration) the aforementioned analytical procedure (1) by assessing linearity, accuracy and precision, recovery, detection and quantitation limits.

4.2. Results and Discussions

4.2.1. Method development

The use of methanol as a solvent was based on its ability to dissolve and efficiently extract fucoxanthin from algal biomass much better than acetone, ethanol and a combination of dichloromethane and methanol (*see* chapter 5).

The relatively nonpolar nature of fucoxanthin limits the solvents that may be used as mobile phase in its analysis by reversed phase HPLC. Since most previous studies used a combination of MeOH and ACN as mobile phase (Jaswir *et* al., 2012) we explored the effect of these mobile phases on the retention behavior of fucoxanthin on an Xterra® 150 x 4.6 mm i.d. (Fig 4.1).

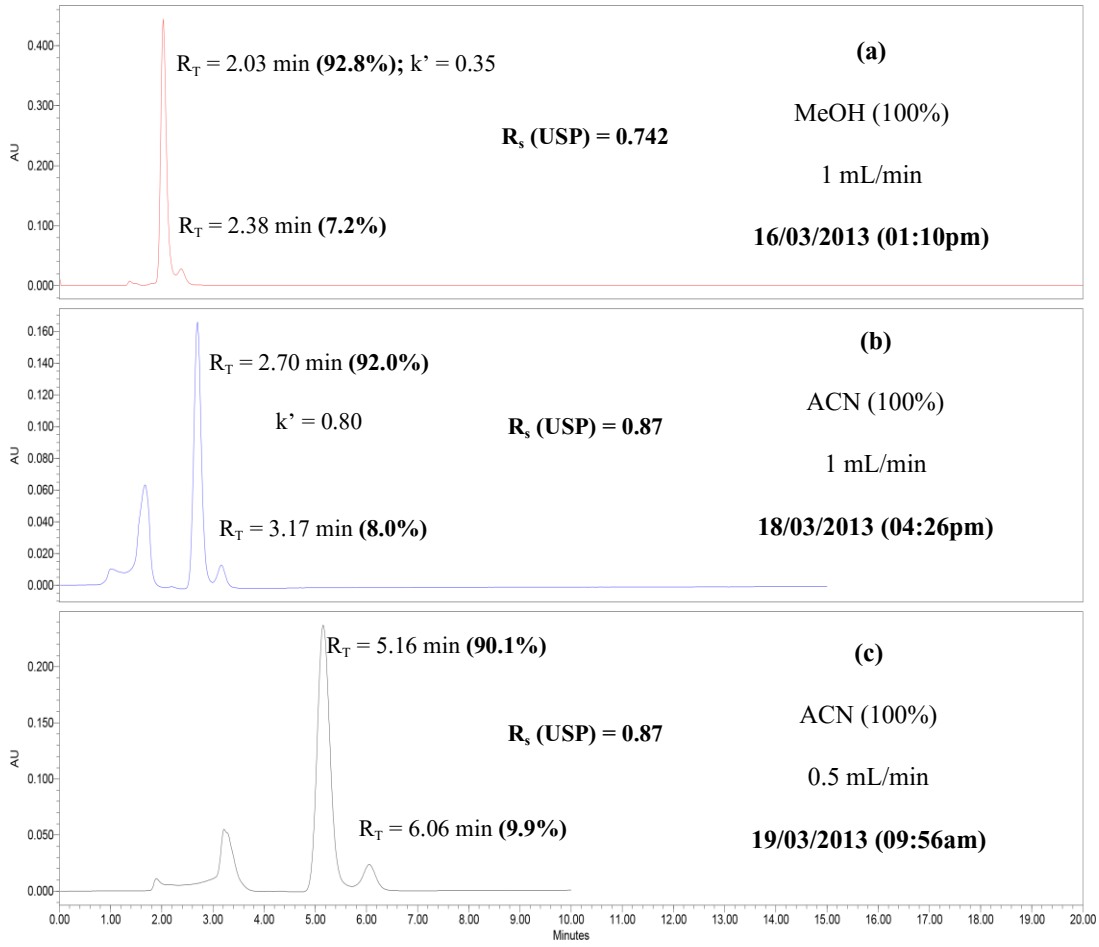

Figure 4.1: (a) The first isocratic run in MeOH (100%) (b) ACN (100%); Sample: **BM13-37c**; Column: Xterra® 150 x 4.6 mm i.d.; Flow rate: 1 mL/min; Detection: UV-Vis at 450 nm; (c) ACN (100%, 0.5 mL/min) Injection volume: 20 µL.

Fucoxanthin eluted at 2.03 minutes, using MeOH as mobile phase (**a**), while a persistent unknown impurity eluted at 2.38 minutes. On changing the mobile phase to ACN (**b**) its retention time increased to 2.70 minutes (k' = 0.80). That was a positive response and attention was briefly focused on to the side peak that was constantly being observed. This peak had already been observed during the semi-preparative chromatographic separations and is likely an isomer[26] of fucoxanthin (Nakazawa *et al.*, 2008). The size slightly increased over time. Fucoxanthin and 'isomer' had peak areas of 92.8% and 7.2% respectively as shown in (Fig 4.1).

A combination MeOH/H_2O was attempted in anticipation of retaining the compound more (Le Lann *et al.*, 2012). That was unsuccessful when MeOH/H_2O (9:1) triggered an automatic safety-fail system stall owing to excessive back pressure (4000 psi). Le Lann (2012) had attempted this mobile phase on a C-6 column.

Decreasing the flow rate to 0.5 mL/min (**c**) increased the retention time to 5.16 minutes, however, no significant improvement in the resolution (R_s = 0.87)[27] between fucoxanthin and the 'isomer' was observed. A number of additional solvent combinations *e.g.* methanol and acetonitrile (**d**) were tested without any significant improvement in separation between fucoxanthin and its 'isomer'. In a final attempt to improve the resolution between fucoxanthin and the 'isomer' a small volume of water (5%) was added to acetonitrile (**e**). The R_s was improved to 1.13. The recommended resolution is $R_s > 1.5$, $R_s > 2.0$ is most ideal but if it is > 1.0 then two adjacent peaks can be successfully differentiated (Ornaf and Dong, 2005). The retention times were 2.94 minutes; k' = 0.96[28] (fucoxanthin) and 3.83 minutes ('isomer'). This method was therefore satisfactory to test on a crude extract although the capacity factor was slightly under 1.0 (recommendation is between 1 and 20, Fig 4.2).

[26] The stability of fucoxanthin under different conditions were studied in chapter 6.

[27] Resolution R_s (US) = $\frac{\Delta tR}{wb}$ where ΔtR is the difference in retention times and wb is the width of the peak.

[28] Capacity/Retention factor: k' = $\frac{(tR - t0)}{t0}$ where tR (retention time of analyte) and t0 (void volume).

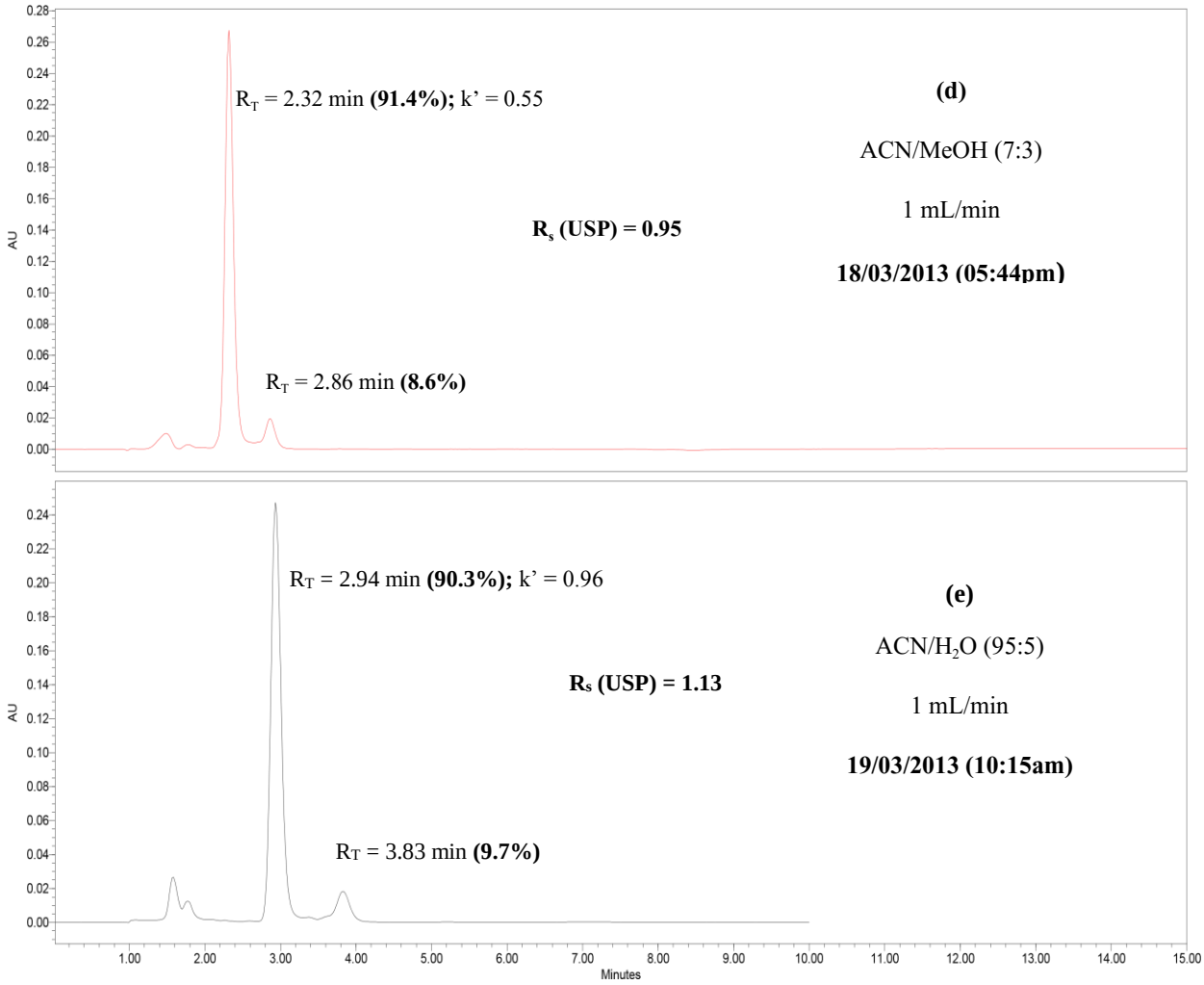

Figure 4.2: (d) ACN/MeOH (7:3) (e) ACN/H2O (95:5); Sample: **BM13-37c**; Column: Xterra® 150 x 4.6 mm i.d.; Flow rate: 1 mL/min; Detection: UV-Vis at 450 nm; Injection volume: 20 µL.

At this stage we were concerned whether this method would adequately separate fucoxanthin from other components in crude extracts. This was an important condition since we wanted to limit any "cleanup" steps prior to analysis. A crude extract of *Sargassum incisifolium* was analyzed under the optimized conditions. A number of co-eluting components (**f**) from the crude extract which would make quantitative analysis very difficult are clearly shown in (Fig 4.3). It was therefore necessary to explore separation of the components in a different way. At this point we decided to change the column to a Phenomenex® Synergi™ 250 x 3.0 mm i.d., 4 µm. Fortunately this change significantly improved the separation of the various components and the resolution between fucoxanthin and its 'isomer' (R_s) increased to 2.0 (**g**), with concomitant increase in retention factor

(k' = 1.3). The total run time for running pure fucoxanthin was 7 minutes and that of a methanolic crude extract was 10 minutes, an acceptable target run time making for a simple, rapid and economical method to analyze fucoxanthin content from a number of brown algae. Smaller particle sized and shorter diameter columns are known to improve resolution and lower detection limits (Ornaf and Dong, 2005).

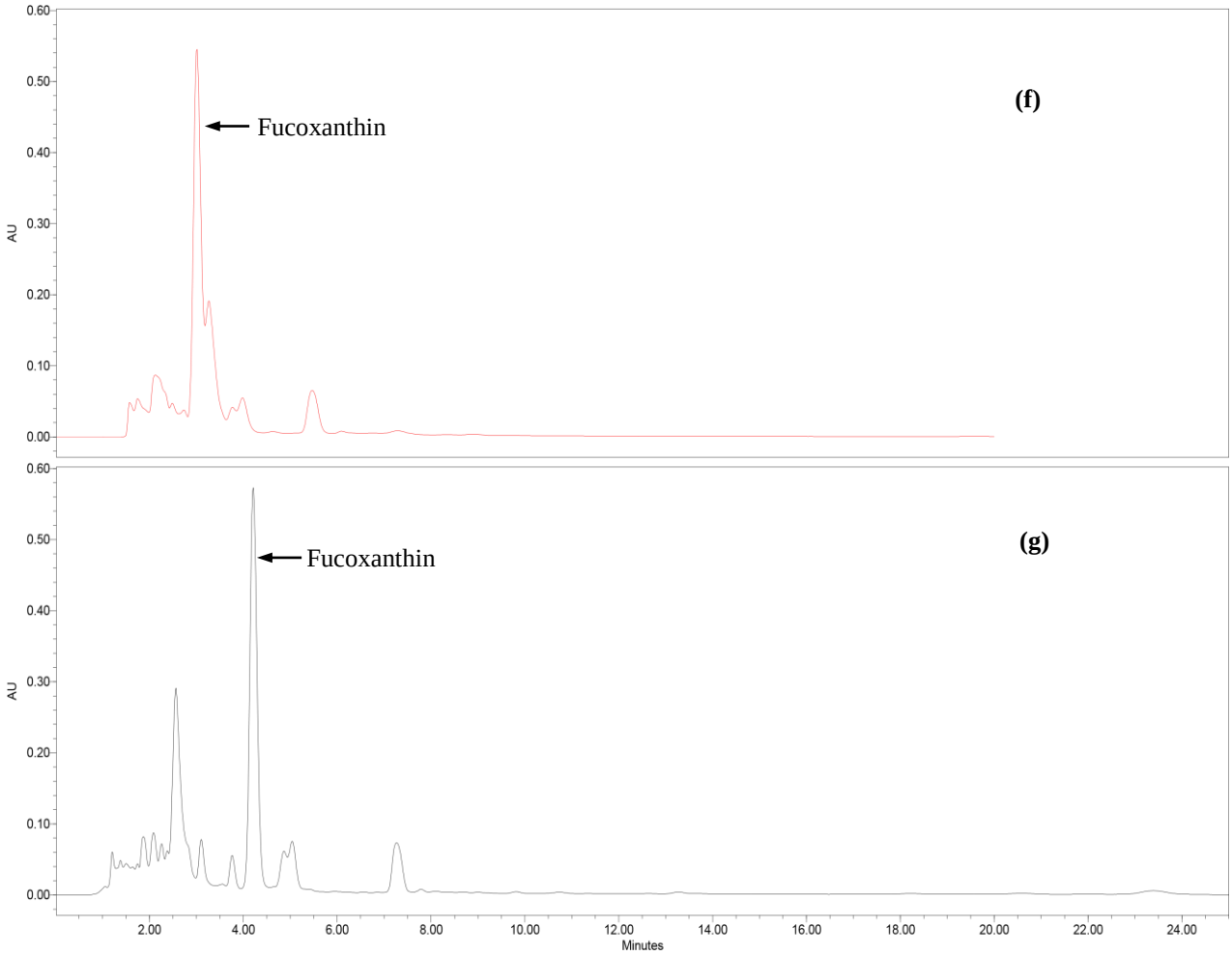

Figure 4.3: The analysis of *S. incisifolium* on the (f) Xterra® 150 x 4.6 mm i.d. (g) Phenomenex® Synergi™ 250 x 3.0 mm i.d., 4 µm; Sample: **BM13-37c**; Flow rate: 1 mL/min; Detection: UV-Vis at 450 nm; Injection volume: 20 µL.

4.2.2. Validation

4.2.2.1. Linearity

The linear range studies were repeated two times (Day 2 and Day 3) to fulfil the requirements of the ICH to have a total of nine determinations. Day 1 studies are shown in (Fig 4.4) and (Table 4.2) whilst (Table 4.3) includes the linear ranges for Day 1-3. The linear relationship is demonstrated with correlation of 0.9998 in all determinations. Between concentrations of 0-50 µg/mL the determinations are linear. The RSD is below the recommended 5%.

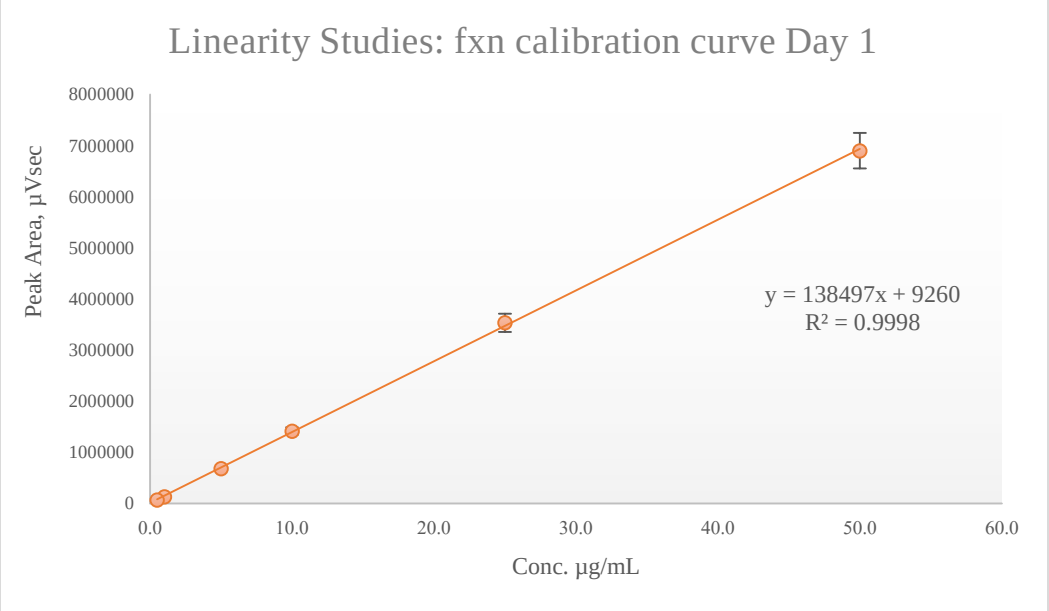

Figure 4.4: Linearity studies on **BM13-73b** (Showing Day 1).

The consistency in linearity throughout experiments carried from day 1 through day 3 is shown in (Table 4.2).

Table 4.2: Linear ranges for **BM13-73b** (Showing Day 1- Day 3).

Analyte	Day	Linear Model; $y = mx + c$	R^2 coefficient
BM13-73b	Day 1	y = 138497x + 9260	0.9998
	Day 2	y = 145164x + 75905	0.9986
	Day 3	y = 144427x + 116105	0.9976

Table 4.3: Linearity studies for **BM13-73b** (Showing Day 1).

Day 1

Conc. (µg/mL)	Conc. (µg/mL) 1	2	3	Mean	SD (n =3)	RSD
50.0	49.0	50.4	49.8	**49.8**	**0.69**	1.4%
25.0	25.7	25.8	24.9	**25.4**	**0.49**	1.9%
10.0	10.2	9.9	10.3	**10.1**	**0.21**	2.0%
5.0	4.9	4.9	4.8	**4.8**	**0.06**	1.2%
1.0	0.9	0.8	0.9	**0.9**	**0.03**	3.0%
0.5	0.4	0.4	0.4	**0.4**	**0.01**	2.7%

4.2.2.2. Accuracy

The accuracy studies were repeated twice (Day 2 and 3) to fulfil the requirements of the ICH to have nine determinations. Day 1 studies are shown in (Table 4.4) and are a true reflection of how accurate the method was. The overall range was 97% – 102%. As mentioned, the precision was for both linearity and accuracy studies was less than 5%, a limit set to demonstrate the reproducibility of the analytical procedure (Ornaf and Dolan, 2005).

Table 4.4: Accuracy studies for **BM13-73b** (Converted to relevant concentrations).

Accuracy Day 1

Conc. (µg/mL)	Conc. (µg/mL) 1	2	3	Mean	Std. dev.	RSD %	Accuracy
50.0 (49.8)	48.6	47.7	48.2	**48.1**	**0.45**	**0.93%**	**97%**
25.0 (25.4)	25.1	24.7	25.0	**24.9**	**0.21**	**0.86%**	**98%**
5.0 (4.8)	5.0	5.0	4.9	**4.9**	**0.08**	**1.69%**	**102%**

4.2.2.3. LOD and LOQ

For LOD and LOQ, the method of calculation using the standard deviation and slope was chosen because the chromatograms were not showing noise bands to adequately use signal to noise ratio as is commonly done. The idea was to pick concentrations at the very low end (Fig 4.5). The concentration was lowered until no significant peak could be detected by the software (Table 4.5). The lowest concentration of fucoxanthin that can be detected was estimated at 10 ng/mL and quantified at 30 ng/mL with reasonable precision. This makes the method relatively sensitive.

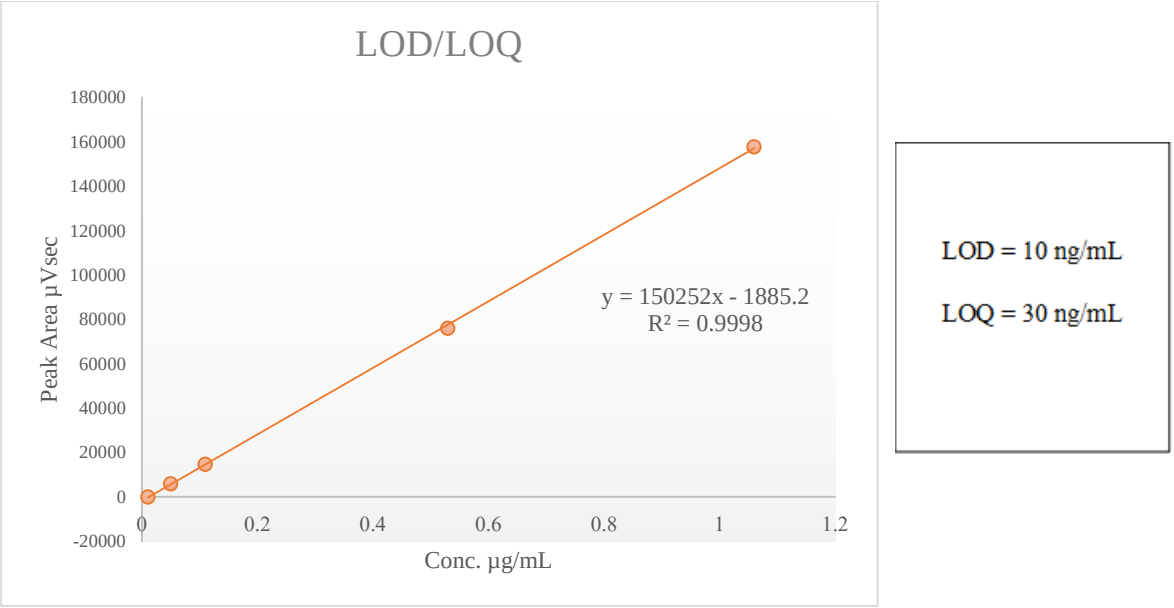

Figure 4.5: LOD and LOQ determinations for **BM13-73b** by calculation using response, standard deviation and slope.

Table 4.5: LOD and LOQ determinations for **BM13-73b** (Corrected to relevant concentrations).

LOD/LOQ determinations Day 1

Conc. (µg/mL)

Conc. (µg/mL)	1	2	3	Mean	Std. Dev.	RSD %
1.06	1.07	1.07	1.06	**1.06**	**0.01**	**0.49%**
0.53	0.50	0.53	0.53	**0.52**	**0.01**	**2.72%**
0.11	0.11	0.11	0.11	**0.11**	**0.00**	**2.70%**
0.05	0.05	0.05	0.05	**0.05**	**0.00**	**2.26%**
0.011	0	0	0	**0**	**0.00**	**0.00%**

4.2.2.4. Recovery

The recovery experiment tested the extraction efficiency of the analytical method hence the crude extract from *S. incisifolium* was used (Table 4.6 and 4.7). The preliminary method was used to test a spiking method. The original intended purpose of the method was to determine the fucoxanthin content of a selected alga after a single methanol extraction. The extent and consistency of the recovery of fucoxanthin from the biomass was therefore very important to determine. The recovery, from the biomass, was about 92% tested on high (300 mg), medium (200 mg) and low (100 mg) to represent the varying amounts of fucoxanthin from different algae. Although the recovery was lower than expected, the extraction was consistent. The controls for low, medium and high showed concentrations of 11, 22 and 33 µg/mL of fucoxanthin for 100, 200 and 300 mg of algal biomass, respectively. When the biomass was doubled the amount of fucoxanthin extracted was doubled whilst tripling it achieved a similar effect. There was therefore no evidence of saturation in the extraction vessel (4 mL) solvent. This overall, was a good indication that the extraction method was adequate. The low recovery % was attributed to spiked fucoxanthin possibly "sticking" onto the biomass as there wasn't any procedure employed to completely wash off the biomass. It was observed that it is important to spike with the reference standard after addition of the solvent (3 mL) to avoid sticking and inconsistent results.

Table 4.6: Preliminary recovery procedure for **BM13-73b** from *S. incisifolium*.

Preliminary recovery procedure

Sample	Conc. (µg/mL)	% Recovery
Standard FXN	24.5	
Control in MeOH (0.39 µg)	18.9	**104%**
Spiked (0.39 µg + 0.31 µg)	45.1	

Table 4.7: Recovery studies for **BM13-73b** from *S. incisifolium.*

Recovery Studies

		Conc. (µg/mL)						
		1	2	3		**Mean Spike**	**Std. Dev.**	**RSD**
Std. fxn (µg/mL)	**27.4**							
Lo Cntrl (µg/mL)					**11.5**			
Lo Spike (µg/mL)		**36.4**	**35.7**	**34.8**		**35.6**	**0.79**	**2.2%**
Recovery %	***92%***							
Std. fxn (µg/mL)	**27.4**							
Med Cntrl (µg/mL)					**22.0**			
Med Spike (µg/mL)		**43.6**	**45.4**	**45.5**		**44.8**	**1.09**	**2.4%**
Recovery %	***91%***							
Std. fxn (µg/mL)	**27.4**							
Hi Cntrl (µg/mL)					**33.0**			
Hi Spike (µg/mL)		**56.4**	**56.0**	**55.9**		**56.1**	**0.26**	**0.5%**
Recovery %	***93%***							

4.2.3. Conclusion

A simple, rapid and reproducible analytical HPLC procedure was successfully developed and validated according to the standards set by the ICH. This method was capable of consistently quantifying fucoxanthin in both pure form and when it is within an extract. The use of a reversed phase column, Phenomenex® Synergi™ 250 x 3.0 mm i.d. in mobile phase ACN/H_2O (95:5) set up the best possible conditions to analyze fucoxanthin in diverse samples of South African marine brown algae (*see* Chapter 5).

4.3. Experimental

4.3.1. General procedures

All analytical experiments were carried out on a Waters® 1525 binary pump and Waters® 2487 dual wavelength absorbance detector (Massachusetts, USA) distributed by Microsep, (South Africa). Pump A and B were set to an isocratic mode. The injection was manual via a Rheodyne® injector which fed the samples into a 20 µL loop. The initial analytical column used was an X-terra® C-18 5 µm (150 x 4.6 mm i.d.) fitted with a Phenomenex® C-18 guard column (4.0 x 3.0 mm i.d.). The final adjustments to the initial method designed were carried out on a Phenomenex® Synergi™ C-18; 4 µm (250 x 3.0 mm i.d.) fitted with a guard cartridge containing a Phenomenex® C-18 (4.0 x 3.0 mm i.d.) guard column. The wavelengths were set at 254 nm (impurities and solvents) and 446 nm (maximum UV-Vis absorption for fucoxanthin as determined in section 3.2.2.2). The computer interface was powered by Windows® XP™ and the software for controlling the instrumentation, peak identification, manipulation and calculation of data was a Waters® Breeze™ software. A 100 µL Hamilton® micro syringe was used for all injections.

Organic solvents were of liquid chromatography grade filtered through 3.0 µm Millipore™ membrane filters from Merck (Darmstadt, Germany) and sonicated for 20 minutes per Liter of solvent. Methanol (Lichrosolv®) from Merck KGaA (Darmstadt, Germany), acetonitrile (HiPerSolv for HPLC™) from VWR International Ltd (Poole, England) and water was MilliQ® quality (milliQ dispenser) from the Biopharmaceutics Research Laboratory (Rhodes University, Grahamstown, South Africa).

4.3.2. Sample preparation

The original sample for all analyses except crude extract experiments was the purified reference standard (*see* section 3.3.3), fucoxanthin **BM13-37c**. The stock solution of 1 mg/mL was prepared in methanol as well as subsequent serial dilutions. All samples were filtered through a 0.2 µm PTFE syringe-filter Lida® (Kenosha, West Indies) before injection into the HPLC system.

4.3.3. Method development

Initial method development was carried out on an X-terra® C-18 (150 x 4.6 mm i.d.) column using different ratios of MeOH, ACN, and water. The final analysis and validation was done on a Phenomenex® Synergi™ C-18 (250 x 3.0 mm i.d.) column using ACN/H_2O (95:5) as mobile phase.

4.3.4. Validation of analytical method

Note: All analyses were carried out under diffuse light and all samples were covered with aluminum foil and stored at -20 °C between analyses.

4.3.4.1. Linearity

The procedure was carried out with the reference standard obtained in (*see* chapter 3) (all-*trans* fucoxanthin, **BM13-73b**) without an internal standard (external calibration method, *see* supplementary chapter). The standard solutions were prepared in methanol (100, 50, 25, 10, 5, 1 and 0.5) µg/mL.

Different concentrations of fucoxanthin were injected in triplicate and linearity was analyzed by plotting peak area (µVsec) against concentration (µg/mL). The calibration curve generated was used to determine the detected concentration. The regression coefficient (R^2) and coefficient of variation (RSD) were also determined.

4.3.4.2. Accuracy

Three concentrations (50, 25 and 5) µg/mL were selected to be the high (Hi), medium (Med) and low (Lo) concentrations.

The concentrations were injected in triplicate and accuracy was assessed by calculating the observed concentration relative to the actual concentration (as a % of the relevant average concentrations as determined in the linearity study). The coefficient of variation (RSD) was also determined.

4.3.4.3. Precision

Precision was assessed from every repetitive procedure and expressed as the coefficient of variation *i.e*. relative standard deviation (RSD).

4.3.4.4. Limits of detection (LOD) and quantitation (LOQ)

BM13-73b was used for the determination of LOD and LOQ. This was done partially by calculation based on observed response at low concentrations between 1.06 µg/mL and 0.011 µg/mL. The samples were prepared as in the previous validation procedures.

Injections were made in triplicate at the lower end of the calibration curve until no significant peak could be detected. The LOD and LOQ were determined by calculation using a set of three calibration curves at the low concentration end using the standard deviation of the response and the slope.

$$LOD = \frac{3.3\sigma}{S}$$

$$LOQ = \frac{10\sigma}{S}$$

Where σ is standard deviation of the response and S is the slope of the curve.[29]

[29] Q2B Validation of analytical procedures: http://www.fda.gov/downloads/Regulator%20yInformation/Guidances/UCM128049.pdf/ [Accessed 22 January 2014]

4.3.4.5. Recovery

The recovery determinations were carried out using freeze-dried algal material (*Sargassum* sp., **PA130427-2**) and the reference standard (**BM13-73b**). A preliminary study was carried out to investigate the plausibility of this approach using 200 mg of algal material and 2 mL of solvent for which 50% of total solvent was spiked with the reference standard. For the final recovery study, three masses of alga material were used to represent low (Lo), medium (Med) and high (Hi) concentrations 100 mg (Lo), 200 mg (Med) and 300 mg (Hi).

Sample preparation:

Alga material (100, 200 and 300) mg was extracted with MeOH (4 mL) by sonication (30 min). The algal material was weighed out into a vial and suspended in MeOH (3 mL) after which the reference standard dissolved in MeOH (1 mL) was added. The mixture was sonicated for 30 minutes and the supernatant (methanolic extract plus pure fucoxanthin) filtered through a 0.22 µm PTFE syringe filter before analysis. The control was un-spiked with the reference standard.

Injections and analysis:

The injections (3 sets) *i.e*. reference standard, control and spiked algal extract were done in triplicate. The calibration curve generated for the recovery experiment was used to calculate the concentration of the aforementioned injections. The equation was;

$$y = 102215x + 259213$$

The recovery was calculated to be the observed fucoxanthin in the spiked sample as a fraction of the total fucoxanthin spiked and extracted from the alga material (reference standard plus control).

4.4. References

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Chapter 5

Screening of brown algae commonly found in South Africa for fucoxanthin content

5.1. Introduction

5.1.1. Distribution of marine brown algae in South Africa

South Africa possesses a large flora of seaweed (approximately 800 species) of which 98 reported brown algae species are found on the west, east and south coasts (Bolton and Stegenga 2002). According to Bolton and Stegenga (2002), the distribution of brown algae is such that most species are found on the north east coast, with marginally more species on the south coast as compared to the west which demonstrated low diversity. The simple brown algae *e.g*. the *Zonaria* spp. and *Dictyopteris* spp. are widespread on the east coast and overlap on to the south coast. The only reported kelp on the east coast, overlapping on to the south is *Ecklonia radiata* (the spiny kelp) while the rest of the kelps of South Africa are found on the west coast (Branch *et al*., 1994). *Sargassum*-like algae are found to almost span the whole coastline; north-east, east, south and south-west coasts. *Bifurcariopsis* spp. are found only on the south-west coast. Other algae not studied in detail are the bladders and stings *e.g*. *Iyengara* sp. may be found on the east coast/south coast overlap (Branch *et al.,* 1994).

5.1.2. Description and classification of the brown algae under study

Color as already established (*see* section 1.2.1) is a key guiding criteria when distinguishing between macroalgae. This is very convenient albeit not always allowing for explicit distinction between seaweeds and may lead to a lot of confusion. Nonetheless, brown algae will tend to appear olive, dark green or brown and are mainly identified by a brown, yellow or yellow-green thallus (Branch *et al.,* 1994). Other than color, morphological structure and reproduction have also been relied on in the description and classification of algae. Phycology has comprehensively provided

some sense of order in a group of organisms with a mixed evolutionary history. A list of the algae that were used in this study with taxonomical information that identify the species as brown algae is shown in (Table 5.1).

Table 5.1: Algae used in the study. Taxonomical information adapted from Algae base™ online.

Collection code	Genus & species	Family	Order/Class
KOS130226-18	*Zonaria subarticulata* (J.V. Lamouroux)	Dictyotaceae	Dictyotales Phaeophyceae
KOS130226-9	*Zonaria tournefortii* (J.V. Lamouroux)	Dictyotaceae	Dictyotales Phaeophyceae
TS130227-2	*Dictyopteris macrocarpa* (Areschoug)	Dictyotaceae	Dictyotales Phaeophyceae
TS130227-5	*Dictyopteris* sp.	Dictyotaceae	Dictyotales Phaeophyceae
PA130427-2	*Sargassum incisifolium* (Turner)	Sargassaceae[30]	Fucales Phaeophyceae
TS130227-8	*Sargassum incisifolium* (Turner)	Sargassaceae	Fucales Phaeophyceae
TS130227-9	*Sargassum incisifolium* (Turner)	Sargassaceae	Fucales Phaeophyceae
TS130227-10	*Sargassum incisifolium* (Turner)	Sargassaceae	Fucales Phaeophyceae
KOS100329-1	*Sargassum* sp.	Sargassaceae	Fucales Phaeophyceae
KOS100329-2	*Sargassum* sp.	Sargassaceae	Fucales Phaeophyceae
KB100213-3	*Bifurcariopsis* sp.	Bifurcariopsidaceae	Fucales Phaeophyceae
GC120901-2	*Bifurcariopsis capensis* (Areschoug)	Bifurcariopsidaceae	Fucales Phaeophyceae
TS130227-2	*Oerstedtia scalaris* (Suhr)	Sargassaceae	Fucales Phaeophyceae
PA130427-3	*Ecklonia radiata* (C.Agardh)	Lessoniceae	Laminarales Phaeophyceae

[30] The most abundant brown algae along the southeast coast belong to the genus *Sargassum*. However, it is not always easy to differentiate between the various *Sargassum* spp. In addition, recent evidence suggests that there may be more *Sargassum* spp. along the South African coast than previously thought (John Bolton, *Personal. Communication* 03 March 2014.). Therefore, all *Sargassum* spp. that appear morphologically different were collected and analyzed separately

The families of brown algae studied were Sargassaceae, Bifurcariopsidaceae, Dictyotaceae and Lessoniceae.

5.1.2.1. Sargassaceae

This family (order Fucales) is described as the brown thallus algae with air bladders (Fig 5.2, **S7**). The species have a distinct hold-fast, thickened stipe (stem/stalk) and flattened blades (Branch *et al*., 1994). *Sargassum incisifolium* also known as the differentiated-leafed *Sargassum* has a bushy yellow brown thallus (Fig 5.1, **S2**) that has a triangular stipe, lower oval-shaped 'leaves' with tooth margins and upper spear-shaped 'leaves' with smooth margins. The specie is common in deep gullies and warm pools and are approximately 25 cm long. *S. incisifolium* also thrives in high tide warm pools exposed to the sun. The Agulhas current brings warm water from the tropics raising the sea surface temperatures of the east coast to about 23-24 °C. The distribution is therefore largely on the eastern coastline of South Africa (Lubke, 1998).

According to Algaebase™ online database[31] there are about 524 species in the order Fucales and 332 of those are from the genus *Sargassum* which is about 10 times more than any other genus belonging to the same family. Rightly so, the genus appears to be one of the most abundant of the seaweeds on the east coast of South Africa (*see* section 5.1.1).

A related specie in South Africa known to occur on the south coast is known as *Sargassum elegans.* It is very similar to *S. incisifolium* but the thallus is cylindrical in section. More of the *S. incisifolium* species identified, were slightly different morphologically. The observed species had ovate upper leaves with marginal teeth of variable size. The upper third is without a midrib and they appeared spiny and flattened (Fig 5.1, **S3**) (Lubke, 1998).

A not so frequently reported member of the family was also found *Oerstedtia scalaris* (Fig 5.2, **S8**). It is very large (may grow up to 70 cm) and hard textured (woody). It is the only species located on the south coast (Algaebase™ online).

