Lipid, fatty acid and sterol content analysis of New Zealand *Undaria pinnatifida*

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Table of content

List of tables

List of figures

Attestation of Authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

……. Date

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Abstract

Marine algae, usually called seaweed, are common aquatic plants found in the oceans (Vazhiyil Venugopal, 2008). They have been used for centuries as food, pharmaceuticals and fertilizers. In many cultures, especially in Asian countries such as Japan, Korea, China, Vietnam, Indonesia and Taiwan seaweeds are a significant part of the diet (Besada et al., 2009. Seaweed is also used in the manufacture of industrial hydrocolloids such as agar, carrageenan and alginates.

The brown seaweed *U. pinnatifida* was accidentally introduced to Wellington harbour by ships from Asia in the late 1980's. Since then, it has spread very quickly around the coast because of two important factors: suitable growth conditions in New Zealand and its strong invasiveness (M. D. Stuart, 2004). However, until 2010 the law in New Zealand did not allow people to harvest or culture *U. pinnatifida* as it was defined as an "unwanted organism" by MAF Biosecurity. In 2010 the government reviewed this policy and have subsequently allowed greater freedom for the marine industry to use this seaweed commercially (MAF.Biochemistry, 2010).

As a species of marine algae, geography and temperature are the two major parameters that determine its growth and nutritional content (Nelson, Phleger, & Nichols, 2002). For example, It has been shown that the total lipid contents of green and red seaweeds may increase at lower temperature (5-10 ^{o}C) (Nelson et al., 2002).

In general, the lipid content of marine algae is lower than other plant species but the lipids are rich in polyunsaturated fatty acids (PUPAs) which are important for human health (Dawczynski, Schubert, & Jahreis, 2007; Norziah & Ching, 2000). Since it is hard for humans to synthesize the long chain n-3 polyunsaturated fatty acids (LC-PUFAs) found in fish oil, sources such as *Undaria pinnatifida* have gained more attention recently, because not only it contain abundant n-3 PUFAs but

also the cost of cultivation is relatively low (Kumari, Kumar, Gupta, Reddy, & Jha, 2010).

The public still have little knowledge on the *Undaria pinnatifida*. They do not realise how valuable the *Undaria pinnatifida* can be. As a result, this project will focus on the lipid and fatty acid content of *Undaria pinnatifida*, which because of the high proportion of PUFAs is of considerable commercial interest.

Results of this research show that the main lipid classes found in *Undaria* are the non-polar lipids, followed by phospholipids and glycolipids. The sporophyll contains 2.34% total lipid by weight, while the blade is 1.53% total lipid by weight.

LC-MS analysis of the lipid extracts showed the presence of many phytochemical compounds and lipids especially glycerophospholipids. This suggests that further analysis is likely to produce more interesting compounds.

The fatty acids determined in this project confirmed pervious research, but with some differences. Polyunsaturated fatty acids and saturated fatty acids are the two major fatty acid classes found in *Undaria*, of which palmitic and henicosanoic are the major saturated fatty acids, while gamma-linolenic and dihomo-g-linolenic are the principal polyunsaturated fatty acids. The sporophyll contains more fatty acids (12.46 mg/g) than the blade (10.85 mg/g).

Analysis of thye non-saponifiable lipids showed that commercially interesting fucosterol is the major sterol found in *Undaria* and the concentration is 0.323 mg/g

Chapter.1 Introduction about seaweed

1.1 Background information of *Undaria*

Seaweeds, sometimes called sea vegetables are the familiar plants that grow on rocks in every sea and oceans all over the world. Generally, they are algae, and the target in this research is the brown algae *Undaria pinnatifida* referred to in this thesis as *Undaria*.

Figure 1 Undaria distribution of world

Figure 1 above shows the *Undraia* distribution by country. According to this map, 12 countries from different continents are pointed out. From left to right, they are United States, Mexico, Argentina, Spain, France, United Kingdom, Russia, Italy, China, Japan, Korea, Australia and New Zealand. As a result, it is obviously that that the distribution of *Undaria pinnatifida* is all over the world.

In general, three major algae species are commercially used in food markets. They are: *U. pinnatifida* (Hare.), Suringar, *U. Undarioides* (Yendo) Okamura, and *U. peterseniana* (Kjellm.) Okamura. People, especially in Asia, consume them in a fresh or dried way and among these species, *U. pinnatifida* is the most popular one and is the most commercially interested. The three species are cultivated in Asia, but *U. pinnatifida* is the only species found so far in New Zealand waters (Blunden, 1991).

U. pinnatifida is known by many different names throughout the world. They are "sea mustrad" in English, "hai-dai" or "wakamé" in Chinese, "fougère des mers" in French and "wakame" in Japanese. According to the algaebase, the taxonomy classification is listed below in *Table 1*:

Table 1 Alage taxonomy classification

Empire	Eukaryota
Kingdom	Chromista
Phylum	Ochrophyta
Class	Phaeophyceae
Order	Laminariales
Family	Alariaceae
Genus	Undaria

(Alagebase, 2013)

Undaria pinnatifida and its by-products are becoming more popular and the FAO (Food and Agriculture Organization of the United Nations) reported that the world production of wakame in 2008 was 1.8 million tonnes (1.8 billion kilograms). (FAO, 2008)

In terms of diet, many essential supplements required by humans are rich in edible seaweed. For example they are polyunsaturated fatty acids, fiber, vitamins, proteins, and carotenoids such as fucoxanthin (MacArtain, 2007).

Research at AUT has shown that "*Undaria* as well as other brown algae such as *Laminaria* sp, and *Hizikia* sp are composed of very high concentrations of oleic acid , alpha-linolenic acid and eicosapentaenoic acid (EPA)" (Boulom, 2012), and these fatty acids are considered very important for human health.

Humans are unable to synthesize long chain n-3 polyunsaturated fatty acids (LC-PUFAs) typically found in marine fish. As a result, these brown algae can be an excellent source of n-3 PUFAS (Simopoulos, 2008).

In addition, it is reported that seaweeds contain an abundant mineral content, because of its specific cell wall structure, inorganic substances are much more easily absorbed from sea water (MacArtain, 2007). Its mineral content is higher compared to other land vegetables and as a result, even small amounts in the diet can provide a good source of minerals (MacArtain, 2007). From a health perspective, it is also thought that the high mineral content can potentially help control hyperlipidaemia, thrombosis, tumours and obesity (V Venugopal, 2008).

Overall, although *Undaria* is said to be a very healthy food for people, there is surprisingly little known about its composition. This thesis will examine the lipid fractions of *Undaria* in detail.

1.1.1 Description of *Undaria pinnatifida*

Undaria is a brown edible seaweed. *Figure 2* shows a typical structure of *Undaria*, where three major parts are pointed out. They are midrib, blade and sporophyll.

The midrib connects the main stipe to the top of the blade. It is elliptical and is usually 1-3 cm wide. Botanically, the blade is *Ecklonia*-like. There are a lot of lobes on it, and the texture of its surface is described as membranous and mucilaginous, (it feels smooth and slippery). The sporophyll is the reproductive part of *Undaria*. It is the structure that bears the sporangia, in which the reproductive zoospores are produced.

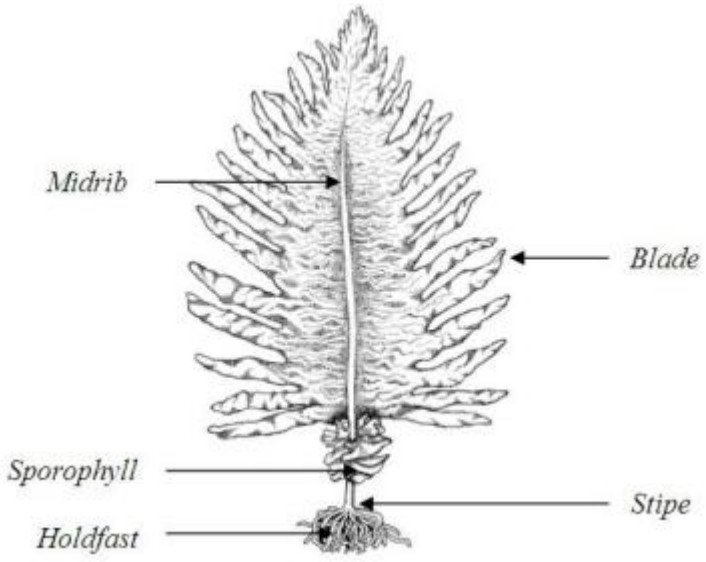

Figure 2 A typical structure of U. Pinnatifida
http://www.mass.gov/czm/invasives/docs/potentialinvaders/u_pinnatifida.pdf

1.1.2 Life history of Undaria pinnatifida

In general, *Undaria* is a kelp-like plant. As a result, the life annual cycle of *Undaria* species is similarly compared to the kelp such as *Laminariales*.

The swimming zoospores are produced by the annually formed sporophyll (*Figure 3*), in which male and female gametophytes are generated. The male and female gametophytes fertilise the oospore, from which a new plumule sporophyll is generated. Finally, it will become to an adult one. A detailed life cycle is presented below (*Figure 3*) (Floc'h, 1991; Ohno, 1993).

In some Asian countries, the sporophylls usually appear between September and November, after that they grow very quickly until the end of the March. The

sporophylls grow is in the meristematic zone between stipe and blade. As the sporophyll grows, the apical parts degenerate at same time and give a rather tattered appearance (Castric-Fey A, 1999).

Figure 3 below shows the two stages of the life cycle of *Undaria.* They are called the macroscopic sporophyte stage and the microscopic gametophyte stage. As an annual plant, the macroscopic sporophyte stage usually lasts 6 months, while the microscopic gametophyte stage can last for over 2 years (Mike D. Stuart, 2004).

1 → zoospore (n);
2 – 4 → the germination of zoospore;
5 – 9 → the formation of male gametophyte;
7 – 9 → the formation of female gametophyte;
10 → fertilised oospore and egg;
11 → the germination of sporophyte;
12 → plumule of young sporophyte;
13 → the formation of adult sporophyte;

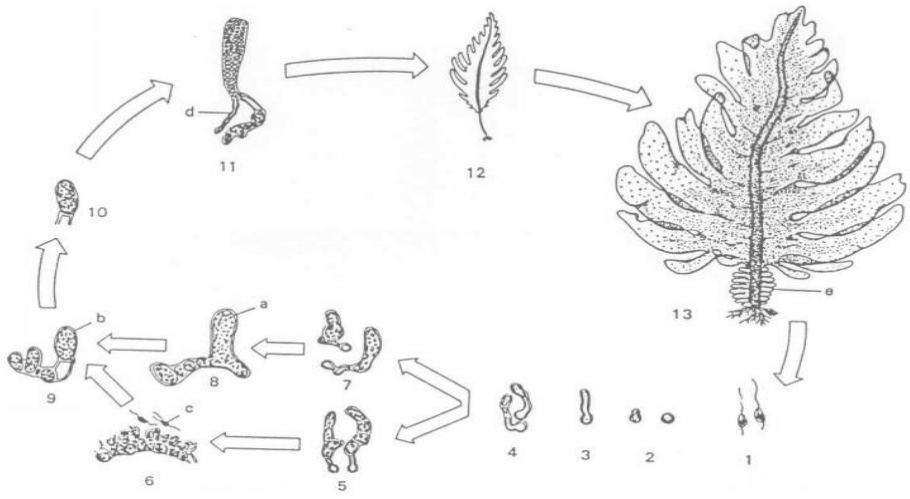

Figure 3 Life history of Undaria

1.2 Geographic and environmental conditions for *Undaria's* growth

As discussed above, the main *Undaria* species cultivated by people is *Undaria pinnatafida*. It can be found on hard rocks including reefs, cobbles, ropes, wharf piles, ship hulls, moorings and other artificial structures in coastline (C. H. Hay & Luckens, 1987). *Undaria* is able to grow and develop in different geographical environments, whose physical properties can be significantly different. As a result, *Undaria* occurs along the different coast lines within different continents and different length. For example, in Japan, *Undaria* hold the record for the longest blade at 3 meters long (Akiyama, 1982), while the longest blade of up to 1 meters are found in New Zealand (Silva, Woodfield, Cohen, Harris, & Goddard, 2002).

Similarly, the length of the sporophyll ranges from 0.2 meters to 3 meters based on the different cultivation stages which can be vary due to different location (Herbreteau, Coiffard, Derrien, & De Roeck-Holtzhauer, 1997). For example, it is reported that the New Zealand *Undaria* sporophylls showed significant difference compared to the Asian *Undaria* sporophylls. The NZ ones range from 70 cm to 1.3 m while the length of Asian sporophyll is from 20-23 cm under the same temperatures (C. Hay, & Luckens, P. A, 1987). Specific research on the different *Undaria* region in Matsushima Bay has reported that algae from the outside region are much bigger than the inside it.

The most important factor influencing the ecology of *Undaria* is probably the water temperature. According to the previous research, the most suitable temperature range for the development of sporophytes is between 5 and 15ºC (FAO, 2011).

Commonly, a life cycle of sporophytes is between early winter (a water temperature approximate 10°C) and early summer (approximately 25°C). It has been found that 14°C and 23°C is the best growing temperature range, while 5 - 14°C is best for

sporophyte formation in Asian countries such as Japan, China and Korea (Wallentinus, 2007).

In addition, there is no obvious influence on the formation of the sporophyll at different temperatures (Saito, 1972). However, when the surface temperature of sea water continues over 14°C for 10 days, a zoospore release will occur soon after the adult sporophytes die. *Table 2* shows suitable temperature ranges for the *Undaria* in terms of different growth stage (Sanderson, 1990).

Table 2 Temperature factors for the generations of Undaria

	Sporophyte	Gametophyte
Lethal temperature	< 0°C, >25°C	<-1°C, >30°C
Growth temperature	3.5°C - 20°C	10°C - 24°C
Reproductive temperature	<7°C, >23°C	<10°C, >24°C

1.3 Global spread of *Undaria* species

Undaria, to some extent, is regarded as one of the most invasive plants in the world. It can spread very widely and rapidly because of its strong colonizing ability. It causes unexpected problems to native marine plants, animals and the environment. However, introductions of this seaweed have been accepted by people, because *Undaria* is valuable in many fields (Wallentinus, 2007).