[31] Algaebase online: http://www.algaebase.org/browse/taxonomy/?id=8389/ [Accessed 21 January 2014]

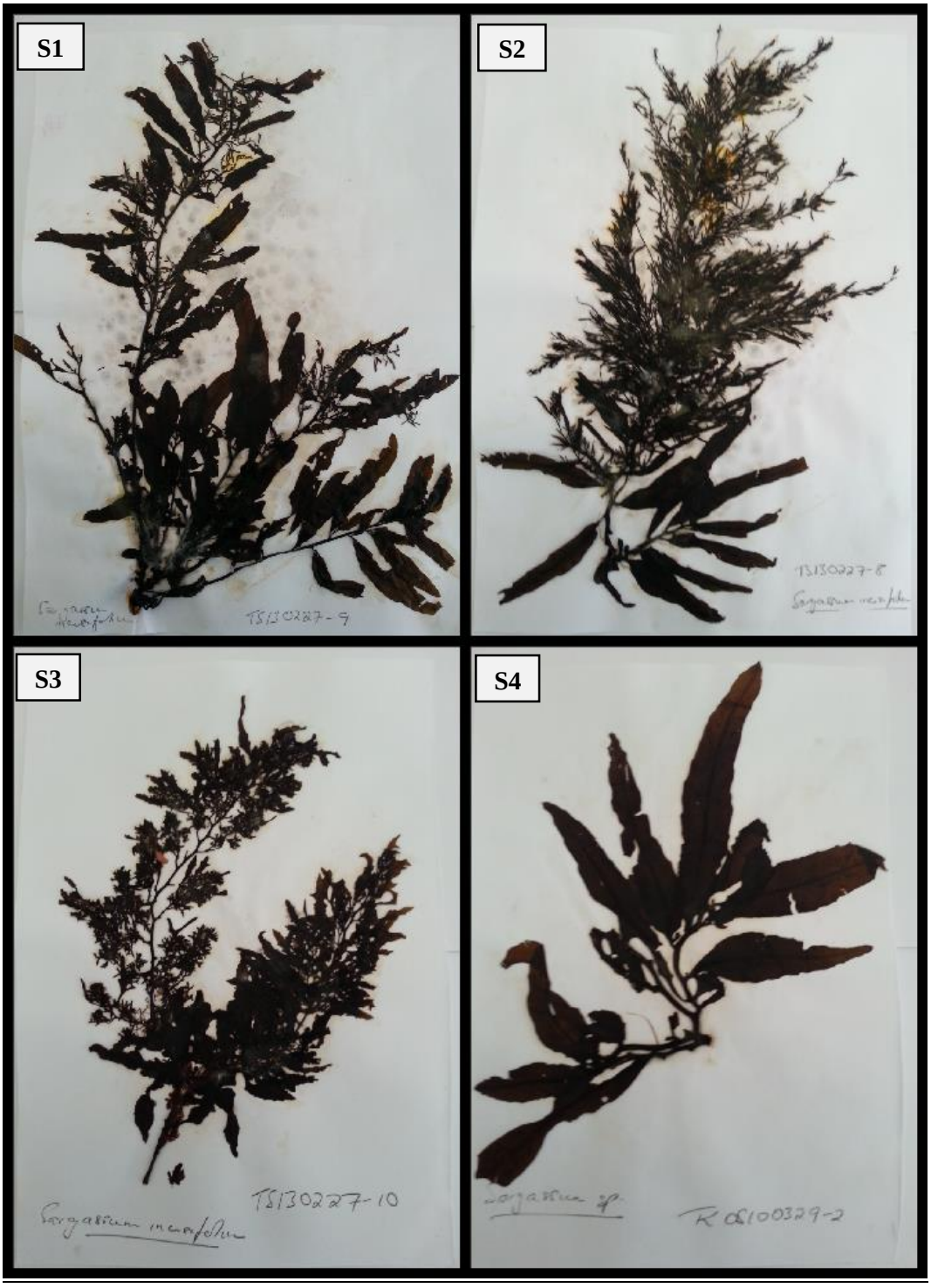


Figure 5.1: The algae belonging to the family Sargassaceae used in the study.

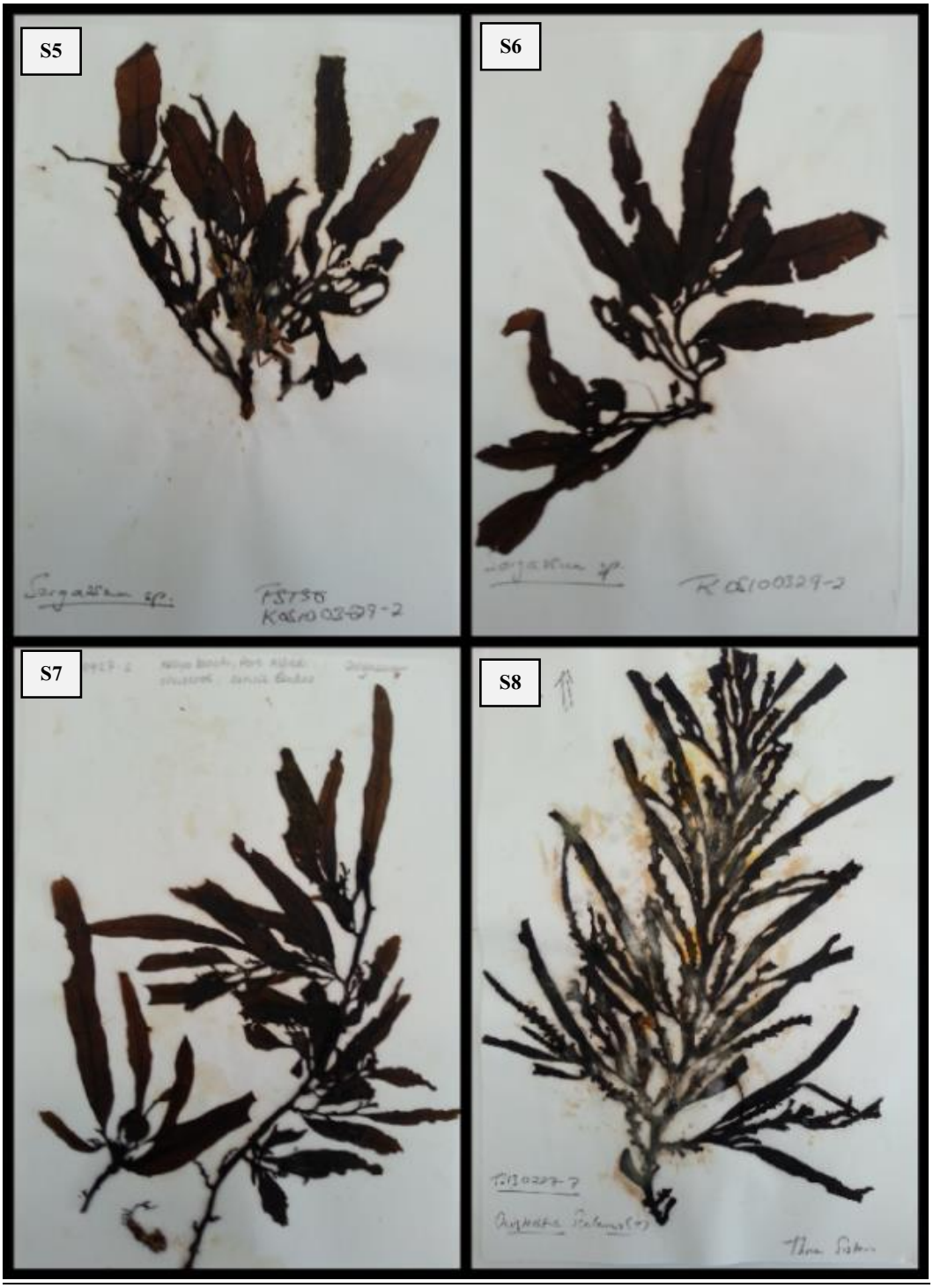


Figure 5.2: More algae belonging to the family Sargassaceae used in the study.

5.1.2.2. Bifurcariopsidaceae

The common name for this family is the wrack (Lubke, 1998). Two species are reported to exist on the west coast of South Africa, *Bifurcariopsis capensis* (upright wrack) and *Bifurcaria brassicaeformis* (hanging wrack). The alga with a conical disc-shaped holdfast which grows into a tough, upright and woody seaweed is *B. capensis*. It grows on the floors of the deep rock pools (Fig 5.3, **B1**). The hanging wrack (Fig 5.3, **B2**) however favors the wave pounded areas. They have creeping holdfasts and like the other member of their family, possess a tough matrix (Lubke, 1998).

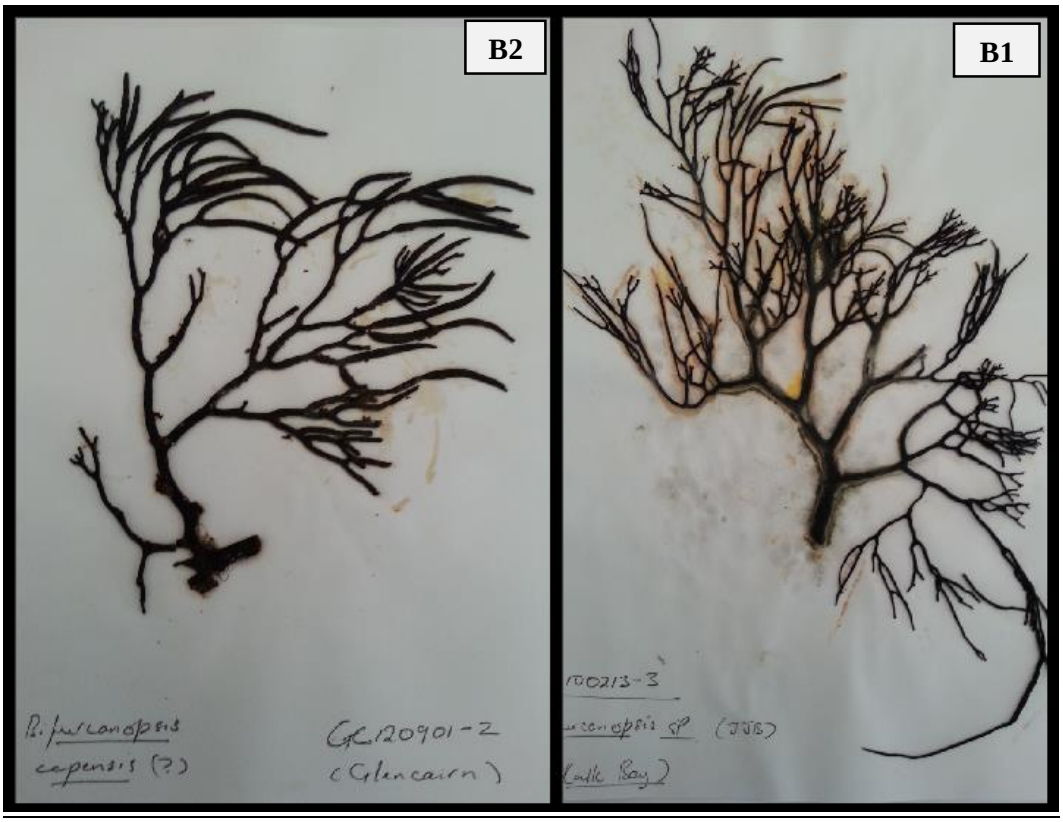


Figure 5.3: The algae belonging to the family Bifurcariopsidaceae used in the study.

5.1.2.3. Dictyotaceae

This group is described as the simple branched and zoned brown algae. They exhibit a small holdfast, short stipe and flattened thallus. Branches may be fan-shaped and/or ribbed 'veined'. The

family is subdivided into the fork branched type with midribs *i.e*. the *Dictyopteris* spp. (Fig 5.4, **D4**) and the *Zonaria* spp. that is zoned or fanned. *Dictyopteris marcrocarpa* has elongated blades with a mid-rib, wavy or split margins and streaks or dark patches and ~ 20 cm long. It is also described as ribbons with a mid-rib (Oliveira, *et al.*, 2005). With *Zonaria subarticulata* (Fig 5.4, **D2**), the branches are fan-shaped and have pale tips. The thallus is thin and leathery with a hairy holdfast. It may reach up to 20 cm and is found in lower tidal regions. *Z. tournefortii* (Fig 5.4, **D1**) is very similar to *Z. subarticulata* but is only about 30 mm high and is multi-fanned (Branch, *et al*., 1994).

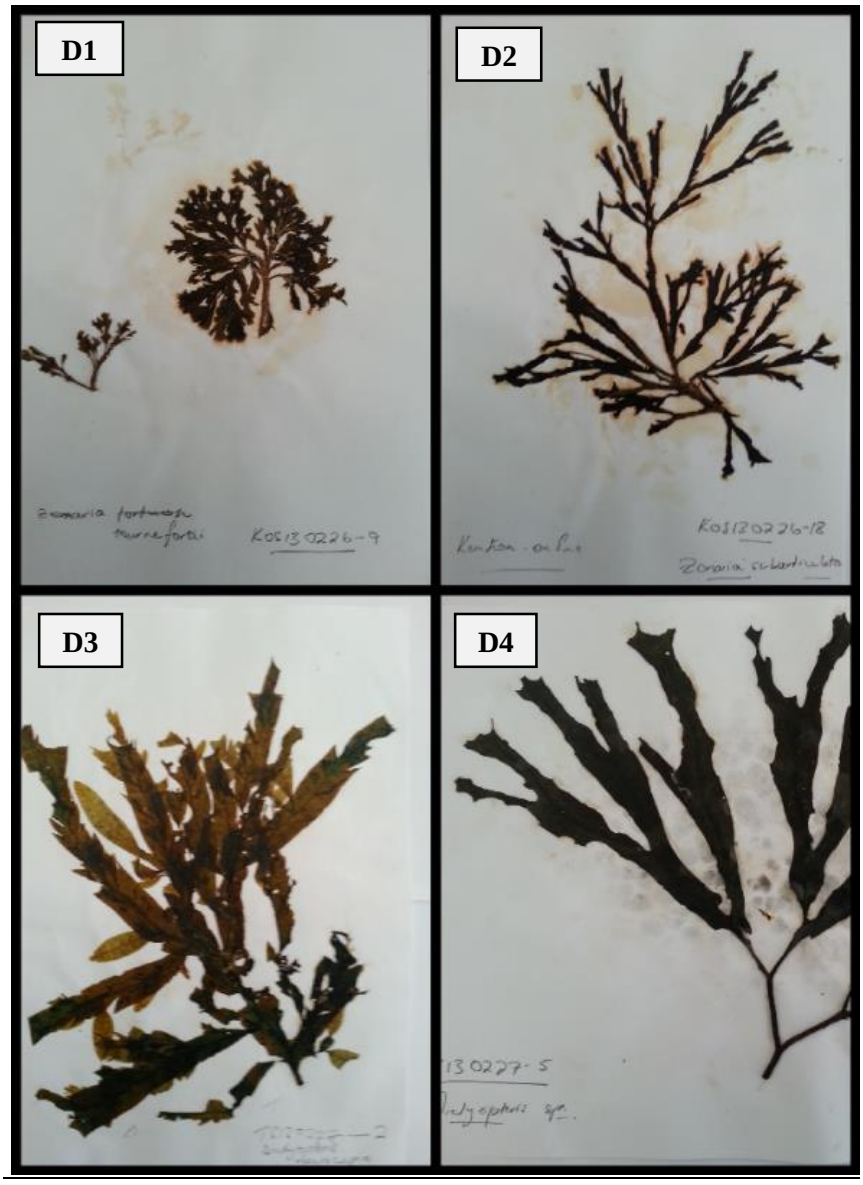


Figure 5.4: The algae belonging to the family Dictyotaceae used in the study.

5.1.2.4. Lessoniceae

This family is one of about seven known families forming the largest and fastest growing brown algae (13 mm a day). They occur in what is commonly referred to as the kelp forests. They have sturdy root-like holdfasts. The kelp shown (Fig 5.5, **L1**) is the spined-kelp, *Ecklonia radiata* and it is the species common along the east-south coast with irregular prickly fronds. It occurs in deep pools as well as shallow gullies. Unlike the other kelps, it hardly forms a solid stand (Lubke, 1998).

Figure 5.5: The alga belonging to the family Lessoniceae used in the study.

5.1.3. Previous studies on fucoxanthin content of brown algae

Terasaki *et al.,* (2009) described the aspects relating to fucoxanthin, fucosterol and fatty acid content in fifteen algae including seasonal variations observed in selected algae from Hokkaido, Japan. The use of DMSO to extract five different brown algae species from Madura Islands of Indonesia and determining fucoxanthin content from their crude extracts was reported by Zailanie and Purnomo (2011). The majority of the reported studies involved pre-isolation and purification of fucoxanthin before analysis (Shang *et al.,* 2011; Jaswir *et al.,* 2012; Kim *et al.*, 2010; Mori *et al*., 2004). The fucoxanthin content previously determined from crude extracts and after clean up steps is shown in (Table 5.2). The study by Terasaki *et al*. (2009) shows a much greater fucoxanthin content for the family Sargassaceae when compared to the one reported by Zailanie and Purnomo (2009) and Mori *et al.,* (2004).

The marked difference in fucoxanthin content between the aforementioned studies may be attributed to different species producing different amounts of the pigment. Geographical and seasonal variations are all possibilities that may play major roles in the amount of fucoxanthin produced by a particular seaweed. Cultured seaweed have been reported to produce more fucoxanthin when compared to their wild counterparts. *S. horneri* for example showed 3.7 mg/g fucoxanthin but the same seaweed (mono-cultured) produced 14.6 mg/g (Terasaki *et* al., 2009). Algae at young stages of their lifecycle showed higher fucoxanthin content, whilst female and male gametes of the *Undaria pinnatifida* showed higher fucoxanthin content as well compared to both the thallus of the seaweed and commercial *U. pinnatifida* (Mori *et al.* 2004). Therefore aspects of morphology, lifecycle and sex are factors that may determine fucoxanthin content. Sample preparation, extraction techniques used and the analytical procedures of choice could all be important factors to be reckoned in determining the amount of fucoxanthin in algae.

Table 5.2: Fucoxanthin content determined from (a) crude extracts and (b) purified fractions of brown algae.

Family	Species	Fucoxanthin (mg/g)	Reference
(a) Determined from crude extracts			
Sargassaceae	*Sargassum horneri*	3.7 ± 1.6	Terasaki *et al.*, 2009.
	Sargassum thunbergii	1.8 ± 1.0	
	Sargassum fusiforme	1.1 ± 0.6	
	Cystoseira hakodatensis	2.4 ± 0.9	
	Sargassum confusum	1.6 ± 0.8	
Laminariaceae (Kelp)	*Saccharina sculpera*	0.7 ± 0.4	
Alariaceae (Kelp)	*Alaria crassifolia*	1.1 ± 0.4	
Chordariaceae (Kelp)	*Sphaerotrichia divaricata*	0.2 ± 0.1	
Dictyotaceae	*Padina australis*	0.27 ± 0.0	Zailanie and Purnomo, 2009.
Sargassaceae	*Turbinaria conoides*	0.21 ± 0.1	
	Sargassum filipendula	0.20 ± 0.1	
	Sargassum echinocarpum	0.16 ± 0.0	
	Sargassum cinereum	0.16 ± 0.0	
(b) Determined from pre-fractionated samples			
Laminariaceae (Kelp)	*Eisenia bicyclis*	0.39 ± 0.0	Shang *et al.*, 2011.
Sargassaceae	*Sargassum binderi*	0.73 ± 0.2	Jaswir *et al.*, 2012.
	Sargassum duplicatum	1.01 ± 0.1	
Alariaceae (Kelp)	*Undaria pinnatifida*	4.2 ± 0.82	Fung *et al.*, 2013.
Alariaceae (Kelp)	*Undaria pinnatifida (male)*	2.6	Mori *et al.*, 2009
Alariaceae (Kelp)	*Undaria pinnatifida (female)*	1.6	

5.1.4. Chapter Aims

The aim of the research in this chapter was to determine which of the selected algae had the most fucoxanthin per gram of dried weight. The developed analytical method from the previous chapter may give an indication as to which of the algae are most likely the highest and lowest fucoxanthin producers and may allow further assessment as to which algae may be a more viable source for large scale fucoxanthin production.

Chapter objectives:

1. Determine the amount of fucoxanthin from fifteen samples of different brown algae commonly found in South Africa.

5.2. Results and Discussions

5.2.1. Preliminary studies

The method developed for the quantification of fucoxanthin was designed to avoid additional pre-fractionation and/or purification steps. The following factors were considered in the development of the extraction method: (i) extraction solvent (ii) extraction technique (iii) efficiency of the extraction method and (iv) the complexity of the crude extract.

The four most common solvents used in the extraction of fucoxanthin were assessed for their extraction capacity using *S. incisifolium* as a representative sample. These were ethanol, methanol, acetone and dichloromethane/methanol (2:1) which were assessed specifically for their extraction efficiency and the amount of fucoxanthin they were able to afford (Table 5.3). The preliminary extractions were done on fresh *S. incisifolium* (~ 200 mg) which were size reduced and extracted under sonication. The mass of the total extract and ratios of fucoxanthin content after three sequential extractions are shown in (Table 5.3. Methanol was selected as the solvent for the small scale extraction step in the analyses of the algae due to the larger amount of organic material and consequently, fucoxanthin isolated. In addition, the methanol extract chromatogram showed all the metabolites eluting within 10 minutes (Fig 5.6).

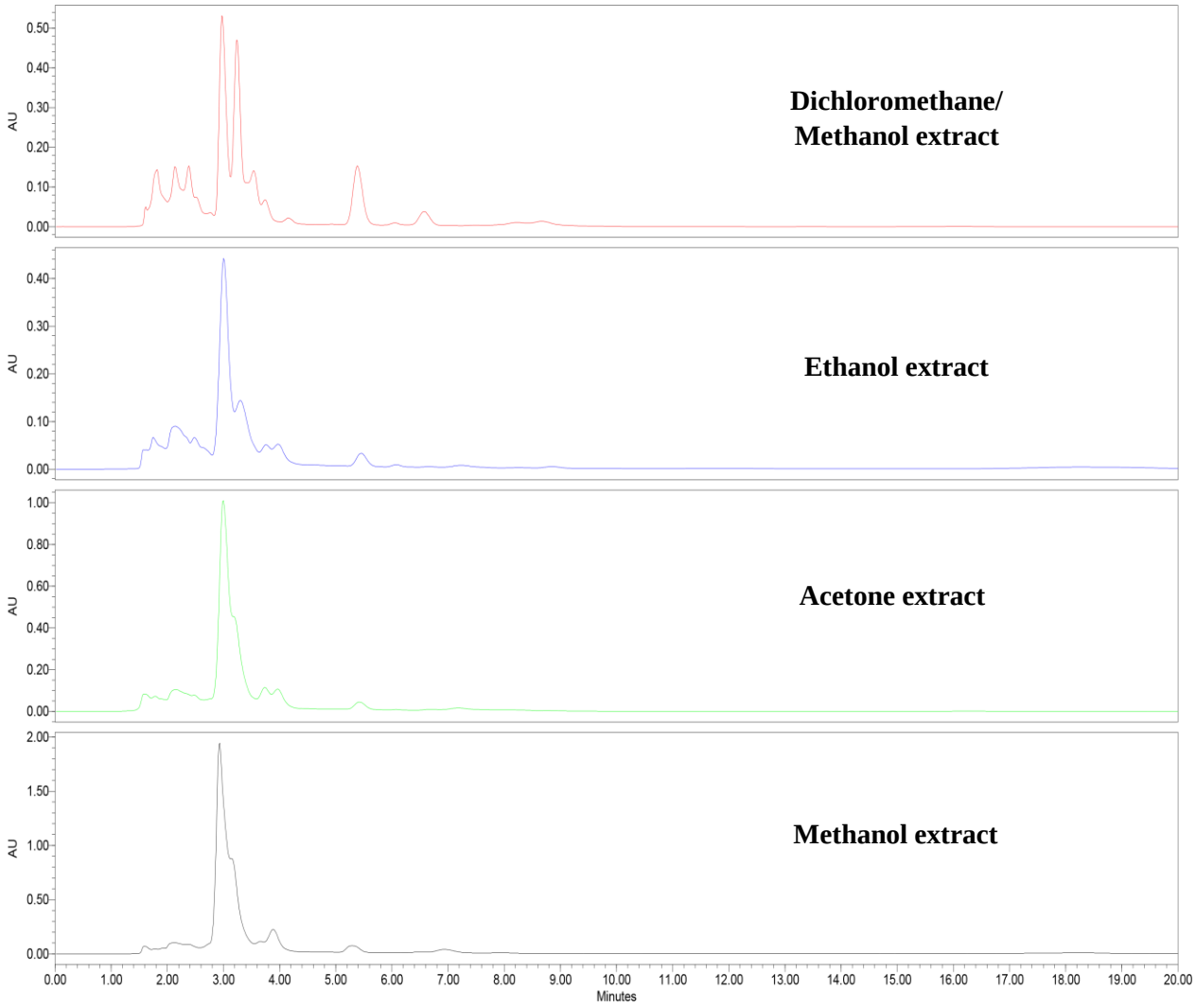


Figure 5.6: Preliminary studies: The extraction efficiency of four solvents; Dichloromethane/methanol combination, ethanol, acetone and methanol. Conditions: Xterra® 150 x 4.6 mm i.d. C-18 column.

The assessment shown in (Fig 5.6) of the extraction efficiency exhibited by the different solvents/solvent combinations was done on an Xterra® 150 x 4.6 mm i.d. RP-column. The fucoxanthin peak eluting at ~3 minutes was the preliminary criteria for identifying methanol as the solvent of choice. Methanol was shown to effectively target fucoxanthin from *S. incisifolium* better than all the tested solvents. Using peak area ratios, we were able to (on a preliminary basis) compare and identify the best solvent to use for our determination of fucoxanthin content in diverse samples of brown seaweed (Table 5.3).

Table 5.3: Solvent extraction efficiency exhibited by different solvents on the extraction of *S. incisifolium* (~200 mg).

Extract type (2 mL)	**Total mass of extract afforded by 200 mg biomass (crushed in N_2)**	**Peak area, ratios (after 3 extractions)[32]**
acetone	7.7 mg	60:30:10
methanol	14.1 mg	47:41:12
ethanol	3.3 mg	35:36:29
dichloromethane/methanol (2:1)	9.3 mg	34:32:34

The use of MeOH was satisfactory and has been reported in literature (Terasaki *et al.*, 2009). The successful use of acetone has been reported in *Zonaria and Padina* species (Rowan, 1989). Several researchers however, have shown that acetone is less efficient compared to other solvents in the extraction of a full range of pigments and a combination of methanol and acetone has been reported to selectively target carotenoids (Sand-Jensen, 1976; Seely et al. 1972; Pechar, 1987). Preliminary results obtained with *S. incisifolium* and *Zonaria tournefortii* (data not shown) indicate that quantitative extraction of fucoxanthin from biomass will require three to five sequential extractions.

In order to address concerns regarding saturation of the extraction solvent (MeOH), the solvent volumes were varied (2 mL, 11 mL, 20 mL) while the effect of different degrees of sonication were investigated (10 minutes, 35 minutes, 60 minutes) in the small scale extraction of ~200 mg of crushed *S. incisifolium*. The preliminary results (Fig 5.7) show that both solvent volume and extraction time may affect extraction efficiency. In addition to the above, consideration was also given to the stability of fucoxanthin during extended sonication times (because of the heat generated during sonication) and time required to evaporate the extraction solvent. As a result, a sonication time of 30 minutes and solvent volume of 4 mL was selected as adequate to carry out our investigation (as will be seen in the final study).

[32] These ratios are for fucoxanthin content determined for each sequential extraction.

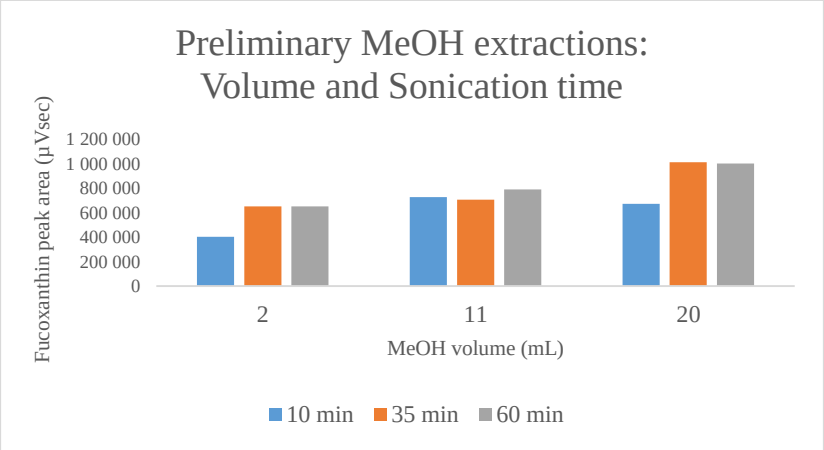


Figure 5.7: Fucoxanthin recovered on the first extraction (varying solvent volume and sonication time); Sample: crushed fresh *S. incisifolium* (~200 mg).

5.2.2. Sample preparation

The samples used were freeze dried and ground to a fine powder of uniform particle size to ensure consistency in each of the 200 mg biomass measurements. Preliminary studies indicated that the particle size may significantly influence the extraction efficiency and the use of chunks of crushed fresh algae may lead to difficulties in reproducing results.

For example, the alga *Oerstedtia scalaris*, made up of a tough matrix, showed a crude ^{1}H NMR spectrum that suggested fucoxanthin to be the major component in the alga (data not shown). On preliminary assessment, extremely low fucoxanthin was recovered, but after pulverization, a considerable amount of fucoxanthin was obtained (estimated at 0.59 mg/g of dry weight, DW). By way of further comparison, the bulk extractions of (*see* chapter 3) afforded about 0.094% (DW) fucoxanthin from wet fronds of *S. incisifolium* whilst the same algae afforded 0.97% (DW) fucoxanthin after freeze drying and pulverization, a 100 times better yield. Preliminary studies for the extraction of a 200 mg biomass showed that the freeze-dried pulverized material released more fucoxanthin (HPLC) compared to the fresh sample. Bidigare *et al*, (2005) reported an improvement of extraction efficiency when algal samples were exposed to liquid N_2 due to its ability to disrupt cells. Most reports suggest some form of drying before analysis of algae *i.e*. oven and air. (Haugan

and Liaane-Jensen, 1989). We used freeze drying, a more rapid process which preserves the alga better (limiting degradation) and improves pulverization (Fig 5.8). Freeze drying removes all the water in the matrix by sublimation without exposing the alga to external agents which may contribute significantly to the degradation of pigments such as air, heat, electromagnetic radiation and moisture.

The ultrasound assisted extraction of the algae was dependent primarily on the solubility of fucoxanthin in methanol. The induced energy improved migration of pigments out of the matrix and disruption of cells. The secondary factors expected to play a major role in the migration of the compound out of the matrix of the biomass were probably the presence of polysaccharides and water. Freeze drying removes water from the matrix of algae leaving that volume free to be occupied by just the extraction solvent (Rowan, 1989). We therefore managed to eliminate water as a limitation to our extraction procedure.

Figure 5.8: Pulverized alga after freeze-drying.

5.2.3. HPLC analysis for quantification of fucoxanthin

The chromatograms for the crude extracts showed that the fucoxanthin peak was accurately and adequately quantified without any interfering peaks. The chromatograms for the examined algae are shown in (Fig 5.9, Fig 5.10 and Fig 5.11). Fucoxanthin has the retention time of 4.2 minutes.

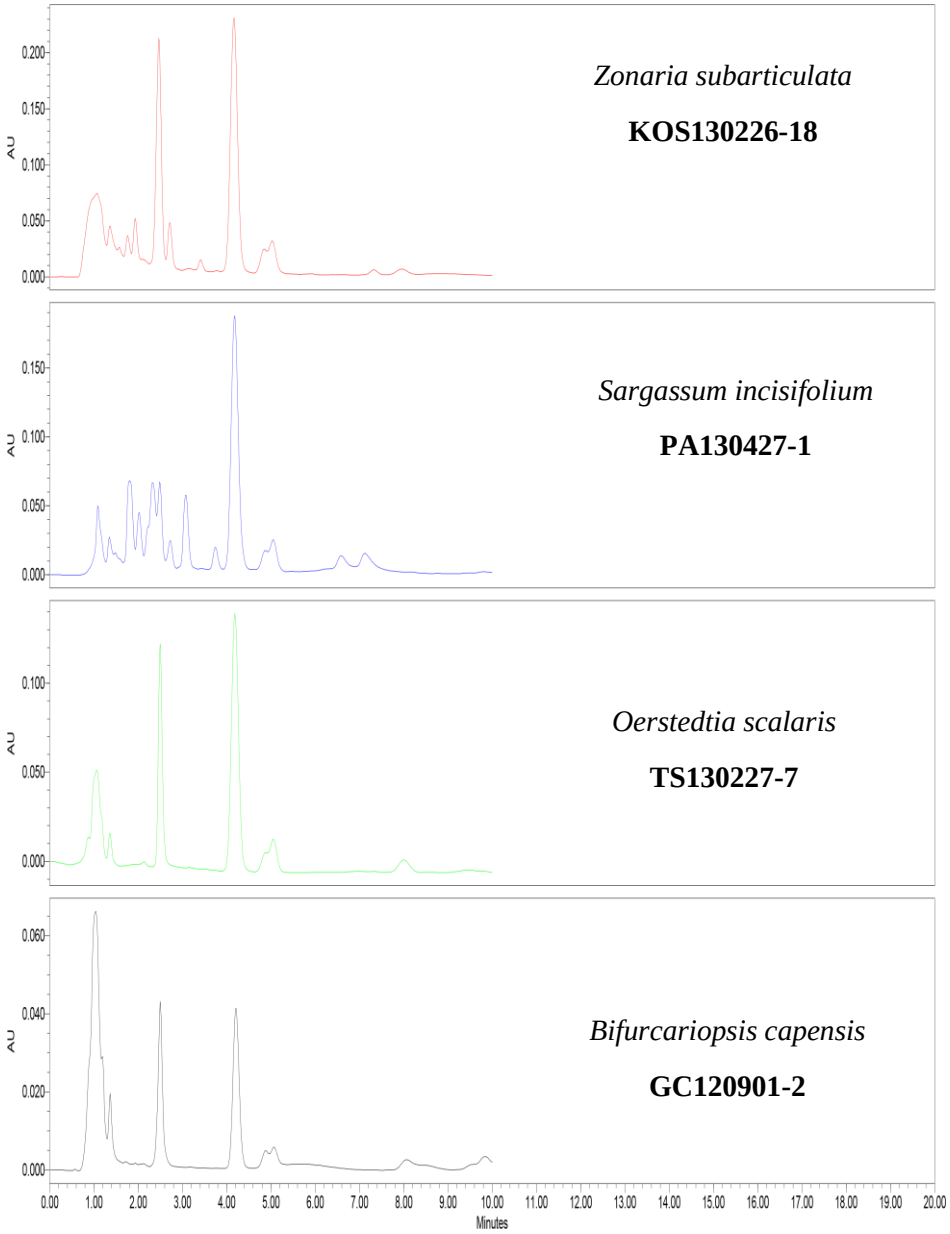
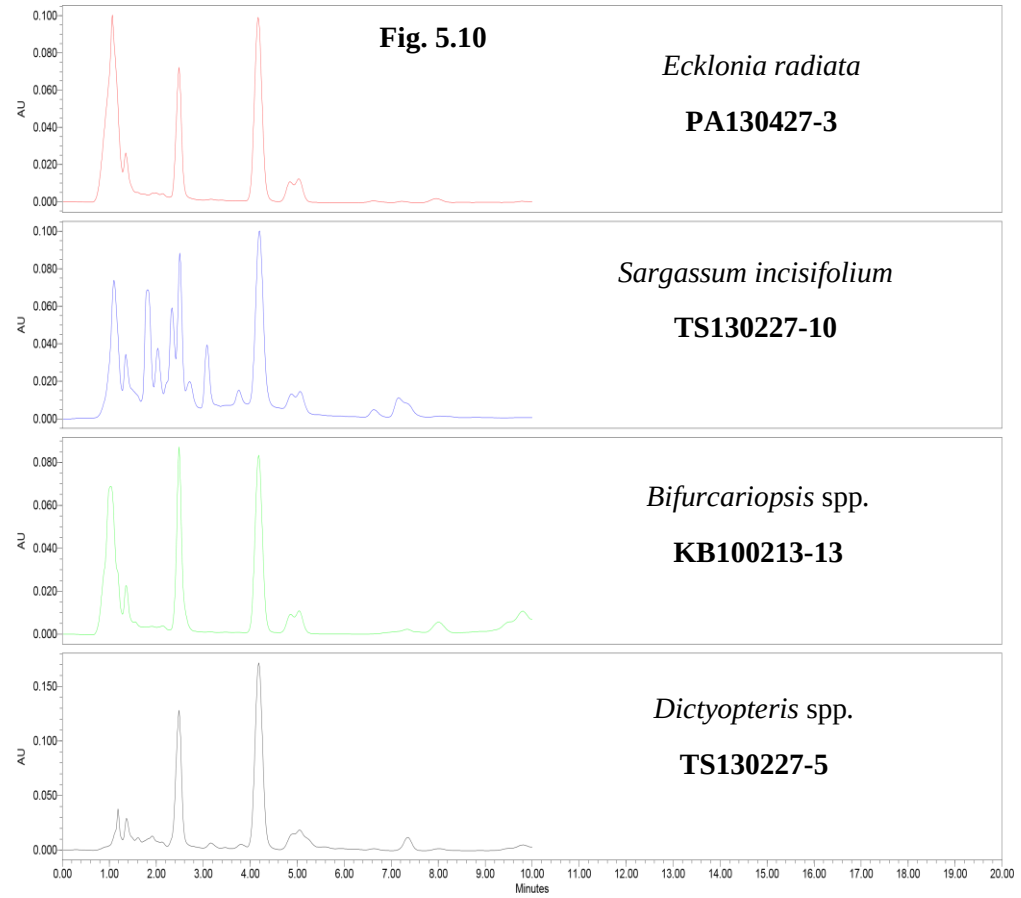


Figure 5.9: HPLC chromatograms showing fucoxanthin content in tested algae. Conditions: Phenomenex® Synergi™ 250 x 3.0 mm i.d. in ACN/H_2O (95:5).



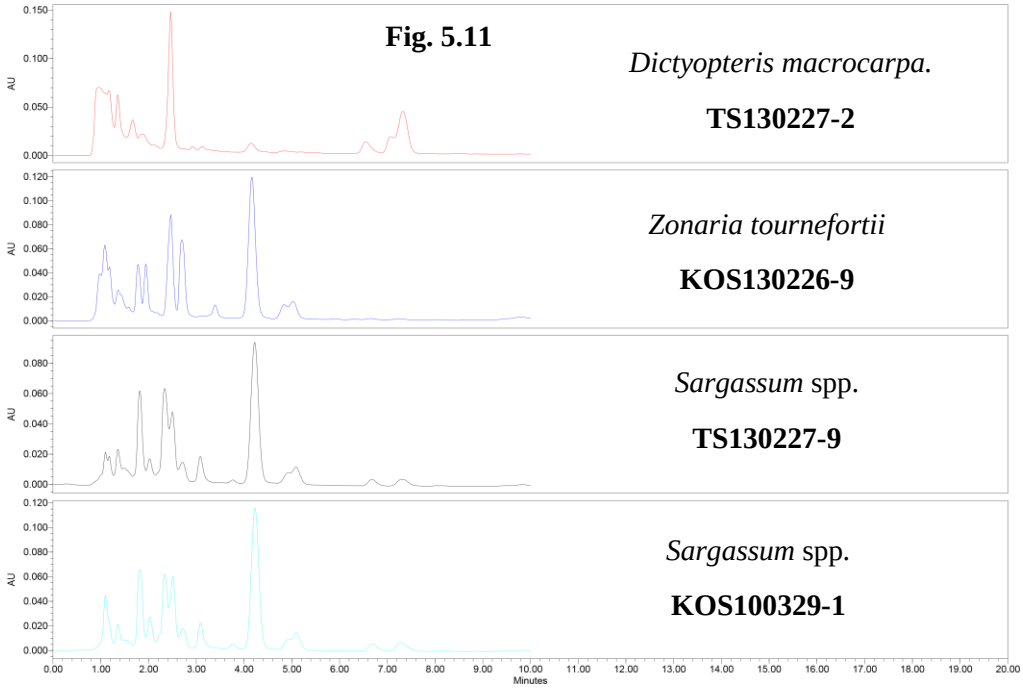


Figure 5.10 (**PA130427-3** – **TS130227-5**) and **Figure 5.11** (**TS130227-2** – **KOS100329-1**): HPLC chromatograms showing fucoxanthin content in tested algae. Conditions: Phenomenex® Synergi™ 250 x 3.0 mm i.d. in ACN/H2O (95:5).

The pigment concentrations are expressed in µg/g of dry lyophilized ground powder (200 mg). The results of triplicate analyses of fucoxanthin and % of crude extract obtained from 200 mg biomasses are shown in (Table 5.4). The content of the pigment was calculated from the equation derived from the calibration curve as previously discussed (*see* section 4.3.3.5).

Moreover, the preliminary study showed that 30 minute extraction may not extract all the fucoxanthin out of the matrix. Using MeOH on 200 mg biomass afforded 47 % on the first, 41 % on the second and 12 % on the final extraction (Table 5.3). The analysis in this study was done based on the first extraction.

The highest quantity of fucoxanthin was extracted from *Z. subarticulata* (**KOS130226-18**, 0.50 mg/g), *S. incisifolium* (**PA130427-1**, 0.44 mg/g), *S. incisifolium* (**PA130427-2**, 0.35 mg/g) and the *Dictyopteris* sp. (**TS130227-5**, 0.33 mg/g). The lowest producers of fucoxanthin (according to our results) were the woody algae from Bifurcariopsidaceae family; *Bifurcariopsis capensis* (**GC120901-2**, 0.05 mg/g) and *Bifurcariopsis* sp. (**KB100213-13**, 0.11 mg/g; most likely *Bifurcaria brassicaeformis*). These algae lack any leaf-like structures.

The other *Sargassum* species showing relatively low fucoxanthin recovery (in the range of 0.15 – 0.20 mg/g) seemed to be at different life stages and were morphologically different. Some had smaller or less fronds, some filament-like branchlets and yet another looked older and shriveled. The morphology seemed to impact the amount of fucoxanthin within the alga. Terasaki *et al*. (2009) examined the distribution of fucoxanthin within selected algae (*Sargassum confusum* and *Cystoseira hakodatensis*). More fucoxanthin is distributed in the fronds and vesicles with considerable amounts in the main and lateral branches as well as the main axis (Terasaki *et al.*, 2009). There are seasonal variations to be considered that may affect fucoxanthin content. Winter and spring were reported to be the time when algae produces the most fucoxanthin as demonstrated by a study on *Sargassum horneri*, *Cystoseira hakodatensis*, *Sargassum thunbergii* and *Sargassum fusiforme.* Variations reported in literature were attributed to varying light intensities (Terasaki *et al.*, 2009).

Table 5.4: Fucoxanthin content expressed in µg/g of dried biomass and % of crude extract.

Collection code	Alga	Mean µg/g	RSD	% fxn (crude extract)
TS130227-2	*Dictyopteris macrocarpa*	-	-	-
KOS130226-9	*Zonaria tournefortii*	235.4	4.0%	**0.67%**
TS130227-10	*Sargassum incisifolium*	243.4	1.1%	**0.35%**
TS130227-9	*Sargassum* sp.	155.9	1.5%	**0.52%**
KOS100329-1	*Sargassum* sp.	199.1	1.9%	**0.32%**
TS130227-5	*Dictyopteris* sp.	332.1	2.5%	**0.73%**
TS130227-7	*Oerstedtia scalaris*	276.6	3.9%	**0.53%**
KB100213-13	*Bifurcariopsis* sp.	109.3	3.1%	**0.24%**
GC120901-2	*Bifurcariopsis capensis*	48.5	3.4%	**0.06%**
TS130227-8	*Sargassum incisifolium*	124.2	5.4%	**0.20%**
KOS100329-8	*Sargassum* sp.	151.0	3.3%	**0.34%**
PA130427-2	*Sargassum incisifolium*	350.0	2.5%	**0.34%**
KOS130226-18	*Zonaria subarticulata*	449.8	1.5%	**0.78%**
PA130427-3	*Ecklonia radiata*	170.0	1.7%	**0.50%**
PA130427-1	*Sargassum incisifolium*	444.0	0.8%	**0.49%**

Note: The calibration equation used in the recovery experiment; ***y = 102215x + 259213*** *was used to quantify fucoxanthin from the crude extracts.*

The highest producing algae *Z. subarticulata* (0.50 mg/g) and *Sargassum incisifolium* (0.45 mg/g) specifically, show relatively low values of fucoxanthin content compared to reported algae (Table 5.2) which showed *Sargassum horneri* to have 3.7 mg/g fucoxanthin content. Terasaki *et al.* (2009) used methanol for extraction for two overnights. We extracted for 30 minutes under sonication. As previously suggested, different species (even in the genus), geographical location, harvesting time and extraction methods may significantly impact on the fucoxanthin content of algae.

5.2.4. Conclusion

The chapter directly addresses the research question, which of the brown algae commonly found in South Africa produces the most fucoxanthin? Fifteen different algae comprising eleven different species and four of the same species collected in different locations were analyzed for fucoxanthin content. The fucoxanthin content was determined (by HPLC; *see* chapter 4) directly from crude methanol extracts of the algae without prior fractionation and expressed in both µg/g of lyophilized ground dry alga powder and as a % of the crude extract. *Zonaria subarticulata* (**KOS130226-18**) from Kenton-On-Sea beach and *Sargassum incisifolium* (**PA130427-2**) from Port Alfred beach, Eastern Cape, South Africa, we found to produce the most fucoxanthin relative to all the brown seaweed tested.

Although the alga producing the largest quantities of fucoxanthin can easily be determined from these results a number of other factors may have to be considered for commercial scale production of this compound. The extraction method used here was developed using *Sargassum incisifolium* as a representative sample, however, one could optimize the extraction method for each individual alga. The complexity of the crude extracts and the abundance of the source material are also factors that need to be considered.

5.3. Experimental

5.3.1. General procedures

All HPLC analyses were done as described before in (*see* section 4.3.1.) *i.e.* the PTFE filtration (0.22 µm) and the HPLC-UV/Vis system. The organic solvent used in this procedure was MeOH (*see* section 4.3.1.1). Liquid nitrogen (liq. N_2) was provided by Rhodes University Chemistry Department.

A standard ceramic mortar and pestle was used to pulverize the algae. The freeze dryer used for all freeze drying processes was a Labconco® Freezone Drying System (Missouri, USA). The centrifuge was a Roto-Uni™ II BHG (Germany).

The algae were identified by Professor John J. Bolton of the Biological Sciences Department (University of Cape Town).

5.3.2. Sample preparation

Each alga (Table 5.4) was frozen in liquid N_2 and crushed in a mortar and pestle. In most cases the outcome was small chunks of fronds which were then transferred into a round bottomed flask for freeze drying (overnight). After freeze drying, the dry chunks were placed back into the mortar, mixed with more liquid N_2 and pulverized in a circular motion to a fine powder. The powder was then passed through a wooden shaker sieve (size 44 mesh). An example of the powdered *Sargassum incisifolium* is shown in (Fig. 5.8).

5.3.3. Algal extraction and analysis

Each sample was analyzed in triplicate as per (*see* Chapter 4, section 4.3.1). The fine powder (200 mg) was weighed into a vial and methanol (4 mL) was added followed by sonication of the mixture for 30 minutes. The resultant mixture was centrifuged at 10 000 rpm for about 3 minutes. An aliquot of the supernatant (3 mL) was removed and filtered through a PTFE syringe filter (0.22 µm) before being transferred into a separate vial. 20 µL of the extract was then injected and analyzed by the HPLC. A further 2 mL of the remaining extract was measured out and transferred into yet another separate pre-weighed vial. The extract was evaporated under reduced pressure and the mass of the extract determined.

5.4. References

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Chapter 6

Stability studies on fucoxanthin

6.1. Introduction

6.1.1. Photostability and ICH guidelines

Fucoxanthin is light sensitive and this aspect has been briefly discussed in previous studies (Hii *et al*., 2010; Piovan *et al*., 2013). The ability of fucoxanthin to withstand degradation from light or not may impact greatly on its biological activity.

It has been almost two decades since the inception of guidelines for harmonization on the procedures for photostability testing of drug products. However, there are still some lingering questions, concerns and problems associated with the current guidelines. Tonnesen (2007), was convinced that due to the complexity of photo-exposure, photostability tests were rather non-straightforward when compared to thermal stability for instance. Issues pertaining to irradiation sources, exposure levels, irradiation level and temperature effects, containers, presentation of samples and interpretation of data were stated as major guideline problems (Drew, 1998).

Irradiation sources:

The ICH recommended two options for irradiation sources. Option 1 was revised to simulating outdoor daylight (D65) or indoor window-filtered daylight (ID65). These are internationally recognized standards. There is an issue of standardizing photo-testing when the building for instance, where the medicinal product is handled or stored, has generally less natural light, less windows, a combination of natural light and fluorescent lamps *etc*. It makes it difficult to standardize the irradiation to accommodate such scenarios. It is however most likely that the ID65 is best appropriate source of irradiation and is preferred to option 2, a cool white fluorescent and near ultraviolet lamp (Tonnesen, 2007). A xenon or metal halide lamp provides that sort of irradiation with satisfactory spectral energy distribution. The lamps give off light in the UV-Vis regions and proprietary lamp suppliers include filters which are capable to exclude irradiation of

less than 320 nm according to the standards for window glass (international organization for standardization 10977).[33]

Exposure level:

The irradiation levels are difficult to simulate and there is the likelihood that a drug product is exposed to varying intensities of light when inside a pharmacy for example. Tonnesen (2007) argues that the standard indoor indirect daylight (ID65) conditions are harsher than natural light. The recommended exposure is an energy of not less than (NLT) 200 W/m^2 in the UV range of 320 – 400 nm (equivalent to 1-2 days on the window-glass filtered daylight on a sunny day). The recommendation for an ID65 lamp in the visible region is a total exposure of 1.2 million lux-hours (equivalent to 3 days on a window sill on a sunny day). According to the author a test run to achieve 1.2 million lux-hours will exceed the stated energy standard of 200 W/m^2 by 2.5 – 3 times *i.e.* it will require energy of between 500 and 600 W/m^2.[34] Higher irradiation levels will allow for less exposure time but may also introduce unwanted temperature effects because irradiance and temperature are dependent variables (Thatcher *et al*., 2001).

Irradiation and temperature effects:

The irradiation level is not explicitly specified by the ICH guidelines. It is therefore dependent on the individual test requirements. When considering irradiation levels, the unwanted effects of temperature must be taken into consideration. Temperature will increase whilst irradiation is increased, therefore in an attempt to reach overall ICH illumination exposure levels of 1.2 million lux-hours one must be careful of temperature effects. The best compromise would be a high enough irradiance level to accelerate the procedure without causing unwanted temperature effects whilst a system that keeps internal ambient temperature in the test chamber at desired levels could be best (Tonnesen, 2007). The cooling system in available equipment may be useful as it is monitored *e.g*. using a black standard thermometer (BST).

[33] *ISO* is an organization that coordinates the development and adoption of numerous international standards. *ISO* 10977 (1993) is the international standard for describing test equipment, test procedures and analytical procedures for predicting image stability. It has been revised to *ISO* 18909 (2006).
[34] These opinions guided the selection of irradiation energy as described in (*see* section 6.3.2.2).

Presentation of samples:

Most importantly, maximum and equal irradiance is critical in obtaining accurate and reliable results. The containers used, the distance from the light source and the sample preparation are all important factors to consider. Solid samples for example are reported to degrade only on the surface and are therefore spread in a single layer as recommended by the ICH guidelines (Tonnesen, 2007). Liquid samples are exposed in clear glass vials or quartz cuvettes. It has been alluded to that liquids offer more uniform irradiation of light when compared to solids and suspensions. Containers must be considered in terms of transmittance characteristics. Quartz for example does not filter off any radiation but glass vials are acceptable (Tonnesen, 2007).

Interpretation of data:

The end goal of photostability tests is to identify the need to take precautions when handling a drug substance during manufacture, packaging and storage. In any case, if there is no change in the test product or sample after exposure then there is no need to have concerns over excluding light during synthesis, packaging and storage. Conversely, if there is such change, then there is every chance that during handling and storage of the sample, protection from light is necessary to preserve the quality of the compound. According to ICH guidelines, if a compound is stable to visible light exposure, then it meets the ICH criteria for a photostable compound. Instruments that satisfy the requirements of the ICH guidelines have been developed and are commercially available.[35]

6.1.2. Basic principles for photostability testing

Photo-lability may be imparted to a molecule because of the chromophoric property and weak covalent bonds in some functional moieties such as carbonyl (C=O), alkene (C=C), polyenes and hydroxyl (O-H) groups. This is much related to the bond energies and corresponding wavelengths of maximum absorption. The chemistry of photo degradation is in susceptible functional groups that make up a molecule. If a molecule absorbs photons having a wavelength equal to 257 nm or

[35] ICH guidelines Q1B: http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q1B/Step4/Q1B_Guideline.pdf/ [Accessed 24 January 2014]

shorter for example, the O-H bond it bears absorbs enough energy for it to undergo photoreaction. The complexity and unpredictable nature of photoreactions is evident because, if this principle holds true then most chemicals in nature would dissipate instantly. There are a variety of competitive dissipative pathways for absorbed energy to compensate for photodegradation. Allen (2007) presents this phenomena concisely;

$$D + h\nu \rightarrow D^*$$

$$D^* \rightarrow \text{degradation products}$$

$$D^* \rightarrow D + \text{heat (internal conversion)}$$

$$D^* \rightarrow D + h\nu' \text{ (fluorescence)}$$

$$D^* \rightarrow D + h\nu'' \text{ (phosphorescence)}$$

$$D^* + M \rightarrow D + M^* \text{ (energy transfer)}$$

Where D = drug molecule; D = drug in electronically excited state; hv = electromagnetic radiation at a given energy, frequency and wavelength, M/M* = acceptor molecule in ground and electronically excited state.*

A compound may absorb light strongly but if there are efficient mechanisms present such as fluorescence or transfer of energy to another molecule within the same system, then it may not undergo photoreactions or photodegradation rather. Indirect photosensitization including reactive free radicals and molecular oxygen is a possibility (Allen, 2007). Samples used in drug photostability testing may be of either a dilute solution (uniform exposure to radiation), suspensions and/or solid material (thin layer of molecules absorbing radiation at the surface of the sample). Liquids allow samples to be maximally exposed to radiation because of minimized scatter and reflection. A drug stable in liquid form, is most likely stable in solid form but the reverse is not always true (Allen, 2007).

6.1.3. Previous studies on photostability of fucoxanthin

There are only a handful of studies regarding the photostability of fucoxanthin (Hii *et al*. 2010; Piovan *et al*., 2013). There is no mentioning of the explicit use of the ICH guidelines in these studies. This is problematic and difficult to correlate the information provided in literature. Zhao (2014) exposed samples of fucoxanthin (95%) purified from *Costaria costata* in an oven maintained at 25 °C and exposed them to an 18 W fluorescent lamp (300 lux and 2000 lux) for 10 weeks. The fucoxanthin was sampled weekly following an extraction procedure on the oil-fucoxanthin mix (0.5 g) by HPLC (C30 YMC; 250 x 4.6 mm i.d; 3 µm) using a gradient mobile phase (MeOH-Water and MTBE). The starting material was approximately 165 µg/mL and after 10 weeks of 2000 lux light with no air only 21.9% of the *all-trans* fucoxanthin had degraded. This is an estimated 3.36 million lux-hour total exposure. The recommended 1.2 million lux-hour was achieved after about 3.5 weeks. At that time only 3.1% fucoxanthin had degraded. The authors alluded to the possibility of the 13 and 13'-*cis* isomers converting to *all-trans* form in the early stages of the treatment as an explanation to the initial increase in *all-trans* fucoxanthin in the first week. An increase in the 9'-*cis* was reported as significant. The degradation was about four times greater when exposed to both 2000 lux and air which in both cases, illumination resulted in the formation of *cis* isomers of the compound.

The other study was carried out on an acetone-methanol extract of *Sargassum binderi* containing fucoxanthin. The details of how the procedure was carried out are absent making the results difficult to comprehend. Fucoxanthin as a carotenoid is expected to be susceptible to light and may lead to a loss of color owing to the disruption of the conjugated double bond system (Hii. *et al*., 2010).

Another recent study investigated the stability of fucoxanthin to photo-exposure using acetone, methanol and acetonitrile crude extracts of *Undaria pinnatifida*. The study assessed the effect of the different constituents owing to the slightly different chemical profiles from each extract on the stability of the pigment (Piovan *et al*., 2013). The fucoxanthin content was diminished by 90 % at the end of their 5 hour study on exposure to direct day light measured as 2500 lux.[36]

[36] The most likely limitation is the lack of standardization *i.e*. varied exposure of energy from direct sunlight and unregulated temperatures. A controlled environment complying with international standards (*ISO* and ICH) may give more reliable results *e.g.* Suntest chamber that has controlled temperature and irradiation energy.

6.1.4. pH stability

The human gut, the immediate environment for all orally taken substances is predominately aqueous and varies in pH. How fucoxanthin behaves under various pH conditions may shed some much needed light on oral formulations containing fucoxanthin and their bioavailability.

pH stability studies are part of stress testing that pharmaceuticals have to undergo to assess their susceptibility to hydrolysis across a wide range of pH values. A conventional way of exposing the sample to various pH conditions suffices as the ICH mention the need for testing under these conditions but do not provide a proper guideline of the procedure (Qui *et al*. 2013).

Carotenoids are mainly used in food industries and are isolated to act as nutraceutical ingredients. The biggest challenge with the incorporation of carotenoids into foods is their low water solubility and pH instability. The use of buffered solutions adjusted to the desired pH ranges using HCl and NaOH has been a method used in the testing of food technologies *e.g*. β-carotene enriched nanoemulsion exposed to various pH solutions (Qian *et al*., 2012).

6.1.5. Previous studies on pH stability of fucoxanthin

Fucoxanthin isolated from *Sargassum binderi* is one study to the best of our knowledge that exposed the pigment to various pH conditions to assess its stability. Using 1 M HCl and 1 M NaOH a pH range (pH 3, pH 5, pH 7, and pH 9) was prepared by adjusting the initial pH of the pigment extract. The analysis carried out in the dark over four weeks showed better stability and it was found that fucoxanthin exhibited greatest and least stability at pH 9 and pH 3 respectively (Hii *et al*., 2010).

6.1.6. Chapter Aims

The aim of the research in this chapter was to understand the extent of stability of fucoxanthin to photoexposure and pH. The pigment fucoxanthin, has a plethora of points of functionality likely to be susceptible to photo- and pH degradation to mention the polyene chain, the carbonyl and

hydroxyl functional groups. Given the chromophoric nature of fucoxanthin it is expected to have at least some photostability issues. The pigment's vulnerability in aqueous conditions and varying pH was also anticipated. Stability is a functionally relevant quality attribute of any biologically active compound. The concerns of instability include a loss of the active component and sometimes bioavailability, formation of toxic degradation products and an almost certain compromised safety (Piechocki and Thoma, 2007). If fucoxanthin is to be pharmaceutically relevant, then the aforementioned factors must be addressed and the stability of the compound clearly established.

Chapter Objectives:

1. Evaluate the degree and possibly the course of photodegradation of fucoxanthin in different forms *i.e*. crude CH_2Cl_2/MeOH extract, step gradient hexane/EtOAc fraction (chapter 3), 99% *all-trans* fucoxanthin (chapter 4) and MeOH extract of *Sargassum incisifolium* (chapter 5) according to ICH guidelines.
2. Evaluate the integrity of fucoxanthin (99%) when exposed to varying relevant (to the human gastrointestinal tract) pH conditions (between pH 1.0 and pH 10.0).

6.2. Results and Discussions

6.2.1. Photostability

The samples of fucoxanthin were prepared *viz* pure fucoxanthin[37] (~100 µg/mL, **A**), step gradient fucoxanthin fraction (~113 µg/mL, **B**), methanol extract of *Sargassum incisifolium* (~78 µg/mL, **C)** and dichloromethane/methanol extract of *Sargassum incisifolium* (~100 µg/mL, **D**). These samples were exposed to xenon lamp irradiation whilst enclosed in a Suntest® chamber for a total of 16 hours of which sampling time intervals were 30 minutes, 1, 2, 4, 8 and 16 hours. We were interested in also investigating the sampling technique. One sample was left in the chamber for the entire duration of the test whilst sampling from it at each time interval. The rest of the samples were sacrificially sampled *i.e.* once sampled at each time interval, the sample was analyzed and not returned into the chamber. No notable difference was observed between the two techniques.

[37] Isolated fucoxanthin (*see* chapter 3)

The fucoxanthin concentrations were monitored throughout the test period using the quantification method of (chapter 4). The changes in concentration of fucoxanthin after photo-exposure (16 hours) are detailed in (Table 6.1) and (Fig 6.1 and 6.2). Over 16 hours, **A** showed the most overall resistance to degradation showing about 59 % loss of all-*trans* fucoxanthin when compared to **B**, **C** and **D** which showed a 100 %, 100 % and 88 % overall loss respectively. The differences in components of **A** – **D** must be defined. In **A**, no other component was present except for fucoxanthin. **B** was mainly fucoxanthin but with a few other components that elute close to the pigment *i.e*. compounds of similar polarity. We expected one other component together with the *cis*-forms of fucoxanthin as previously reported in literature (Nakazawa *et al*., 2008). In **C**, several polar components (not determined in this study) found in the alga were most likely present together with fucoxanthin. **D** was expected to contain almost all the components within the alga, from non-polar to polar compounds (not determined in this study). The chemistry therefore and environment that fucoxanthin was being illuminated under, was significantly different from **A** – **D**.

Surprisingly, pure fucoxanthin (**A**) was more stable over the 16 h period than the crude or partially purified fractions. After 16 hours of exposure, 40% of the original content was left. The degradation rate was steady and much slower than any other sample and the degradation was ascribable to *cis*-isomerization *i.e*. formation of more oxidizable *cis*-forms of fucoxanthin (Piovan, 2013). In our study, slight formation of what we expect to be what Nakazawa, (2008) reported as isomers of fucoxanthin was observed (Fig 6.1) eluting after fucoxanthin.

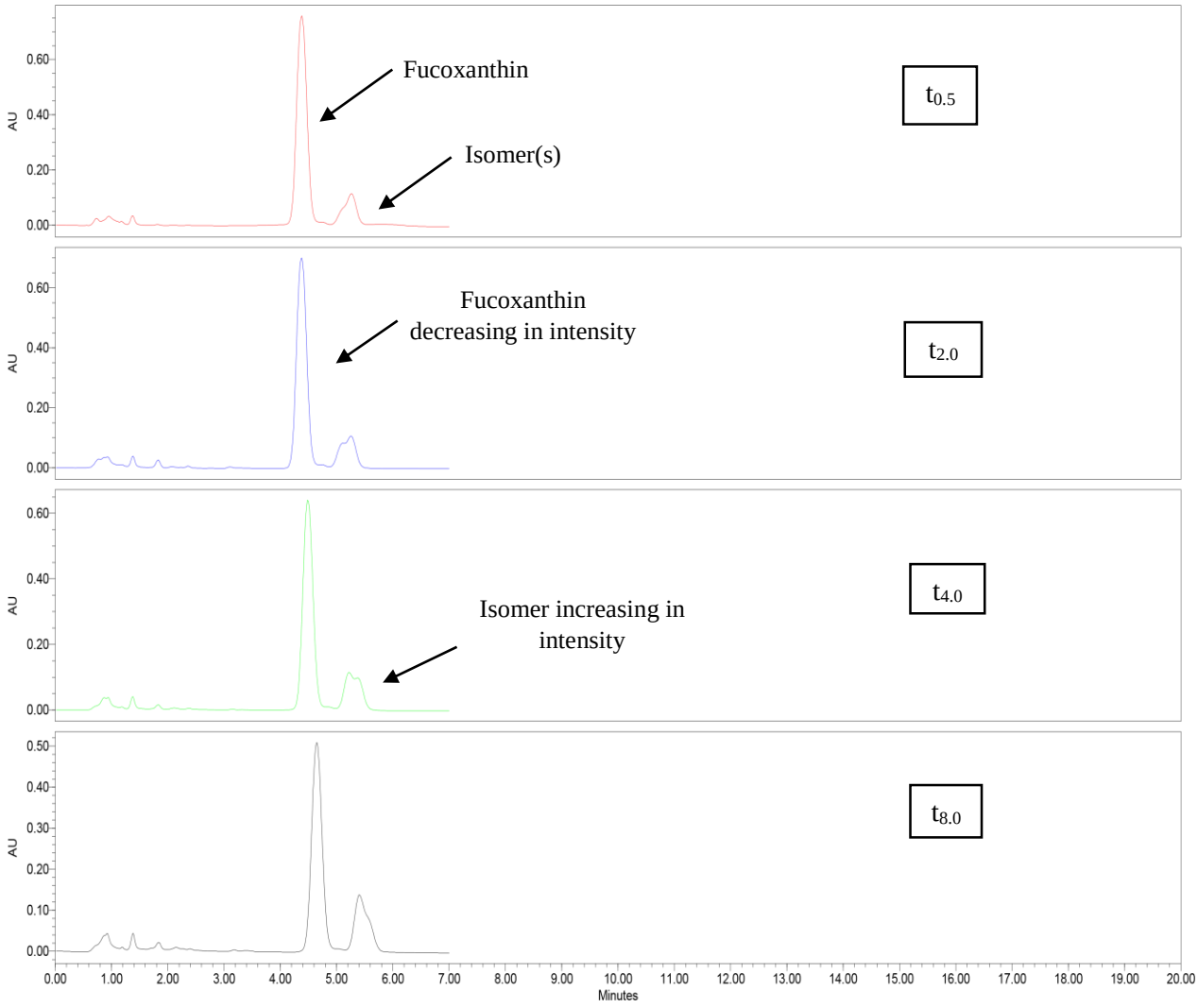


Figure 6.1: Progressive depletion of the fucoxanthin peak and slight increase in the "isomer(s)" peak. Conditions: Phenomenex® Synergi™ 250 x 3.0 mm i.d. in ACN/H_2O (95:5).

Piovan *et al*. (2013) proposed that components present in conventional crude extracts containing fucoxanthin may accelerate the reactions leading to the degradation of fucoxanthin. Certainly, the crude and partially purified fractions (**B** – **D**) demonstrated a decreased stability of fucoxanthin on light exposure. The degradation of fucoxanthin was somewhat accelerated in crude extracts but rather steady in **B** and **D**. The protective effects of antioxidants were also investigated and were shown to play a major role in retardation of the degradation of fucoxanthin (Piovan *et al*., 2013).

Brown seaweed extracts have been found to contain at least one more identified carotenoid, β-carotene and chlorophylls (Haugan and Liaaen-Jensen 1989). The same can be expected in extracts of *S. incisifolium* as was observed in the step-gradient fractionation of (*see* chapter 3). The effect of these compounds can be postulated and used to explain differing rates of fucoxanthin degradation between samples under study such the antioxidant properties of co-extractives reported by Afolayan *et al*. (2008). The major metabolites reported from the crude extracts of *Sargassum heterophyllum*[38] are sargaquinoic acid, sargahydroquinoic acid and sargaquinal (Afolayan *et al*., 2008) and the antioxidant activity of these compounds has been reported and may play an immense role in providing some protection in the degradation of fucoxanthin as described by Piovan *et al*. (2013).[39] However, crude extracts in this study seem to show accelerated degradation of fucoxanthin.

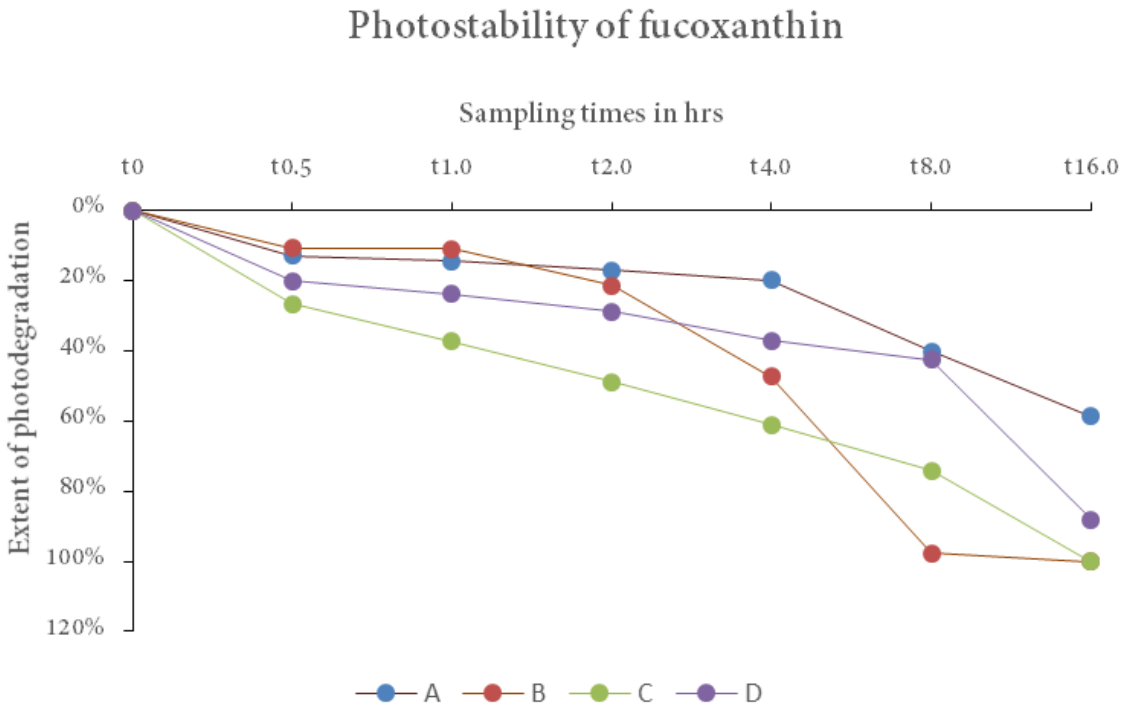


Figure 6.2: A graphical representation of the extent of photodegradation of fucoxanthin in **A** (pure form, 99%), **B** (step-gradient fraction, ~87%), **C** (MeOH extract of freeze dried *Sargassum incisifolium*) and **D** (crude CH_2Cl_2/MeOH extract of *Sargassum incisifolium*).

[38] The heterotypic synonym for *Sargassum incisifolium*

[39] There is however not enough evidence as to what was the cause of increased degradation amidst reports of the photo-protective effects different extracts may possess (Piovan *et al.*, 2013) and the isomers have not been characterized. More investigations are required. The focus was placed mainly on the loss of fucoxanthin during photo exposure.

Table 6.1: Changes in fucoxanthin concentration after 16 hours of ID65 exposure in a Suntest CPS+ chamber.

sample	sample times t_h	fucoxanthin mean conc. µg/mL	%RSD	%Δ	sample	sample times t_h	fucoxanthin mean conc. µg/mL	%RSD	%Δ
A		102.6	2.08%	0%	**A**		82.2	0.57%	20%
B		113.4	0.44%	0%	**B**		59.8	0.38%	47%
C	t_0	78.4	1.45%	0%	**C**	$t_{4.0}$	30.5	1.23%	61%
D		102.2	1.50%	0%	**D**		64.3	1.17%	37%
A		89.3	4.47%	13%	**A**		61.4	1.20%	40%
B		101.3	0.92%	11%	**B**		2.7	4.54%	98%
C	$t_{0.5}$	57.5	1.83%	27%	**C**	$t_{8.0}$	20.3	1.27%	74%
D		81.6	0.96%	20%	**D**		58.7	0.59%	43%
A		87.9	1.86%	14%	**A**		42.3	2.57%	59%
B		101.1	2.30%	11%	**B**		0.0	0.00%	100%
C	$t_{1.0}$	49.1	0.58%	37%	**C**	$t_{16.0}$	0.0	0.00%	100%
D		77.8	0.60%	24%	**D**		12.2	0.79%	88%
A		85.1	3.11%	17%	**A**		102.7	2%	0%
B		89.2	0.37%	21%	**B**		91.2	1%	20%
C	$t_{2.0}$	40.1	2.20%	49%	**C**	t_{cntrl}	76.2	1%	3%
D		72.7	0.27%	29%	**D**		100.7	0%	1%

Interestingly, **B** turned from a bright orange color to colorless after the duration of the test. Fucoxanthin has been reported in several studies to absorb light and transfer it to chlorophyll molecules in what is known as a fucoxanthin-chlorophyll protein complex (Papagiannakis, 2005). This is a light harvesting complex which exists in brown and other algae containing fucoxanthin (*in vivo*). Whether the same arrangement can be attained *in vitro* by solubilization of protein

complexes (during normal extraction of pigments) or having fucoxanthin molecules adjacent to chlorophyll molecules remains to be investigated. This phenomena may provide a different dimension in understanding fucoxanthin degradation, at least our ability to detect it at 446 nm. In addition to excitation energy transfer (EET) to chlorophyll *a* and *c*, carotenoids exhibit excitation state absorption (ESA) at higher wavelengths to cover the blue/green region where chlorophyll cannot absorb, all in part of their natural role as accessory pigments. Fucoxanthin specifically as a carotenoid with a carbonyl conjugated backbone, is characterized by a low-lying intramolecular charge transfer (ICT) state that results in an ESA at 600 nm in polar solvents (Zigmantas, 2004).

The path of photoreactions is very complicated and sometimes unpredictable. Reports support the exhibition of a different absorption spectrum in the presence of chlorophyll on photoexposure which is known as a blue wavelength shift. If this kind of reaction and/or rearrangement occurred during the photo exposure then it is highly likely that detection of excited molecules was impossible at a wavelength of 446 nm further contributing to the decreased detection of fucoxanthin with time. Bleaching occurs with fucoxanthin on photoexposure and it may or may not return to ground state after excitation (leading to restoration of color) as observed with **B** which remained bleached.

The initial path of degradation is understood to be via the conversion of fucoxanthin to its *cis* forms. The HPLC chromatograms for the progressive analysis revealed an increase in peak area of the isomers that coincided with a decrease in peak area of fucoxanthin peak by the same extent (Fig 6.1. The isomers of fucoxanthin have been identified and have been demonstrated to elute immediately after all-*trans* fucoxanthin, (9'-*cis*), and two conjoined peaks (13' and 13-*cis*). Systematic conversion to the 13 and 13'-*cis* isomers followed by conversion to 9-*cis* has been reported to be a consequence of photo exposure (Zhao, 2014).

6.2.2. pH stability

The sample used was pure fucoxanthin (*see* chapter 3). Eight samples of concentration 1 mg/mL were prepared, one for each tested pH and the MeOH control. Fucoxanthin was solubilized in MeOH before being added to prepared pH solutions (pH 1.0, 2.0, 3.0, 7.0, 8.0, 9.0 and 10.0).

Materials
99% pure fucoxanthin
1 mL 10 % MeOH aqueous solutions (pH1.0, pH2.0, pH3.0, pH7.0, pH8.0, pH9.0, pH10.0)
Calibration equation: *y=102215x + 259213*

The fucoxanthin concentration was also monitored using the HPLC analytical method developed in (*see* chapter 4). The changes in concentration after exposure to varying pH conditions are detailed in (Table 6.2) and (Fig 6.3). After overall exposure (8 hours), fucoxanthin was most stable at pH 10.0 and showed poor stability in almost all the other pH conditions tested. The nature of the instability was in that fucoxanthin was precipitating out of the solution into fluffy orange crystals. Fucoxanthin at pH 1.0 showed relatively rapid degradation. There was evidence of a unique reaction where the crystals turned from orange to blue. The instability of carotenoids has been reported and demonstrated by the reaction of fucoxanthin with strong acids (Haugan and Liaaen-Jensen, 1994). The reaction led to the formation of blue oxonium ions. At pH 2.0 the product was brownish in color whilst other pH conditions had an orange precipitate. A bathochromic shift of λ_{max} to 720 nm has been reported confirming that fucoxanthin could not be detected at the set wavelength, 446 nm. An intermediate, isofucoxanthin is also reported. The active part of the molecule is reported to be the 5, 6-monoepoxide moiety (Haugan and Liaane-Jensen, 1994, *see* Appendix 6.1).

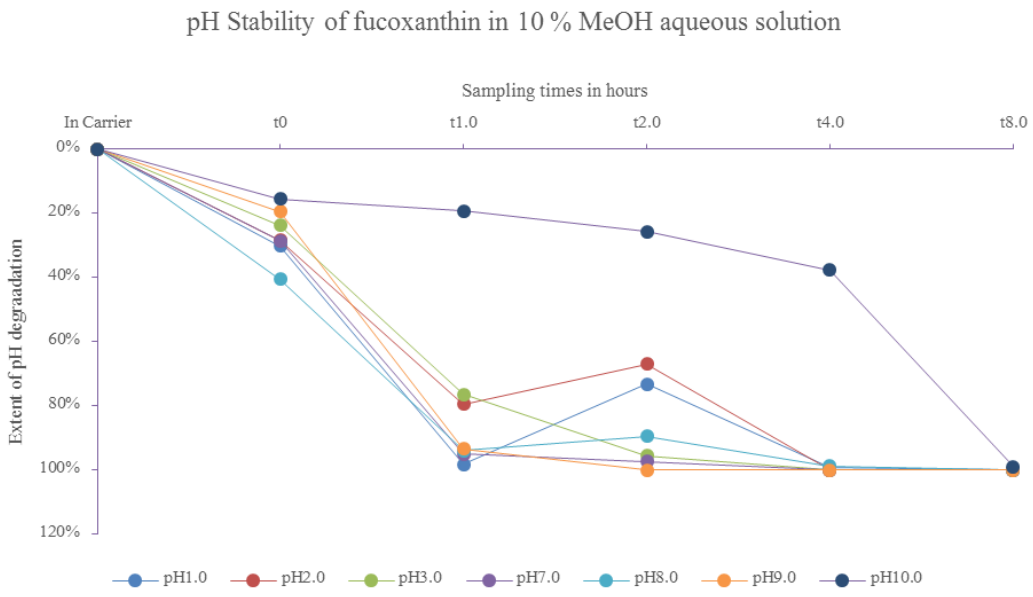


Figure 6.3: A graphical representation of the extent of degradation owing to varying pH conditions.

Table 6.2: Changes in fucoxanthin concentration owing to varying pH conditions over 8 hours.

Note: Starting concentration before mixing with aqueous solutions = 124.1 µg/mL

	sample times t_h	**fucoxanthin conc. µg/mL triplicates**			**Mean conc. µg/mL**	**std. dev.**	**%RSD**	**%Δ**
		1	**2**	**3**				
pH1.0		86.6	87.1	86.3	86.7	0.4	0.47%	30%
pH2.0		89.2	89.2	88.4	89.0	0.4	0.51%	28%
pH3.0		94.9	94.2	95.1	94.7	0.5	0.49%	24%
pH7.0		88.3	88.9	88.6	88.6	0.3	0.33%	29%
pH8.0	t_0	73.2	75.1	73.6	74.0	1.0	1.41%	40%
pH9.0		99.7	100.9	99.6	100.1	0.7	0.71%	19%
pH10.0		104.2	105.8	104.6	104.9	0.8	0.78%	15%

pH1.0		2.2	2.3	2.3	2.3	0.1	2.37%	98%
pH2.0		25.7	25.6	25.7	25.6	0.1	0.20%	79%
pH3.0		28.8	30.2	29.1	29.4	0.7	2.41%	76%
pH7.0		6.3	6.2	6.5	6.3	0.2	2.49%	95%
pH8.0	$t_{1.0}$	7.6	7.7	7.6	7.6	0.0	0.51%	94%
pH9.0		8.2	8.5	8.4	8.4	0.1	1.27%	93%
pH10.0		100.4	100.3	100.0	100.2	0.2	0.19%	19%
pH1.0		32.8	34.1	33.3	33.4	0.6	1.93%	73%
pH2.0		42.6	40.2	40.6	41.1	1.3	3.10%	67%
pH3.0		5.7	5.7	5.6	5.6	0.1	1.07%	95%
pH7.0		3.4	3.4	3.4	3.4	0.0	0.38%	97%
pH8.0	$t_{2.0}$	13.0	13.0	13.5	13.1	0.3	2.11%	89%
pH9.0		-1.4	-1.5	-1.5	0.0	0.0	0.00%	100%
pH10.0		92.1	92.0	92.8	92.3	0.4	0.47%	26%
pH1.0		1.0	0.9	1.0	1.0	0.0	1.96%	99%
pH2.0		0.0	0.0	0.0	0.0	0.0	0.0%	100%
pH3.0		0.0	0.0	0.0	0.0	0.0	0.00%	100%
pH7.0		0.0	0.0	0.0	0.0	0.0	0.00%	100%
pH8.0	$t_{4.0}$	1.7	1.7	1.8	1.7	0.1	3.60%	99%
pH9.0		0.0	0.0	0.0	0.0	0.0	0.00%	100%
pH10.0		77.0	77.0	78.2	77.4	0.7	0.89%	38%
pH1.0		0.0	0.0	0.0	0.0	0.0	0.00%	100%
pH2.0		0.0	0.0	0.0	0.0	0.0	0.00%	100%
pH3.0		0.0	0.0	0.0	0.0	0.0	0.00%	100%
pH7.0		0.0	0.0	0.0	0.0	0.0	0.00%	100%
pH8.0	$t_{8.0}$	0.0	0.0	0.0	0.0	0.0	0.00%	100%
pH9.0		0.0	0.0	0.0	0.0	0.0	0.00%	100%
pH10.0		1.5	1.5	1.5	1.5	0.0	0.41%	99%
MeOH	t_{cntrl}	120.4	121.4	122.4	121.4	1.0	0.84%	2%

Fucoxanthin has previously been reported to be stable at pH 9 (Hii *et al*., 2010). In another study conducted by Chen *et al*. (1995) xanthophylls and carotenes showed slower degradation at pH 10 and pH 8 compared to pH 5 and pH 6. Our study partially confirms these claims with fucoxanthin degrading extremely slowly at pH 10.0 and extremely quick in all the other pH conditions tested. There were some erratic concentrations observed in the first 2 hours or so but a general loss of pigment was observed. It was marquee to observe that after 4 hours all of the pigment had been lost except for pH 10.0 where only 38% of fucoxanthin had degraded. The alkali instability of the pigment has been reported where a complex mixture of products was observed when fucoxanthin was treated to differing concentrations of potassium hydroxide (KOH). The products found included fucoxanthinol, fucoxanthin hemiketal, isofucoxanthin and isofucoxanthinol which may be what we observe as the orange precipitate in the alkaline vessels (Haugan *et al*., 1992, *see* Appendix 6.1). No further studies of our own have been done to analyze these products at this stage.