Generally, *Undaria* have been introduced accidentally by two pathways namely commercial vessels and aqua-cultural animals. In 1971, a report suggested that it is very possible that Asia ships firstly bought *Undaria* to the northern Mediterranean and France (Herbreteau et al., 1997). After that this *Undaria* was considered to be commercially interesting in France and was cultivated firstly in the north Atlantic in

1983. From the Atlantic, *Undaria* was finally made its way to Spain, England and the Netherlands between 1990 and 1999 (Bourdouresque, 1985; Manfredi, 1995).

As discussed above, *Undaria* was first reported in New Zealand in the late of 1980s near the Oriental Bay in Lambton Harbour, Wellington (C. H. Hay & Luckens, 1987; Mike D. Stuart, 2004). From there *Undaria* is thought to have been introduced to other New Zealand harbours by ships: Lyttelton (Hay 1990), Timaru (Hay 1990, Brown & Lamare1994), Oamaru (Hay 1990), Picton (Nelson et al. 1992, Brown & Lamare 1994), Porirua (Hay &Villouta 1993), Otago harbour and Port Chalmers (Hay & Villouta 1993, Brown & Lamare 1994, Anon. 1994). *Undaria* has also become established in Tasmania (Sanderson, 1990). It was discovered later that the New Zealand climate conditions are particularly suitable for the growth of *Undaria*. The relatively low sea temperature (around 10 ^{o}C) throughout the year is the key factor, which is thought to be the ideal temperature for growth of *Undaria* (C. H. Hay & Luckens, 1987). *Figure 4* shows the locations where some of the earlier discoveries of *Undaria* were made in New Zealand.

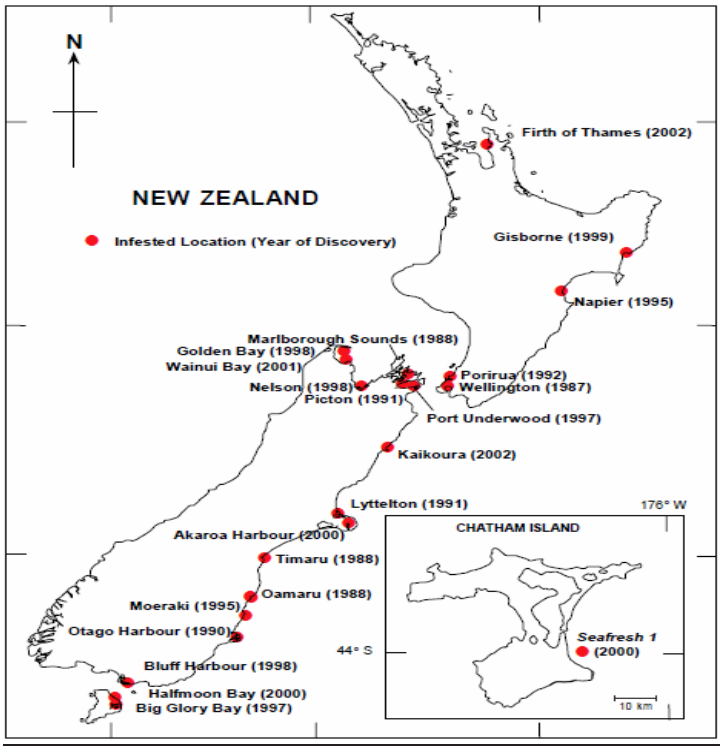

Figure 4 Localities in New Zealand where Undaria was discovered

1.4 History of *Undaria* species in New Zealand and attempts to control its spread.

Many issues were of concern in New Zealand after the discovery and spread of *Undaria*, because its ability to colonize has a significant effect on the native marine animals and plants (C. H. Hay & Luckens, 1987; Mike D. Stuart, 2004). For example, it inhabits the areas of other species and its thick layer in the sea can easily block the light. As a result, *Undaria* is defined as an "unwanted organism" by MAF Biosecurity, and was targeted in order to slow down the speed of its growth and spread in New Zealand. In recent years, many methods are used to try to control *Undaria*.

One of the most successful techniques was so called "purification of *Undaria"*. This method is based on the elimination of *Undaria* during the sporophyte stage and it significantly reduces large number of sporophytes. Other useful techniques include the heat treatment which use hot steam or water (around 70°C for a while) to ruin *Undaria* gametophytes in vitro and a vessel control system to identify whether the ships were contaminated with *Undaria* species or not (Mike D. Stuart, 2004).

After 2010 the government conceded that this policy was not effective and begin to gradually encourage the marine industry to use *Undaria* commercially (MAF.Biochemistry, The commercial use of Undaria - An exotic Asian Seaweed).

This new 2010 policy had two principal points:

1. *Undaria* can be cultivated in specific areas.
2. Collection of *Undaria* can be performed on specific surfaces such as marina and sea farms.

1.5 Cultivation methods and global production of *Undaria* species

The history of *Undaria* farming can be back to 1930s in China, Korea and Japan (C. Hay & Gibbs, 1996). Asian seaweed farmers seed the spores directly to collectors such as ropes or frames between May and June. Then they put this frames in a light-blocked tank of water for approximately four months. When they become visible, the plants are then taken out into the sunshine and open sea. Generally, this method is the most typical one and it can be concluded into three steps:

1. the production of seeds
2. the pre-culture in tank
3. the cultivation in the open sea

It should be pointed out that water must be changed once a week and the collectors must be cleared from other species such as mussels and diatoms by manual cleaning As a result, this is a very expensive culturing method.

France was the first European country try to cultivate *Undaria* (Perez, Kaas, & Barbarroux, 1984). The French Institute for Exploitation of the Sea (IFREMER) applied a new method using a vegetative gametophyte technique in flasks, namely as the free-living technique. The advantage of this method is that it can generate a large number of male and female gametophytes from only a few selected spores.

Depending on the intended use, the vegetative and reproductive development have somewhat different morphologies (Gibbs, Hay, & Dodgshun, 1998). *Undaria* has the ability to increase both the length and width of the stipe during the vegetable stage, while the thickness of the blade and stipe increase during the sporophyll growth (H. G. Choi, Kim, Lee, & Nam, 2007).

According to the new policy in section 1.4, it is clearly that the NZ government has realized the commercial importance of seaweed.

A summary *(Figure 5)* of the global production and markets of *Undraia pinnatifida* of Food and Agriculture Organisation of the United Nations, Fisheries and Aquaculture Department shows a considerable increase in *Undaria* cultivation of the past 10 years. The total amount of aquatic production which is dominated by seaweeds ("99.6% of the total production of aquatic plants") (FAO, 2008) was globally 15 million tonnes at the end of 2010.

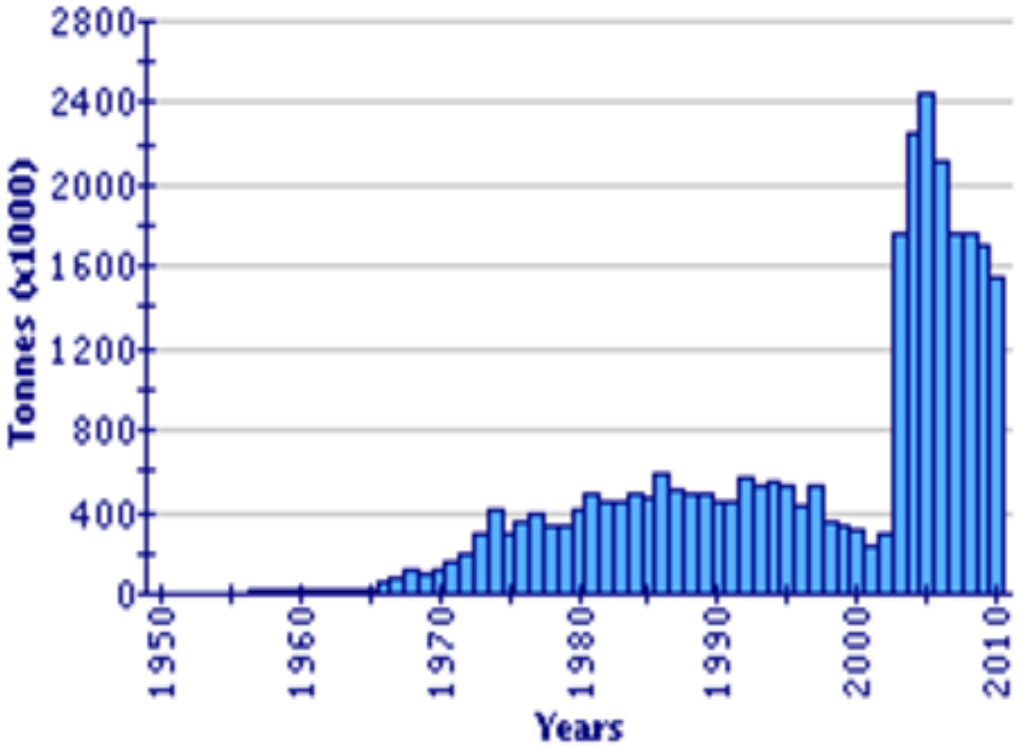

Figure 5 Globa production of Undaria (FAO Fisherly Satatisics)

Figure 6 shows four aspects of the global seaweed production. In the main, China is the biggest seaweed producer and market followed by other Asian countries. The total production of aquatic plants increased from 8 million tonnes in 1997 to nearly 15 million tonnes in 2008, this represents about a $2.5 billion (US) increase over this period. The other three graphs show the value (in GWon) production of three different algae species in Korea. Although it seems no significant difference among these three species, it should be still pointed out that the production of *Undraia pinnatifida* is

higher than others, which means it is the most popular seaweed. The pie charts in each graph shows the proportion of the harvest lost to diseases each year since the data collection began (Korean Fisheries, 2009). The major function of most aquatic products was used for human diet. Nevertheless, some algae is also produced as raw materials for various industries. For example, *Eucheuma*, a major seaweed species was produced in Southeast Asia and used for carrageenan extraction (Boulom, 2011).

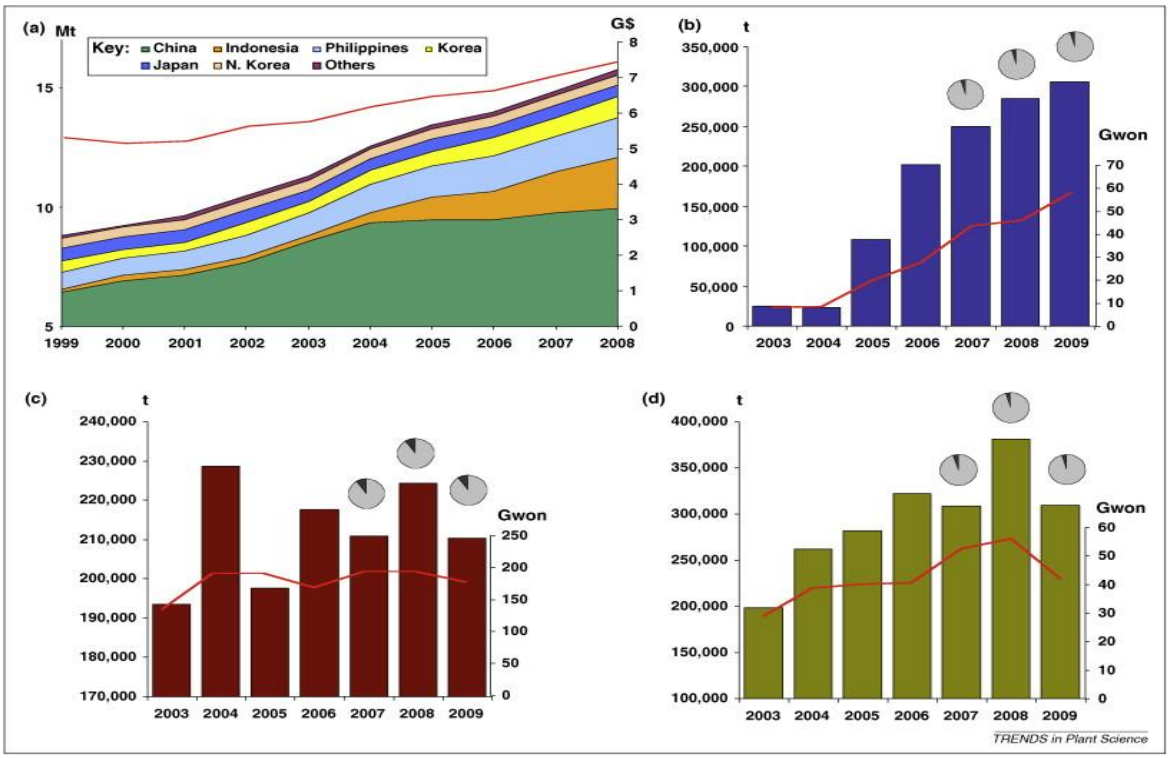

Figure 6 (a) Production of aquatic plants by country (in Mt of fresh weight). (b) Production of the Laminaria *spp. (d) Production of* Undaria*, and (c)Production of laver Porphyra spp. In Korea*

In New Zealand, the harvesting and marketing of *Undaria* is very new. *Undaria* is being collected as a nuisance by-product of mussel farming. It fouls the mussel lines and needs to be removed regularly. However, although there is considerable potential value in selling this by-product, in 2011 only 4,000 tonnes of fresh *Undaria* were exported to other countries (Aquaculture, 2011).

1.6 Application of seaweed

Seaweed is mainly consumed as food for people and many factors are involved in determining the quality of seaweed products. They are texture (hardness), colour, stability during storage and smell (Yamanaka & Akiyama, 1993). Generally, hardness effects the taste of the food and is effected by the cultivation conditions. Customers prefer green color to other colored seaweed and it was found that product of blanching temperature ranged from 80°C to 90°C within 30 to 60 seconds generated the ideal colour (Balbas, 2012). Blanching for a long time and high temperature can result in the chlorophyll being degraded to phaeophytin, which gives a brown colour (Yamanaka & Akiyama, 1993). There are however many different processing methods for seaweed. Among them, the two most common are sun drying and ash-drying (haiboshi). The following is a table from Balbas, which gives the different processing methods for the *Undaria,* giving the various traditional varieties of wakame products (*Table 3*).