6.2.3. Conclusion

The chapter was on the stability of fucoxanthin when exposed to external environmental agents *e.g*. light, heat, air and pH. The focus was on light (photostability) and acid/base conditions (pH stability). These type of studies attempt to establish a sense on how handling and storage of fucoxanthin may have an impact on its quality. The stability of the pigment was compared at different stages of extraction *i.e*. the crude CH_2Cl_2/MeOH extract, step-gradient fucoxanthin rich fraction (*see* Chapter 3), pure fucoxanthin from *Sargassum incisifolium* (*see* Chapter 4) and the MeOH extract from (*see* Chapter 5) to ascertain how other components contribute to the stability of fucoxanthin. The samples were exposed to a controlled light source for a reasonable period of time (16 hours) in compliance with ICH guidelines as much as possible to determine the degree and certainly the course of photodegradation. The purified fucoxanthin was also exposed to varying acidic and basic pH conditions in order to possibly predict the stability of the compound within a biological system before absorption by simulating the stomach and small intestines.

Fucoxanthin from the results obtained, is clearly photo-labile and unstable across wide range of pH conditions. Our investigations showed that in all stages of extraction, protection of fucoxanthin

from light should be an important consideration. This precaution including protection from air and elevated temperatures (not in this study), may contribute significantly to improved isolation of fucoxanthin in terms of yield and limiting isomerization. The pH studies confirm that any contemplation of orally taking fucoxanthin as is currently being suggested by nutraceutical developers (*see* section 2.6), should be extended to the protection of fucoxanthin during its transit in the gastro-intestinal tract which may prove to be a harsh environment for the pigment. More work is however required to further understand the differences observed in the rates of degradation and the products thereof, in both the photostability and pH stability tests carried out.

6.3. Experimental

6.3.1. General procedures

The photostability testing for samples of fucoxanthin was carried out in a controlled stability chamber, an ATLAS® Suntest CPS+™ (Linsengericht, Germany). The instrument was fitted with a xenon lamp, optical quartz filters, sensor for measurement and control of black standard temperature (BST) at specimen level and program controller. The pH stability of fucoxanthin was carried out in solutions comprised of buffer tablets from UNILAB® buffer tablets for pH 7.0 and pH 4.0, Saarchem (Pty) Ltd (Krugersdorp, South Africa) and standard bench reagents of HCL (0.1 M) and NaOH (0.1 M). Organic MeOH described in (*see* section 4.3) was used for all samples in photostability testing and solubilization required in pH stability testing. All the pH readings were recorded by a PCSTestr 35™ Waterproof Multi parameter Tester (pH meter), (Australia).

All samples for photostability testing were in Supelco® 4 mL screw cap glass vials from Sigma Aldrich® (Germany). All pH stability testing were carried out in Eppendorf tubes® (1.5 mL), (Hamburg, Germany).

6.3.2. Photostability testing

6.3.2.1. Sample preparation

The samples for photostability testing (**A - D**) were designed to have as much fucoxanthin as possible within an absorbance of 1.00 as per the criteria set in the analytical procedure described

in (*see* chapter 4). For **A** (pure fucoxanthin) and **B** (step-gradient fraction), a stock solution targeting 100 µg/mL (10 mL) fucoxanthin was prepared. For **C** (methanol extract), 464 mg from freeze dried *S. incisifolium* and 1.2 mg of **D** (CH_2Cl_2/MeOH extract) from fresh *S. incisifolium* obtained from the storage discussed in (*see* chapter 3) were also prepared.

6.3.2.2. Photoexposure

The experiment was designed to run initially for eight hours with six sampling points at 0.5, 1.0, 2.0, 4.0 and 8.0 hours. Seven replica samples for **A** – **D** (1 mL) were prepared to account for the six time points and a control (vial covered in aluminum foil). The stock solution for each sample was first injected into the HPLC (triplicate) to obtain t_0. A visual perspective of how the samples were arranged into the Suntest chamber is given in (Fig 6.4). After the first 30 min, the ($t_{0.5}$) samples were taken out of the Suntest and were immediately analyzed by HPLC. The same procedure was repeated for ($t_{1.0} – t_{8.0}$) at the relevant times. The $t_{0.5}$ samples however, were returned back into Suntest chamber immediately after sampling for the duration of the test period. These samples were periodically sampled (100 µL) the same way as $t_{1.0} – t_{8.0}$. At the end of the 8 hour phase, the $t_{0.5}$ samples were exposed for another 8 hour phase to provide $t_{16.0}$ and represent an extended exposure. At the end of the test period, the control samples were also sampled in the same way as described for the other samples.

Parameters for Suntest CPS+

Lamp: Xenon

Energy: 700 W/m^2

Filter: G (Solar ID65) a simulation of solar radiation behind a 6 mm window glass (ICH guideline for pharmaceuticals).

Wavelength: 380 – 800 nm

Exposure time: 16 hours

Figure 6.4: Suntest CPS+ and presentation of samples in the test chamber.

6.3.3. pH stability testing

6.3.3.1. Sample preparation

A stock solution (100 µg/mL; 10 mL) was prepared. Aliquots (1 mL) were transferred to seven size 6 Pu vials and dried using a rotavapor. Each vial contained approximately 100 µg fucoxanthin. A stock solution of pH 4.0 and pH 7.0 was prepared by dissolving a buffer tablet (pH 4.0) and (7.0) respectively in 100 mL of deionized water. An aliquot of pH 4.0 solution was adjusted with HCl (0.1 M) to give separate solutions of pH 1.0, pH 2.0 and pH 3.0 whilst an aliquot of pH 7.0 solution was adjusted with NaOH (0.1 M) to give solution of pH 8.0, pH 9.0 and pH 10.0. To each of the aforementioned vials containing 100 µg fucoxanthin, 100 µL of MeOH (carrier) was added and 900 µL of the respective pH solutions. The control was 100 µg fucoxanthin dissolved in 1 mL MeOH. Each of the 1 mL solutions were quantitatively transferred into the Eppendorf tubes (1.5 mL) for the duration of the test.

6.3.3.2. pH exposure

Note: The final preparation of the samples was staggered with 7 min (HPLC run time) in between them.

The experiment was designed to run for an 8 hour period with sampling from times t_0, $t_{1.0}$, $t_{2.0}$, $t_{4.0}$, and $t_{8.0}$. After sample preparation, each sample was immediately analyzed for fucoxanthin content (t_0) before being transferred into the freezer. After the first hour the first sample was centrifuged at 10 000 rpm for 5 min before being analyzed by HPLC ($t_{1.0}$). The same procedure was carried out for the rest of the samples in sequence ($t_{1.0} – t_{8.0}$). The samples are shown below (Fig 6.5).

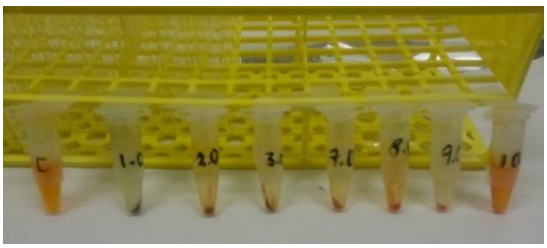
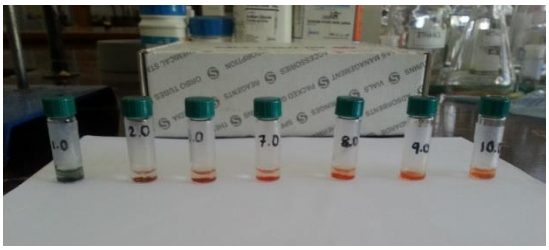
(a)  **(b)** 

Figure 6.5: pH stability samples (a) during testing (b) post testing.

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Chapter 7

7.1. Conclusions

The changing face of natural products has probably been responsible for the increased resurgence of not a new, but refreshed and revitalized interest in naturally derived bioactives. Algae is currently receiving attention as potential sources for not only raw materials (as we have come to expect from industries such as those that produce algin for example) but pharmaceutically relevant bioactive compounds. A considerable number of brown algae are edible and metabolites such as fucoxanthin have been attributed to the additional health benefits discovered from consuming brown seaweeds. The concept of nutraceuticals inspired by societies such as from within Japan, China, India *etc*. has since developed into a standalone industry which could potentially be worth up to US $ 50 billion with the carotenoid market alone being projected at US $ 1.4 billion (2018). [40] The increased market and industrial zeal has resulted in several concerns being raised regarding the safety, quality and efficacy of nutraceuticals, something that has triggered some form of regulation from the main regulatory bodies such as the WHO, USA's FDA, Europe's EMEA, the ICH and South Africa's MCC. Regulation of herbal preparations is still a work in progress but it is inclined to treating nutraceuticals as allopathic medicines at least in terms of their preparation and analysis. Nutraceuticals still do not need to go through clinical trials, neither are they registered as safe or effective. Nutraceuticals and natural products from marine resources as a whole, may have a new lease of life after all, considering they still possess structural novelty, diversity, binding efficiency and the propensity to interact with molecular targets.

Fucoxanthin is an inherent pigment in brown algae. It is both important (for photosynthesis) and characteristic (marker compound) to brown algae, making this type of seaweed an ideal source of the xanthophyll carotenoid. Fucoxanthin is purported to be a multifunctional biomolecule interacting with several targets within a biological system. This claim has emanated from the

[40] Carotenoid Market Forecast: http://newhope360.com/supply-news-amp-analysis/carotenoid-market-forecast-grow-14-billion-2018/ [Accessed 29 January 2014]

multiple tests that have been carried out in rodents and cultured human cells where the antiobesity, anticancer, antidiabetic and antioxidant activities have consistently been reported (Peng *et* al., 2011) from the past two decades. Several other potential bioactivities have been identified such as antiplasmodial, cerebrovascular, hepatotoxic and ocular protection (Peng *et al*., 2011). The unique structure of fucoxanthin might as well be responsible for some of the superior biological activities exhibited by the molecule. There are only a couple of human studies reported where its antiobesity effects have been investigated (Abidov *et al*., 2010). These studies however have not gained official recognition from the main regulatory bodies because nutraceutical companies still claim that their specifications have not yet been approved by the FDA, neither have they been corroborated by any other regulatory body. However, since it is not required for herbal preparations to undergo the stringent clinical trials allopathic or synthetic medicine do, nutraceutical companies have developed and marketed formulations containing crude plant extract in the form of soft gels, capsules and patches to deliver fucoxanthin for slimming purposes. Examples are products such as FucoThin™.

The synthesis of fucoxanthin has so far proved challenging in terms of time, resources and yields. There has thus been a lack of highly stable or high quantity reference standards. Extraction of the pigment from natural sources on the other hand, has proved reliable and relatively effective (Kanazawa *et al*., 2008). We needed a reference standard for the development of the analytical method that would be used to quantify fucoxanthin from diverse samples of different brown seaweed extracts. Fucoxanthin (**BM13-73b**) of high purity (99%) was successfully isolated from *Sargassum incisifolium* and kept stable by improved handling and storage conditions. The precautions when isolating fucoxanthin, have to be adhered to from the plant material and crude extracts to the final product. These include, working under reduced light, exclusion of air and light by flushing storage vessels with inert gas and covering them with aluminum foil whilst maintaining storage temperatures of -20°C. The use of step gradient chromatography followed by reversed phase semi-preparative HPLC was effective in repeatedly isolating and purifying high quality fucoxanthin. The NMR, IR, UV characteristics confirmed the isolated product, **BM13-73b**, to be authentic fucoxanthin when compared to both literature and when the same tests were carried out on the standard acquired from Sigma Aldrich® (*see* chapter 3).

A simple, rapid, and reproducible HPLC analytical procedure for the quantification of fucoxanthin in crude extracts was successfully developed and validated following the standards set by the ICH. HPLC-UV/Vis in a continuous and repetitive manner, is able to distinguish between components (including pigments) in an alga crude extract (methanolic or dichloromethane) and quantitate the individual components in a manner that is both simple to interpret and communicate. The versatility of the equipment (HPLC) demonstrated by the ability to change columns and effect a desired outcome, makes it a very ideal analytical procedure to determine the amount of fucoxanthin produced by different brown algae common to South Africa. The use of hyphenated systems may be very useful, especially coupling the HPLC system with an atmospheric pressure chemical ionization mass spectrum (HPLC-UV/Vis-APCI-MS) to confirm the identity of both fucoxanthin and of other components detected at the same wavelength (*see* Chapter 4).

The main species commonly found in large enough masses on the coastline of South Africa are from the genera *Sargassum, Zonaria, Ecklonia, Bifurcariopsis, Dictyopteris and Oerstedtia.* A lot more other species (kelps) can be found on the south west coast. However from the selected species, *Zonaria subarticulata* (**KOS130226-18**) *and Sargassum incisifolium* (**PA130427-1**) from Kenton-on-Sea and Port Alfred respectively were found to have high fucoxanthin content. *Sargassum* spp. have been a reliable source of fucoxanthin from previous studies our research group has embarked on, but *Zonaria* spp. sometimes occurring in very simple structures relative to the *Sargassum* spp., may prove to be an equally good source. However to fully determine the best source of the pigment, several factors have to be considered. The life stage of the algae (*Sargassum* spp.), the morphology (leafy algae versus branchlike algae), growth rate (Laminariaceae and Kelp forests) and the season in which the identified algae produces peak levels of fucoxanthin may be some of the important factors. An independent study solely focusing on these factors, may be very important before targeting a particular algae for innovative and/or commercial production of fucoxanthin (cultivation) (*see* Chapter 5).

The stability of fucoxanthin affects this study quantitatively in that if the compound degrades during handling, unreliable results are obtained especially during the method development and validation procedures. Degradation from external agents can be averted but it has to be understood and the rate at which it occurs, established. Our selected factors (light and pH) shed some much needed light on the degradation of fucoxanthin and how to control it. Fucoxanthin is light sensitive

and must be protected from light at every stage from extract to pure form. It is however more photostable in pure form than in extract form. The initial degradation products are *cis* isomers of the pigment before unknown species dominate which are possibly responsible for the accelerated degradation of fucoxanthin we observed with time. Fucoxanthin is unstable in aqueous environments. Alkaline conditions (specifically, pH 10) however, may impart some stability to the molecule. The behavior of the molecule in acidic conditions may impact significantly on the application of fucoxanthin in oral formulations where the conditions of the stomach are acidic for instance. Other external agents to concurrently protect fucoxanthin from, include air and high temperatures (*see* Chapter 6).

The overall aim of the study was to find out which of the brown algae common to South Africa was the best suitable source of fucoxanthin. We wanted to achieve this using a simple, reproducible and effective analytical method to quantify the pigment from diverse samples of South African marine brown algae. The objectives to this aim where achieved and the knowledge gained opened up opportunities for future studies;

1. To reliably source, harness and handle fucoxanthin (in appropriate conditions) in order to support our medicinal chemistry interests such as antiplasmodial and cytotoxicity activities. These may involve trying to understand the molecule's pharmacophoric pattern and whether potent analogs can be synthesized.
2. To analyze several commercial products of fucoxanthin *e.g.* FucoThin™ in order to determine how much fucoxanthin is actually being delivered in available formulations and whether this is consistently being achieved.
3. To explore the possibility of manipulating the production of fucoxanthin from brown algae *i.e*. cultivation of seaweed for the purposes of harnessing larger amounts of fucoxanthin.

7.2. References

Abidov, M., Ramazanov, Z., Seifulla, R. and Grachev, S. The effects of Xanthigen in the weight management of obese premenopausal women with non-alcoholic fatty liver disease and normal liver fat. *Diabetes Obesity and Metabolism* **2010**, 12: 72-81.

Kanazawa, K., Ozaki, Y., Hashimoto, T., Das, S.K., Matsushita, S., Hirano, M., Okada, T., Komoto, A., Mori, N. and Nakatsuka, M. Commercial-scale preparation of bio-functional fucoxanthin from waste parts of brown sea algae *Laminaria japonica. Food Science and Technology Research* **2008**, 14: 573-582.

Peng, J., Yuan, J. and Wang, J. Fucoxanthin, a marine carotenoid present in brown seaweeds and diatoms: Metabolism and bioactivities relevant to human health. *Marine Drugs* **2011**, 9: 1806-1828.

“The real voyage of discovery consists not in new landscapes, but in having new eyes”.

Marcel Proust

Appendix 1.1

Footnote websites

Carotenoid market

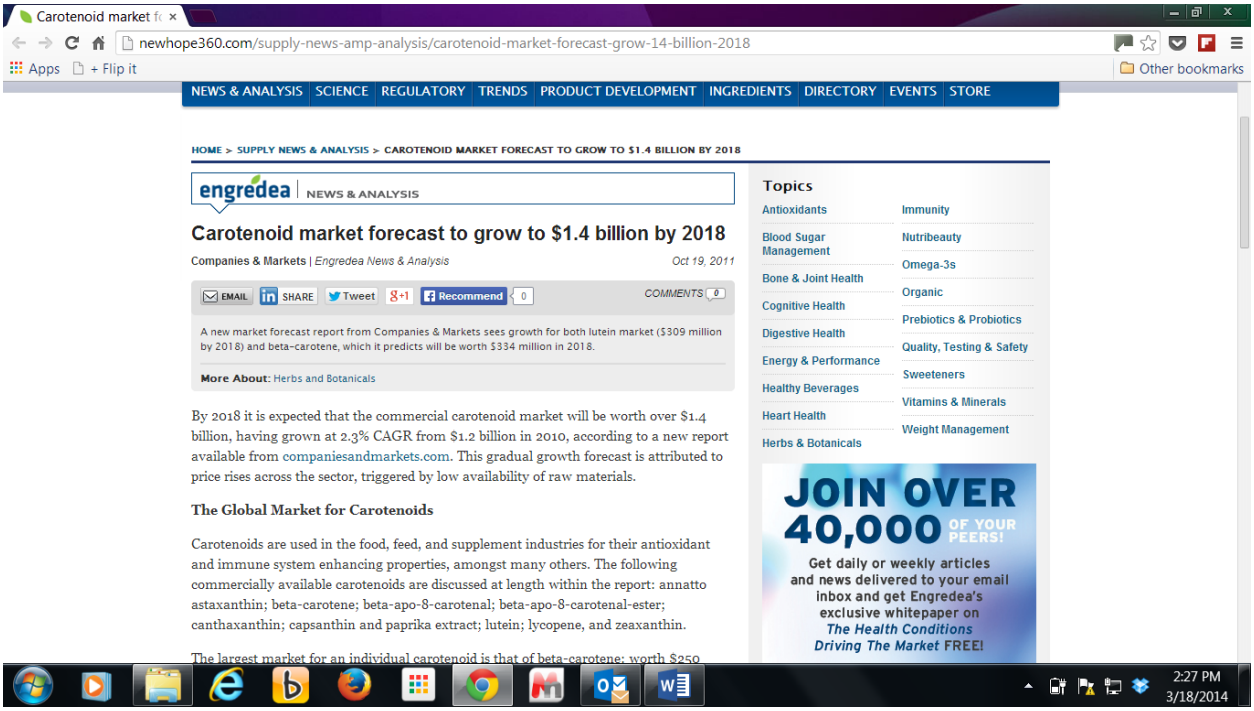


Figure A1.1: Carotenoid market projection to US$1.4b in 2018

Complimentary medicines and the MCC.

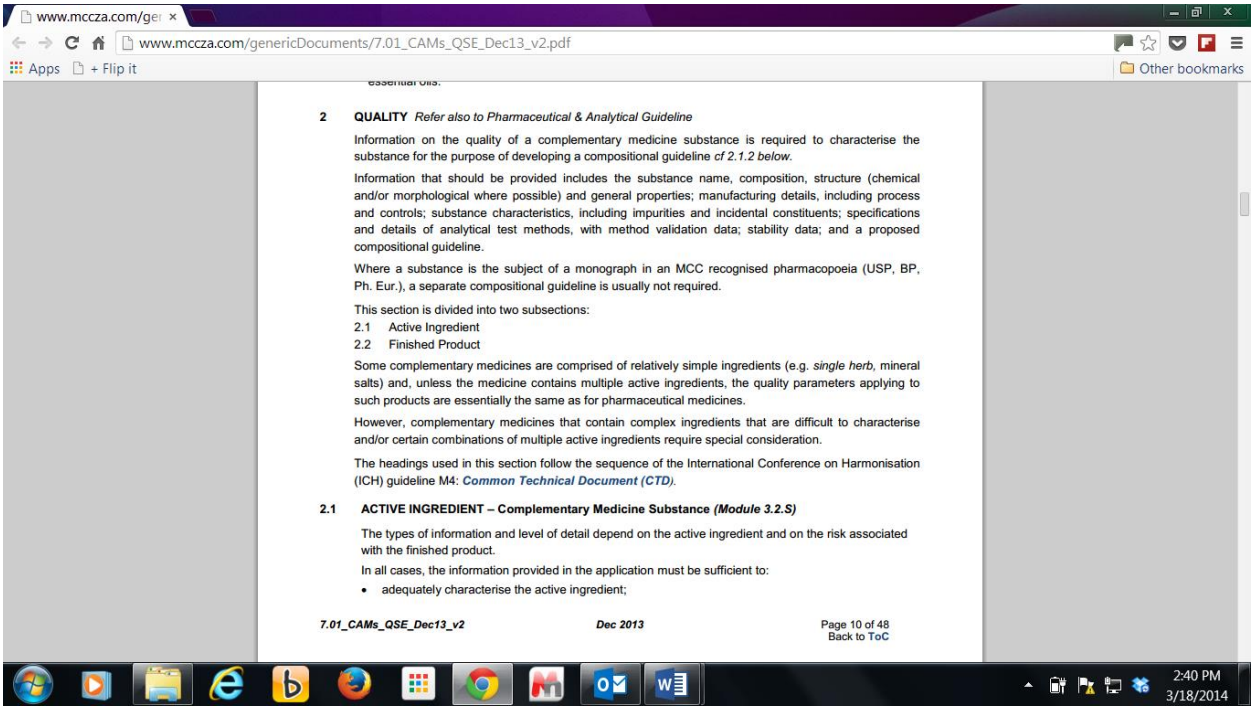


Figure A1.2: The MCC and standard expected for complimentary medicines.

Physicochemical properties from Sigma-Aldrich®

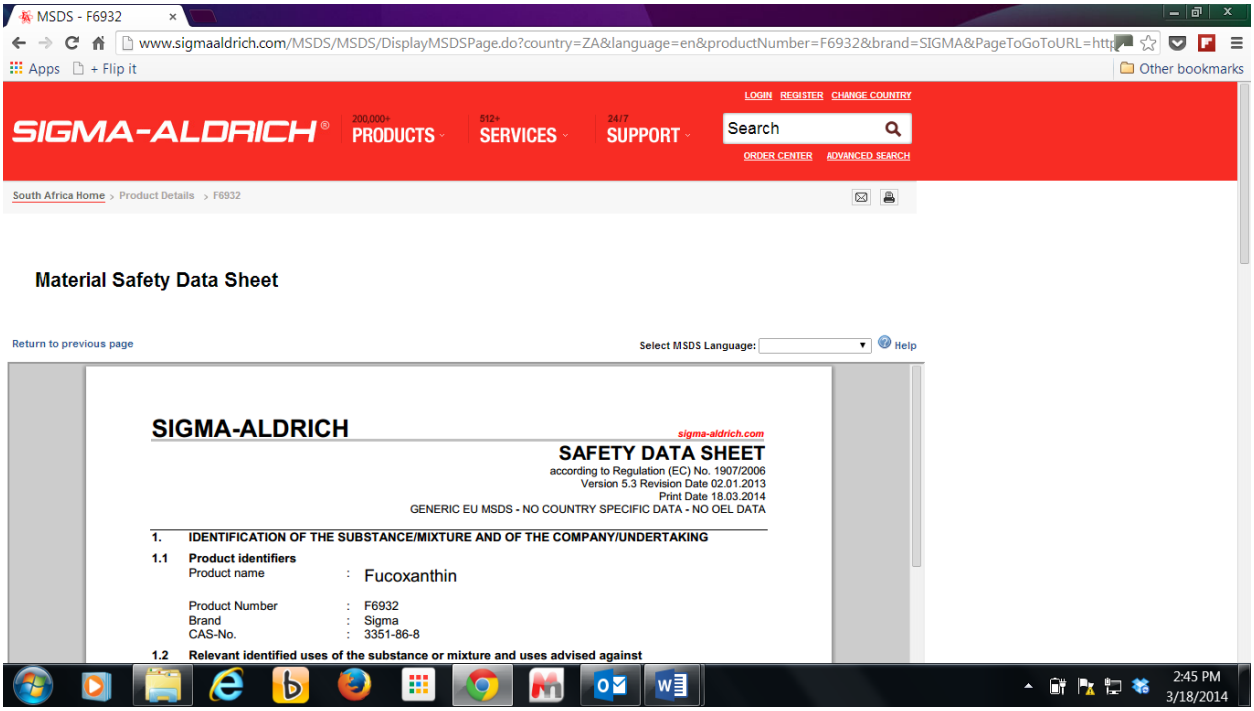


Figure A1.3: MSDS for fucoxanthin from Sigma-Aldrich®.

Physicochemical properties of fucoxanthin from Cayman-Chemicals®

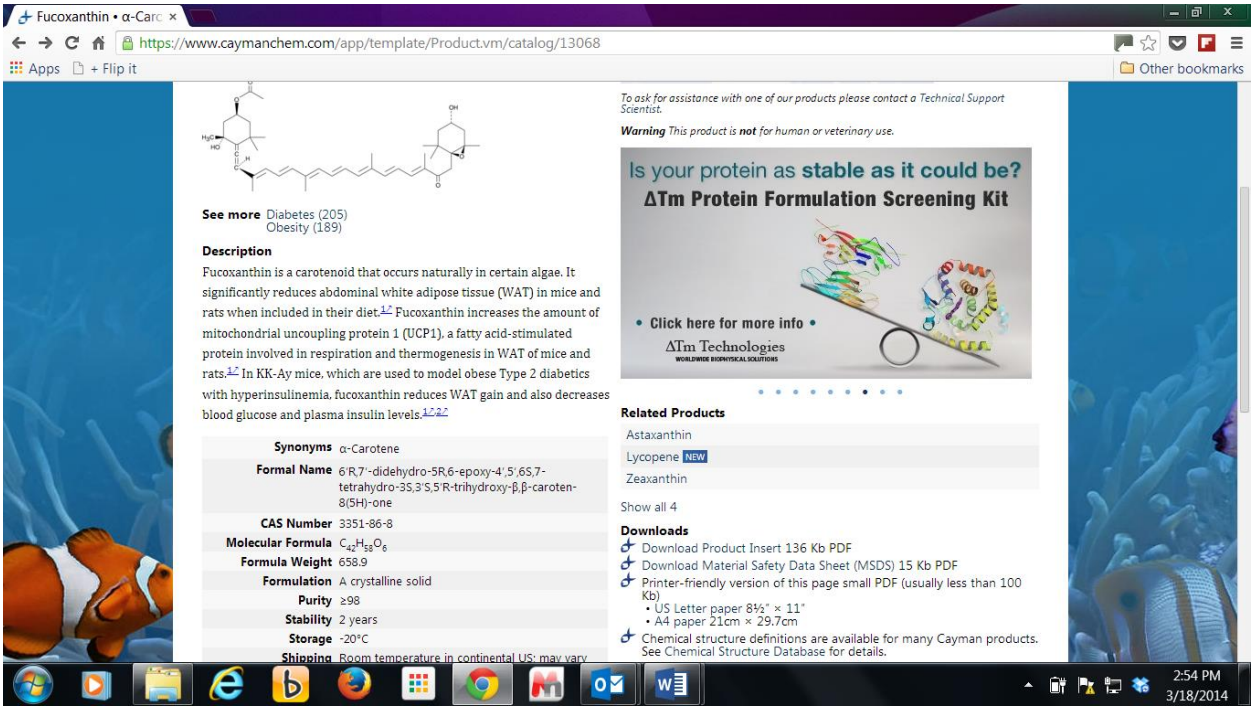


Figure A1.4: Physicochemical data for fucoxanthin from Cayman-chemicals®.

FucoThin™

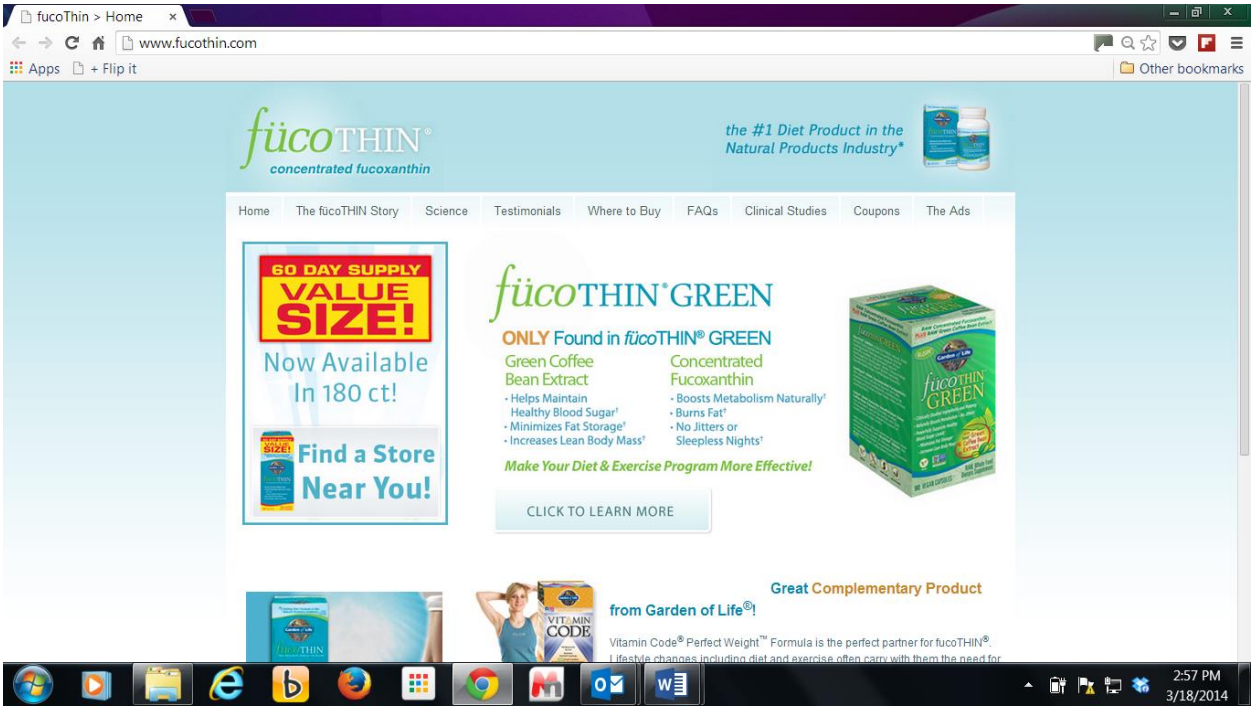


Figure A1.5: FucoThin website.

Brown seaweed plus™

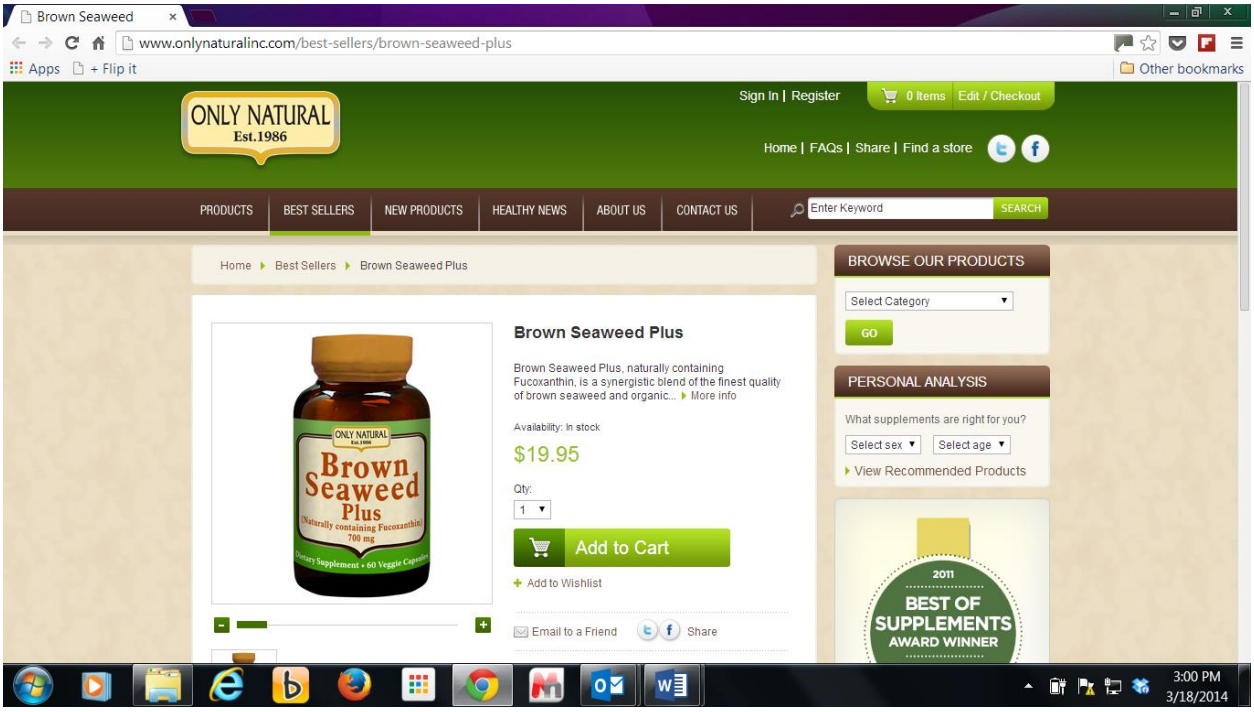


Figure A1.6: Brown seaweed plus™.

Fucoxanthin-Slim™

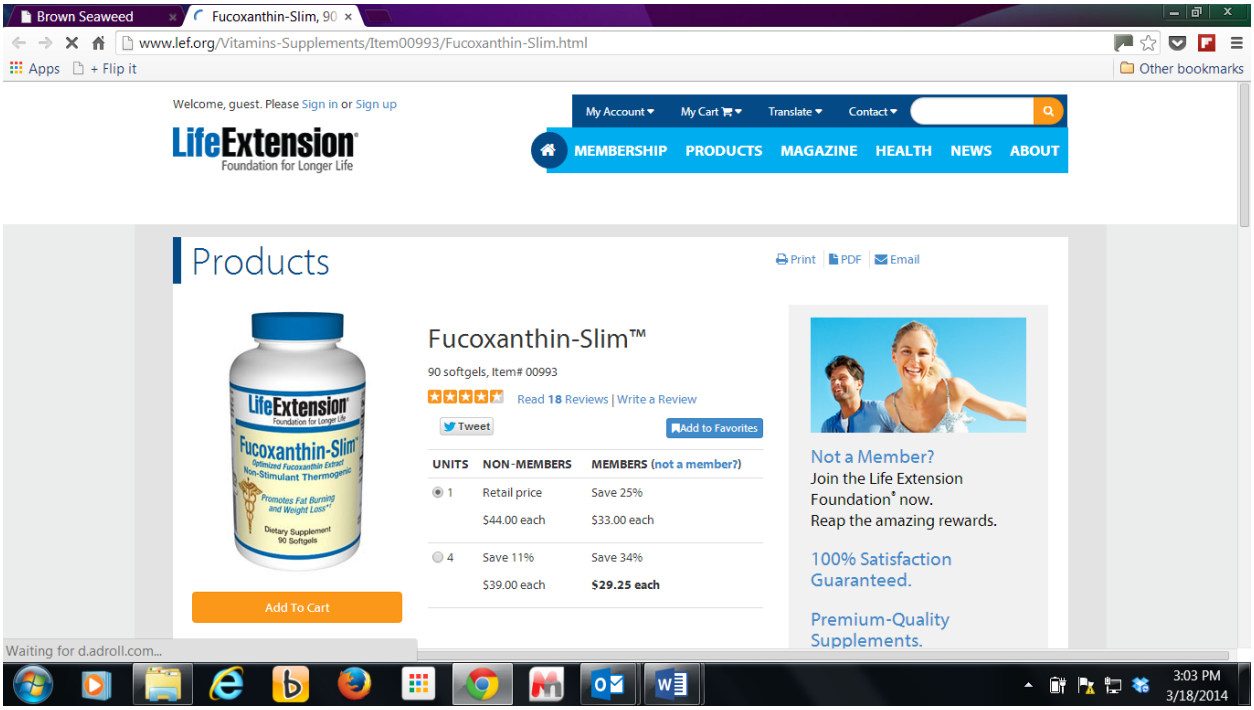


Figure A1.7: Fucoxanthin-Slim™ website.

Fucoxanthin Diet Patch

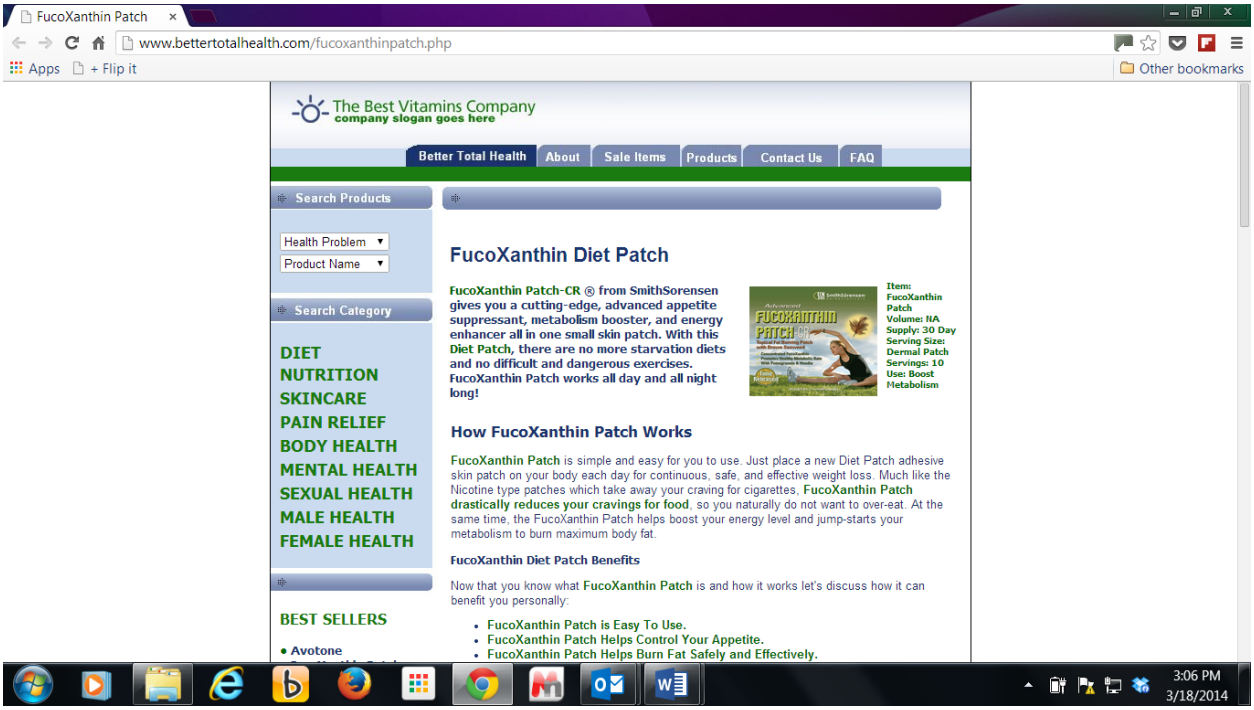


Figure A1.8: Fucoxanthin Diet Patch.

EMEA guidelines regarding herbal products.

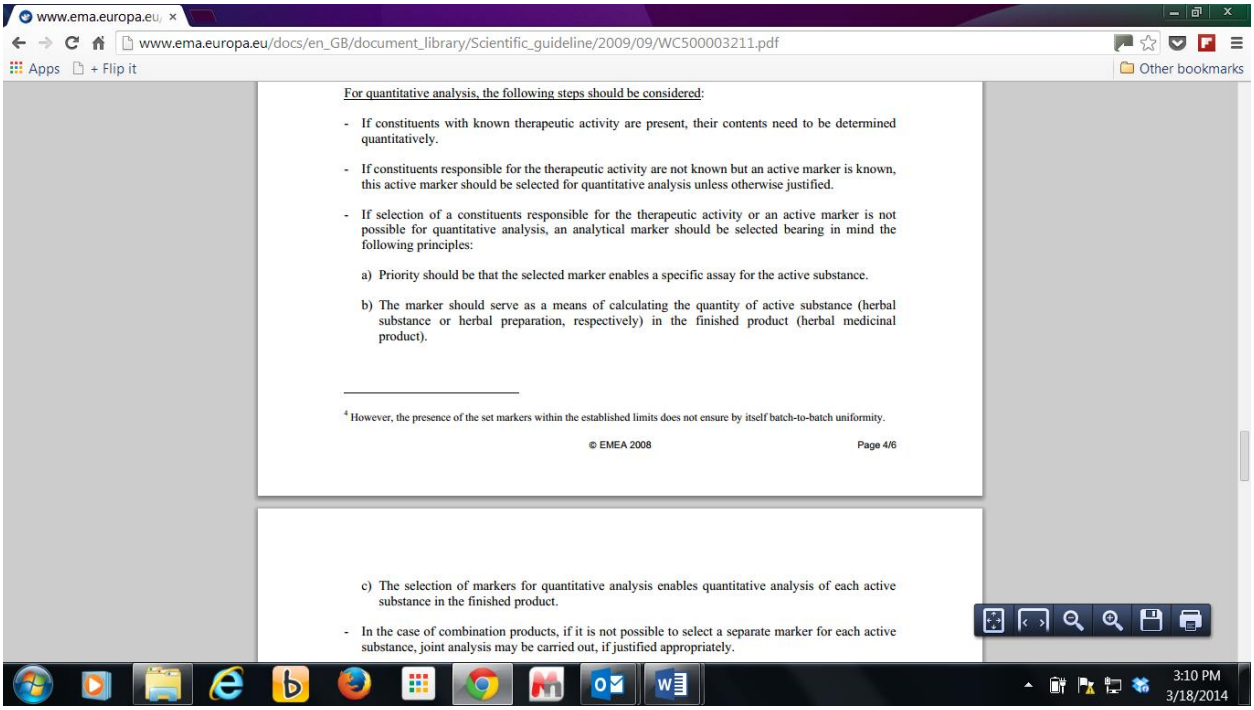


Figure A1.9: EMEA guidelines for herbal products.

WHO recommendation of HPLC in herbal extract analysis

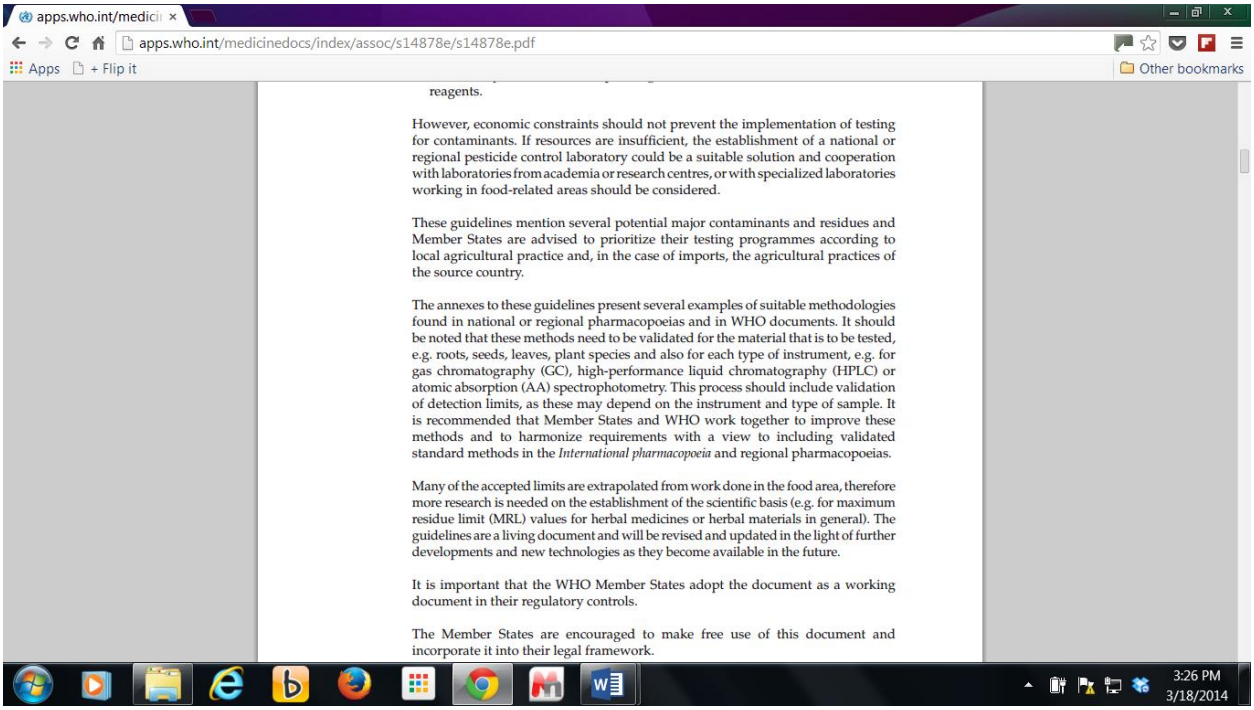


Figure A1.10: HPLC recommendation by the WHO for herbal extract analysis.

HPLC method development with Agilent technologies

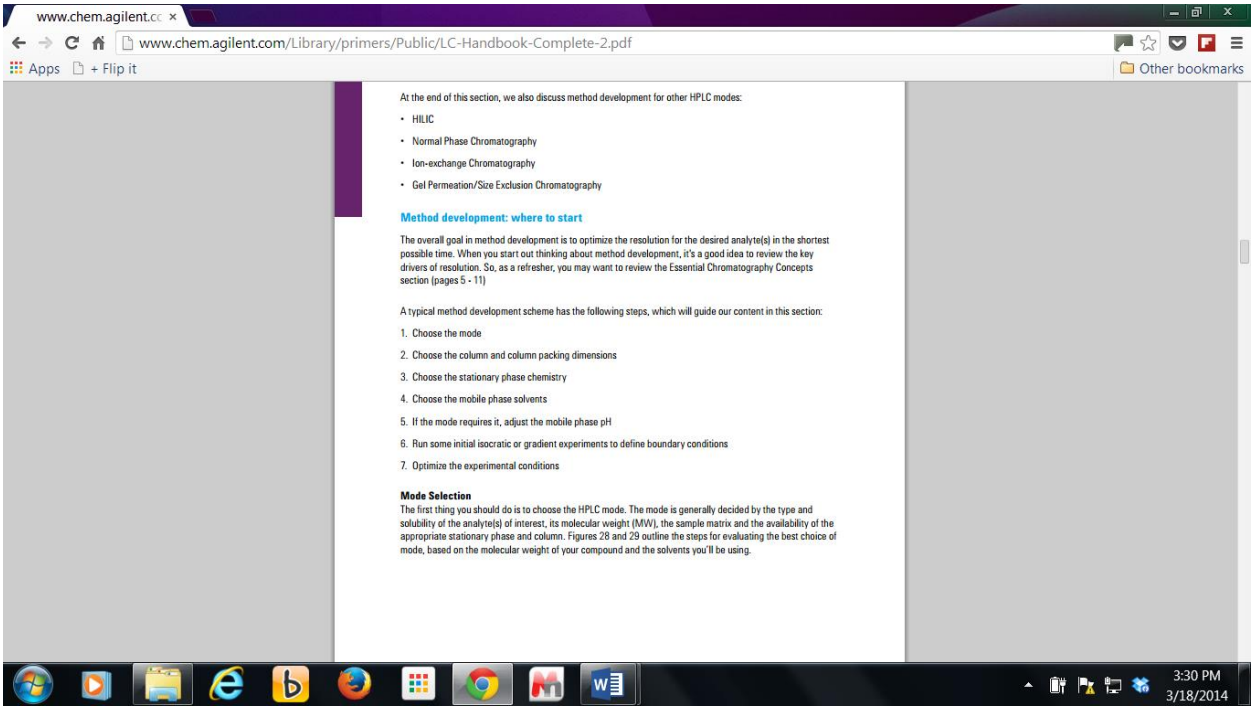


Figure A1.11: Guiding principles during HPLC method development by Agilent technologies

Analytical procedures: Method validation.

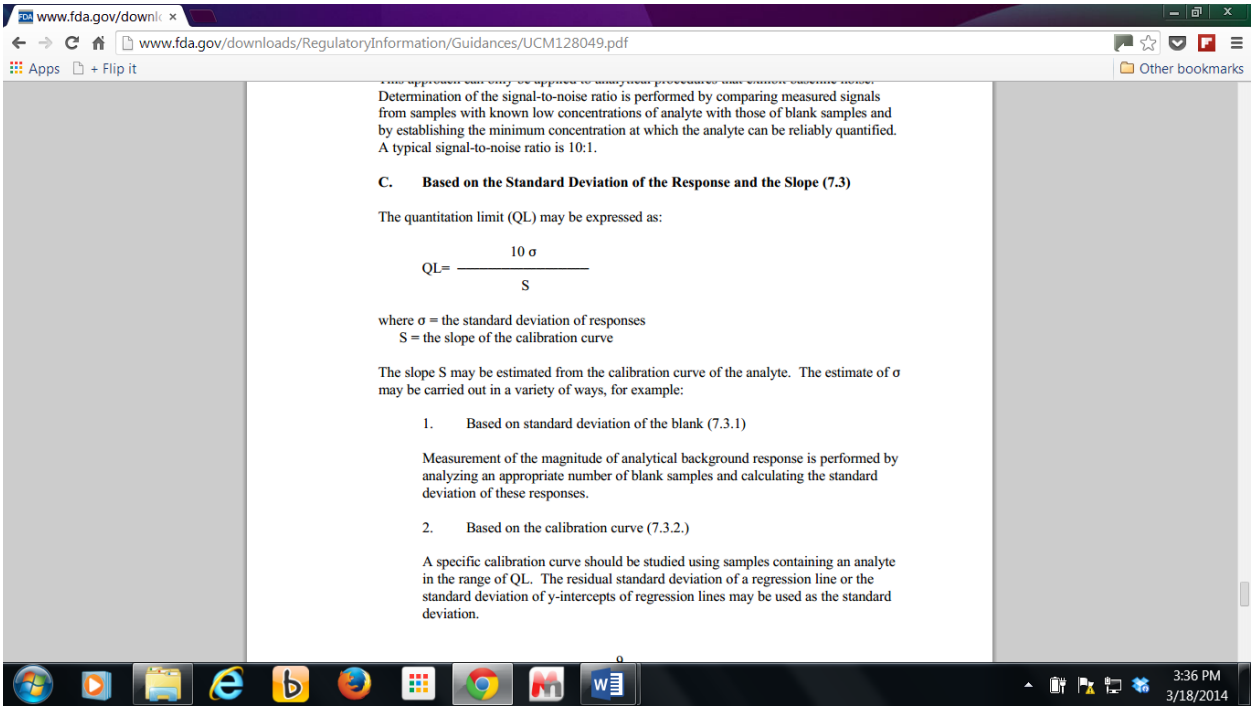


Figure A1.12: Quantitation limit equation.

Appendix 2.1

The synthesis of fucoxanthin (Adapted from Ito *et al*., 1994)

Formation of the C_{15}-8-oxo compound

(i) Synthesis of the C_{15}-α-acetylenic alcohol from (4R, 6R)-4-hydroxy-2, 2, 6-trimethyl-cyclohexanone:

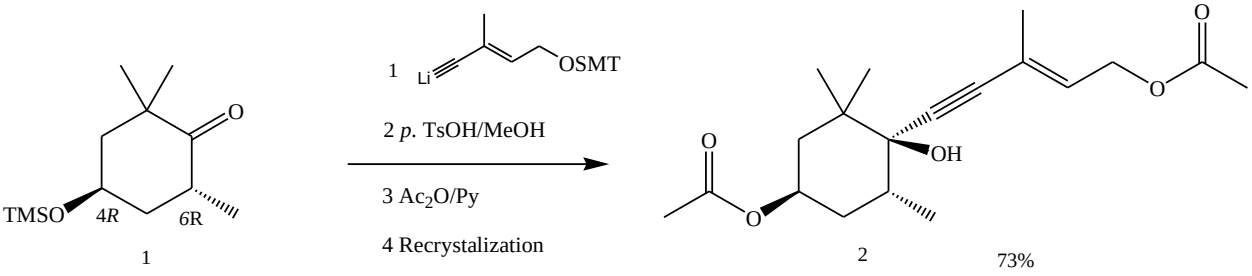


Figure A2.1: Synthesis of the C_{15}-α-acetylenic alcohol

(ii) Reaction of the α-acetylenic alcohol (2) with tris (triphenylsilyl) vanadate/triphenysilylsilanol in refluxing xylene containing small amounts of benzoic acid. This reaction converts the α-acetylenic alcohol to an α-β- and β-γ-unsaturated ketones. The α-β-unsaturated ketone (3) is converted to β-γ-unsaturated ketone (4) by treatment in iodine in refluxing heptane.

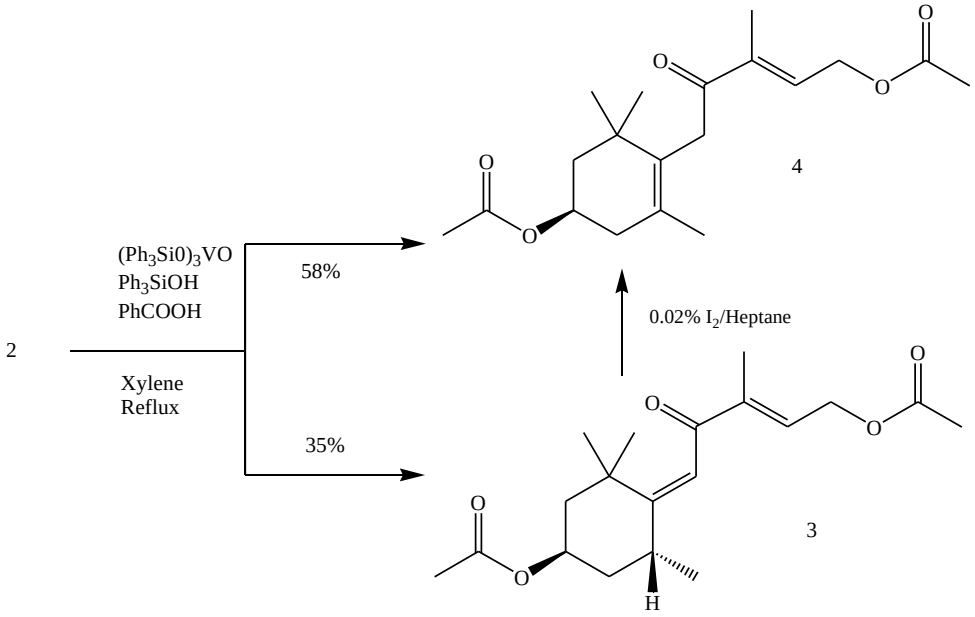


Figure A2.2: Synthesis of the α-β- and β-γ-unsaturated ketones

(iii) *Formation of the C_{15}-8-oxo-Wittig salt (A part in scheme 2.2). The β-γ-unsaturated ketone undergoes mild hydrolysis by potassium carbonate (K_2CO_3) followed by lithium chloride-methane sulfonyl chloride complex (LiCl-MsCl) then triphenylphosphine (PPh_3) refluxed in chloroform.*

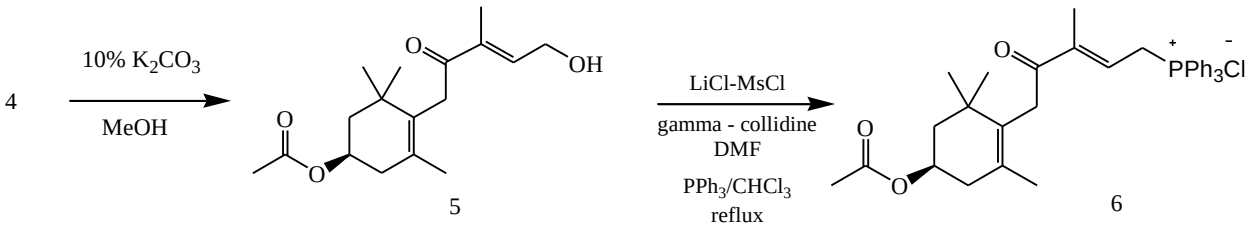


Figure A2.3: Synthesis of the C_{15}-8-oxo-Wittig salt

Formation of the C_{15}-allenic phosphonium chloride

(i) The C_{15}-α-acetylenic alcohol/acetylenic diacetate (13) was converted into an allenic di-hydroxy aldehyde before being acetylated and reduced by $NaBH_4$ to give the allenic alcohol. The alcohol was treated with (LiCl-MsCl) and reacted with PPh_3 to give the C_{15}-allenic phosphonium chloride.

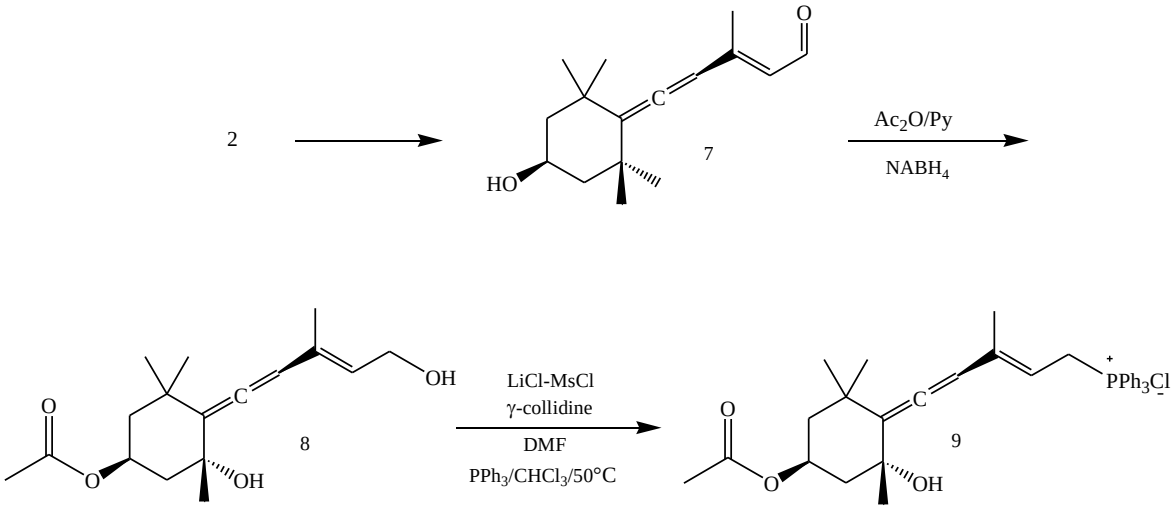


Figure A2.4: Synthesis of the C_{15}-allenic phosphonium chloride

Synthesis of optically active fucoxanthin

(i) The Wittig salt (A-part) was condensed with a C_{10} di-aldehyde in the presence of sodium methoxide (NaOMe) followed by the hydrolysis with 5% sodium hydroxide (NaOH). A mixture of (all-E)-8-oxo-apocarotenal and the 11Z-isomer was afforded. The latter was isomerized to the former on treatment with bis (acetonitrile) dichloropalladium (II) ($PdCl_2(CH_3CN)_2$)

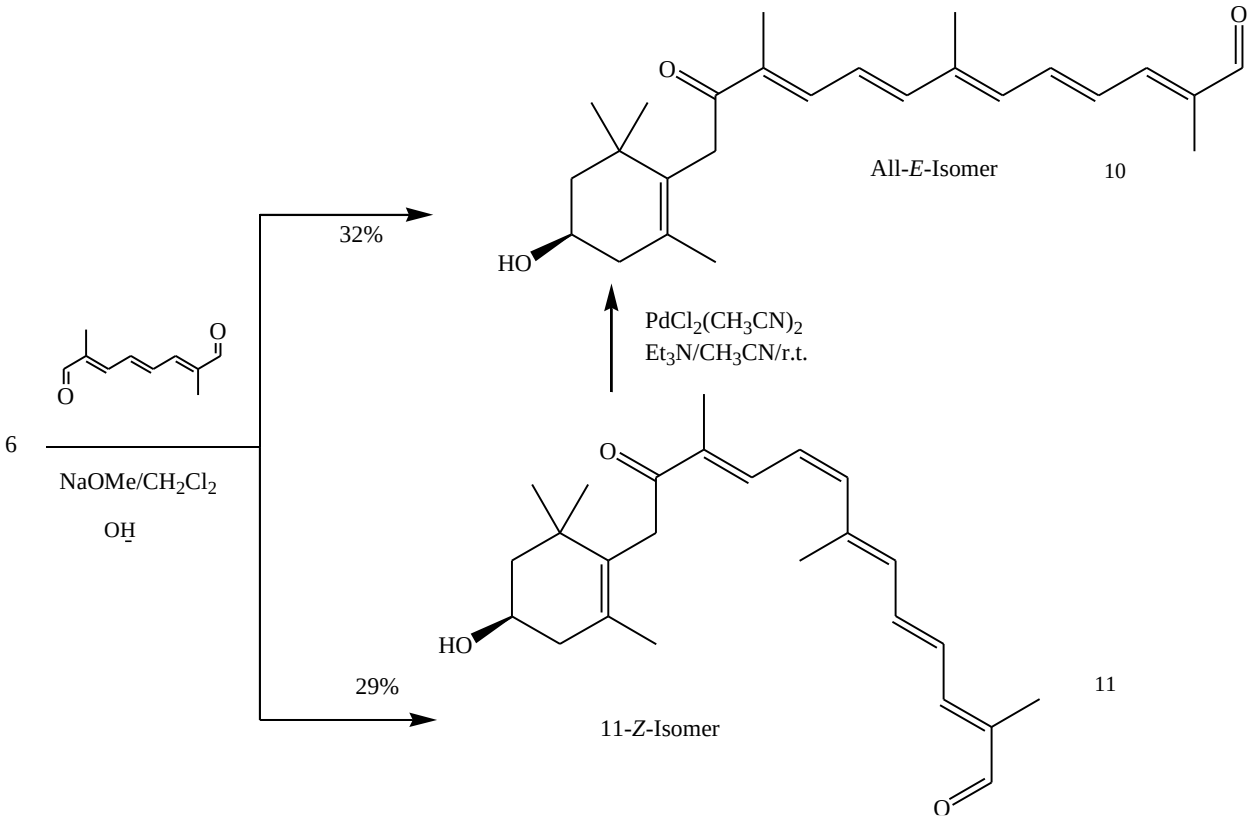


Figure A2.5: Synthesis of the *(all-E)*-8-oxo-apocarotenol

(ii) The hydroxyl on the (all-E)-8-oxo-apocarotenol (21) was protected by triethylsilyl trifluoromethanesulfonate/γ-collidine (TESOtf/γ-collidine) and then condensed with C_{15}-allenic phosphonium chloride (B-part) with sodium methoxide as a base. The condensed products where acetylate and desilylated with tetra-n-butylammonium fluoride $(n\text{-}bu)_4NF$ (TBAF) to provide (all-E)-fucoxanthin skeleton. The skeleton was then epoxidized with meta-chloroperoxybenzoic acid (MPCBA) to give a mixture of the syn-epoxide and anti-epoxide in the ratio of 7:2.

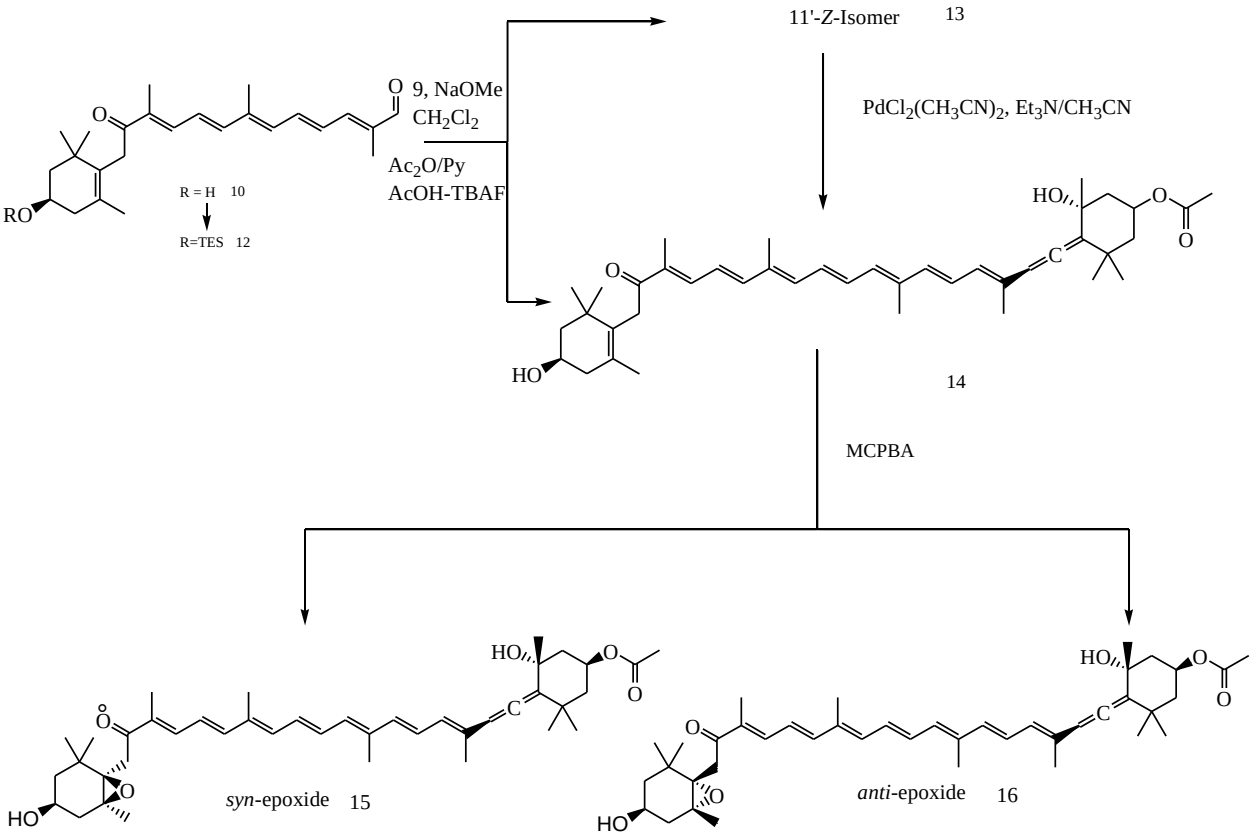


Figure A2.6: Synthesis of the *syn*- and *anti*-epoxide fucoxanthin

In summary, the synthesis of fucoxanthin follows the building principle of condensing C_{15} (A part) + C_{10} (di-aldehyde) + C_{15} (B Part) to give a C_{40} backbone (see section 2.4).

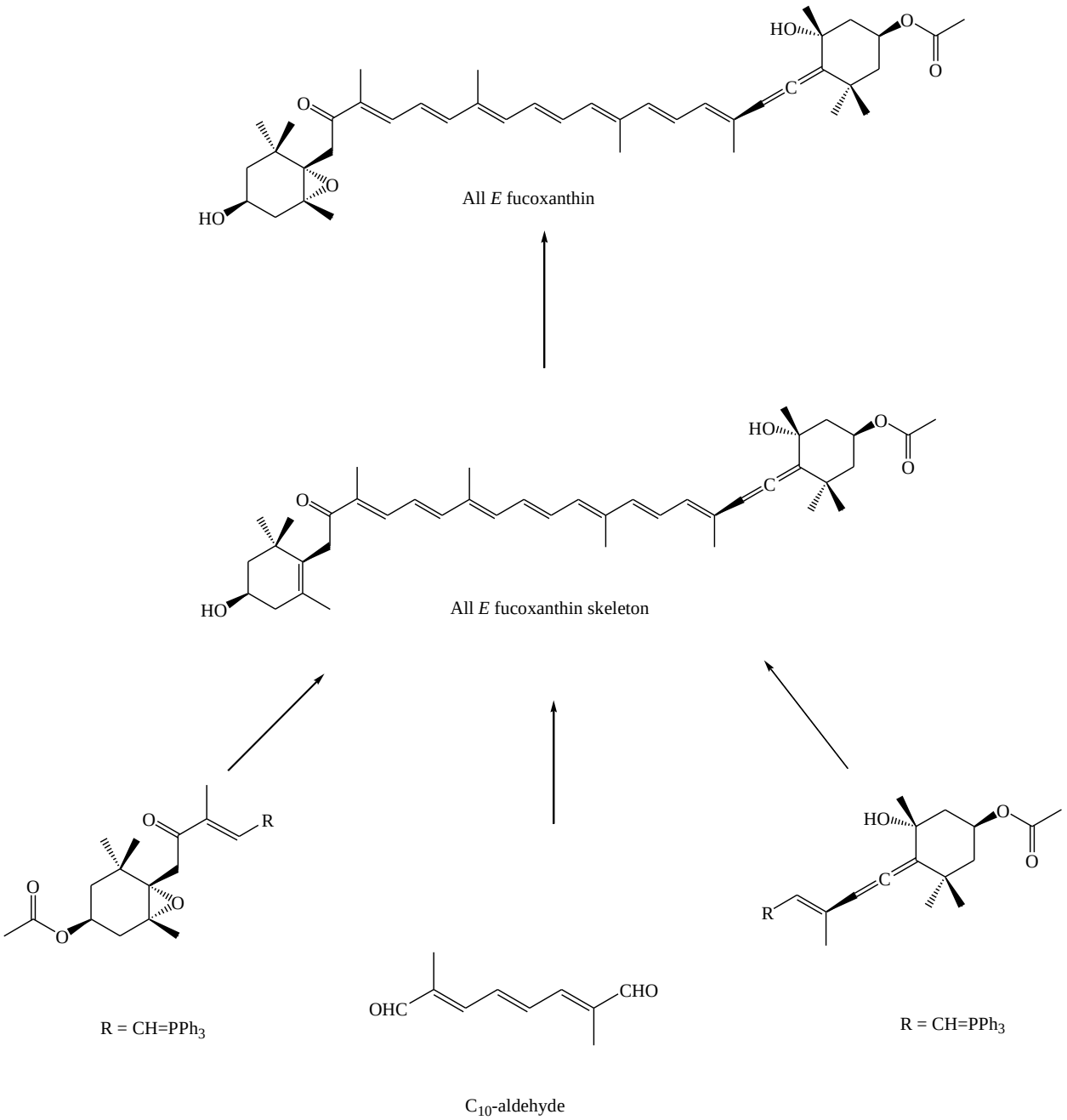


Figure A2.7: Summary of the synthesis of (all-E)-fucoxanthin

Reference

Ito, M., Yamano, Y., Sumiya, S. and Wada, A. Recent progress in carotenoid and retinoid synthesis. *Pure and Applied Chemistry* **1994**, 66: 939-939.

Appendix 3.1.

NMR spectra for fucoxanthin

Preliminary step-gradient fractions for *Sargassum incisifolium* (BM13-crude (16)

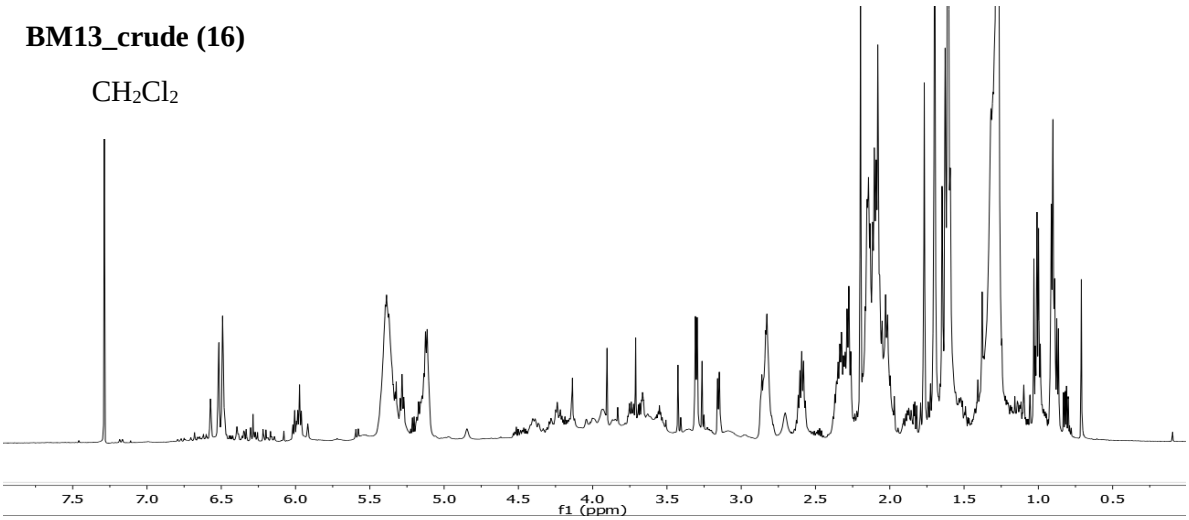


Figure A3.1: ^{1}H NMR for *S. incisifolium* crude extract ($CDCl_3$, 600 MHz)

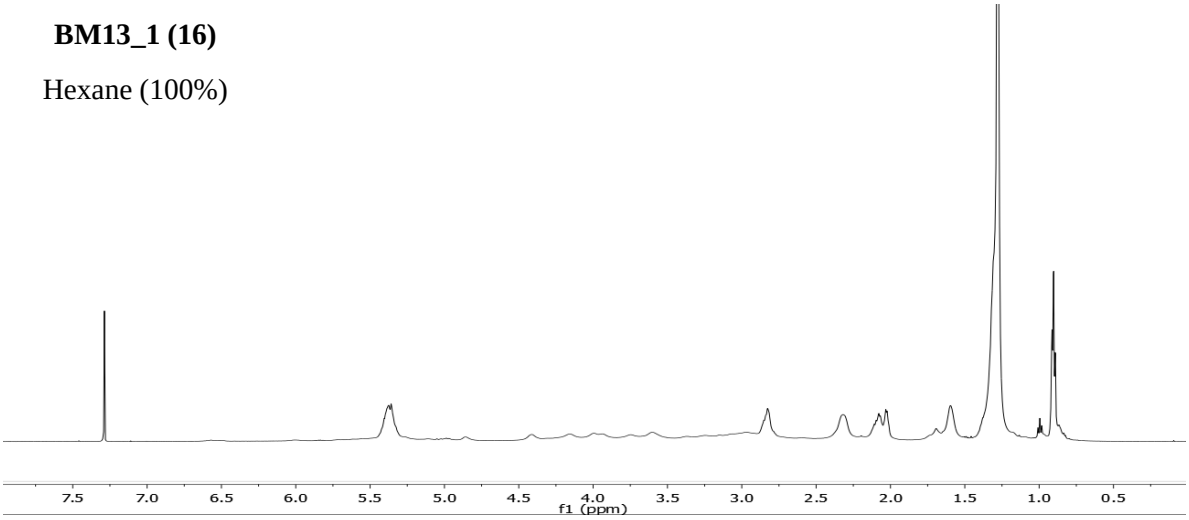


Figure A3.2: ^{1}H NMR for fraction 1 ($CDCl_3$, 600 MHz)

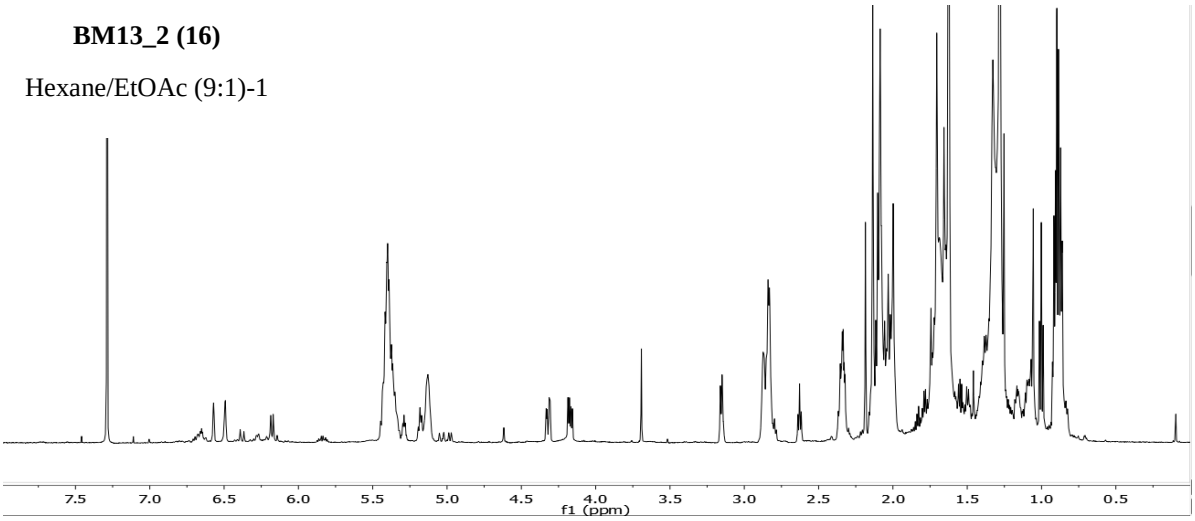


Figure A3.3: ^{1}H NMR for fraction 2 ($CDCl_3$, 600 MHz)

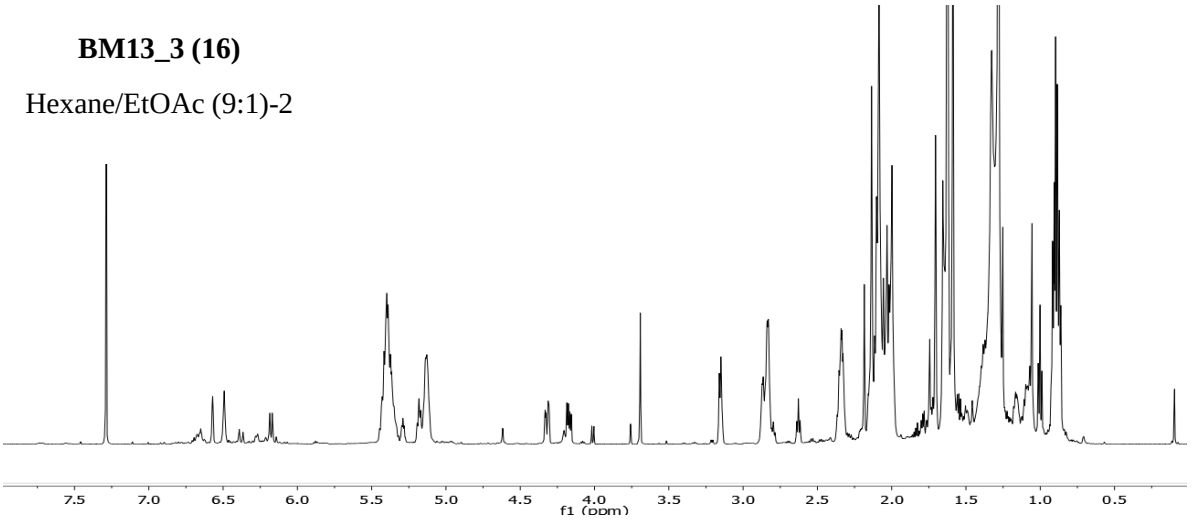


Figure A3.4: ^{1}H NMR for fraction 3 ($CDCl_3$, 600 MHz).