Table 3 Different wakame products in Japan (Balbas, 2012)

Wakame Variety	**Process**	**Quality**
Suboshi	Raw → sun-dried	Brownish green High in foreign materials Poor storage quality
Haiboshi	Raw → mixed with ash →sun-dried → washed → sun-dried	Fresh green High in foreign materials Poor storage quality
Salted	Raw → salted → dehydrated → midrib removed → visual selection → packaging	Brownish green
Boiled and salted	Raw → boiled → cooled → salted → dehydrated → midrib removed → visual	Fresh green

	selection → packaging	
Dried cut	Boiled and salted → sifted → washed → dehydrated → cut → washed → dehydrated → salt removed → dried via rolling dryer → mechanical selection → visual check → metal detection → packaging	Fresh green Low in foreign materials Good storage quality

Seaweed is an important material for industry, as well. Various seaweed species are used to produce hydrocolloids like agar, carrageenan, and alginate. Further, alginate fiber is extracted from the brown seaweed *Sargassum confusum*. The seaweed derived hydrocolloids have many diverse applications such as ice cream stabilisers, dental impression materials, and textile sizes (Tseng, 2001).

1.6.1 Pharmaceutical Uses

Undaria has recognized pharmaceutical uses in many different fields, mainly as an iodine supplement, neuroprotective products and drugs.

1.6.1.1 Supplement of iodine

Iodine is an important element in the human diet. The iodine containing thyroid hormones are essential metabolism regulators. Marine algae are typically rich in iodine, especially in *Laminaria* species (Madhusudan, Manaj, Rahul, & Rishi, 2011). Recently, the Chinese government recommended the use of the *Laminaria* species to instead of the *Sargassum* species as a raw material for the production of alginate. As a result of this change, it has relieved chronic iodine deficiency for almost its 40% population (Tseng, 2001).

1.6.1.2 Neuroprotective products

Many algae have been regarded as neuroprotective agents, which have potentially therapeutic function for the nervous system disease such as neuroinflammation. For example, one marine algae called *Ulva conglobata* is used as a treatment for murine hippocamal and microglial cells. Brown algae is full of PUFA with the ratio 1:1 between omega-6 and omega-3, which are considered very possibly to prevent inflammatory, cardiovascular and nervous system disorders (van Ginneken, Helsper, de Visser, van Keulen, & Branderburg, 2011).

1.6.1.3 Drugs

Because of the compounds with functional groups contained in algae, many drugs can be made of them.. For instance, due to the sulfated polyanions present in *Undaria*, it has been suggested that these are very likely to be the basis of some of the reported therapeutic effects of *Undaria* (Fitton, 2003).

However, the most important bioactive compound contained in *Undaria* is thought to be the polysaccharide fucoidin. Fucoidin has many reported functions such as anticoagulant, antithrombotic, antivirus, antitumor, immunomodulatory, antioxidant, and anti-inflammatory (B. Li, Lu, F., Wei, X., & Zhao, R, 2008). For example, fucoxanthin and fucoidan found in brown algae behaves as an antioxidant and may have anti-cancer properties (Terasaki et al., 2009) and decrease potential carcinogens (Gudiel-Urbano & Goni, 2002).

While there is little scientific evidence for their efficacy, seaweeds are also used widely for cosmetic purposes such as a facial mask, face-washing cream and marine mud (Fitton & Irhimeh, 2008). These creams and masks are also believed to have the ability to relieve rheumatic pain and eliminate cellulite (Fitton, 2003). The hydrocolloids in seaweeds are claimed have the ability to recover the elasticity and suppleness of the skin, and act as a moisturisers (Bang et al., 2011). *Undaria* is also

believed to contain anti-obesity compounds (J. H. Choi, Kim, Kim, Kim, & Kim, 1999)

1.6.2 Agricultural Uses

Seaweeds can be used as good materials for agriculture. They have been applied as fertilizers, animal foods and soil conditioners . There is a long tradition in many countries of using seaweeds, especially the brown algae to fertilize land. Seaweed species such as *Ascophyllum*, *Ecklonia* and *Fucus* are commonly applied as soil fertilzer and soil conditioner, because seaweed is rich in nitrogen and potassium.

Moreover, large amounts of insoluble carbohydrates in brown seaweeds make it a soil conditioner which improves soil structure and keeps essential moisture retention properties.

1.6.3 Human consumption of seaweeds

Because of its beneficial nutrients, seaweed consumption in Asian countries especially in China, Japan and Korea is very large (FAO, 2011). Since the beginning of the history, seaweed has already been thought as a kind of vegetable food for people and animals in those countries (Honya, Kinoshita, Ishikawa, Mori, & Nisizawa, 1994). More than 1.6 kg of dry weight per capita is consumed annually in Japan (Dawczynski et al., 2007; M. Murata & Nakazoe, 2001).

It should be also pointed out that different part of the seaweed could be eaten in different ways. *Undaria* is commonly used in seaweed salad and miso, while the dried cut seaweed can be eaten with water after rehydrating (Onodera, Yoshie-Stark, & Suzuki, 2008). Cut and dried wakame can be used in instant noodles and the sushi sheet can be made from the midrib part of *Undaria* (Nisizawa, Noda, Kikuchi, & Watanabe, 1987).

Nowadays, lots of consumers are seeking good quality products in terms of consistency and nutritional benefits (Dawczynski et al., 2007). In New Zealand, the consumption of *Undaria* has also increased both because of its pharmaceutical application in terms of potential antiviral properties and nutraceutical value (Plaza, Cifuentes, & Ibáñez, 2008).

1.6.4 Animal Feed

It has been a long time for seaweed has been used as food for animals living in coastal areas in European countries. Nowadays, the consumption of seaweed for animals has increased with the increased of total seaweed production. It is dried and milled to a powder as meal for the animals.

Generally, the seaweed species used for animal feed including *Ascophyllum nodosum (Norway), Laminaria digitata (*France), *Ascophyllum* and *Laminaria (*Iceland), and *Ascophyllum (*the United Kingdom).

1.6.5 Wastewater treatment

Seaweeds have been shown to have the potential ability for the treatment of wastewater for two main areas. One is the ability of control agricultural wastes by removing the nitrogen and phosphorus compounds before these waters are released into rivers. Another is the treatment of heavy metals from industrial wastewater. Both applications are involved the introduction of seaweeds to the sewage. For example, red and brown seaweed are used for the removal of nutrient in wastewater source (Aderhold, 1996).

Eutrophication of waters with nutrients including phosphorus and nitrogen contained compounds and minerals results in certain unexpected results such as the excessive of marine plant growth. Further, this problem can occur naturally, and it becomes more

common nowadays. However, this problem can be resolved by the introduction of sewage effluent into waters, or let the water full of fertilizers.

Interestingly, unlike terrestrial plants, seaweeds prefer to absorb ammonium for their growth, while ammonium is the commonly compounds of nitrogen in most agriculture waste water. In addition, research suggested that seaweed are likely to absorb more phosphorus than they need. As a result, seaweed has the potential ability for the treatment of agriculture wastewater.

The idea about using seaweeds for the accumulation of heavy metals including copper, lead, nickel, zinc and cadmium comes from the observation that large brown seaweed have a surprisingly high heavy metal content. This can be vary due to the different location (how far to the industrial waste outlets). Now, this technique has been successfully applied by using brown seaweed such as *Sargassum* *Laminaria* and *Ecklonia* and the green seaweeds *Ulva* and *Enteromorpha*.

It should be pointed out that both fresh seaweed and seaweed extracts are used for the applications discussed above. For example, an insoluble waste product from brown seaweed extracts has the ability to absorb toxic metals as well (Aderhold, 1996).

Chapter 2 Compounds in *Undaria*

2.1 Lipid

Lipids, as well as proteins and carbohydrate are fundamental components in living organisms. They are essential parts of the cell structure and are used as energy stores. Various classes of lipids are found in plants and plant membranes. In my project, the total lipid content of *Undaria* is investigated and the various lipid fractions are compared in *Undaria*.

Lipids is made up of a group of naturally molecules such as fats, waxes, sterols, fat-soluble vitamins, monoglycerides, diglycerides, triglycerides, phospholipids, and others. Its major biological functions are energy storage, signalling and structure cell membranes (Fahy E, 2009). Lipids can also be applied in various fields such as cosmetic, food industries and nanotechnology (Mashaghi S., 2013).

Generally, lipid can be divided into two groups namely saponifiable lipids and non-saponifiable lipids.

Saponifiable lipids are the lipids that can generate alcohols and acidic compounds by hydrolysed under alkaline or acidic environments (O'Keefe, 2008). They are triglycerides, phospholipids, glycolipids and sphingolipids. (O'Keefe, 2008)

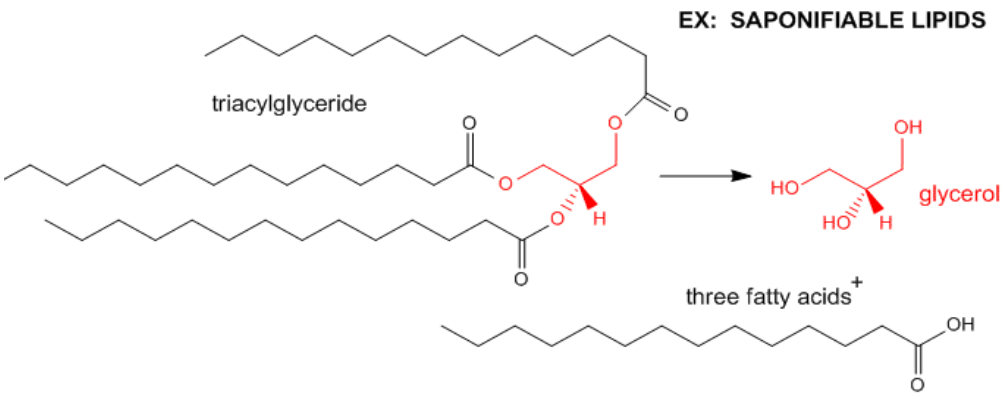

Figure 7 Saponifiable lipids (Koshland, 2000)

In contrast, there are also some non-saponifiable lipids existed. They are sterols, fatty alcohols, carotenoids, phospholipids, vitamin A, D, E and K (Holdt & Kraan, 2011).

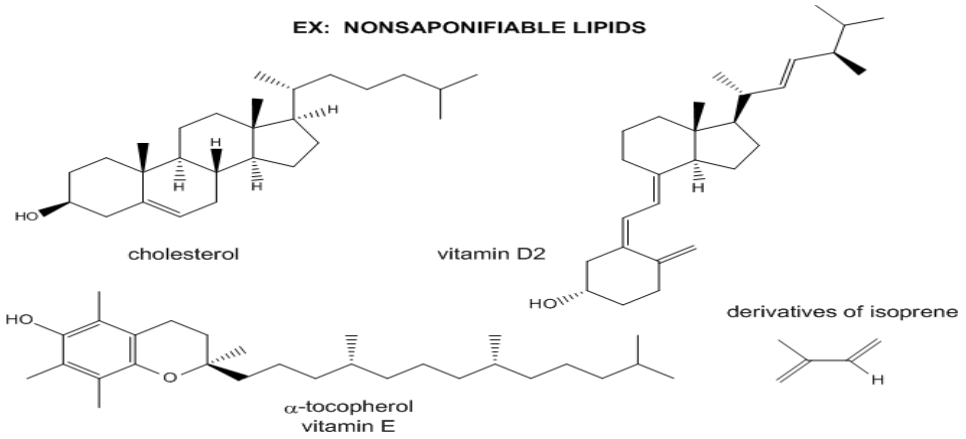

Figure 8 Examples of non-sponifiable lipids (Koshland, 2000)

Although, compared to other plants, the total amounts of lipids in marine algae are relatively low. Nevertheless, these lipids are equally very important because they are the important constituents of omega-3 fatty acids, phytosterol and fat soluble-vitamins.

There is still confusion in the literature about the lipid composition in seaweeds (Bhaskar, 2004). On one hand, phospholipids were found as the major component in seaweed (M. Murata, & Nakazoe, J, 2001), while the other research demonstrates that compared to neutral and phospholipids, the glycolipids are the major lipid class

2.2 Lipids other than fatty acid glycerides.

2.2.1 Phospholipids

Phospholipids are the major component of all animal and vegetable cell membranes as they can form lipid bilayers (Holdt & Kraan, 2011). The common structure of most phospholipids contains a diglyceride, a phosphate group, plus a simple organic

molecule such as choline (Mashaghi S., 2013). The chemical characteristics of phospholipids are hydrophilic and hydrophobic, which means it can be dissolved in fat and water. The major responsibility of phospholipids in all cells is for material transportation and steadying the molecular structure (Heyden, 2006). The phospholipids content in different seaweeds ranges from 10% to 20% of the total lipid content (Holdt & Kraan, 2011).

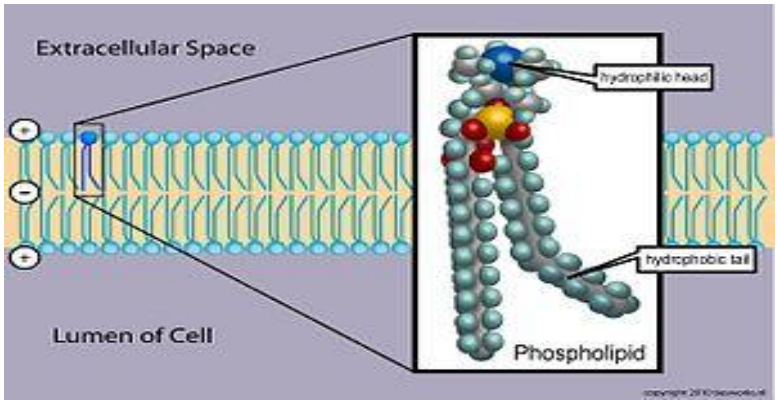

Figure 9 Chemical structure of phospholipid

2.2.2 Glycolipids

Glycolipids are named according to their chemical structure: practically all glycolipids are derivatives of ceramides (GLmetab, 2007). They are the lipids with a carbohydrate attached, whose function is for the energy supplement and serves as markers for cellular recognition . Glycolipids can also act as receptors at the surface of cells, which serve as markers for the identification of cells, an example is the identification of human blood types. There are many different types of glycolipids such as glyceroglycolipids, glycosphingolipids, sulfatides, gangliosides, globosides and glycosylphosphatidylinositols. Among them the most important one is cerebroside which is found in the human brain and nerve tissues. Its major function is supplement of proper conduction of a nervous impulse. It can be significantly different for the glycolipids components in different algae. For example, in brown seaweed, the content of monoglycosyldiacylglycerol (MGDG) ranges from 26% to

47%, while the diglycosyldiacylglycerol (DGDG) ones range from 20% to 44%, of total glycolipids (Dembitsky, 1990).