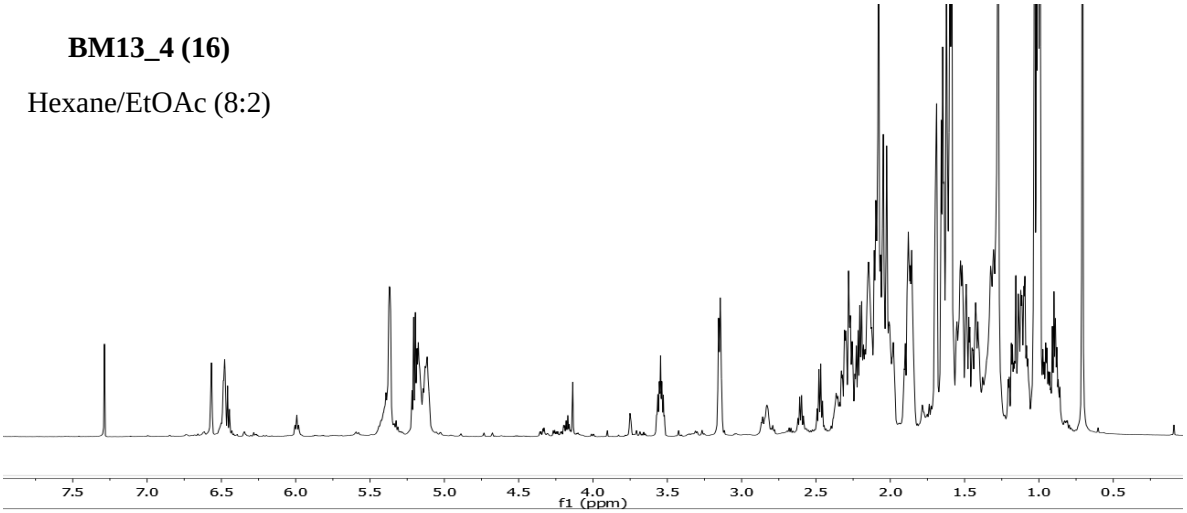


Figure A3.5: ^{1}H NMR for fraction 4 ($CDCl_3$, 600 MHz)

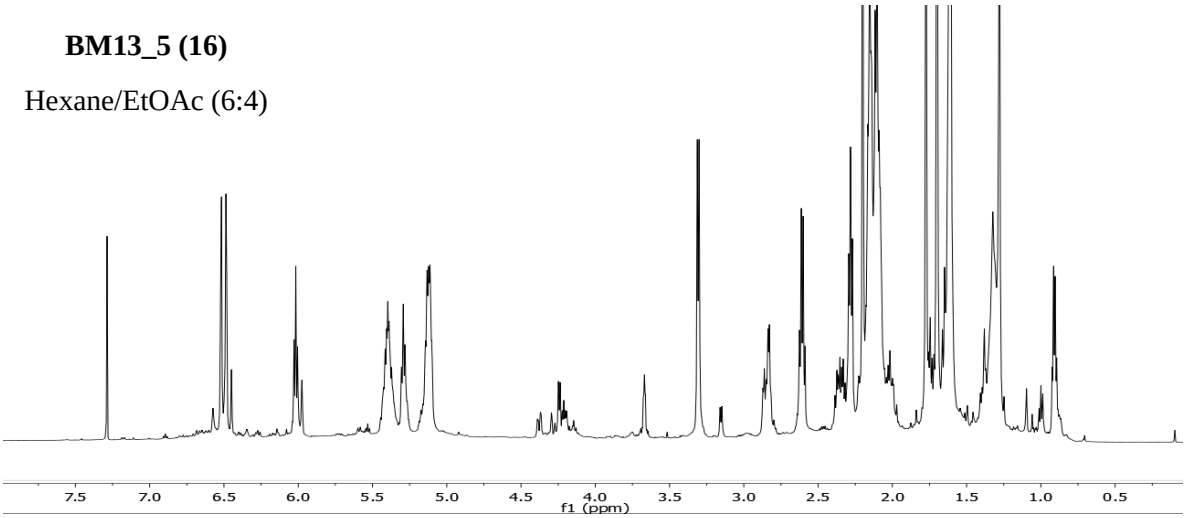


Figure A3.6: ^{1}H NMR for fraction 5 ($CDCl_3$, 600 MHz)

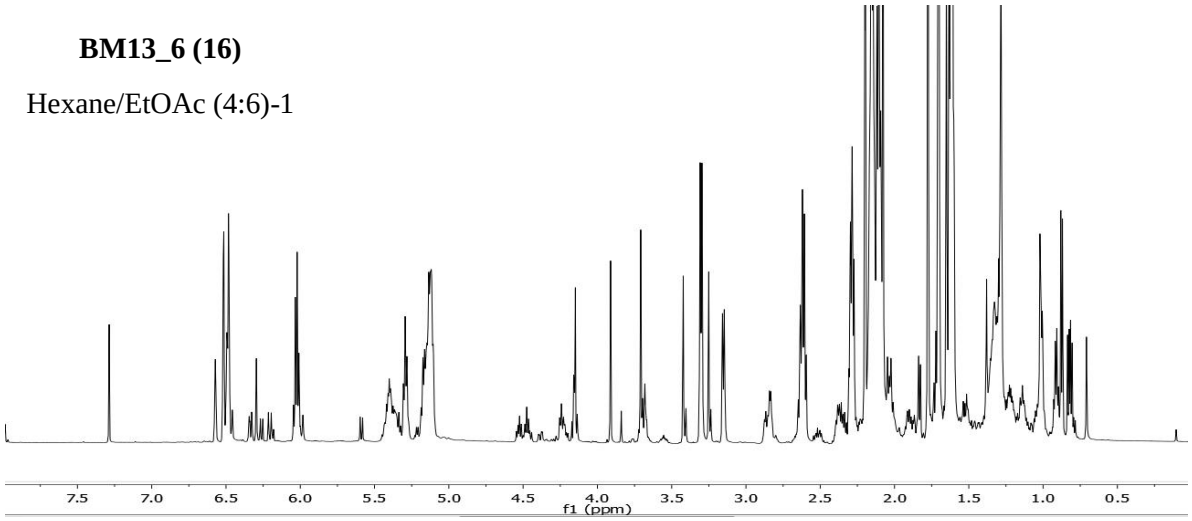


Figure A3.7: ^{1}H NMR for fraction 6 ($CDCl_3$, 600MHz)

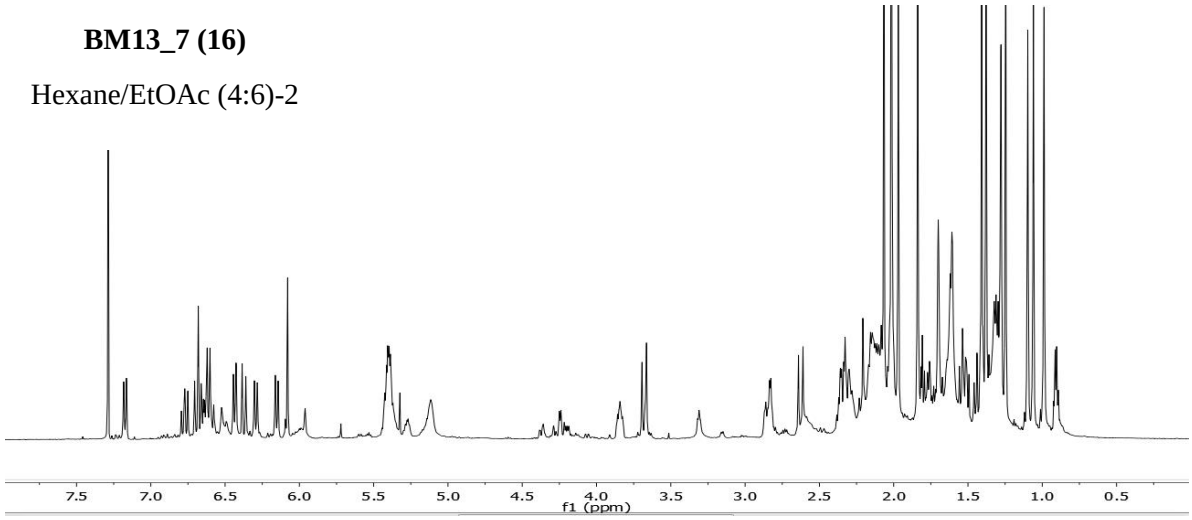


Figure A3.8: ^{1}H NMR for fraction 7, fucoxanthin rich ($CDCl_3$, 600 MHz).

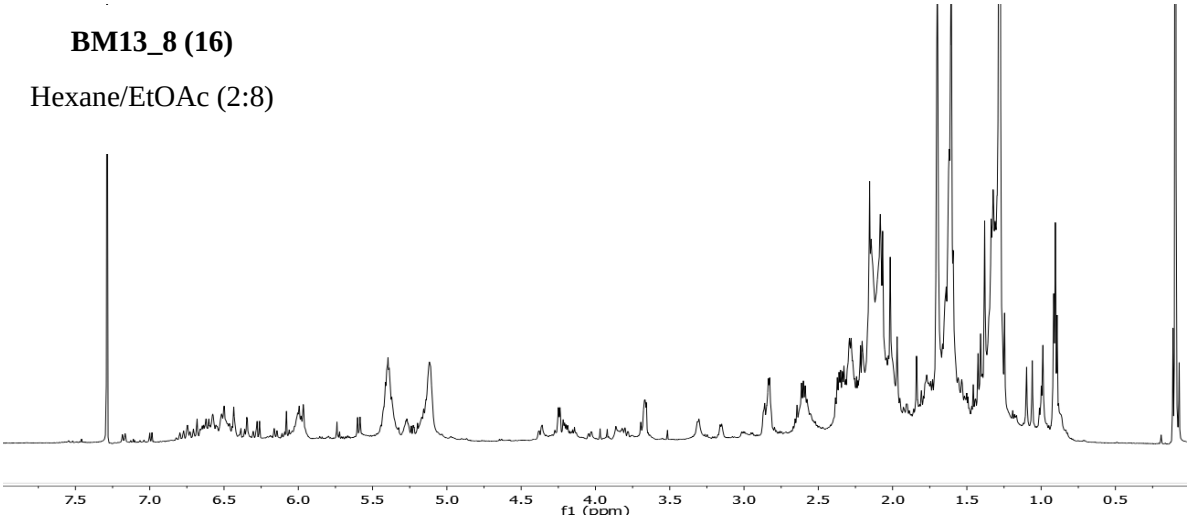


Figure A3.9: ^{1}H NMR for fraction 8 ($CDCl_3$, 600 MHz)

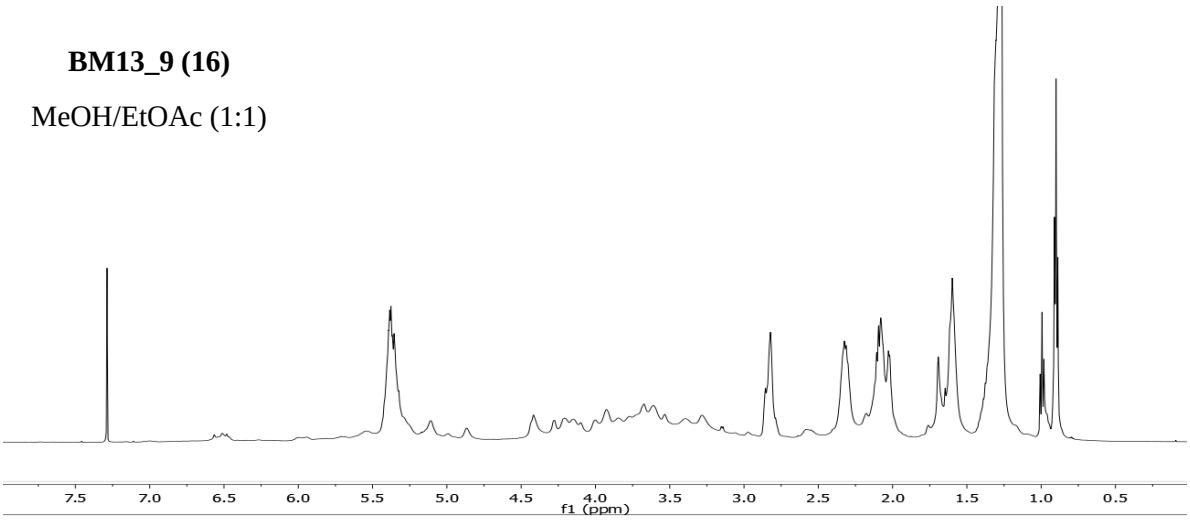


Figure A3.10: ^{1}H NMR for fraction 9 ($CDCl_3$, 600 MHz)

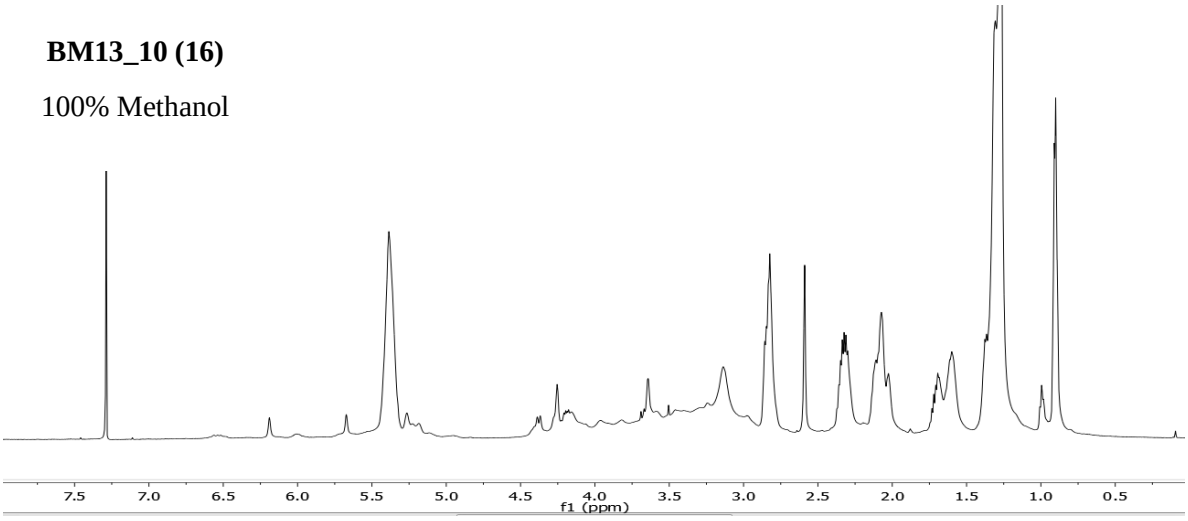


Figure A3.11: ^{1}H NMR for fraction 10 ($CDCl_3$, 600 MHz)

More step gradient fractions (used in the study) for *Sargassum incisifolium* BM13_ (22)

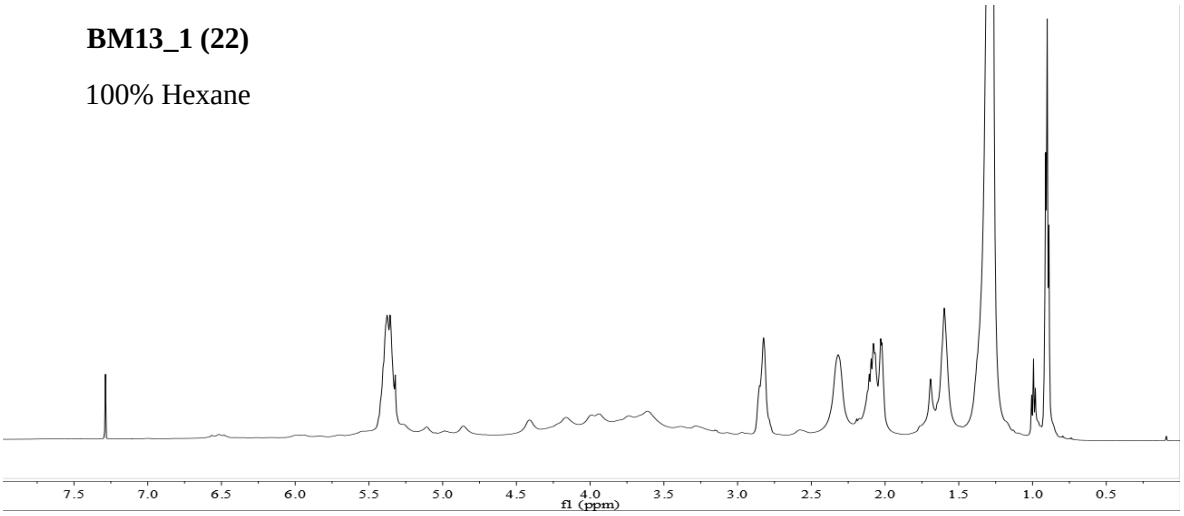


Figure A3.12: ^{1}H NMR for fraction 1 ($CDCl_3$, 600 MHz)

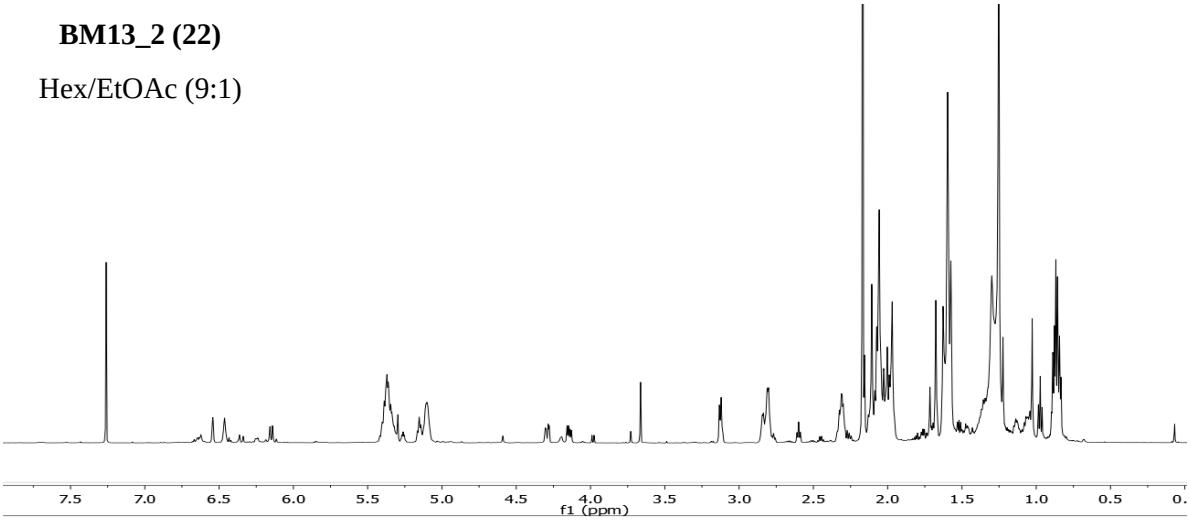


Figure A3.13: ^{1}H NMR for fraction 2 ($CDCl_3$, 600 MHz)

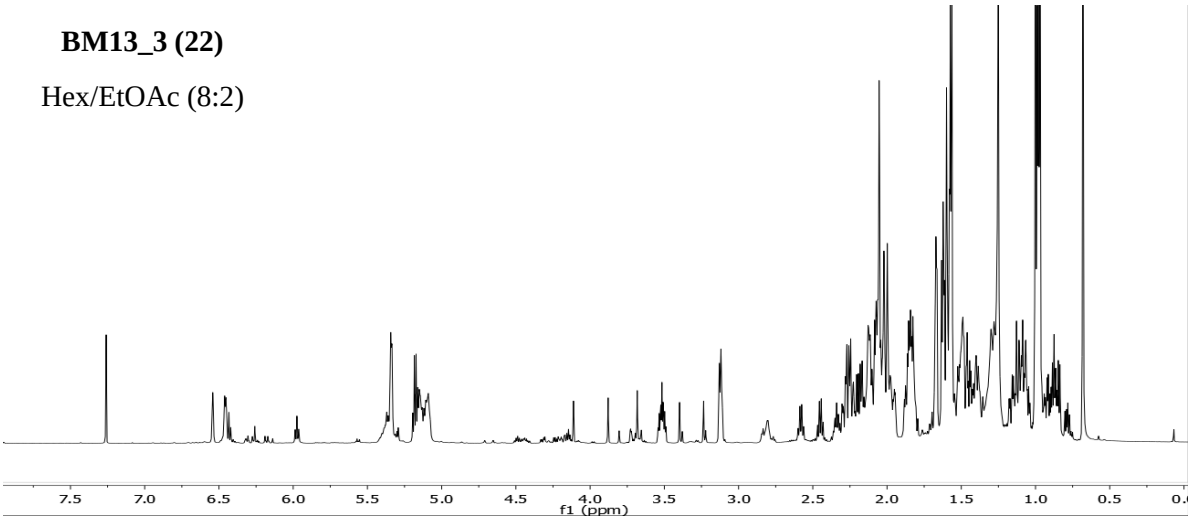


Figure A3.14: ^{1}H NMR for fraction 3 ($CDCl_3$, 600 MHz)

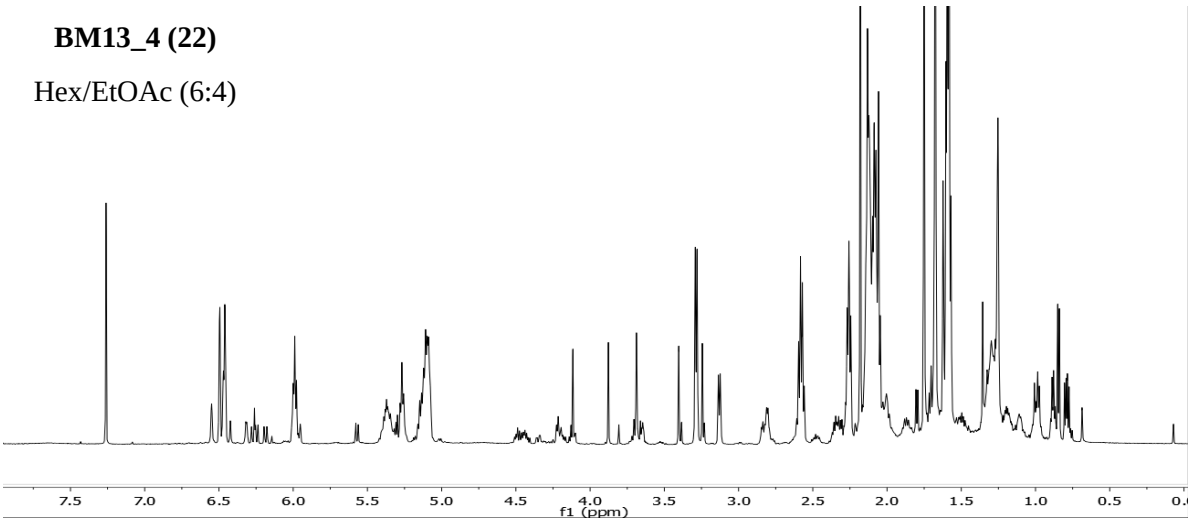


Figure A3.15: ^{1}H NMR for fraction 4 ($CDCl_3$, 600 MHz)

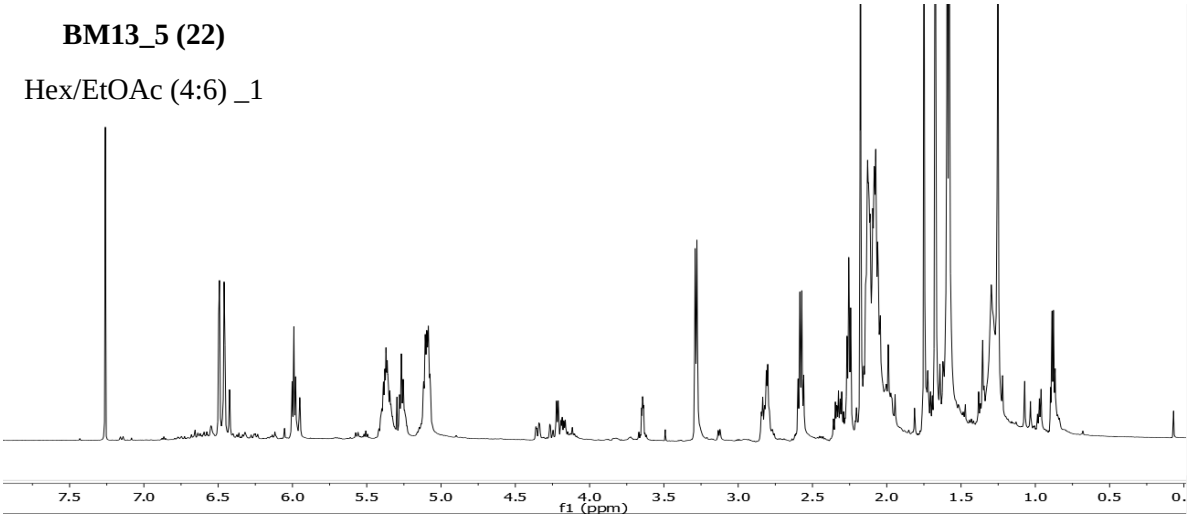


Figure A3.16: ^{1}H NMR for fraction 5 ($CDCl_3$, 600 MHz)

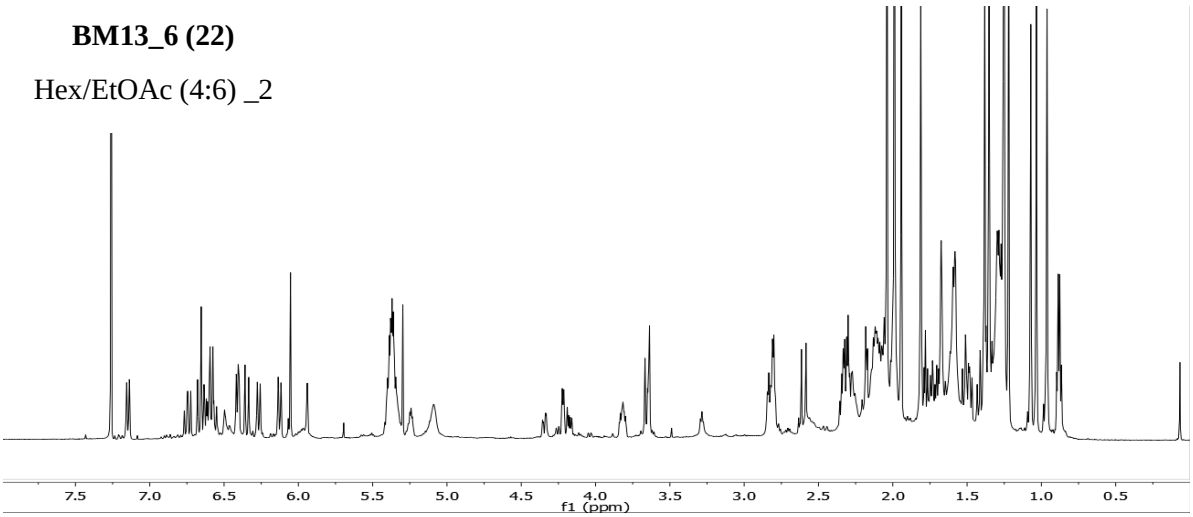


Figure A3.17: ^{1}H NMR for fraction 6, fucoxanthin-rich ($CDCl_3$, 600 MHz)

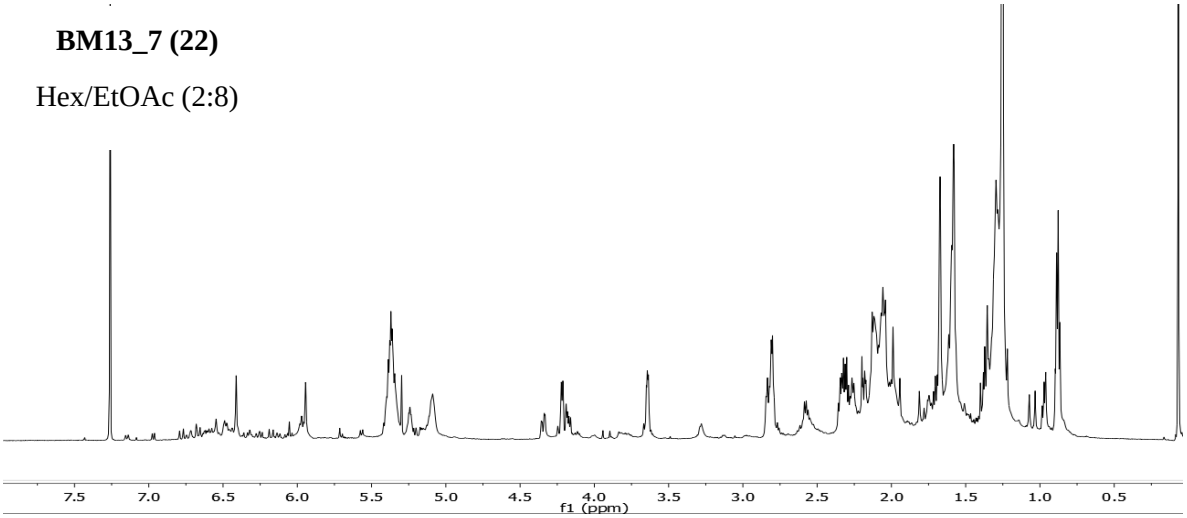


Figure A3.18: ^{1}H NMR for fraction 7 ($CDCl_3$, 600 MHz)

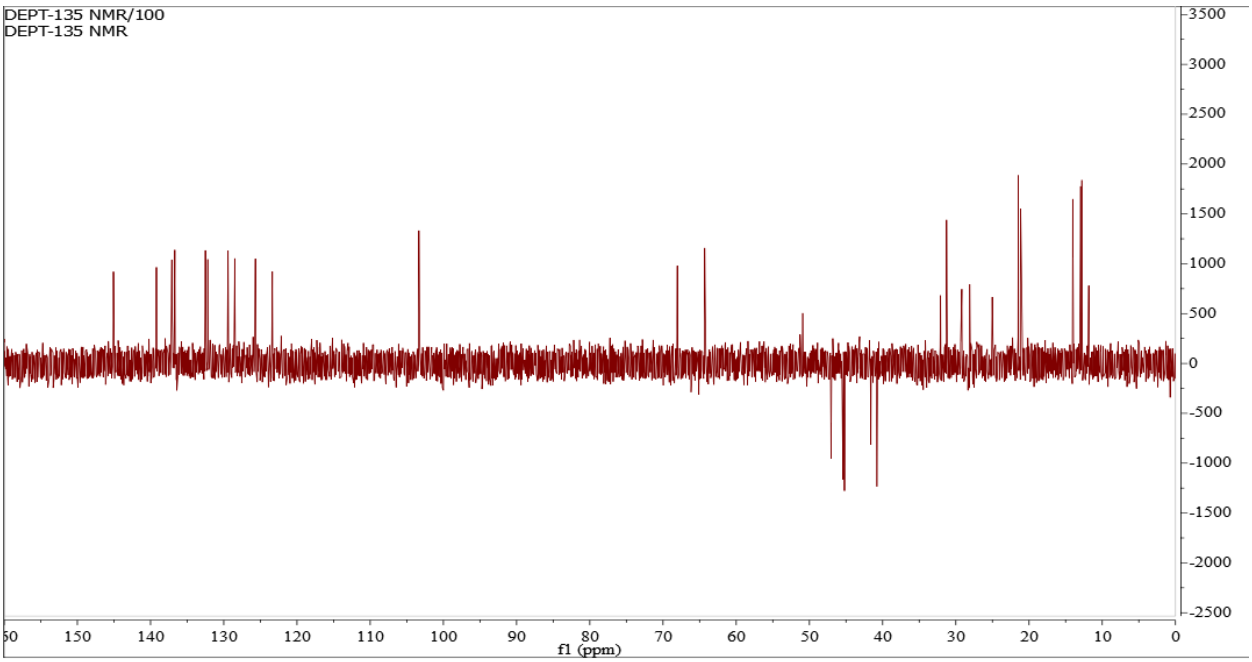


Figure A3.19: DEPT-135 spectrum for **BM13-73b** ($CDCl_3$).

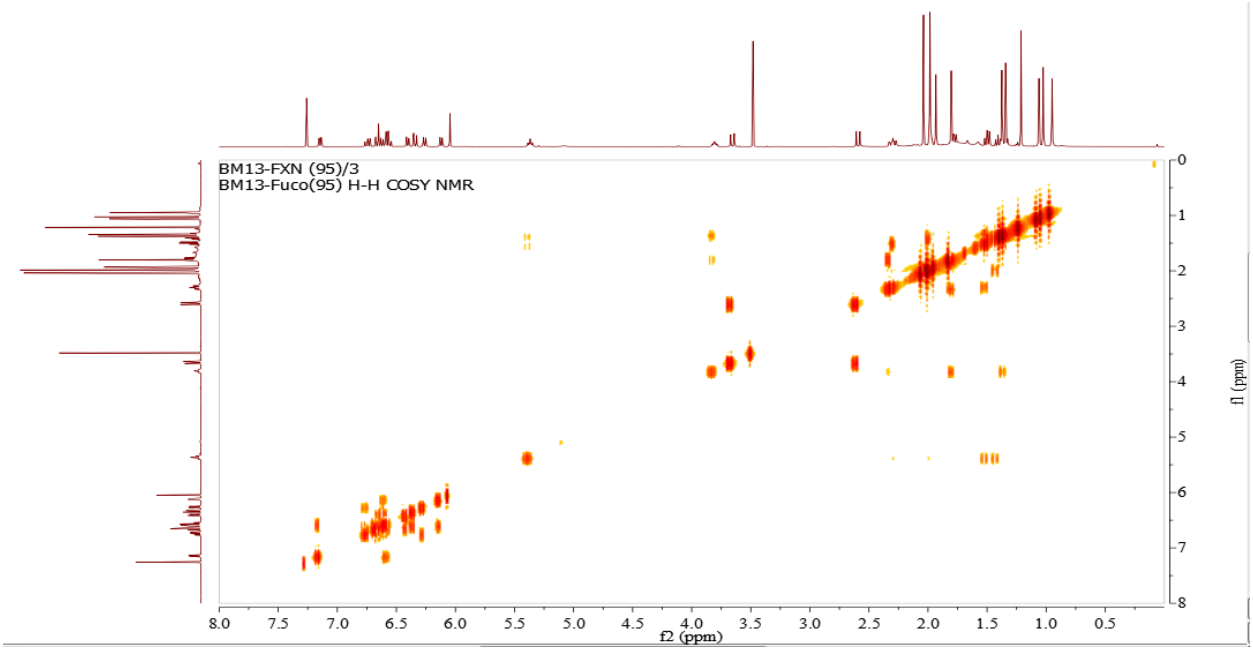


Figure A3.20: COSY spectrum for **BM13-73b** ($CDCl_3$).

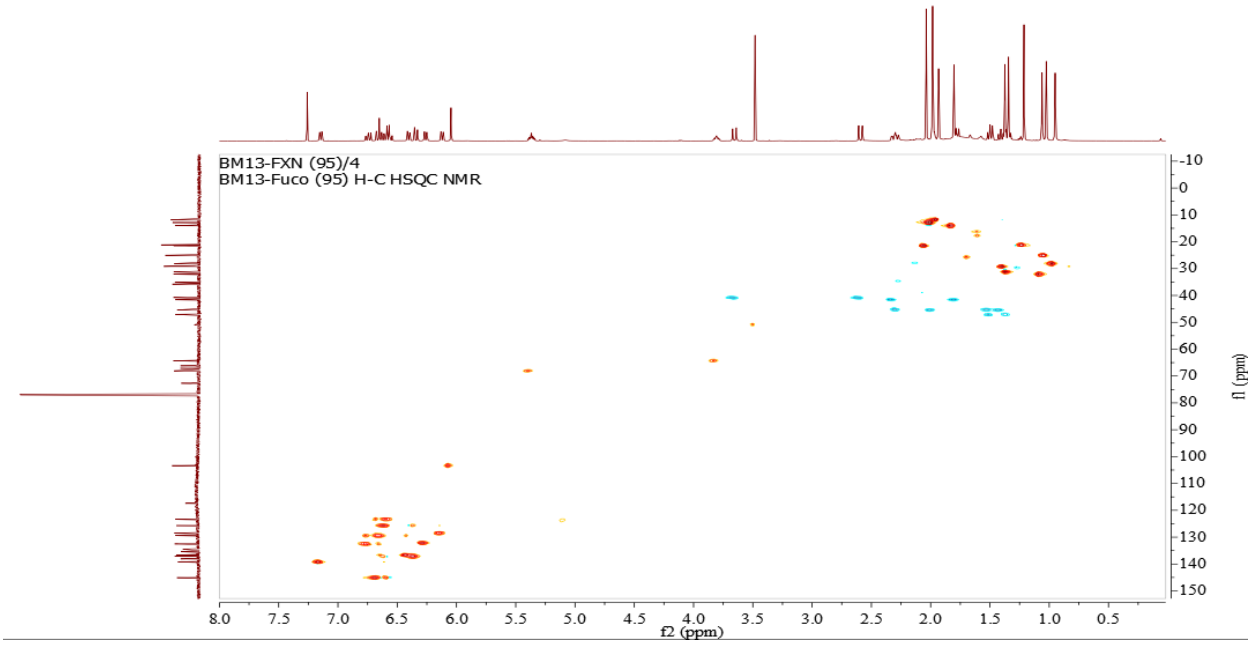


Figure A3.21: HSQC spectrum for **BM13-73b** ($CDCl_3$).

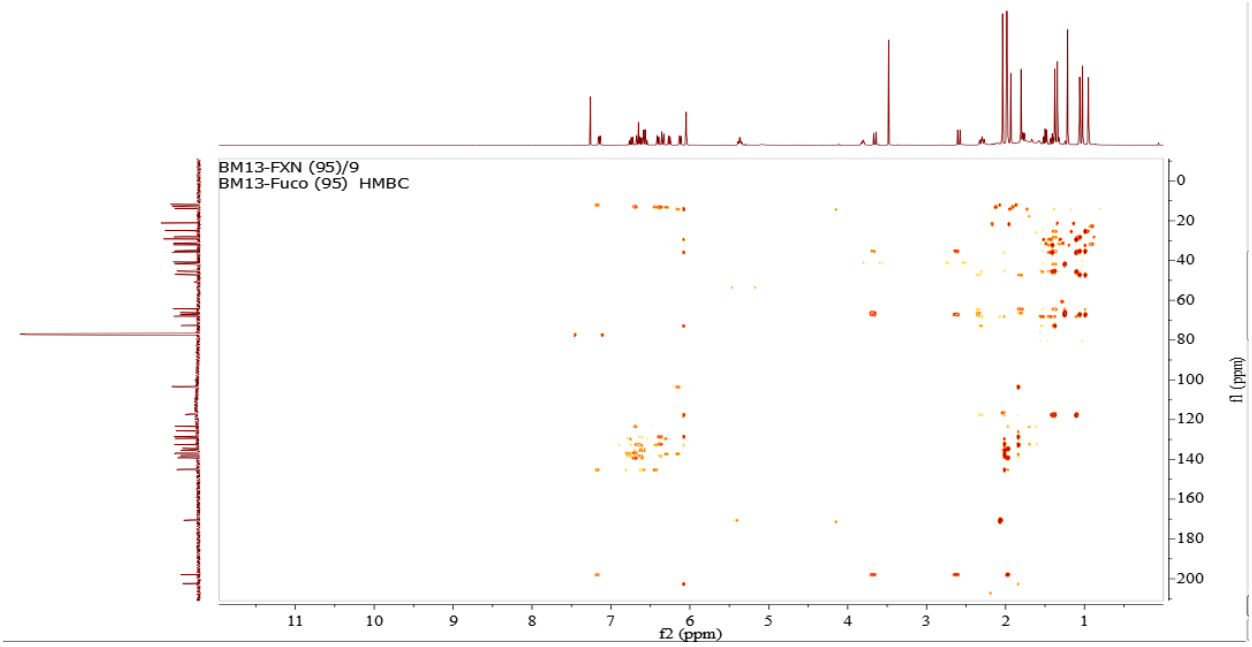


Figure A3.22: HMBC spectrum for **BM13-73b** ($CDCl_3$).

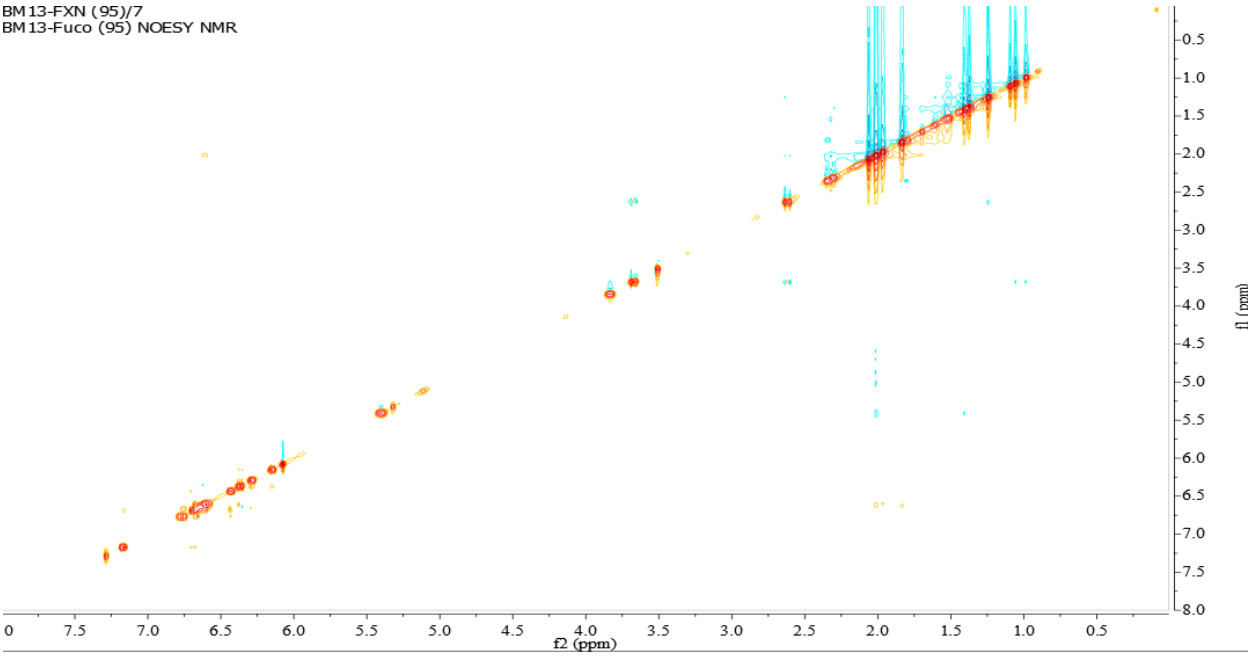


Figure A3.23: NOESY spectrum for **BM13-73b** (CDCl3).

Appendix 3.2

UV/Vis and FT-IR spectra for fucoxanthin

(Adapted from Rajauria and Abu-Ghannam, 2013).

Rajauria and Abu-Ghannam (2013) compared standard fucoxanthin to the sample they had isolated from *Himanthalia elongata*. Our sample **BM13-73b**, agreed with their findings (*see* section 3.2.2.2).

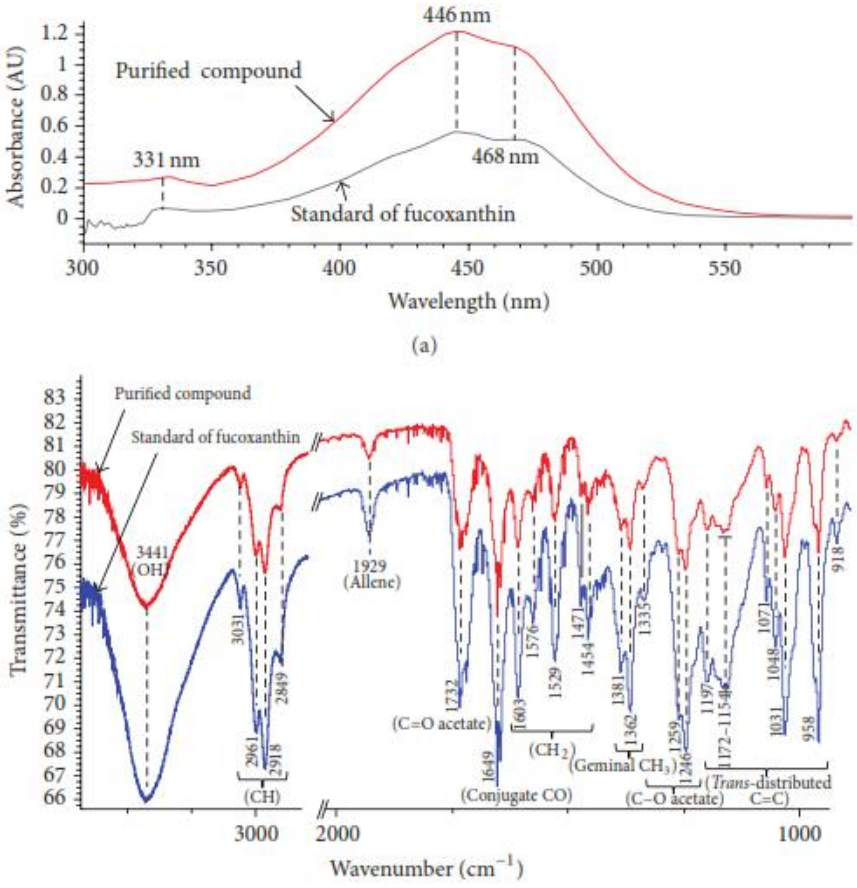


Figure A3.24: UV-Vis and FT-IR comparisons of fucoxanthin isolated from *Himanthalia elongata* and a standard. Adapted from Rajauria and Abu-Ghannam (2013).

Reference

Rajauria, G. and Abu-Ghannam, N. Isolation and partial characterization of bioactive fucoxanthin from *Himanthalia elongata* brown seaweed: A TLC-based approach. *International Journal of Analytical Chemistry* **2013**, 2013: 1-6.

Appendix 4.1

Method Validation

Some of the studies *i.e.* linearity, accuracy, LOD and LOQ tests were carried out over three days in order to fulfill the requirements of the ICH guidelines. All these studies require nine determinations therefore each concentration was replicated 3 x 3 times. Day 1 results are shown in the main manuscript (*see* section 4.2.2.1, 4.2.2.2 and 4.2.2.3). Data for Days 2 and 3 and shown here.

Linearity Studies

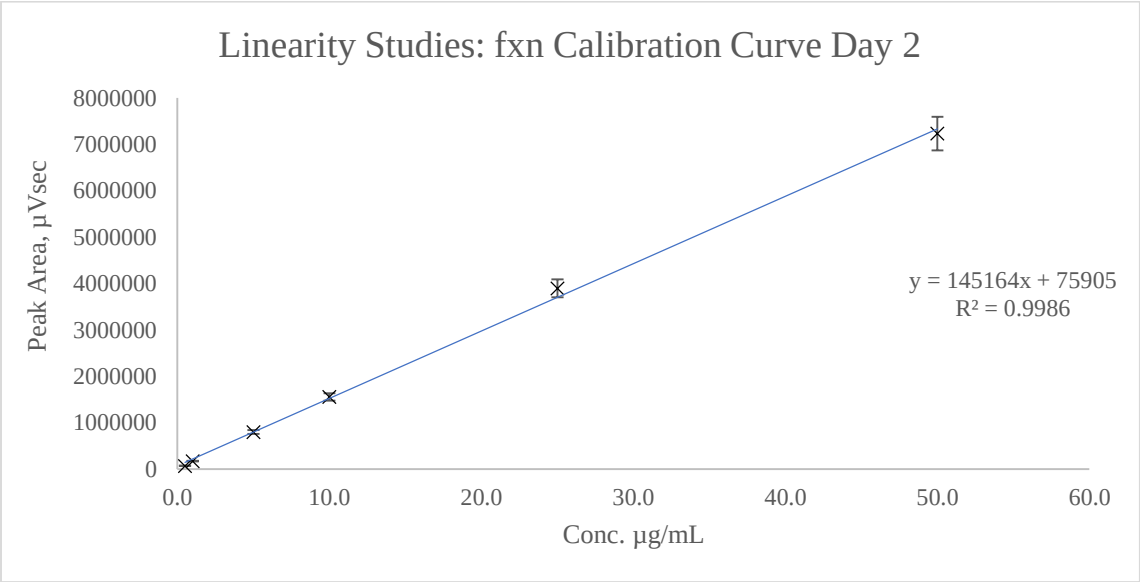


Figure A4.1: Linearity studies for **BM13-73b** – Day 2.

Table A4.1: Linearity studies for **BM13-73b** – Day 2.

Day 2						
	Conc. (µg/mL)					
Conc. (µg/mL)	**1**	**2**	**3**	**Mean**	**SD (n =3)**	**RSD**
50.0	50.4	48.7	48.8	**49.3**	0.95	1.93
25.0	27.2	25.6	26.1	**26.3**	0.81	3.07
10.0	10.1	10.2	10.3	**10.2**	0.12	1.17
5.0	5.3	4.8	5.0	**5.0**	0.24	4.83
1.0	0.9	1.0	0.9	**0.9**	0.02	2.45
0.5	0	0	0	**0**	0	0.00

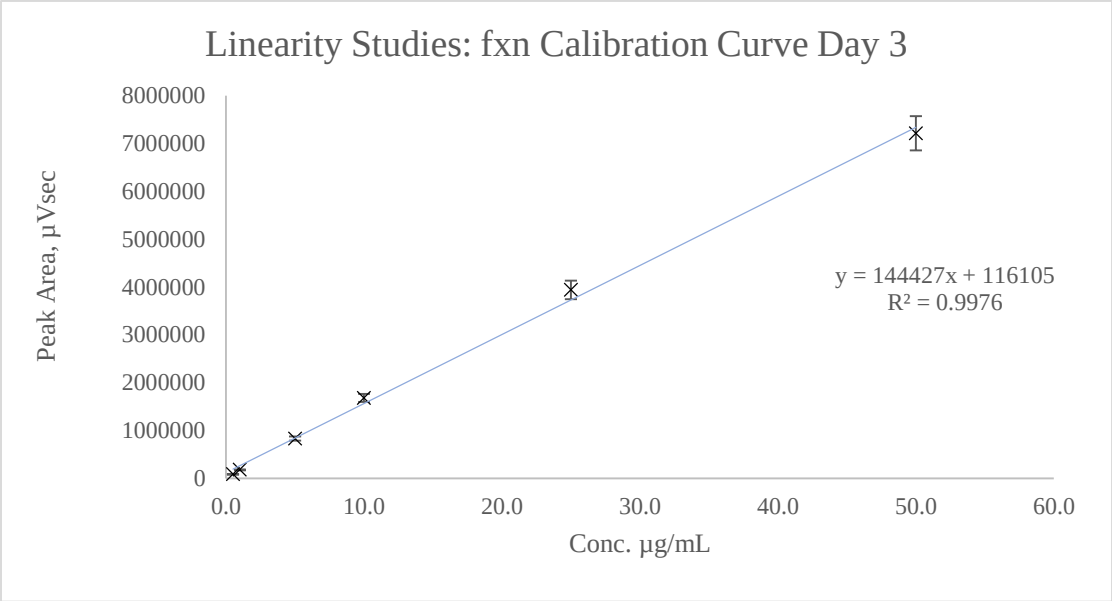


Figure A4.2: Linearity studies for **BM13-73b** – Day 3.

Table A4.2: Linearity studies for **BM13-73b** – Day 3

Day 3

Conc. (µg/mL)	Conc. (µg/mL) 1	2	3	Mean	SD	RSD
50.0	51.3	51.0	50.6	**51.0**	0.34	0.66
25.0	25.1	25.1	25.7	**25.3**	0.33	1.31
10.0	10.5	10.6	10.7	**10.6**	0.12	1.09
5.0	5.1	5.0	4.9	**5.0**	0.09	1.71
1.0	1.0	1.0	1.0	**1.0**	0.03	2.73
0.5	0.5	0.5	0.53	**0.5**	0.00	0.42

Accuracy studies

Table A4.3: Accuracy studies for **BM13-73b** – Day 2

Accuracy Day 2

Conc. µg/mL	Conc. (µg/mL) 1	2	3	Mean	Std. dev.	RSD	Accuracy %
50 (49.3)	48.8	48.6	48.1	**48.5**	**0.37**	**0.76**	**98.3**
25 (26.3)	26.5	27.0	26.3	**26.6**	**0.37**	**1.39**	**101.1**
5 (5.0)	5.0	4.9	4.9	**4.9**	**0.02**	**0.47**	**98.9**

Table A4.4: Accuracy studies for **BM13-73b** – Day 3

Accuracy Day 3

Conc. µg/mL	Conc. (µg/mL) 1	2	3	Mean	Std. dev.	RSD	Accuracy %
50 (51.0)	50.2	49.9	50.1	**50.1**	**0.14**	**0.28**	**98.2**
25 (25.3)	25.2	25.5	24.8	**25.2**	**0.37**	**1.45**	**99.4**
5 (5.0)	5.0	4.9	4.9	**4.9**	**0.05**	**1.09**	**100.9**

LOD and LOQ studies

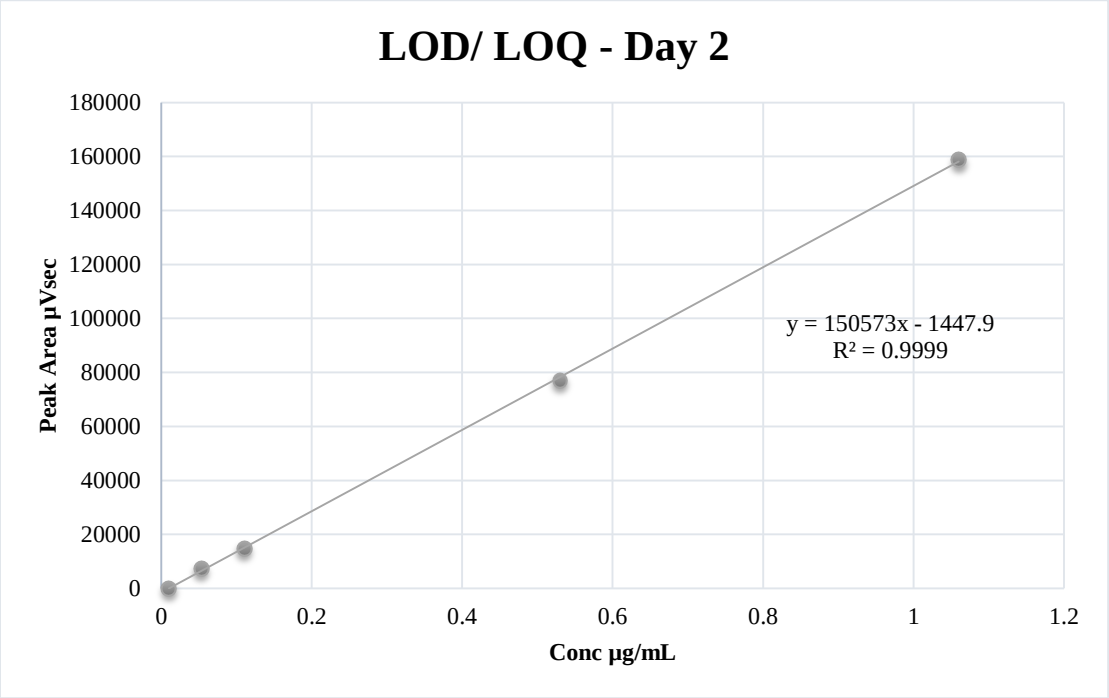


Figure A4.3: LOD and LOQ determinations for **BM13-73b** using response, standard deviation and slope – Day 2.

Table A4.5: LOD and LOQ determinations for **BM13-73b** – Day 2

LOD/LOQ determinations Day 2

Conc. µg/mL	Conc. (µg/mL)			Mean	Std. dev.	RSD
1.06	1.06	1.06	1.06	**1.06**	**0.002**	**0.17**
0.53	0.52	0.52	0.53	**0.52**	**0.004**	**0.75**
0.11	0.11	0.11	0.11	**0.11**	**0.002**	**1.95**
0.05	0.05	0.05	0.05	**0.05**	**0.001**	**1.13**
0.01	0.01	0.01	0.01	**0.01**	**0.000**	**0.00**

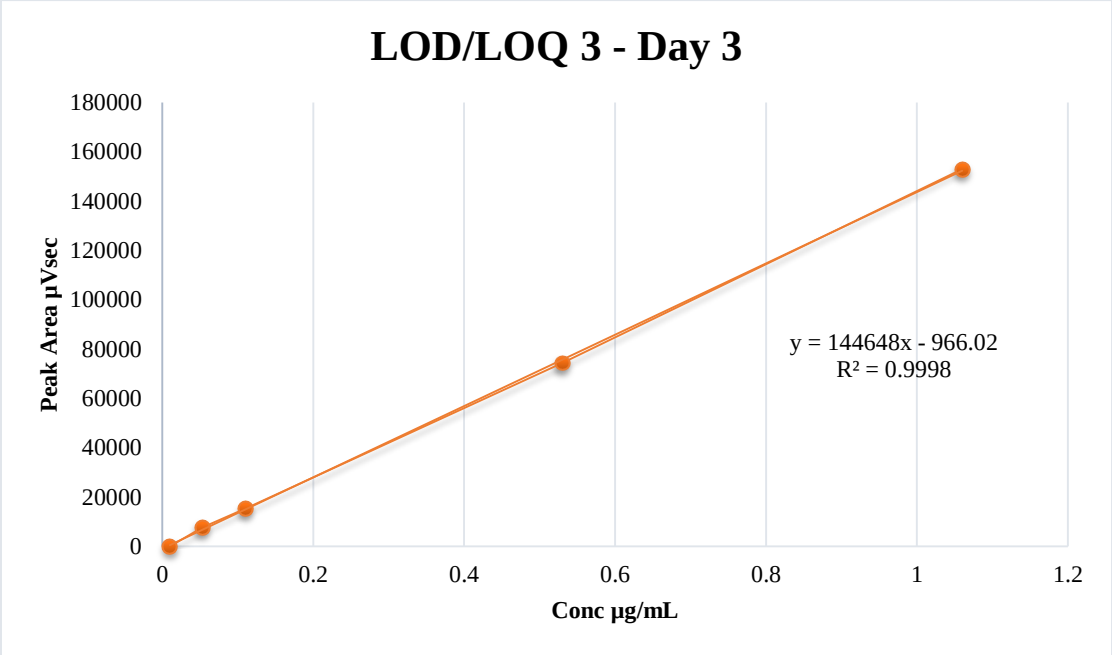


Figure A4.4: LOD and LOQ determinations for **BM13-73b** by calculation using response, standard deviation and slope – Day 3.

Table A4.6: LOD and LOQ determinations for **BM13-73b** – Day 3

LOD/LOQ determinations Day 3

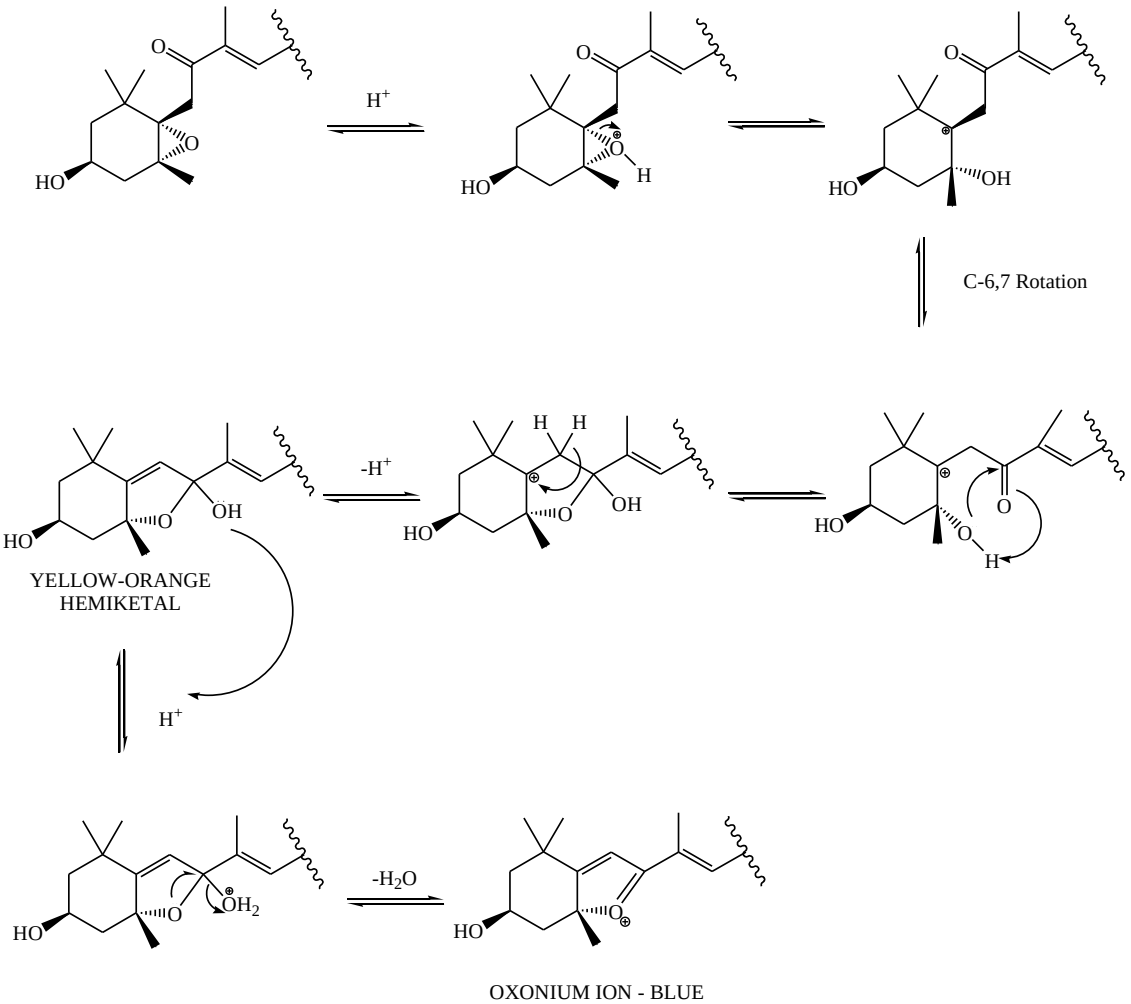
Conc. µg/mL	Conc. µg/mL			Mean	Std. dev.	RSD
1.06	1.06	1.06	1.06	**1.06**	**0.001**	**0.07**
0.53	0.52	0.53	0.53	**0.53**	**0.005**	**0.85**
0.11	0.11	0.11	0.11	**0.11**	**0.001**	**0.82**
0.05	0.05	0.05	0.05	**0.05**	**0.000**	**0.34**
0.01	0.01	0.01	0.01	**0.01**	**0.000**	**0.00**

Appendix 6.1

pH degradation of fucoxanthin: Products

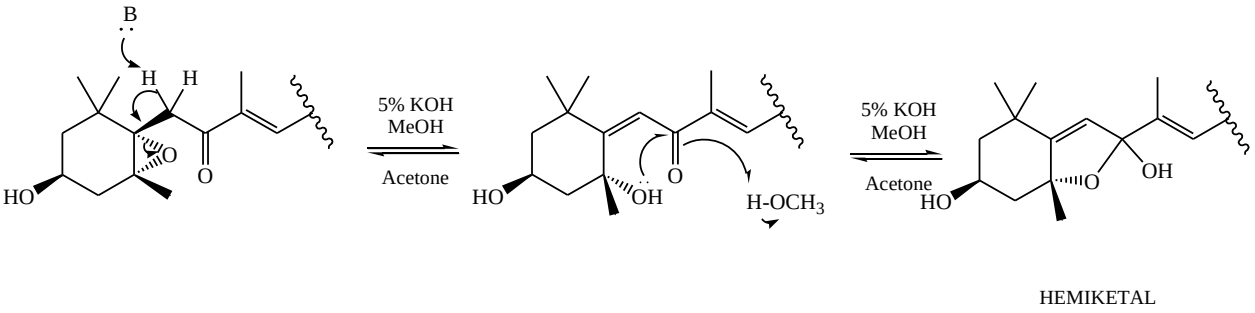
Reactions of fucoxanthin in acidic conditions

Fucoxanthin is extremely unstable in acidic conditions as discussed in (*see* section 6.2.2). Under pH 1.0 conditions, the product was a blue precipitate. The formation of blue oxonium ions has been reported in literature when fucoxanthin was exposed to strong acids. Weaker acids produce the yellow to orange hemiketal (Haugan and Leanne-Jensen, 1994; Leanne-Jensen and Andersen, 2008).



Scheme A6.1: The formation of oxonium ions (blue) and hemiketals (yellow-orange) under acidic conditions

Reactions of fucoxanthin in basic conditions



Scheme A6.2: The formation of the hemiketal under basic conditions

Other products reported by Haugan *et al*., (1992) including fucoxanthinol and its hemiketal, isofucoxanthin and its isofucoxanthinol are shown in (Fig A6.1 – 6.5).

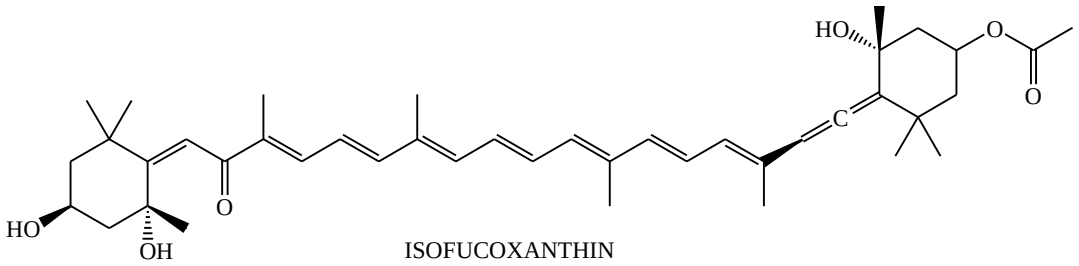


Figure A6.1: The structure of isofucoxanthin

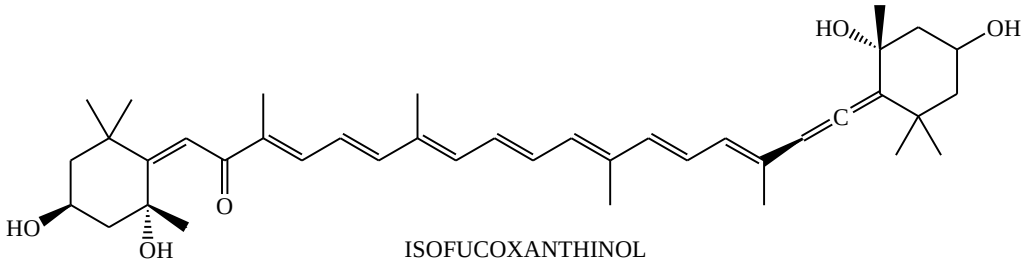
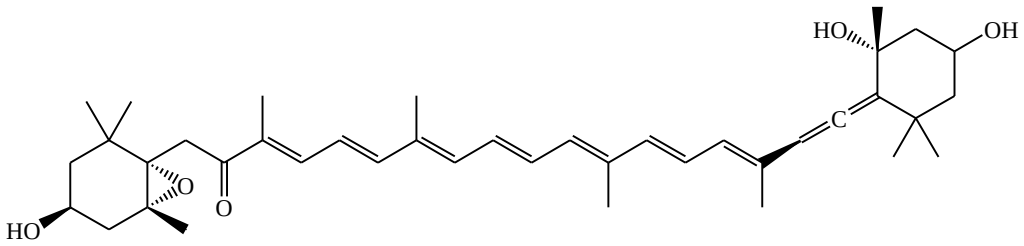
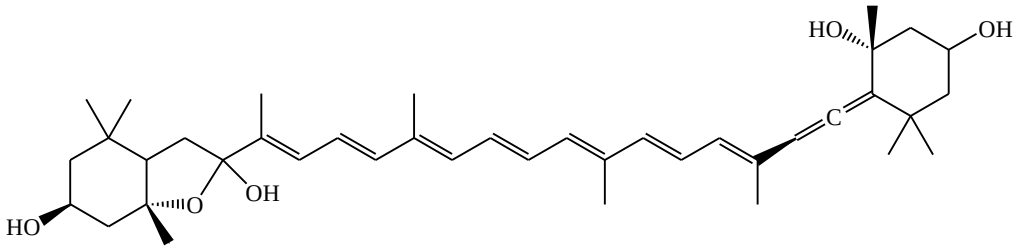


Figure A6.2: The structure of isofucoxanthinol



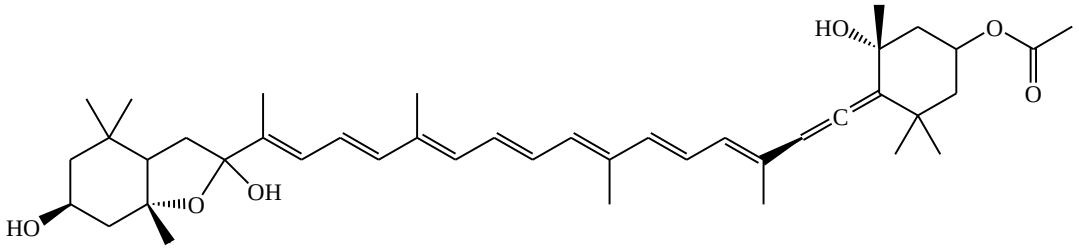
FUCOXANTHINOL

Figure A6.3: The structure of fucoxanthinol.



FUCOXANTHINOL HEMIKETAL

Figure A6.4: The structure of fucoxanthinol hemiketal.



FUCOXANTHIN HEMIKETAL

Figure A6.5: The structure of fucoxanthin hemiketal

References

Haugan, J.A., Englert, G. and Liaaen-Jensen, S. Algal carotenoids 50. Alkali lability of fucoxanthin - reactions and products. *Acta Chemica Scandanavica* **1992**, 46: 614-624.

Haugan, J.A. and Liaaen-Jensen, S. Algal carotenoids 54. Carotenoids of brown algae. *Acta Chemica Scandanavica* **1994**, 22: 31-41.

Liaaen-Jensen, S. and Kildahl-Andersen, G. Blue carotenoids. *Arkivoc* **2008**, 6: 5-25.

Supplementary Chapter

HPLC chromatograms

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Standard fucoxanthin from Sigma Aldrich® (94%)

Fucoxanthin acquired from Sigma (R587/mg) was not used as a reference standard because it was possible to isolate and purify sufficient quantities of fucoxanthin (99%) from Sargassum incisifolium (see Chapter 3). Isolation of our own fucoxanthin was both cheaper and made it possible to have sufficient quantities for the method development process.

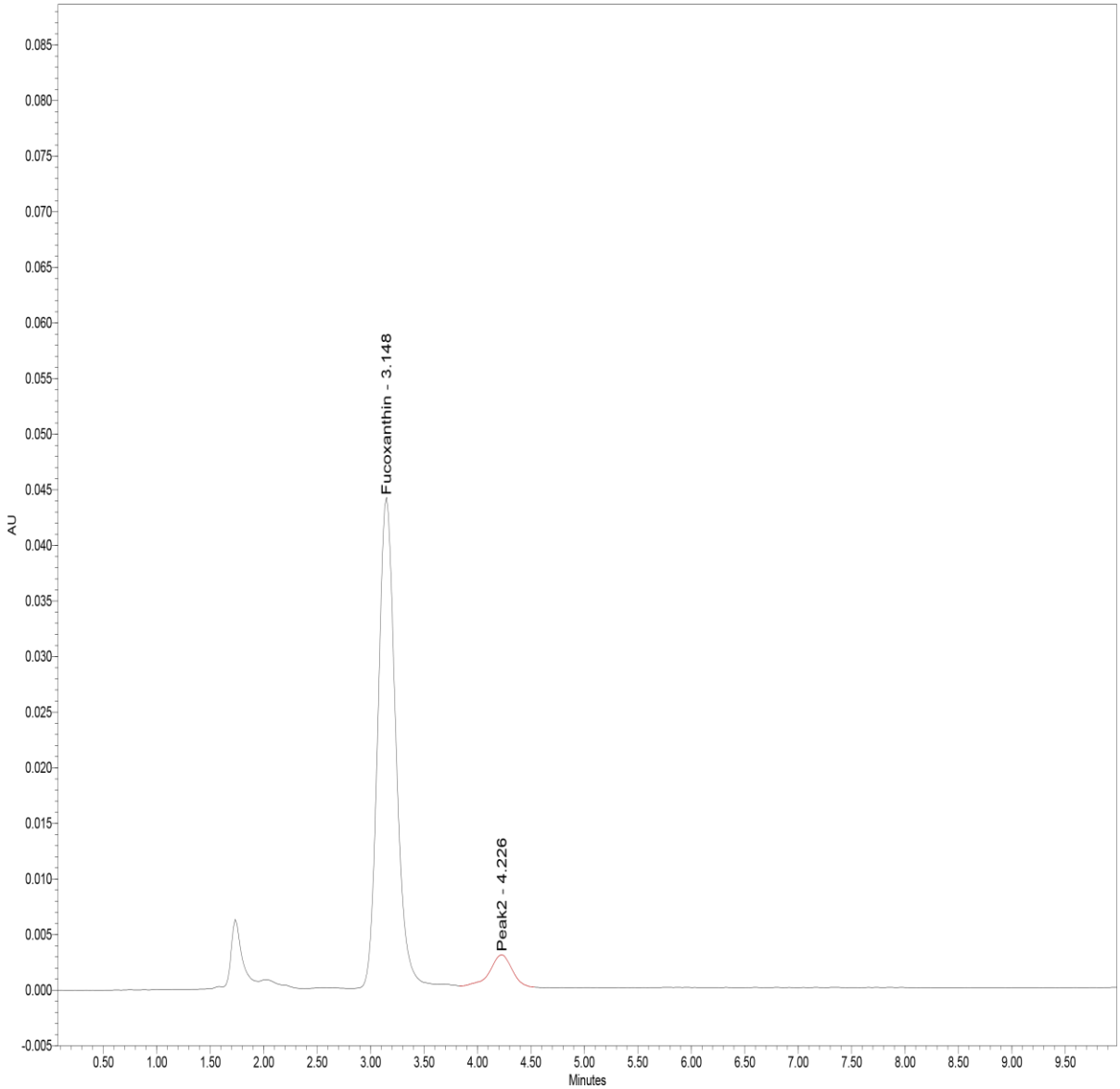


Figure S1: Fucoxanthin from Sigma (94%)

Standard β-carotene from Sigma Aldrich® (95%)

β-carotene could not be used as an internal standard because the carotenoid is also found in most brown algae. The corresponding peaks would therefore interact and distort quantification (see Chapter 3).

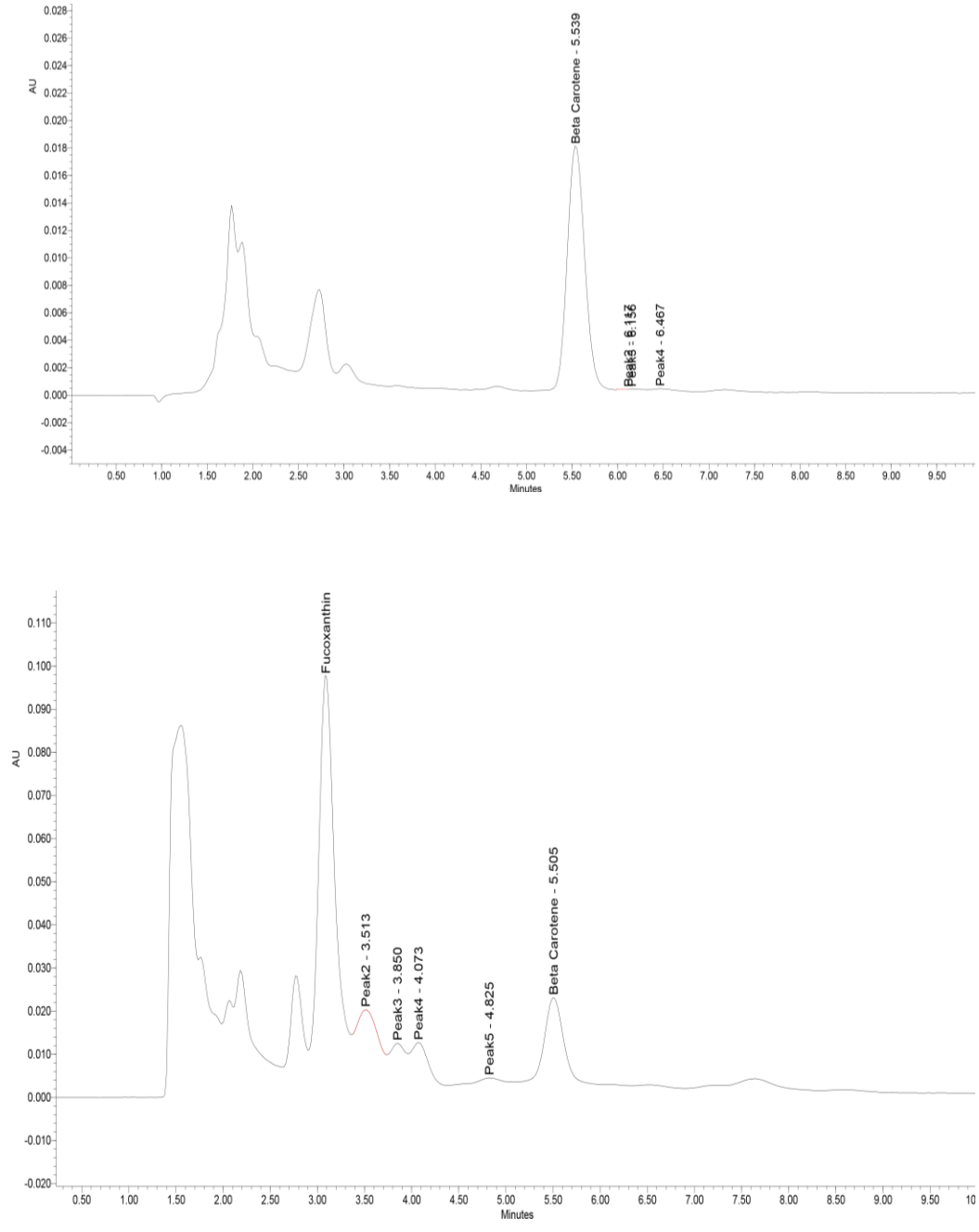


Figure S2: (a) Standard β-carotene from Sigma (b) *Zonaria subarticulata* methanol extract containing β-carotene

Standard canthaxanthin from Sigma Aldrich® (94%).

The use of canthaxanthin as an internal standard was also attempted. Solubility of both β-carotene and canthaxanthin in MeOH was poor and the best chromatogram for canthaxanthin required the use of 10% TCM or THF in ACN as a solvent. Two issues were identified (i) the use of canthaxanthin would require altering the developed method in terms of solvent and mobile phase and (ii) would result in long retention times. External calibration was therefore preferred.