2.3 Fatty acids

The chemical structure of a fatty acid is a carboxylic acid plus a long aliphatic tail (chain). It is defined by length of chain, the configuration and position of double bonds and the other function groups. The two major classes of fatty acids are saturated fatty acids, with no double bonds and unsaturated fatty acids with one or more double bonds. Fatty acids with three or more double bonds are commonly referred to as polyunsaturated fatty acids (PUFAs).

Different plant species contain different fatty acids. For example, the major component of fatty acids in oil seeds are C-16 and C-18 carbon fatty acids, while algae contains mainly saturated and unsaturated14-22 carbon fatty acids (Holdt & Kraan, 2011).

2.3.1 Unsaturated fatty acids

In general, because of its commercial value, people are more interested in unsaturated fatty acids than saturated fatty acids. According to Herbreteau et al. (1997), *Underia* contains relatively high levels of PUFAs with the C18:1o9 and C20:4o6.

As is discussed before, as well as fish oils, long-chain polyunsaturated fatty acids (LC-PUFAs) are also found in algae (Fleurence, Gutbier, Mabeau, & Leray, 1994). The constituent of these LC-PUFAs including omega-6 and omega-3, and are considered as important nutrition for human beings. From the previous research, the main PUFAs in *Undaria* are listed in the following *(Table 4)*.

Table 4 Main PUFA found in U. pinnatifida (Boulom, 2012)

Structure	**Name**
C18:2n-6	linoleic acid
C18:3n-6	γ-linolenic acid
C18:3n-3	α-linolenic acid
C18:4n-3	stearidonic acid
C20:4n-6	Arachidonic acid
C20:3n-5	Eicosapentaenoic acid

In addition, PUFA seems to have the ability to reduce inflammation, prevent cancer, obesity and cardiovascular disease

2.3.2 Essential fatty acids

Essential fatty acids (EFAs) are fatty acids that humans cannot synthesize, so they can only be obtained from diet. They are necessary for the human body in many different ways such as repair of the skin, blood vessels, and nervous system. They build, maintain, and repair cell membranes. Without them, there will be troubles in liver and kidneys and the absence of blood supplement. The omega fatty acids contribute to maintaining a fully-functioning immune system. Without enough of these fatty acids, unwanted results such as heart disease, stroke and the atherosclerosis can increase (Shils, 1980).

For example, the long chain omega-3 PUFA arachidonic acid (AA) and eicosapentaenoic acid (EPA) above are thought to be the most important supplements for children’s growth

The two most important essential fatty acids are alpha-linolenic acid (an omega-3 fatty acid) and linoleic acid (an omega-6 fatty acid) (Burr, 1930). Other fatty acids that considered as "conditionally essential" are gamma-linolenic acid (an omega-6 fatty acid), lauric acid (a saturated fatty acid), and palmitoleic acid (a monounsaturated fatty acid) (Mary, 2005).

2.4 Sterols

Sterols, or steroid alcohols, are a subgroup of the steroids and is an important class of organic molecules. They are hydroxylated steroid alcohols with a hydroxyl typically in the 3 position on the A ring. (Parish, Li, & Bell, 2008). A general structure of a sterol is shown in *Figure 10*. They exist widely in natural plants (campesterol, sitosterol, and stigmasterol), animals (cholesterols), and fungi (ergosterol). Probably the most common sterols we familiar are cholesterols found in animal cells and skin oils. It is extremely important for the cellular function (Lampe, 1983).

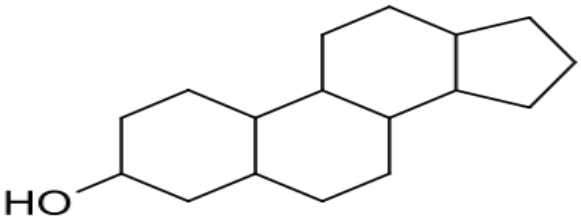

Figure 10 Chemical structure of sterol

However, it should be pointed out that high cholesterol levels in the blood can result in several diseases such as angina, coronary heart disease, heart attack, and stroke.

2.4.1 Phytosterols

Phytosterols is a generic term for sterols found in plants (Piironen, Toivo, Puupponen-Pimia, & Lampi, 2003). 24-methylenecholesterol and fucosterol are two common phytosterols. The structure of phytosterols is similar to that of cholesterol.

The functions or roles they play in a plant include control the fluidity and permeability of cell membranes, help plant growth and help with the synthesis of many secondary plant metabolites and being a precursor of compounds involved in secondary plant metabolites (Piironen et al., 2003). Further, the mixture of sterols and proteins can play essential functions in plant cells such as enzymes, receptors and signal transduction components (Piironen et al., 2003).

Phytosterols in algae or seaweeds can be vary. There are seven phytosterol compounds have been already identified in seaweed. Work by Nobuo Ikekawa suggested that cholesterol, 24-methylene-cholesterol and fucosterol can be found in *Phaeophyta* (brown algae), while cholesterol, brassicasterol and β-sitosterol can be found in *Chlorophyta* (green algae). In addition, Heilbron reported that fucosterol and chondrillasterol are widely distributed in *Phaeophyceae* (marine brown algae), *Chlorophyceae* (green algae) and some *Rhodophyceae* (red algae). Generally, the main phytosterols in brown seaweeds are fucosterol and fucosterol derivatives. It accounts over 88% of total unsaponifiable lipid fractions (Sánchez-Machado, López-Cervantes, López-Hernández, & Paseiro-Losada, 2004b).

Some researchers have also indicated that the phytosterols might be able to block cholesterol absorption sites in human body. This is because the absorption of phytosterols in human diets can result in the increase of hydrophobicity, which will redistribute the equilibrium between phytosterol and micelles, leading to the decrease cholesterol absorption (Lagarda, Garcia-Llatas, & Farre, 2006). For example, daily intake of 2–3 g of phytosterols has been reported to reduce LDL-cholesterol levels by 9% to 20%, with considerable individual variability (Lichtenstein, 2000).

As a result, phytosterols are currently used as a food additive in United States for the reduction of cholesterol, but some concerns have risen that it is possible for the phytosterols to block other nutrients as well (Ostlund RE Jr, 2002).

Another paper byYankah (2006) reported that fucosterol found in different algal species including *Undaria* has the potential ability for anti-diabetic, anti-osteoporotic and anti-oxidant. It was demonstrated that fucosterol can increase the anti-oxidative enzymes such as superoxide dismutase, catalase and glutathione peroxidise, which have the function of oxygen detoxification and can possibly reduce the risk of chronic diseases caused by free radicals (Lee, Lee, Jung, Kang, & Shin, 2003).

2.5 Importance of lipids and fatty acids for human health

Lipids are the main and basic ingredient of foods, and are considered important for humans in many different fields. In general, they play an important role for providing both energy and essential lipid supplements (Umass, 2010).

As discussed above, long chain fatty acids, particularly LC-PUFAs and n-3 PUFAs can bring several health benefits and biological effects for human and animal. The health effects of transport long chain unsaturated and essential fatty acids include adverse effects on lipid risk factors for heart disease, and have ability of anticancer, anti-obesity and antioxidant (Y. Li & Watkins, 2006).

However, over-consumption of lipids and fatty acids are thought not be very good for health. For example, the westerners consume more omega-6 than omega-3 (a ratio of between 15:1 and 17:1). Many diseases including cancer, inflammatory and autoimmune diseases can be the result of such an excess of n-6. There is still a doubt about the ideal ratio of n-6/n-3 essential fatty acids for people intake. According to

the European Nutritional Societies, a 5:1 ratio of n-6/n-3 will be good for people (Simopoulos, 2008), while the World Health Organization (WHO) argued that it is healthy for the ratio of n-6/n-3to be under 10 (Sanchez-Machado, 2004).

Chapter.3 Literature review of lipid and fatty acid analysis

3.1 Lipid analysis method

Lipids are organic-soluble substances with varying solubility in different solvent systems, thus it is necessary for us to choose the right solvents for the extraction. Generally, lipids in plants can be divided into three groups namely triglycerides, phospholipids and glycolipids. Among them, triglycerides are generally the least polar lipids, while the other two groups are generally more polar lipids.

Many different extraction methods have been used for lipid analysis. Although a lot of extraction methods have been regarded as "effective" or "useful" for the analysis of lipids, the most popular one is a modified Folch's procedure of Bligh and Dyer (1959). The traditional Bligh and Dyer procedure uses chloroform/methanol based procedures that include phase partitioning into the organic layer. The improved Folch method includes two solvent extractions. The sample is first extracted with a mixture of methanol/chloroform/water (2:1:1 v/v/v), and then it was re-extracted again with a mixture of methanol/chloroform/water (1:1:1 v/v/v) (Bligh & Dyer, 1959). The lipid mixture is extracted into the bottom layer, which is mostly chloroform . Another very popular extraction method is soxhlet extraction. This procedure removes lipids from samples by refluxing them with specific solvents in a few hours. Then the lipid content was collected by evaporating the solvent.

Although there are various methods have been recommended for lipid analysis, it is still necessary for us to find the most accurate one. According to of Seog June Lee (1998), many different extraction methods and solvent systems have been compared. From *Figure 11* it is clear that the most effective extraction method is bead-beater method, while the most effective solvent system is chloroform/methanol with a 1:1 ratio (*Figure 12*). In *Undaria*, total lipid content was found to vary from 1 to 6.4 g/100g (Dawczynski et al., 2007; Fleurence et al., 1994).

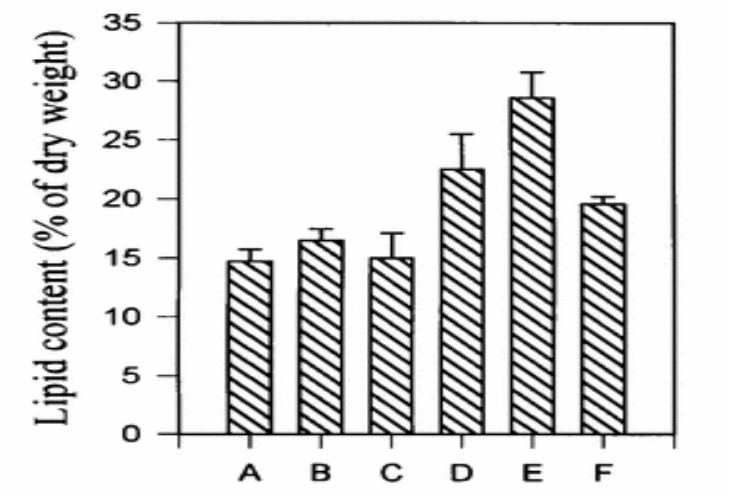

Figure 11 Comparison of extraction methods. Data are represented as mean S.D. A, direct extraction; B, sonication; C, homogenization; D, French press; E, nead-beater extraction; F, lyophilization

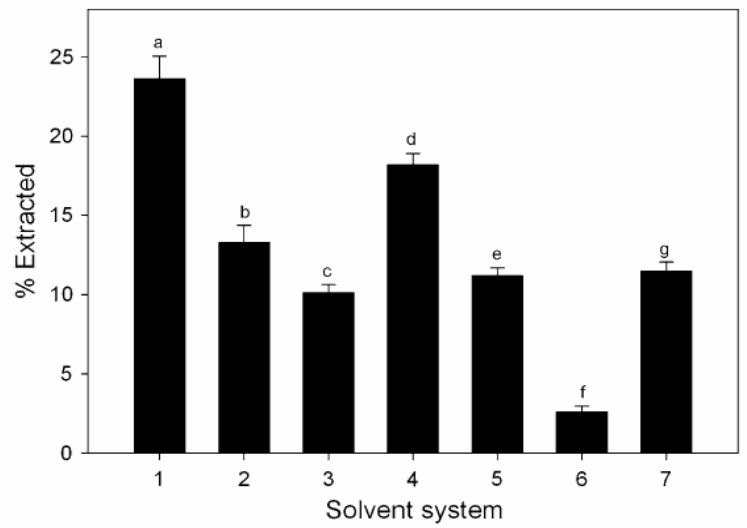

Figure 12 Comparison of solvent system on the total lipid recovery (mean ± SD; n = 3; total of three extractions) from C. vulgaris. Solvent systems: 1 chloroform–methanol 1:1, 2 dichloromethane-ethanol 1:1, 3 hexane–isopropanol 3:2, 4 chloroform–methanol 2:1, 5 acetone, 6 diethyl ether, 7 methyl-tert-butyl ether–methanol 10:3. a, b show significance of difference (Ryckebosch, 2012)

3.2 GC and HPLC analysis for lipid and fatty acids

3.2.1 Gas Chromatography

The working principle of *gas chromatography* (GC) is a form of partition chromatography where the stationary phase is a immobile, usually siloxane, polymer

bound to the inside of a very small diameter quartz tube (called a column for historical reasons) and the mobile phase is an inert gas. The column is kept in a heated oven. A sample is injected into the gas phase where it is volatilised and partitions with the bound phase as it flows through the column. Different compounds spend different times in the mobile phase and the stationary phase, depending on their relative affinities for the latter, and exit the column after different times. These compounds are detected by some means which converts the concentration of the component in the gas phase into an electrical signal, which is amplified and passed to a continuous recorder, so that the progress of the separation can be monitored and quantified. In these laboratories we use either the simple, reliable, flame ionisation detection (FID) or if more information is needed mass spectrometer detection (MS), see below for more discussion on MS.

3.2.2 High Performance Liquid Chromatography

In general, the technique of GC (gas chromatography) and HPLC (high performance liquid chromatography) are almost same and for many analyses can be used interchangeably. They are both techniques for the identification and determination of the component contained in samples quantitatively and qualitatively. Although, the idea is same, it is still some differences between these two methods.

In HPLC, the mobile phase is a liquid solvent mixture such as acetonitrile and water, and as a result, HPLC is commonly used for less volatile sample analysis, or samples that are thermally labile and GC is more commonly used for the more volatile samples. In HPLC, columns are usually kept at a stable temperature typically room temperature.

3.2.3 Mass spectrometric Detection

Both techniques can be coupled to a mass spectrometer as a detector. There are many types of mass spectrometers (MS). The simplest is one equipped with 70 eV

electron ionisations and a single quadrupole ion analyser. This yields classic mass spectra which can be interpreted and molecules identified using well established techniques and libraries. The laboratory has a Thermo GC-MS system and an Argilent LC-MS system.

The LC-MS uses a classic MS/MS arrangement where ions are generated with a soft ionisation technique (electrospray), the ions are scanned and selected by the first quadruple, passed through a gas collision cell and analysed by the second quadruple. This technique yields low resolution (m/z resolution about 0.1) molecular ions. Caution needs to be used in interpreting results but they are rich in information.