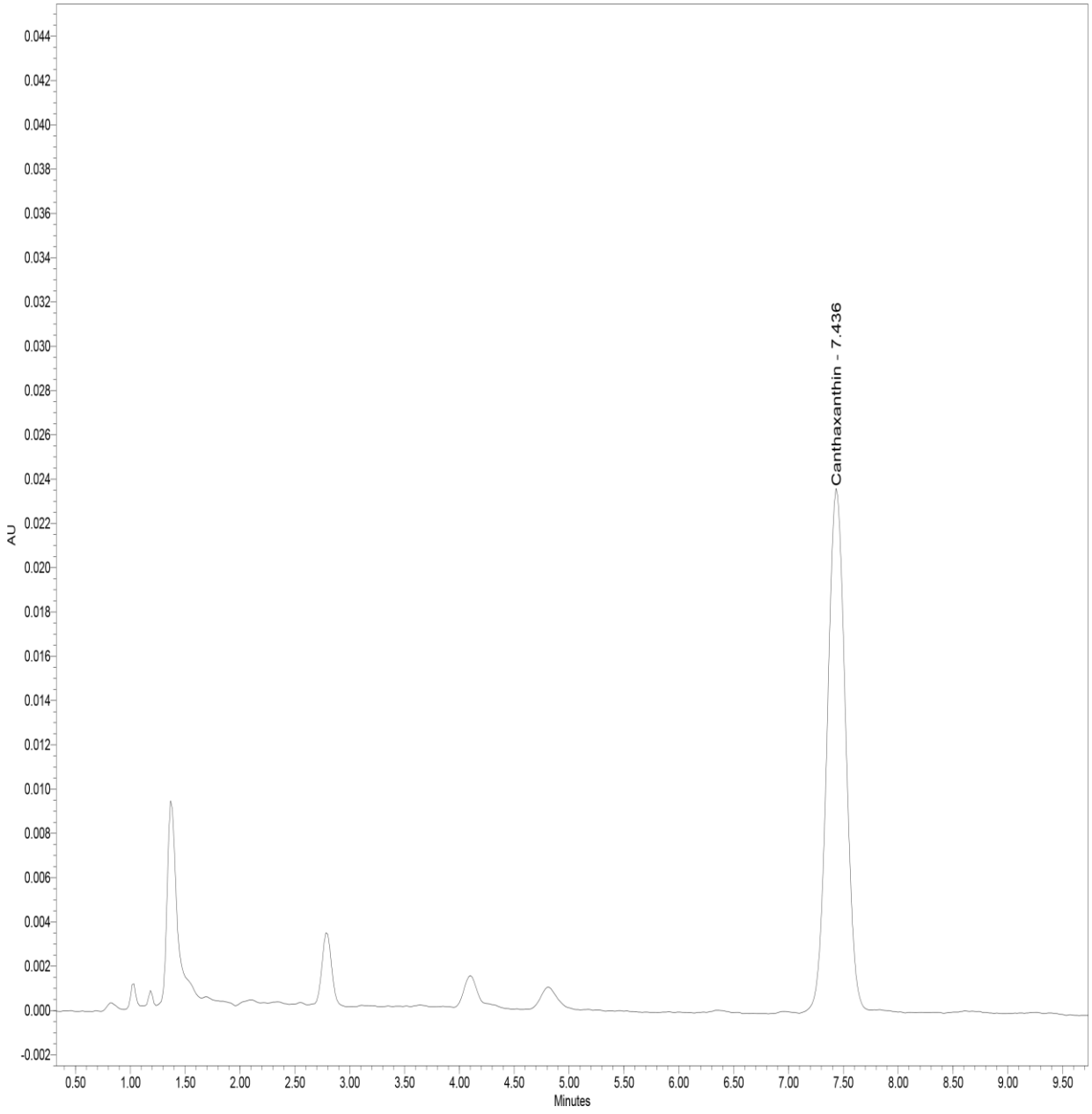


Figure S3: Canthaxanthin dissolved in MeOH.

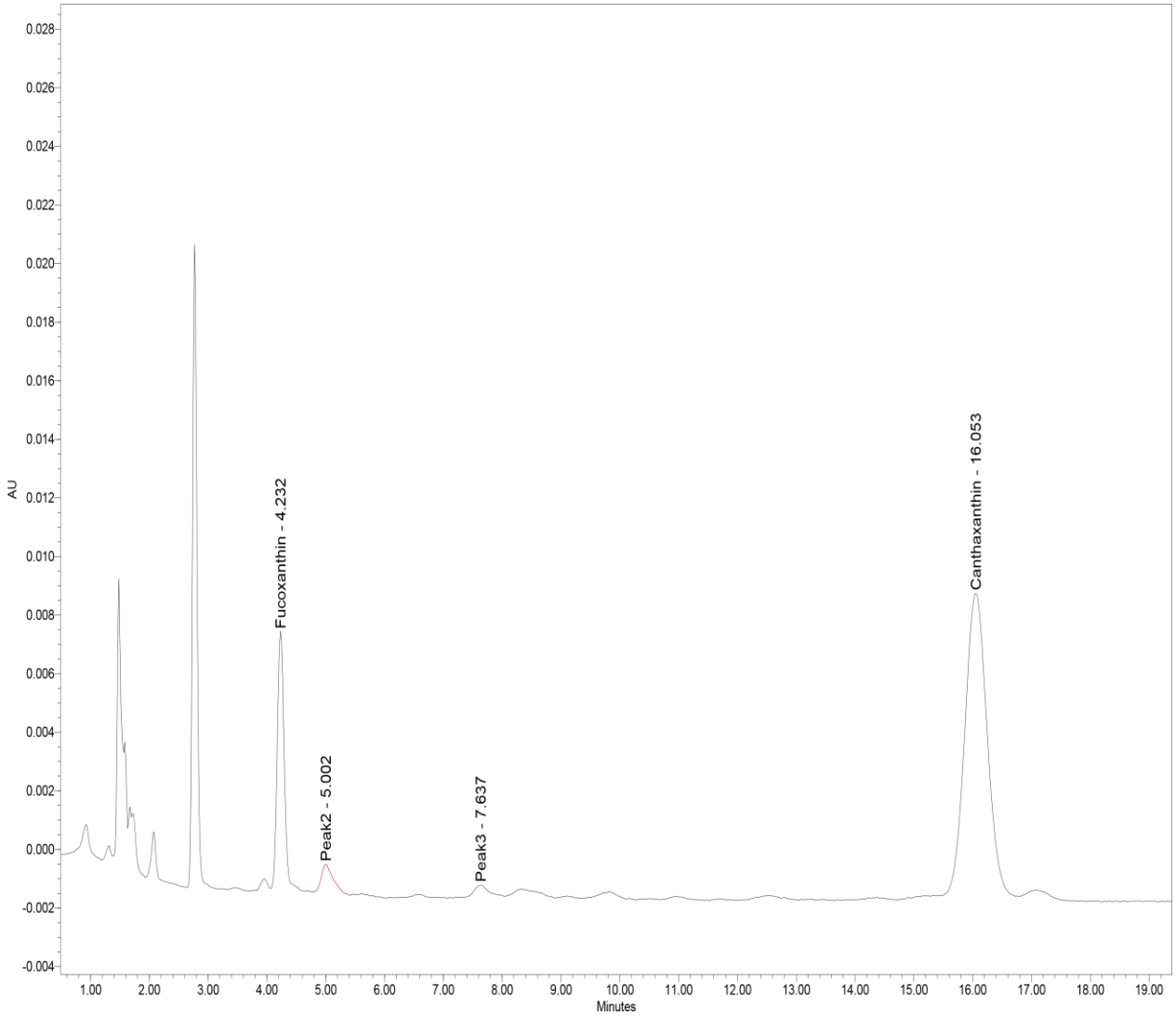


Figure S4: Canthaxanthin dissolved in THF: ACN (1:9)

A crude methanol extract spiked with pure fucoxanthin

A crude methanol extract of Sargassum incisifolium was spiked with pure fucoxanthin to demonstrate the presence of fucoxanthin in the extract. An summative peak height and area was observed.

Figure S6: (a) *Sargassum incisifolium* MeOH extract (b) *Sargassum incisifolium* MeOH extract spiked with pure fucoxanthin

A comparison of profiles from acetone, ethanol, methanol and dichloromethane/methanol extracts of *Sargassum incisifolium*

*A comparison was made on the extraction powers of different solvents. This was done to assess the best solvent for the extraction of fucoxanthin in a crude extract. MeOH (**c**)afforded the most fucoxanthin.*

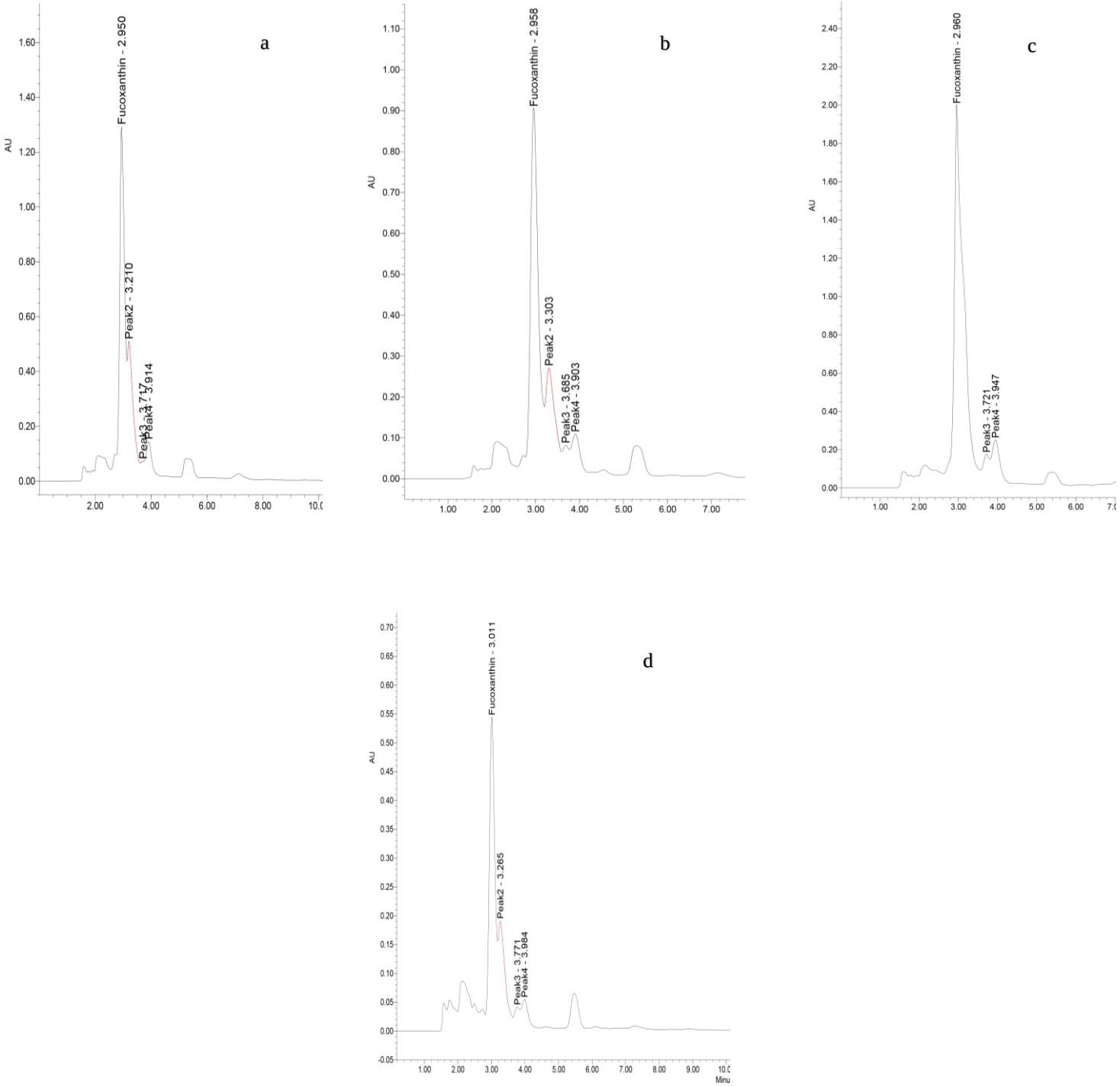


Figure S7: (a) Dichloromethane/Methanol extract (b) Acetone extract (c) Methanol extract (d) Ethanol extract

Crude extract analysis of several brown algae

The brown algae under study were screened for fucoxanthin content. In the preliminary study, the algae was simply crushed using liq. N_2 whilst in the final study the algae were systematically size reduced by crushing using liq. N_2, freeze dried and ground to a fine powder of uniform particle size. The effect of pulverization and changing columns was observed across all genera to improve analysis both qualitatively and quantitatively.

Zonaria subarticulata

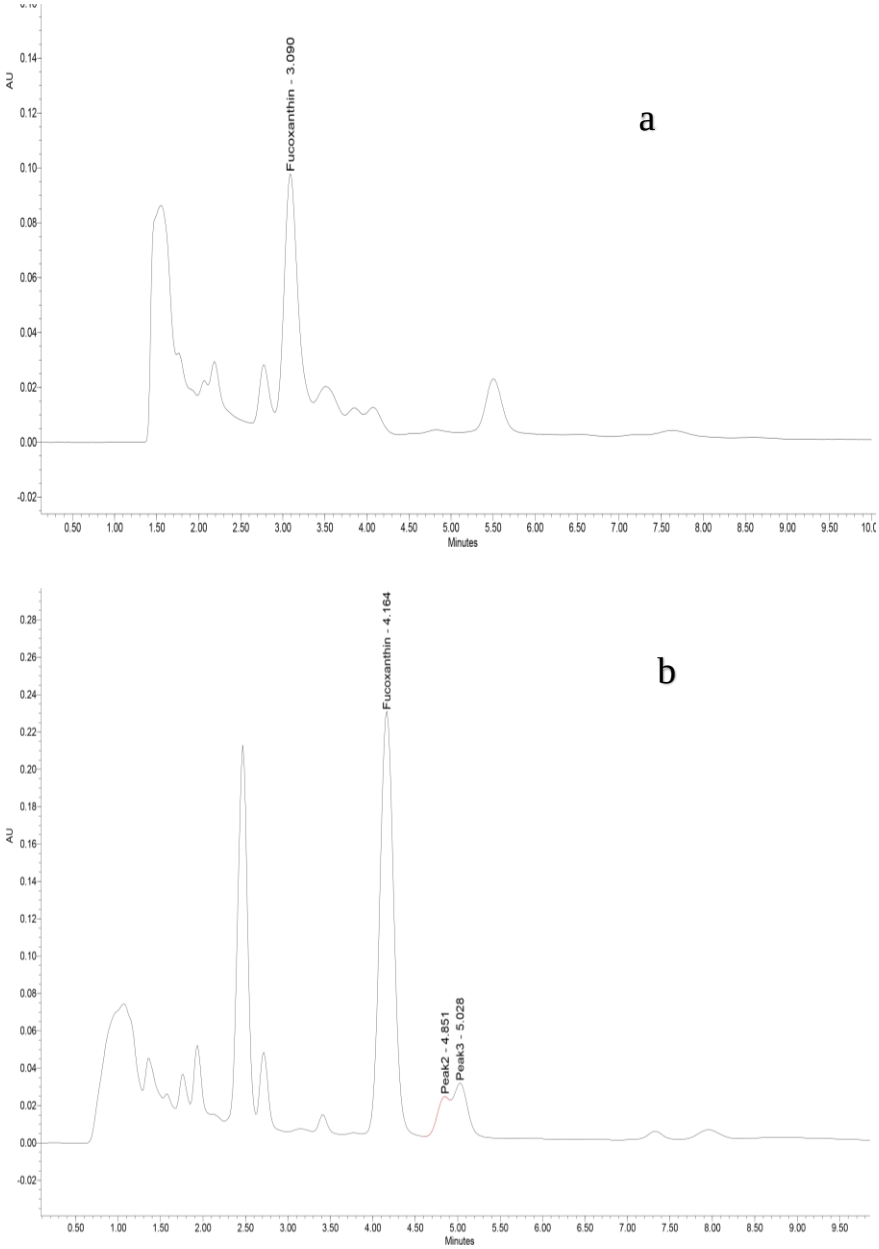


Figure S8: (a) on an Xterra® 150 x 4.6 mm i.d. from crushed alga (b) on a Phenomenex® Synergi™ 250 x 3.0 mm i.d. from freeze dried pulverized alga

Zonaria tournefortii

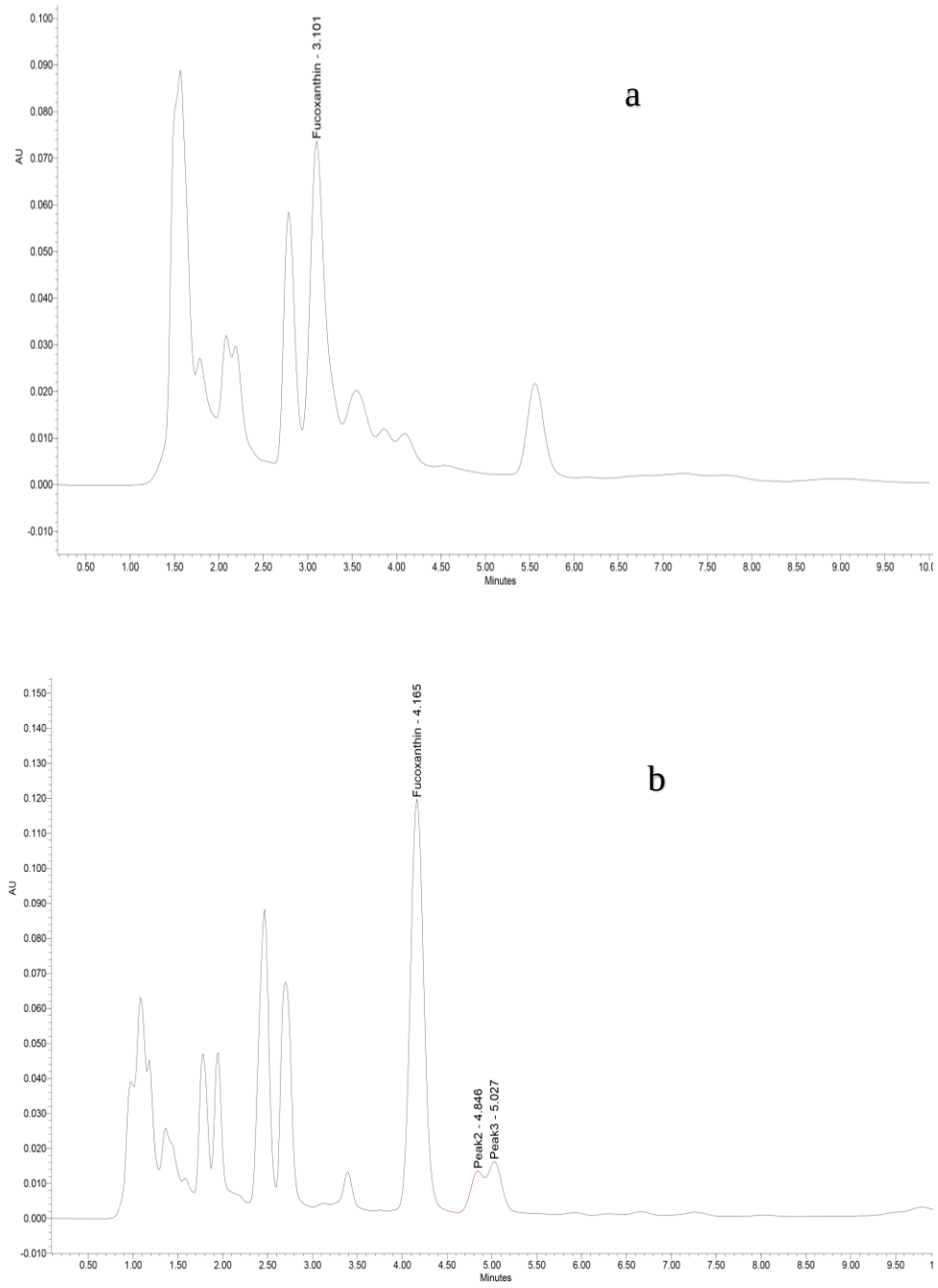


Figure S9: (a) on an Xterra® 150 x 4.6 mm i.d. from crushed alga (b) on a Phenomenex® Synergi™ 250 x 3.0 mm i.d. from freeze dried pulverized alga

Sargassum incisifolium

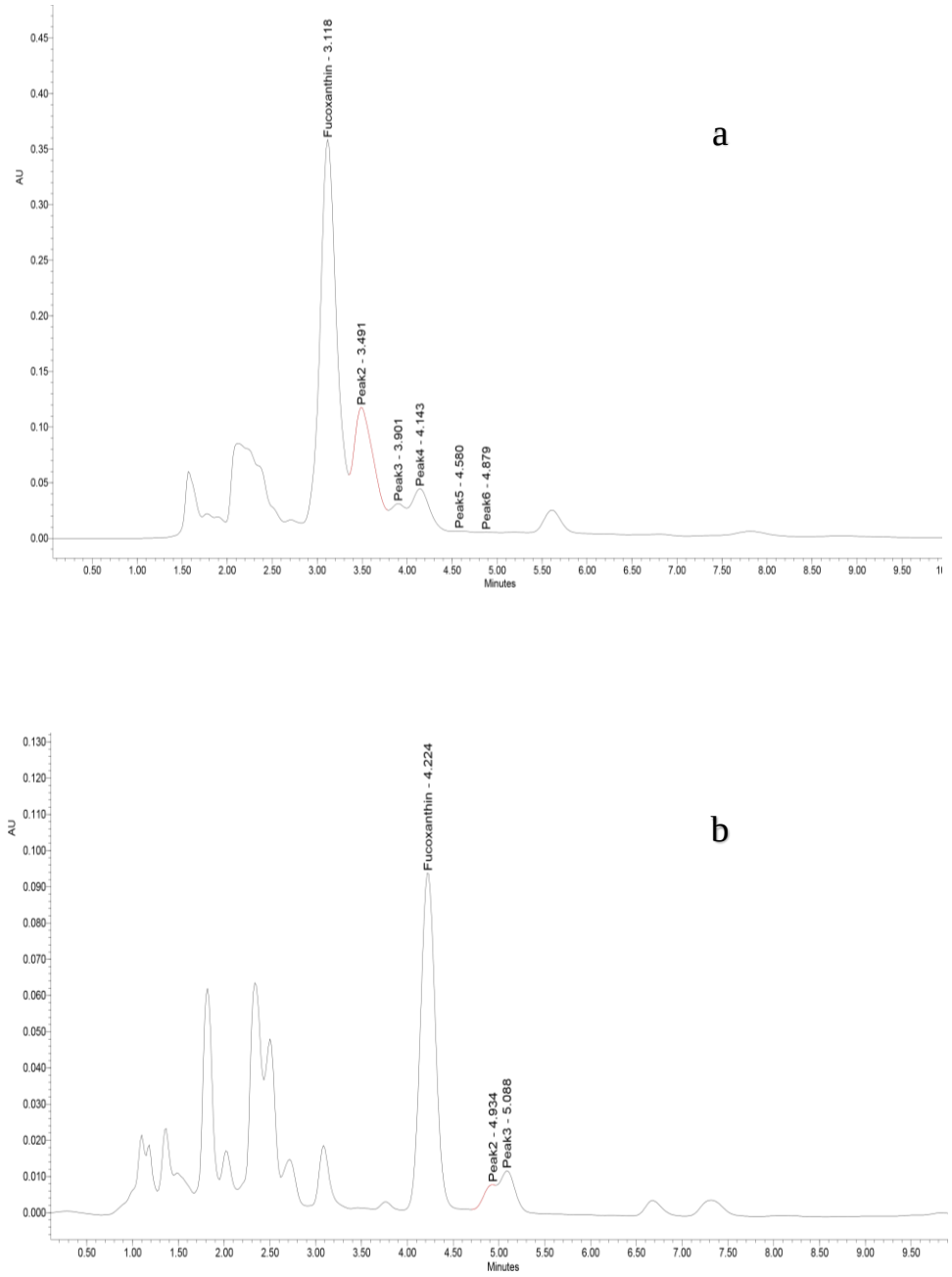
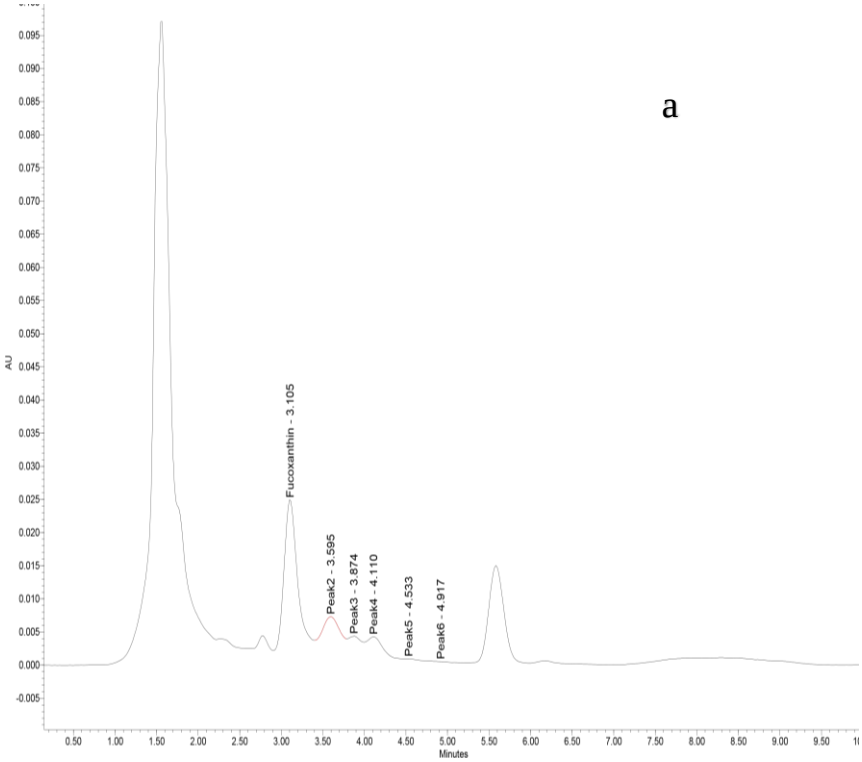


Figure S10: (a) on an Xterra® 150 x 4.6 mm i.d. from crushed alga (b) on a Phenomenex® Synergi™ 250 x 3.0 mm i.d. from freeze dried pulverized alga.

Ecklonia radiata



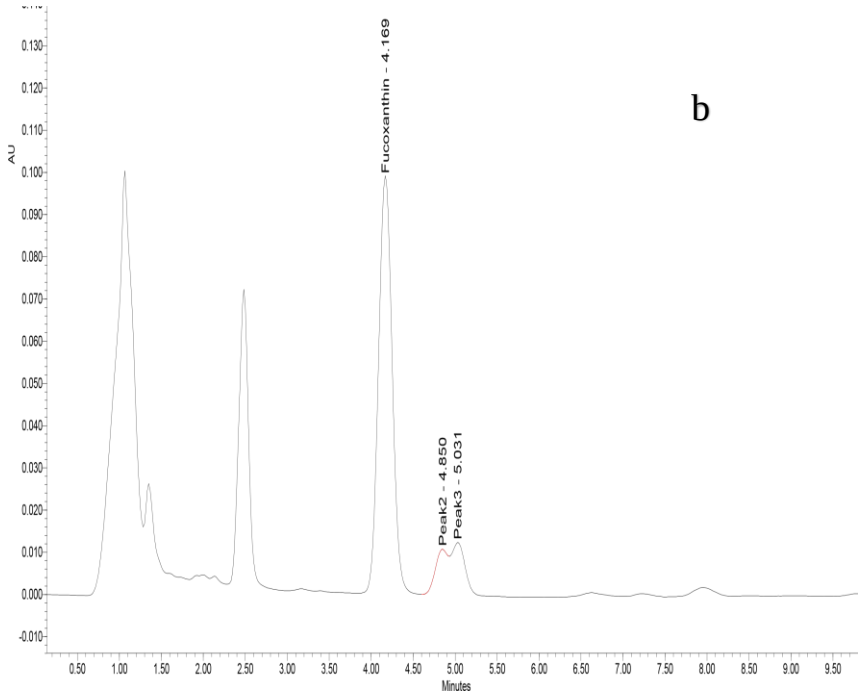


Figure S11: (a) on an Xterra® 150 x 4.6 mm i.d. from crushed alga (b) on a Phenomenex® Synergi™ 250 x 3.0 mm i.d. from freeze dried pulverized alga.

Bifurcariopsis capensis

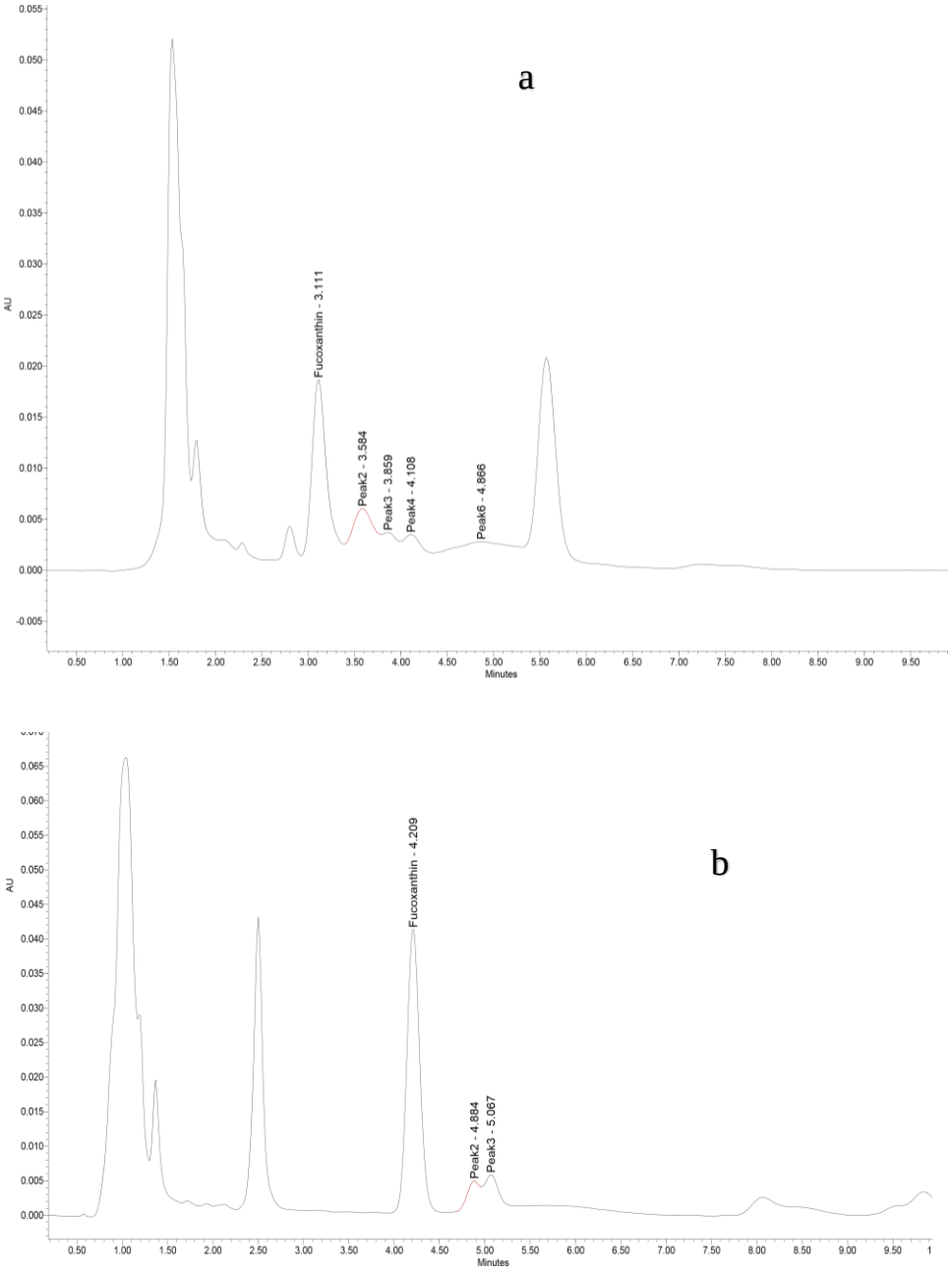


Figure S12: (a) on an Xterra® 150 x 4.6 mm i.d. from crushed alga (b) on a Phenomenex® Synergi™ 250 x 3.0 mm i.d. from freeze dried pulverized alga.

Dictyopteris spp.

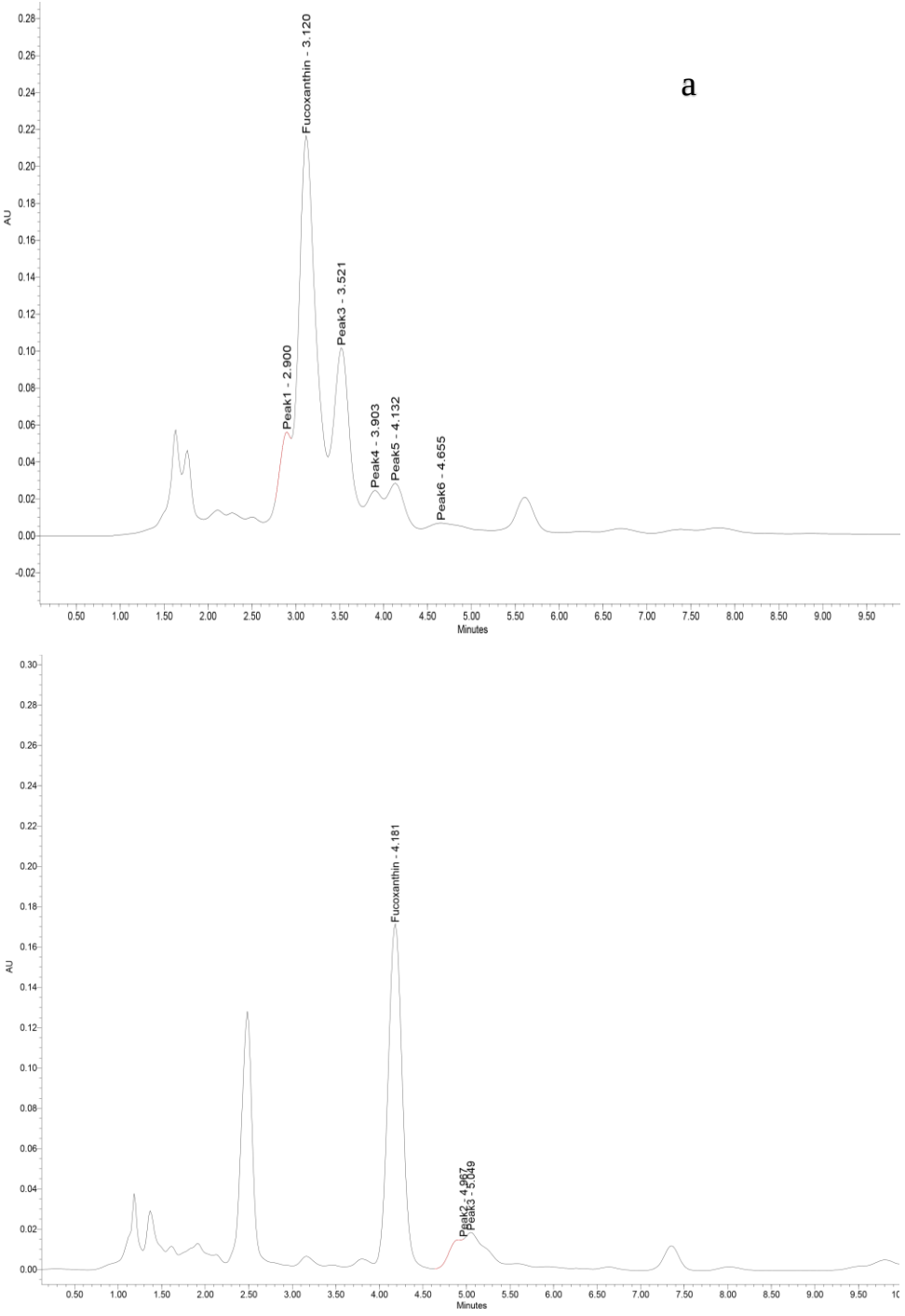


Figure S13: (a) on an Xterra® 150 x 4.6 mm i.d. from crushed alga (b) on a Phenomenex® Synergi™ 250 x 3.0 mm i.d. from freeze dried pulverized alga

Oerstedtia scalaris

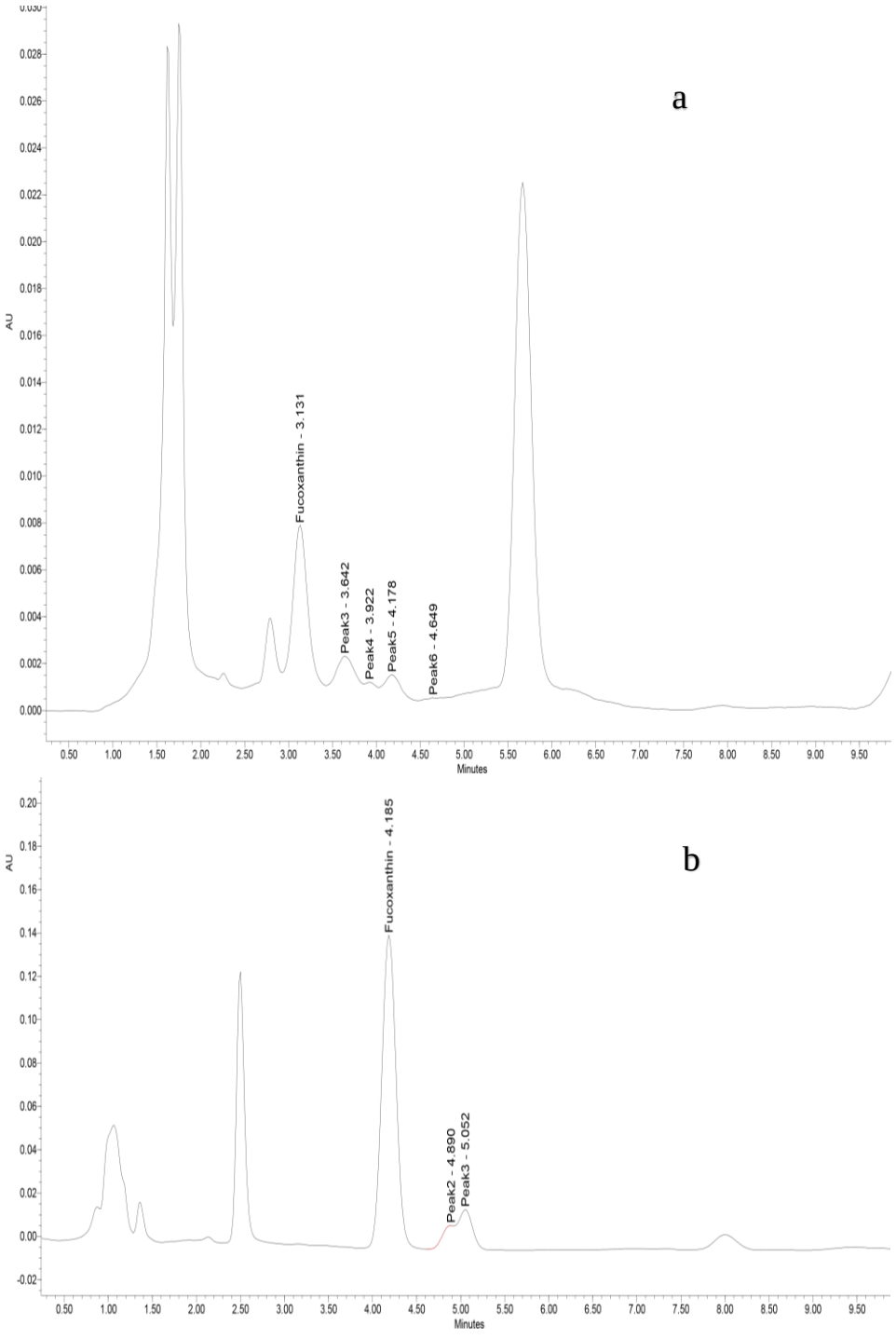


Figure S14: (a) on an Xterra® 150 x 4.6 mm i.d. from crushed alga (b) on a Phenomenex® Synergi™ 250 x 3.0 mm i.d. from freeze dried pulverized alga