3.2.3.1 Lipid analysis by UPLC with charged surface hybrid technology

Ultra Performance Liquid Chromatography (UPLC) with Charged Surface Hybrid (CSH) C_{18} Technology is a more suitable for lipid analysis. When combined with mass spectrometry (MS), especially MS/MS techniques can provide extensive information. In general, UPLC is fast and gives high resolution separation of lipids. It provides an attractive solution for analyzing complex lipids samples. The following is an example chromatogram of the liver lipid samples. The lipids can be detected in the positive ion mode, while some fatty acids are better detected in negative ion mode. As a result, both positive and negative ions are detected in this project.

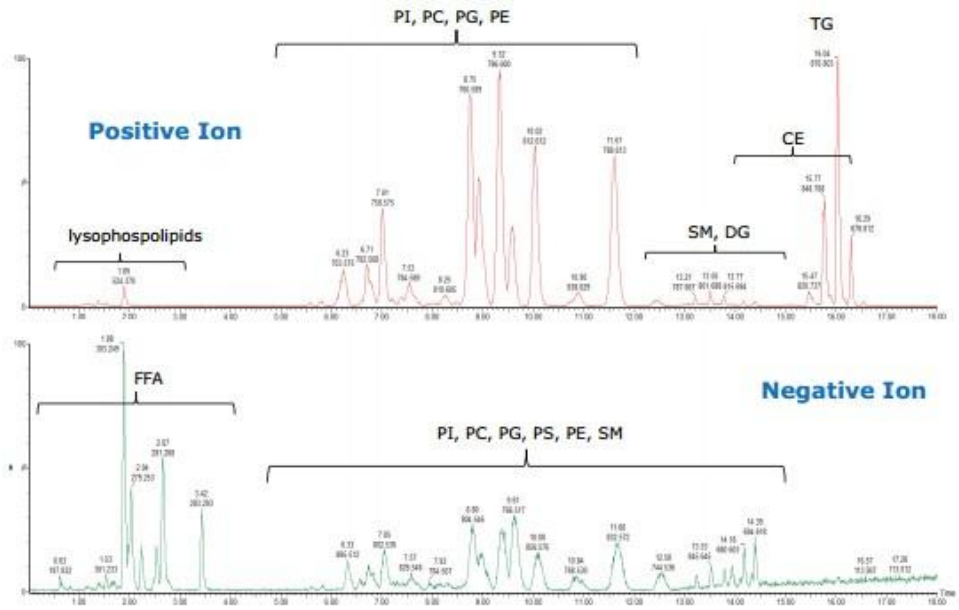

Figure 13 Total bovine liver extract acquired in both positive and negative ionziation modes

3.3 Fatty acid analysis method

In general, the typical fatty acid analysis can be concluded into three steps. At first they are extracted from food and plants by various methods. Then, fat is extracted into ether, followed by methylated to fatty acid methyl esters (FAME). These FAMEs are sufficiently stable and volatile to be analysed quantitatively by gas chromatography and compared to authentic standard mixtures.

Among these steps, probably the most important one is the esterification which results in the formation of the FAME. This reaction *(Figure 14*) is done in either acidic direct transesterification, alkaline hydrolysis followed by acidic esterification or direct alkaline transesterification. This breaks the glyceride esters into individual methyl esters. (Meier, Mjøs, Joensen, & Grahl-Nielsen, 2006). The reaction can also be achieved with the help of enzymes (biocatalysts) particularly lipases.

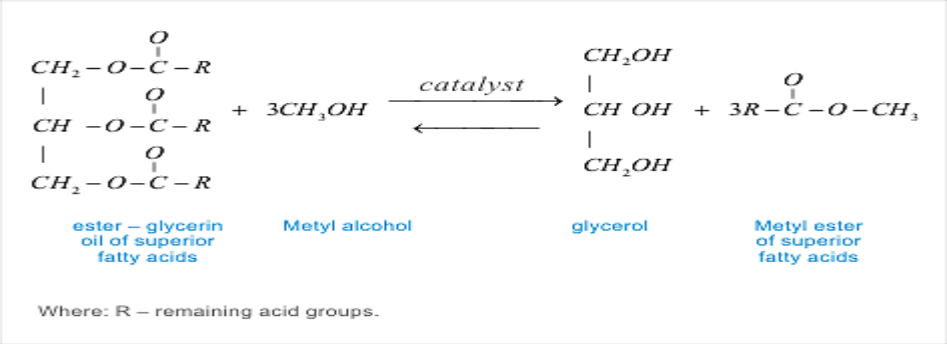

Figure 14 Mechanism of transterification (Meier & Joensen , 2006)

Many reagents including both methanolic (*Figure 15*) and alkaline *(Figure 16*) are applied for the preparation of fatty acid methyl esters (FAME). They are BF_3 (10% - 14%, 1 h, 80° C); HCl (0.5 M, 1 h, 80° C); BCl_3 (10%, 1 h, 80° C) from acidic catalysts and KOH (0.2 M, 15-60 min, 50° C); $NaOCH_3$ (0.5 M, 15-60 min, 50° C) from alkaline catalysts.

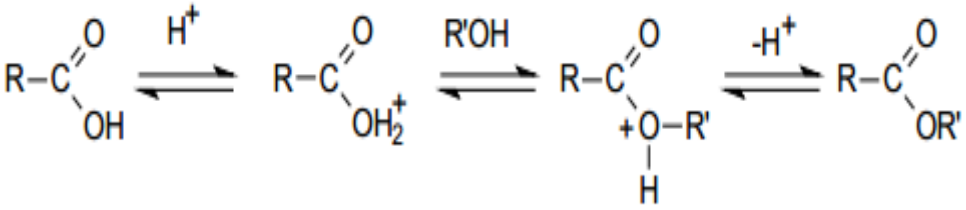

Figure 15 Reaction in acidic condition

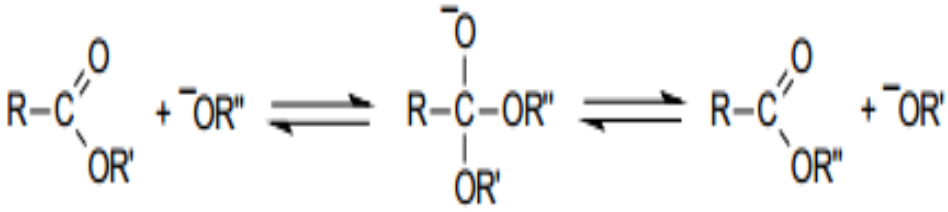

Figure 16 Reaction in alkaline condition

Each method has its advantages and disadvantages. As a result, it is necessary to choose a right reagent for the right target. For example, according to the research of C.M. Murrieta (2003), it is suggested that the alkaline catalysts should be used for

transesterification of muscle lipids in order to maintain the existence of conjugated linoleic acid (CLA), a conjugated fatty acid. However, these catalysts are not suitable for the lipids found in sphingolipids (Christie, 1990).

Methanolic BF_3 and BCl_3 can rapidly and cleanly form FAMEs, but these were no used in my work, because it may results in a decrease of PUFA (de La Cruz, Lopez Hernandez, & Simal Lozano, 2000). In contrast, it is suggested that the Methanolic HCl method has the ability to get more saturated fatty acids (SFA), monounsaturated fatty acids and polyunsaturated fatty acids (PUFA). In addition, it is economy cheap and chemical stable. As a result, methanolic HCl method was choose in this study, since it combines two outstanding factors: fast, convenient process and the prevention of the loss of necessary compounds.

3.3.1 Quantification of fatty acid profile

The calculation formula used is from Boulom's research:

$$\mathbf{Ci} = \frac{\mathbf{Pi} \times \mathbf{Cis} \times \mathbf{L} \times \mathbf{100}}{\mathbf{Pis} \times \mathbf{W}}$$

Ci : concentration of individual analyte (g/100g DW)

Cis : Concentration of internal standard (mg/mL)

L : total reagents (mL)

Pis : peak area of internal standard

Pi : peak area of analyte

W: sample weight (g)

3.4 Sterol analysis methods

In general, the analysis of sterols needs several key factors. These are extraction, isolation, separation, purification, and detection (Wrolstad et al., 2005). Sterols have been isolated from plant tissues or oilseeds by various methods followed by a variety

of clean-up and chromatographic procedures including column chromatography (Ce), gas chromatography (GC), thin-layer chromatography (TLC), normal phase high-performance liquid chromatography (HPLC), reversed-phase HPLC and capillary electro-chromatography (CEC), which are finally detected by GC, GC-MS and LC-MS (S.L. Abidi, 2001).

3.4.1 Isolation and enrichment procedures

The separation and isolation technique can be vary among the different nature of the sample source. Solvent extraction including chloroform-methanol, hexane, methylene chloride and acetone is commonly used to isolate plant sterols, which is usually followed by saponification and chromatographic purification for obtaining enriched total sterols. Generally, in a typical saponification experiment, the plant oil sample was mixed with 1 M ethanolic potassium hydroxide for overnight, the mixture was diluted with water and extracted with three portions of diethyl ether. Finally, the sample was washed with distilled water many times. This removal of solvent yields an non-saponifiable residue suitable for the further chromatographic quantification of sterols.

3.4.2 Purification techniques

Many purification techniques have been used for the final isolation/separation of sterols. Among all these techniques, column chromatography (CC) and thin layer chromatography (TLC) techniques are still the most accessible and affordable. It is Samples more than 200 mg in size can be cleaned by column chromatography, while sample under 200 mg can be conveniently purified by thin layer chromatography (S. L Abidi, 2001)

3.5 Solid-phase extraction for the analysis of lipid classes

In many experiments, before the quantitative determination can be done, it is necessary for us to isolate the sample from its interfering compounds. Thus, in the lipid analysis, liquid-liquid or liquid-solid extractions are basic techniques to separating different lipid classes found in oil or fats.

Solid-phase extraction (SPE) is a an extraction method whose working principle is similarly compared with high-performance liquid chromatography (HPLC) (Tippins, 1987). It was firstly invented in the 1970s, and in 1978 Waters promoted the Set-Pak cartridges, a widely used silica-based column (Mills, 1998). This has many advantages for lipid separations such as simple, quick, compact and relatively inexpensive. This means SPE can be applied for many different aims. For example, it is an effect method for sample purification, sample separation and removal of reagent excess (Wachob, 1991).

The choice of phase depends on what the sample is and what your objective is. Here is a detailed properties of the applications in SPE.

Table 5 Various properties of applications in SPE

	Separation mode	Phase		Properties
Normal silica phase	Normal phase	Silica	-Si-OH	The support is polar and the matrix non-polar; thus, non-polar compounds pass through the cartridge depending on the eluent used
Modified silica phases	Normal or reverse phase	NH (aminopropyl)	$-Si-(CH_2)_3NH_2$	Moderately polar, they are used as an alternative to silica
		CN (cyanopropyl)	$-Si-(CH_3)(CH_2)_3=CN$	
		Diol (2,3-dihydroxypropoxypropyl)	$-Si-(CH_2)_3OCH_2CH(OH)CH_2OH$	
	Reverse phase	C_{18}(octadecyl)	$-Si-C_{18}H_{37}$	It has strong hydrophobicity and used to adsorb analytes from aqueous solutions
		C_8(octyl)	$-Si-C_8H_{17}$	It has moderate hydrophobicity and retains less than C_{18}
		C_2(ethyl)	$-Si-C_2H_5$	They have low hydrophobic character
		Phenyl	–Si–Ph	
	Ion exchange	Quaternary amine	$-Si-(CH_2)_3N^+(CH_3)_3$	Strong anion exchanger used to extract compounds capable of carrying a negative charge
		Propylbenzenesulfonate	$-Si-(CH_2)_3C_6H_4-SO_3$	Strong cation exchanger used to extract positively charged basic compounds
		Propylsulfonate	$-Si-(CH_2)_3SO_3$	

Generally, for the separation of non-polar and polar lipid fractions, silica gel is the most common choice. A variety of solvent systems are also involved for fractionating non-polar and polar lipids. For example, Prieto applied a single Si phase column and 17 solvent mixtures to separate the lipid factions from wheat flour ((J.A. Prieto, 1992). In addition, Bhaskar used chloroform (1:10 w/v of lipid), acetone-methanol (9:1 v/v; 1:15 w/v of lipids) and methanol (1:10 w/v of lipid) to get neutral-lipid, glycol-lipid and phosphor-lipid, respectively.

Chapter.4 Methods and Materials

4.1 Samples

Both fresh and stored one year *Undaria* are used in this project.

Generally, the stale *Undaria* was collected from mussel farms in South Island in New Zealand, and it was already been dried as powders, which is stored very well. This *Undaria* was mainly used for the method development.

The fresh sample was collected from Great Barrier Island. It was stored in the fridge at first, and was dried in the both freeze-dry and oven-dry for half a week or longer. The dried sample was then milled to powder as well. This fresh *Undaria* was the main sample used in all the experiments. Because of the different location and freshness between these two *Undraia*, a comparison of FAME content was also processed in this thesis.

4.2 Materials

HPLC or GC-grade chloroform, methanol, dichloromethane, acetone, hexane, isopropanol, ethanol and 2-ethoxyethanol were purchased from Sigma-Aldrich. For fatty acid analysis, tridecanoic acid (internal standard) and toluene are ordered from Sigma-Aldrich, while methanolic HCl and K_2CO_3 solution are prepared in laboratory. PTFE filters were obtained from Waters Corp.

4.2.1 Analytical Instruments

For the FAME analysis, a gas chromatograph with the flame ionisation detector (FID) (Shimadzu GC-2010) was used. The capillary column was a Zebron *ZB-Wax* capillary column (30 m x 0.25 mm x 0.25 μm) from Phenomenex. The GC conditions were: Carrier gas was nitrogen of 20 cm/sec. Temperature programme: 100 ºC to 250 ºC at 5ºC/min and hold for 20 min. The injection was split mode with a 20:1 split and 3 mL/min septum purge.

For the lipid extract analysis using a Thermo Trace GC Ultra coupled to the mass selective detector Thermo TRACE DSQ quadripole. The instrument was controlled by the *Xcalibur* software. The mass range of GC-MS was set between 50 and 500. The column of GC was a Phemomenex Zebron ZB-5 0.25 mm inner diameter and 0.25 µm film thickness. The temperature program is set up at 180^{o}C for 3 minutes with the increase of 6^{o}C/min to 245^{o}C followed by 3^{o}C /min to 275^{o}C and hold for 14 minutes. The temperature of injector was 290^{o}C and the transfer line 300^{o}C respectively.

The Ultra Performance Liquid Chromatography (UPLC) used an Agilent 6420 series LC-Triple quad MS coupled to 1200 series LC system with 2.0 kV for positive ions and 1.0 kV for negative ions. The column was a Charged Surface Hybrid ACQUITY UPLC CSH C_{18} 2.1 x 100 mm, 1.7 µm. The mobile phases were Acetonitrile/water (60:40) with 10 mM of ammonium formate and 0.1% formic acid (A phase) and Isopropanol/acetonitrile (90:10) with 10 mM ammonium formate and 0.1% formic acid (B phase) (*Table 6*). The HPLC column temperature was kept at 55^{o}C and a 400 µL/min flow rate. The injection volume was 5 µL.

Table 6 HPLC Gradient

Time	Water %	MeCN %	IPA %
0	50	50	0
2	10	90	0
10	5	0	95

MeCN: Acetonitrile/water (60:40) with 10 mM ammonium formate and 0.1% formic acid, IPA: Isopropanol/acetonitrile (90:10) with 10 mM ammonium formate and 0.1% formic acid

4.3 Comparison of different extraction methods on total lipid extraction

The amount of extracted total lipid is highly dependant on the solvent mixture used. Usually, a solvent system contains a polar and non-polar solvent results in good extraction of lipids. The function of the polar solvent is to release the lipids from their protein-lipid complexes, and then those lipids were gradually dissolved in the non-polar solvent phase (Spanner.S, 1973).

Initially, three extraction methods have tested and compared todecide on a standard method in this project. They are soxhlet extraction, modified Bligh and Dyer (mB/D) extraction and direct extraction.

4.3.1 Soxhlet extraction

About 5 g *Undaria* powder was refluxed 2.5 hours in a soxhletor with the 70 mL chloroform-methanol (1:1) solvent. The solution is collected after reflux and the extract was removed by nitrogen steam under normal temperature. Then the amount of crude lipid extract was determined by weight difference.

4.3.2 Modified bligh and dyer (mB/D) extraction

Samples were firstly dried and approximately 3 g placed in a separatory funnel with the solvent system. A single phase solvent system is prepared by 35 mL chloroform, 70 mL methanol and 28 mL 50 mM phosphate buffer solution (pH 7.4). The sample was shaken in this solvent for 15 minutes and it was left to stand for 24 hours for the emulsion to separate. After that, 35 mL chloroform and 35 mL distilled water was added, shaken and left for about 16 hours to separate. The bottom layer, the chloroform phase was removed, filtered through a Whatman No. 1 filter paper then dried under a stream of nitrogen. The weight of the Lipid was then determined.

4.3.3 Direct extraction

Approximate 1 g of freeze dried *Undaria* powder was placed in round bottom flask and heated with 75 mL chloroform-methanol (1:1) at 80^{o}C for three hours. The mixture was then placed in ultrasonic bath machine at 50^{o}C for 30 min to remove the air bubbles. After that, the solution was put into the centrifuge with 3000 rpm for 10 min and the upper phase collected, dried by nitrogen steam and the weight determined.

4.4 Comparison of different solvent systems on total lipid extraction

Due to the extraction efficiency of solvent mixtures, it is extremely important to choose suitable solvent systems for lipid extraction in algae. Although some references have suggested the best solvent systems for the lipid extraction in algae, however it is still necessary for me to prove the authenticity of that story. In this part, 4 different solvent systems namely, chloroform-methanol (1:1), hexane-isopropanol (3:2), dichloromethane-ethanol (1:1) and 2-ethoxyethanol have been tested with a typical soxhlet extraction method. Samples were taken approximate 5 g and the final results were determined gravity. In order to ensure the accuracy, each experiment has been processed three times.

4.5 Comparison of different samples (pre-treatments) on total lipid extraction

Different pre-treatments can also result in the different lipid contents. In this part, 4 different pre-treatments are compared. Solvent system used is chloroform-methanol (1:1). Soxlhet method is used for solvent extraction, and each extraction lasts for 2.5 hours. The samples compared are listed below:

- Directly analysis on wet-sub fresh samples
- Analysis on freeze-dried fresh samples (-20°C)
- Analysis on oven-dried fresh samples (60°C and 100°C)
- Analysis on oven-dried samples which have been stored for a year
- Analysis on *fresh Undaria* sporophyll

4.6 Lipid fraction analysis

In this part, a silica solid phase extraction (SPE) was performed to analyze the lipid fraction from different samples (mentioned in 3.3).

Briefly, certain silica gel (approximate 500 mg) are weighted and transferred into a long Pasteur pipette (with the help of glass gel). Then, this SPE column was rinsed by a portion of chloroform (approximate 10 mL). Let the chloroform level to fall down the top of silica gel. The lipid sample was weighted about 10 mg and was mixed with 1 mL chloroform and then it will be moved into SPE column. Non-polar lipids would be generated by elution with chloroform, while polar lipids including phosphild and glycoplipid would be yielded by elution with methanol and acetone separately.

In general, each elution can be confirmed by spotting on the thin-layer chromatography (TLC). Spots can be visualized through the use of rhodamine 6G solution in ethanol (0.5 g/L).

4.7 Fatty acid profile analysis

An approximate 25 mg of sample was weighed ± 0.1 mg and was placed into a 10 mL test tube. Then a 10 µl volume of a 2g solution of tridecanoic acid was added as an internal standard before the addition of a further 490 µl of toluene and 750 µl of freshly prepared 5% methanolic HCl. The mixture was mixed on a vortex for a while, after that the headspace of each tube was filled with nitrogen followed by the heating at 70 for two hours in water bath. Tubes were then cooled to the room temperature and 1 mL of 6% aqueous K_2CO_3 and 500 µl of toluene were added and mixed on vortex. The mixture was centrifuged at 3000 g for 5 minutes and the organic phase was removed with a glass Pasteur pipette for further analysis in gas chromatraphy (GC).

4.8 Sterol profile analysis

This analysis can be divided into two parts, one is the chromatogram comparison between the *Undaria* extract and cholesterol standard, another is the identification of sterol in *Undaria.*

For the first experiment, the total seaweed extract were compared with a standard cholesterol solution by using the GC-FID under the same GC conditions. Basically, the cholesterol solution was prepared by approximate 3 g cholesterol powder dissolved in 10 mL chloroform. The column of GC was a Zebron ZB-5 0.25 mm inner diameter and 0.25 μm film thickness of Phemomenex. The temperature program is set up at 180 ^{o}C for 3 minutes with the increase of 6 ^{o}C per minute to 245*C followed by 3 ^{o}C per minute to 275 ^{o}C and keep it for 14 minutes. The temperature of injector and detector are also changed to 290 ^{o}C and 300 ^{o}C respectively.

For the second experiment, approximate 1 g dried *Undaria* sample was extracted by 27 mL of ethanolic KOH solution. Then the mixture was put in the water bath for 30 min at 80^{o}C. After cooling to the room temperature, the mixture was filtered with Whatman No1 paper, and 20 mL of hexane and Milli Q water were added to the mixture. This mixture was then centrifuged at 3000 rpm for 5 minutes, and the phytosterols were extracted into hexane (the top layer). It should be pointed out that the GC conditions for the identification of non-saponifiable fractions is a bit different compared with the former one. The temperature program is set up as same as the lipid extract one.

4.9 Statistical analysis

Where possible experiments were repeated in triplicates, The standard deviation (means ± SD, n=3) was used to express the difference between results.

Chapter.5 Results and Discussion

5.1 Comparison of different solvent systems on lipid extraction

Table 7 Total lipid content of different solvent systems

	Total lipid content
Chloroform-methanol (1:1)	1.18%
Hexane-isopropanol (3:2)	0.36%
Dichloromethane-ethanol (1:1)	0.45%
2-Ethoxyethanol	0.27%
Chloroform-methanol (2:1)	0.87%

A soxhlext method was applied to compare the different solvent systems. The total lipid content *(Table 7)* was listed above by the percentage of the dry weight.

Sample used was stored and collected in last two years and was oven dried and homogenised. It is clearly that chloroform-methanol (1:1) gave the highest lipid recovery, while the 2-Ethoxyethanol resulted in the lowest. Chloroform-methanol (1:2) gave the only 73.5% total lipid recovery of the lipids extracted by 1:1 mixture. This result corresponds with the result of Eline Ryckebosch (Ryckebosch, 2012) and it proves that the solvent ratio does have some significant effect on lipid recovery.

Thus, the chloroform-methanol (1:1) was used for further analyses.

5.2 Comparison of different extraction methods on lipid

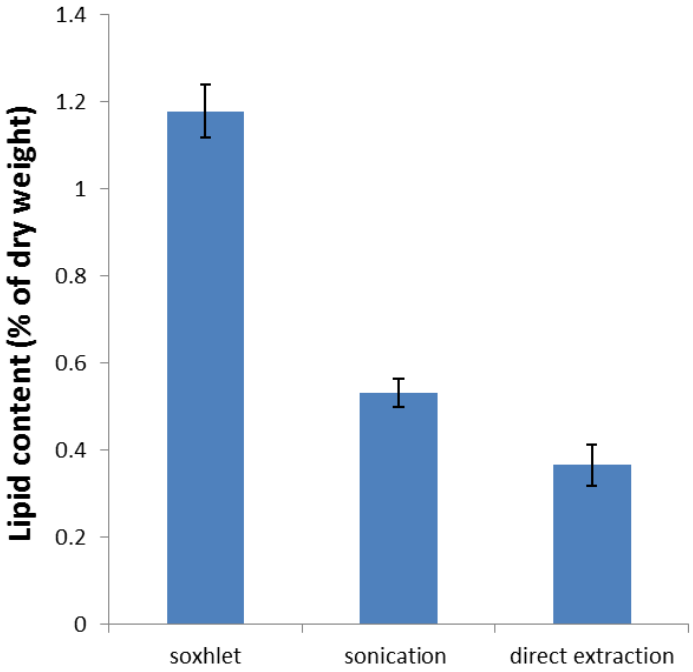

Figure 17 Comparison of different extraction methods on lipid extraction from Undaria (mean SD; n=3; total of three extractions)

In this part, three extraction methods were compared. Solvent systems used in this comparison was chloroform-methanol (1:1) and samples used in these experiments are freeze-dried fresh *Undaria.*

According to *Figure 17*, it is clear that the soxhlet method increases the lipid recovery compared to the other two methods. It is up to 1.2% lipid content, approximately two times compared with others. In addition, it seems that sonication did not give a better lipid recovery in comparison to direct extraction. However, it should be pointed out that soxhlet may cause partial hydrolysis and pre-esterification. But, because of the significantly better lipid yield, the soxhlet method was used for further analysis.

5.3 Comparison of different pre-treatments on lipid extraction

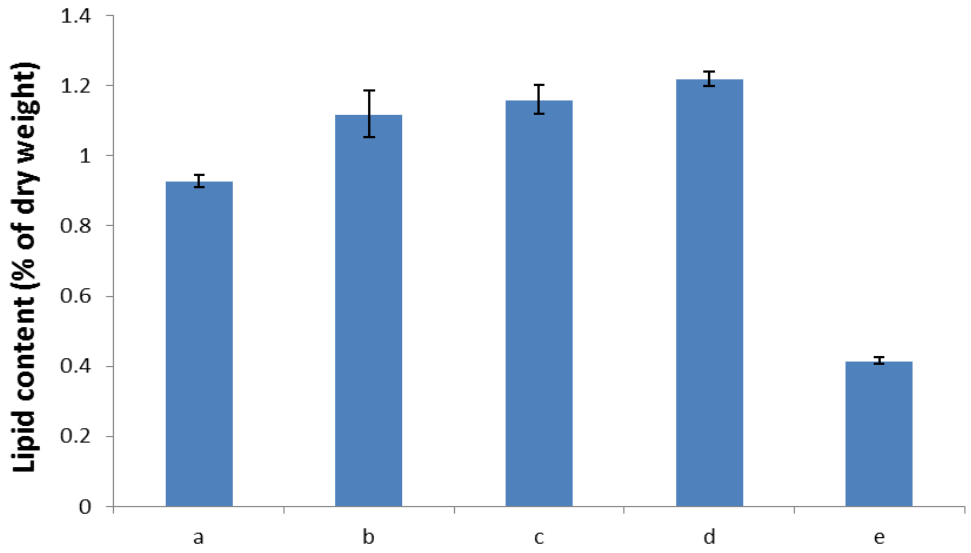

Figure 18 Comparison of different pre-treatments on lipid extraction (mean SD; n=3; total of three extractions) from Undaria. Pre-treatments: a, oven-dried stored sample; b, oven-dried fresh sample at 100 **°C***; c, oven-dried fresh sample at 60* **°C***; d, freeze-dried .fresh sample at -20* **°C**

A large quantity of frozen fresh and dried *Undaria* was available. In this part, five different pre-treatments namely, oven-dried and stored, oven-dried at 100^{o}C, oven-dried at 60^{o}C, freeze-dried at -20^{o}C and direct extraction on wet sample were compared; solvent systems and extraction methods were used as mentioned above (soxhlet method). Each analysis was replicated three times, and the results was converted into dry weight and averaged (expect wet-sub sample).

According to *Figure 18*, the wet sample yields the lowest lipid, while freeze-dried method gives the best lipid recovery (1.22%), which is very close to other research. (N.Bhasrak, 2004) Both sides are reasonable because the water might be contained in the web-sub sample, thus it increases the total weight, results in the decrease of total lipid weight.

The lipids may subject to a thermal degradation under high temperature, which may leads to the decrease of lipid amount. In other words, the freeze-dry method do not

has such a disadvantage, so it is expected to yield higher lipid content than other technique.

Nevertheless, the oven-dry methods seems can be a useful and simple method as well. There is no significant difference among these three samples (b,c,d) in terms of the standard deviation and lipid amount, and the different temperature between 60 and 100 do not make any sense, either.

5.4 Lipid fraction analysis on different samples

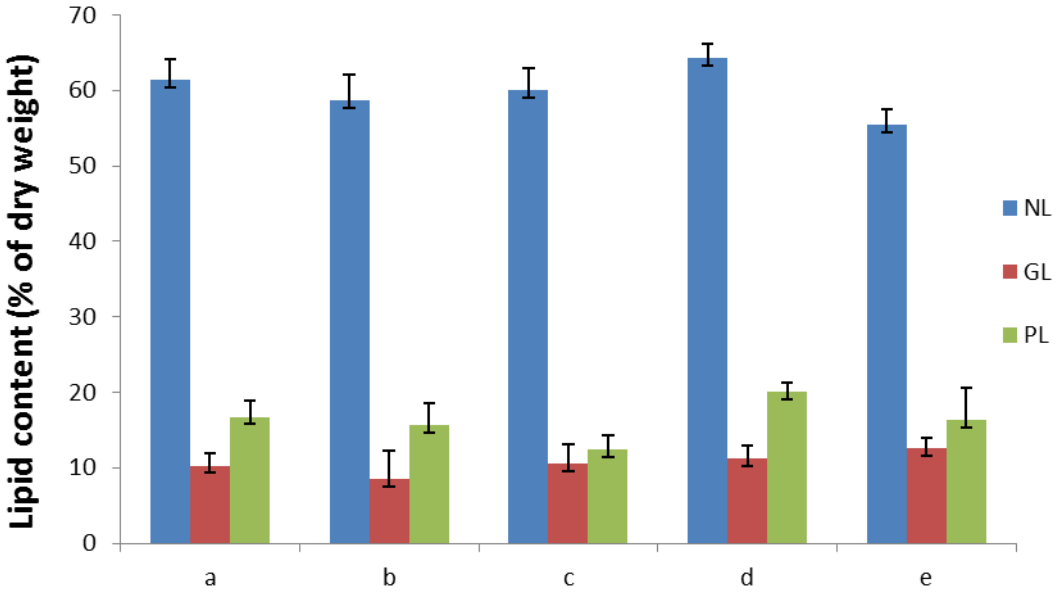

Figure 19 Lipid fraction analysis on different samples (mean SD; n=3; total of three extractions) from Undaria. NL= non-polar lipid GL= glycolipids PL= phospholipids

The lipid fraction was analysed and compared among many different samples in terms of pre-treatments. Solvent systems and extraction methods were used as mentioned above. All the experiments were repeated three times, and the results were determined by weight.

In general, there is no significant difference among these five samples (*Figure 19*). It is obviously that non-polar lipids are the major component of lipid fraction in

seaweed. This result might cause controversy, because it is not corresponding with the result of either Holdt & Kraan's or Murata & Nakazoe's. However, it should be pointed out that the researches of Eline Ryckebosch (2012) and James B (1988) observed the same results. The amount of non-polar lipids occupied approximate 65% in previous, which is very close to mine. It is also found that the amount of phospholipids is a bit more than the glycolipid one in their researches,

On the other hand, this experiment is not a complicated one which requires very simple instrument. In other words, some results have higher standard deviation which means it is possible that the results found in this experiment might not be very accurate. Because, the identification of the different lipids is based on the observation of thin-layer chromatography (TLC) and measurement of volume, some error might be caused during these processes. However, this method is easy for operating and if necessary it should be completed more in further research.

5.5 Lipid content comparison of blade and sporophyll

It is interested that compared with blade, sporophyll is also applied in commercial markets in Asian countries. As a result, a comparison of total lipid extraction on blade and sporophyll, and the lipid fraction analysis on these two samples are processed. Samples were oven dried at 60 ^{o}C for 24 hours and homogliled. The solvent systems and method used were mentioned above.

Table 8 Comparison of total lipid content

	Total lipid content (by weight)
Sporophyll	2.34%
Blade	1.53%

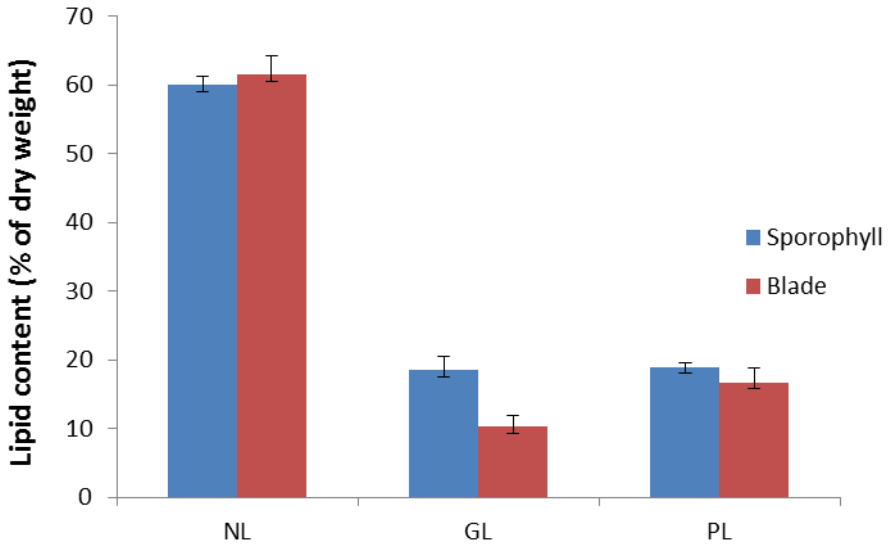

Figure 20 Lipid fraction comparison of blade and sporophyll (mean SD; n=3; total of three extractions) a:sporophyll b: blade NL = non-polar lipid GL = glycolipids PL = phospholipids

It is clearly that sprophyoll generated significant higher amount (almost doubles) of lipid than the blade did *(Table 8).* This result should be reasonable, because many previous researches suggested that *Undaria* can store more energy as lipid form in sporophyll for reproduction (Sánchez-Machado, López-Cervantes, López-Hernández, & Paseiro-Losada, 2004a). In addition, it was also suggested that total lipid contents in *U. pinnatifida* is ranged between 10.5 (Sánchez-Machado et al., 2004a) and 45 mg/g of blade (Dawczynski et al., 2007), which is also close to my result.

According to the *Figure 20*, it is similarly in comparison with the lipid fractions between these two samples. The standard deviations are quiet small, which means there was no significant difference among these results.

5.6 Fatty acid profile analysis on different samples

The analysis of fatty acid profile is done by the comparison of FAMEs standards bought from Sigma Company. Unknown compounds are compared by the retention time from known FAME mixed standards.

5.6.1 Comparison of fatty acid profile on different samples

Table 9 Comparison of fatty acid profile on three different samples by GC-FID

Freeze-dried blade	Sporophyll	Oven-dried blade
capric	capric	capric
nd	caprylic	nd
tridecanoic	tridecanoic	tridecanoic
myristic	myristic	myristic
myristoleic	nd	nd
palmitic	palmitic	palmitic
nd	nd	palmitoleic
cis-10- heptadecenoic	nd	nd
stearic	stearic	stearic
linoleic	linoleic	linoleic
oleic/elaidic	oleic/elaidic	oleic/elaidic
gamma-linolenic	gamma-linolenic	gamma-linolenic
alpha-linolenic	alpha-linolenic	alpha-linolenic
arachidic	arachidic	arachidic
cis-11-eicosenoic	nd	nd
cis-8, 11,14-eicosadienoic	cis-8, 11,14-eicosadienoic	cis-8, 11,14-eicosadienoic
arachidonic	arachidonic	arachidonic

cis-5,8,11,14,17-eicosapentaenoic	nd	nd
henicosanoic	henicosanoic	henicosanoic
nd	erucic	nd
cis-13,16-docosadienoic	nd	nd
tricosanoic	nd	nd
nervonic	nd	nd

nd: not detected

In this *Table 9*, tridecanoic acid is the internal standard. It is clear that the freeze-dried sample contains more fatty acids than other samples. High temperature might result in some thermal reaction, which means it is possible to lose certain fatty acids. However, it should be pointed out that samples under higher temperature also contain some specific fatty acids, whereas these fatty acids are not observed in freeze-dried samples. This is a quite interesting phenomena, and it can be explained that the fatty acids found in high temperature might happen some bio-reaction. For example, palmitoleic acid found in oven-dried blade is biosynthesized from palmitic acid by the action of the enzyme delta-9 desaturase (Zhi-Hong Yang*, 2010).

By the way, the sporophyll sample also contains two unique fatty acids namely caprylic acid and erucic acid.

In order to ensure the accuracy of the FAME experiment, an identification of fatty acid from NZ *Undaria* by O'Keefe is used and compared. Here is the list of fatty acids found in his project *(Table 10).*

Table 10 Identification of fatty acid in edible NZ Undaria according to retention time by O'Keefe

Peak	Common name	Systematic name*	Fatty acid	RT
1	Capric acid	Decanoic acid	C10	6.980
2	Lauric acid	Dodecanoic acid	C12	11.123
3	Myristic acid	Tetradecanoic acid	C14	16.566
4	Myristoleic acid	Cis-9-tetradecenoic acid	C14:1	18.258
5	Palmitic acid	Hexadecanoic acid	C16	22.299
6	Palmitoleic acid	Cis-9-hexadecenoic acid	C16:1	22.628
7	Margaric acid	Heptadecanoic acid	C17	23.717
8	No trivial name	Cis-10-heptadecenoic acid	C17:1	25.759
9	Stearic acid	Octadecanoic acid	C18	27.600
10	Oleic acid	Cis-9-octadecanoic acid	C18:1n-9c	28.319
11	Linoleic acid	9,12 octadecadienoic acid	C18:2n-6c	29.549
12	γ-linolenic acid	6,9,12-Octadecatrienoic	C18:3n-6	30.357
13	α-linolenic acid	9,12,15-octadecatrienoic acid	C18:3n-3	31.229
14	Stearidonic acid	Octadeca-6,9,12,15-tetraenoic acid	C18:4n-3	32.145
15	Arachidic acid	Eicosanoic acid	C20	32.930
16	Dihomo-g-linolenic	Cis-8, 11, 14-eicosatrienoic acid	C20:3n-6	35.313
17	Arachidonic acid	5,8,11,14- Eicosatetraenoic acid	C20:4n-6	36.129
18	Eicosapentaenoic acid	Cis-5,8,11,14,17-Eicosapentaenic acid	C20:5n-3	37.858

*RT: Retention time, *:(O'Keefe, 2008)*

If we compare these two results, it is clear that most fatty acids identified by O'Keefe are also confirmed in my project. However, there is still a slight difference between both. For example, gondoic acid, nervonic acid and docosadienoic acid are found in one or two of my samples while they are not confirmed by other research. Anyway, a

detailed table of unique identified fatty acids found in my project with their systematic name and retention time is shown in *Table 11*.

Table 11 Identification of unique fatty acid in edible NZ Undaria according to retention time

Peak	Common name	Systematic name*	Structure
1	caprylic	octanoic acid	C8
2	heneicosylic	henicosanoic acid	C21
3	docosadienoic	all-cis-13,16-docosadienoic acid	C22:2 (n-6)
4	tricosanoic	nk	C23
5	nervonic	cis-9-tetradecenoic acid	C24:1
6	cis-11-eicosenoic	gondoic acid	C24:1
7	erucic acid	nk	C22:1(n-9)

nk; not known

5.6.2 Comparison of fatty acid concentration between different pretreatment

Table 12Comparison of fatty acid concentration from different pretreatments

Fatty acid	Oven dried at 60	Freeze dried at -80
capric	0.24 ± 0.04	0.26 ± 0.01
myristic	0.34 ± 0.03	0.27 ± 0.03
palmitic	1.99 ± 0.08	2.04 ± 0.10
stearic	0.26 ± 0.02	0.18± 0.06
arachidic	0.04 ± 0.01	0.06 ± 0.02
henicosanoic	1.84 ± 0.13	1.96 ± 0.08
tricosanoic	nd	0.l3 ± 0.10
∑SFA	**4.71 ± 0.31**	**4.9 ± 0.4**
myristoleic	nd	0.05 ± 0.01
palmitoleic	0.07 ± 0.02	0.07 ± 0.01
cis-10- heptadecenoic	nd	0.13 ± 0.01
oleic/elaidic	0.5 ± 0.09	0.7 ± 0.48

gondoic acid	nd	0.056 ± 0.00
nervonic	nd	0.057 ± 0.00
∑MUFA	**0.57 ± 0.11**	**1.11 ± 0.51**
linoleic	0.60 ± 0.03	0.59 ± 0.04
alpha-linolenic	0.34 ± 0.01	0.23 ± 0.01
gamma-linolenic	1.11 ± 0.11	1.46 ± 0.46
dihomo-g-linolenic	1.72 ± 0.32	1.9 ± 0.25
arachidonic	0.62 ± 0.23	0.51 ±0.21
docosadienoic	nd	0.09 ±0.27
eicosapentaenoic acid	nd	0.06 ±0.08
∑PUFA	**4.39 ± 0.7**	**4.84 ± 1.32**
Total fatty acid	**9.67 ± 1.12**	**10.85 ± 2.23**

Results are given with mean ± standard error (mg/g dry weight, n=3). ∑SFA: sum of saturated fatty acid, ∑MUFA: sum of monounsaturated fatty acid, and ∑PUFA: sum of polyunsaturated fatty acids. FA: fatty acids. n-3: omega-3. n-6: omega-6. nd: not detected.

The results of *Table 12* showed a comparison between oven-dry and freeze-dry method of blade. In general, the concentrations of all the fatty acids in freeze-dry method was higher and presumably represents a better recovery than the oven-dry method does. However it must be noted that this is not a simple factor. For example, the amount of SFA and PUFA of freeze-dry method gives more fatty acids and presumably a slightly better recovery while the concentration of MUFA is significant difference between both.

These results are perhaps not surprising because previous research has already suggested that fatty acid may cause some to thermally decompose during the high temperatures in terms of the drying or extraction, which results in some losses. For example, according to the results, tricosanoic in saturated fatty acid; myristoleic, cis-10- heptadecenoic, gondoic and nervonic acids in monounsaturated fatty acid; docosadienoic and eicosapentaenoic acids in polyunsaturated fatty acids are only

found in samples from the freeze-dry method. As a result, the absence of these fatty acids makes the big difference in total fatty acid concentrations between two samples.

On the other hand, it should be pointed out that polyunsaturated fatty acids and saturated fatty acids are the two major fatty acids found in both samples. They make up almost 80% of all the fatty acid concentration while the monounsaturated fatty acids only account for less than 20%. Palmitic and henicosanoic are the major saturated fatty acids, and gamma-Linolenic and dihomo-g-linolenic are the principal polyunsaturated fatty acids.

5.6.3 Comparison of fatty acid concentration between blade and sporophyll

As discussed in introduction, all parts of algae could be consumed by people. For example, the blade could be used in sushi sheet, while the sporophyll is very popular seaweed product in Korean markets. As a result, it is interesting to compare the fatty acid composition of these two parts of the seaweed.

Table 13 Comparison of fatty acid concentration between different morphological

Fatty acid	Sporophyll	Blade
capric	0.23 ± 0.04	0.26 ± 0.01
caprylic	0.02 ± 0.00	nd
myristic	0.38 ± 0.03	0.27 ± 0.03
palmitic	3.62 ± 0.08	2.04 ± 0.10
stearic	0.40 ± 0.02	0.18± 0.06
arachidic	0.08 ± 0.01	0.06 ± 0.02
henicosanoic	1.18 ± 0.13	1.96 ± 0.08
tricosanoic	nd	0.l3 ± 0.10
∑SFA	**5.91 ± 0.31**	**4.9 ± 0.4**
myristoleic	nd	0.05 ± 0.01

palmitoleic	nd	0.07 ± 0.01
cis-10- heptadecenoic	nd	0.13 ± 0.01
oleic/elaidic	1.50 ± 0.33	0.7 ± 0.28
gondoic acid	0.055 ± 0.01	0.056 ± 0.00
nervonic	nd	0.057 ± 0.00
∑MUFA	**1.555 ± 0.34**	**1.11 ± 0.51**
linoleic	0.56 ± 0.03	0.59 ± 0.04
alpha-linolenic	0.38 ± 0.02	0.23 ± 0.01
gamma-linolenic	1.11 ± 0.13	1.46 ± 0.26
dihomo-g-linolenic	2.08 ± 0.25	1.9 ± 0.15
arachidonic	0.67 ± 0.13	0.51 ±0.11
docosadienoic	nd	0.09 ±0.27
eicosapentaenoic acid	nd	0.06 ±0.08
∑PUFA	**5.0 ± 0.56**	**4.84 ± 1.32**
Total fatty acid	**12.46 ± 1.21**	**10.85 ± 2.23**

Results are given with mean ± standard error (mg/g dry weight, n=3). ∑SFA: sum of saturated fatty acid, ∑MUFA: sum of monounsaturated fatty acid, and ∑PUFA: sum of polyunsaturated fatty acids. FA: fatty acids. nd: not detected

Generally, the concentration of total fatty acid in sporophyll is found much more than Blade's. This result can be illustrated more detailed that the amount of each fatty acid class of blade is less than the sample of sporophyll. Total SFA contents gives the biggest difference (1.01 mg/g), followed by MUFA (0.44 mg/g) and PUFA (0.16 mg/g).

Further, it is quite interesting that more fatty acids found in blade do not make any sense in terms of the total fatty acid amount. It is concluded that seven fatty acids are detected additional in blade. They are tricosanoic in saturated fatty acid; myristoleic, palmitoleic, cis-10- heptadecenoic and nervonic in monounsaturated fatty acid, and docosadienoic and eicosapentaenoic acid in polyunsaturated fatty

acid. In contrast, there is also a unique fatty acid found in sporophyll namely capric acid.

Qualitatively, the major difference between these two samples is the oleic acid in MUFA, whose content is approximately double in sporophyll compared with blade one.

At last, a simple table *(Table 14)* is made in order to compare the difference of the major fatty acids found in both samples.

Table 14 Comparison of major fatty acids between different sample

	SFA	MUFA	PUFA
Major Fatty acids (blade)	palmitic and henicosanoic	/	gamma-linolenic dihomo-g-linolenic
Major Fatty acids (sporophyll)	palmitic and henicosanoic	oleic/elaidic	gamma-linolenic dihomo-g-linolenic

5.6.4 Discussion of FAME results

In these results, the predominant saturated fatty acid is palmitic acid. This is consistent with the findings of other researchers in Japanese *Undaria* (Terasaki et al., 2009). However it was found that different parts of the plant gave the different concentration of palmitic acid. According to the table above, the sporophyll has 3.62 ± 0.08 mg/g palmitic acid (31% of the total fatty acids), while the blade is 2.04 ± 0.10 mg/g (20% of the total fatty acids). Again, result is consistent with the results from other groups which reported concentrations of palmitic acid was between 13.5 and 49.6 % of the total fatty acids (Kim, Dubacq, Thomas, & Giraud, 1996).

For the monounsaturated part, oleic acid was the most abundant one. It accounts 13% of total fatty acids in the sporophyll and 7% in blade. Many other reports also suggested that oleic acid is the major one in monounsaturated class (Cheruvanky & Thummala, 1991; Moser, 2008).

Finally, polyunsaturated fatty acids are an important component of Undaria because of their human nutritional importance, and their high content in *Undraia*. PUFA's were found to be 43% of total fatty acid in the sporophyll and 50% in blade. Gamma-linolenic and dihomo-g-linolenic are the main components. However, it should be pointed out that this result does not agree with Boulum's work (Boulom, 2012). He identified the characteristic polyunsaturated fatty acids as linoleic acid and arachidonic acid.

5.7 Identification of lipid extract of different sample by GC-MS

5.7.1 Identification of blade extract by GC-MS

Table 15 Identification of compounds in blade

	Compounds	CAS.Number
A	2-pentadecanone.6.10.14-trimethyl	502-69-2
B	hexadecanoic acid. methyl ester	112-39-0
C	tetradecanoic acid	124-10-7
D	cholest-5-en-3-ol. 24-propyidene	56362-45-9
E	1-eicosanol	629-96-9
F	24-Methylenecholest-5-en-3b-ol	473-63-5
G	phytol	150-86-7
H	hexadecanoic acid. 2.3-dihydroxypropyl ester	19670-51-0
I	stigamasta-5.24(28)-dien-3-ol	481-14-1

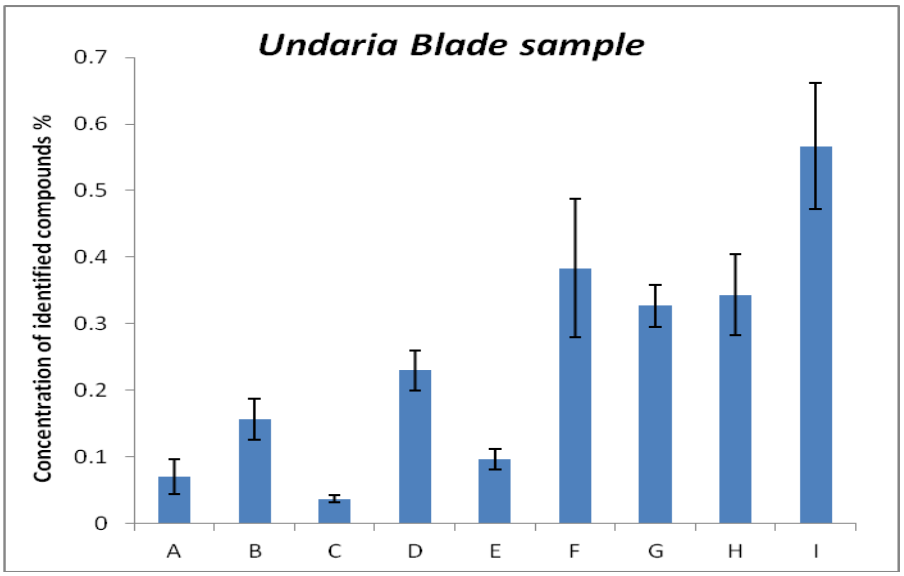

Figure 21 Concentration of identified compounds in blade (mean SD; n=3; total of three extractions)

According to the (*Figure 21*) and (*Table 15*) above, hexadecanoic acid and its methyl ester were found as major fatty acids in *Undaria*, followed by tetradecanoic acid or myristic acid. The same result was also observed by Pakistan (Pakistan, 1987) in two other algae namely *A.dendroides* and *H.porphyroides*. Miristic acid is found in many different algae species such as *Kezuri-konbu*, *Purple laver* and *Green caviar*. It is not surprising to get fatty alcohols (e.g. 1-Eicosanol) in *Undaria*, because the published research by Anitha (Anitha A.S) has already reported that 1-eicosanol along with many other fatty alcohols could be found in *Chaetoceros* (Shaleesha A.Stanley, 2010). Further, two sterol compounds namely cholest-5-en-3-ol 24-propyidene and stigamasta-5.24(28)-dien-3-ol are also observed in the sample extract. This result will be discussed later.

5.7.2 Identification of sporophyll extract by GC-MS

Table 16 Identification of compounds in sporpyhll sample

	Compounds	CAS.Number
A	cholest-5-en-3-ol. 24-propyidene	56362-45-9
B	hexadecanoic acid. Methyl ester	112-39-0
C	phytol	150-86-7
D	hexadecanoic acid. 2.3-dihydroxypropyl ester	19670-51-0
E	stigamasta-5.24(28)-dien-3-ol	481-14-1
F	24-Methylenecholest-5-en-3b-ol	473-63-5

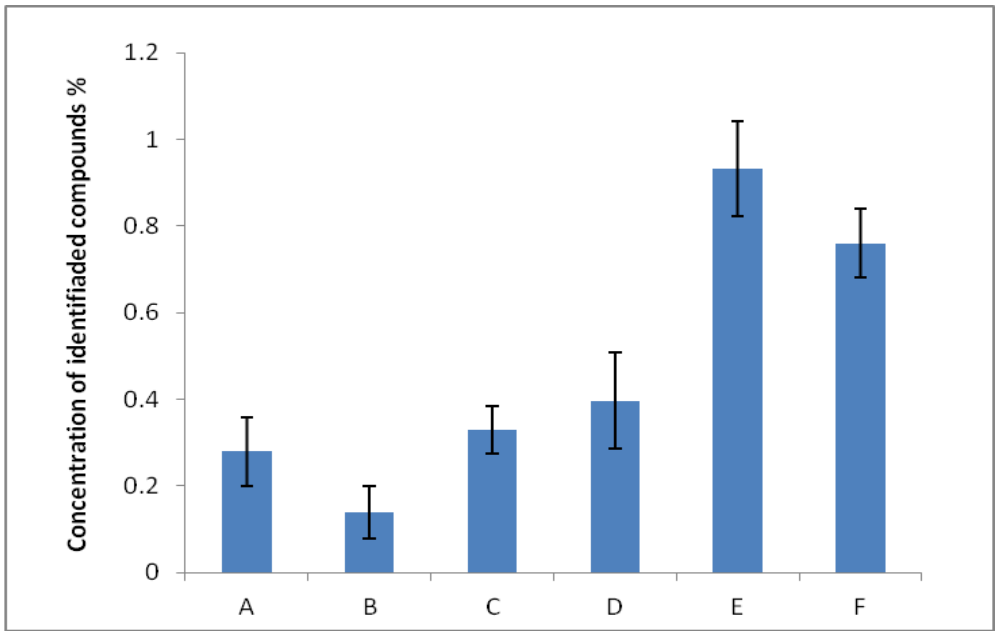

Figure 22 Concentration of identified compounds in sporophyll sample (mean SD; n=3; total of three extractions)

The analysis on sporophyll is also proceeded in order to compare the extract profile with blade. Then from *(Table 16)* and *(Figure 22)* above, the very similarly results are observed and compared with the blade. Almost same compounds have been identified in both sporophyll and blade extracts according to the two pictures above.

Stigamasta-5.24(28)-dien-3-ol is found to be the most abundant compounds in both samples. However, it should be pointed out that the fatty alcohol and Mtristic acid could not been detected in sporophyll sample.

5.8 Comparison of sterol profiles of different samples by GC-MS

It is already proved that sterols could be found in *Undraia.p*. Since, phytosterol is commercially interesting and very popular in Asian markets. Then, a specific analysis on sterol profile in *Undaria* is preceded. *Table 17* shows a comparison of identified sterols between blade and sporopyll samples. From the table, it is clearly that there is no significant difference between these two samples in terms of the quantification.

Table 17 Quantification and qualification of non-saponifiable fractions in different samples by GC

Compounds	CAS.Number	RT.Time (min)	In Blade Con. (mg/g)	In Sporoll Con. (mg/g)
Cholest-5-en-3-ol. 24-propylidene	502-69-2	30.16	0.064 ± 0.03	0.082 ± 0.07
24-Methylenecholest-5-en-3b-ol	473-63-5	27.24	0.123 ± 0.01	0.118 ± 0.04
Stigmasta-5.24(28)-dien-3-ol	56362-45-9	33.16	0.323 ± 0.05	0.387 ± 0.08
Phytol	150-86-7	9.87	0.921 ± 0.06	0.98 ± 0.02

Results are given with mean ± standard error (mg/g dry weight, n=3)

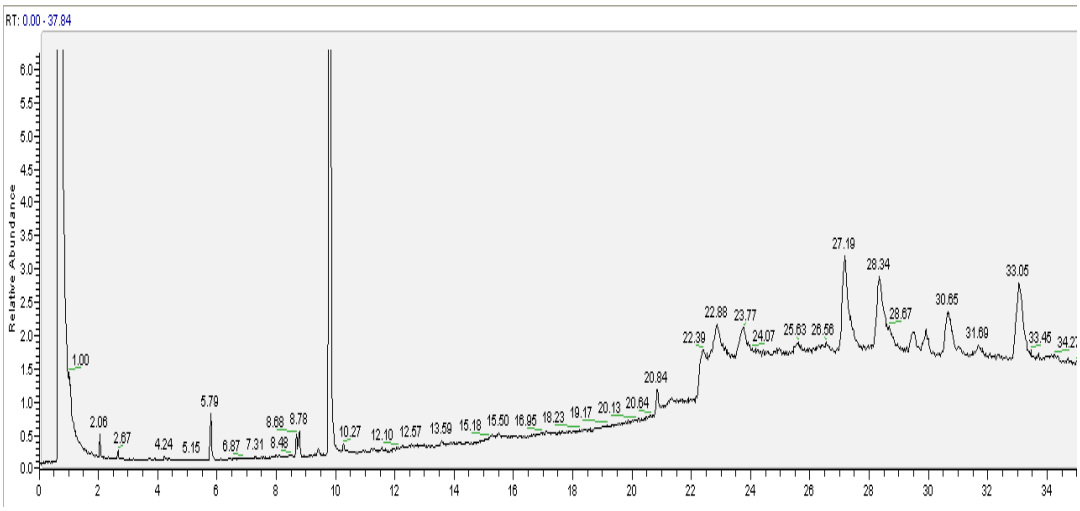

*Figure 23 Chromatogram of **blade** extract on sterol profile by GC-MS*

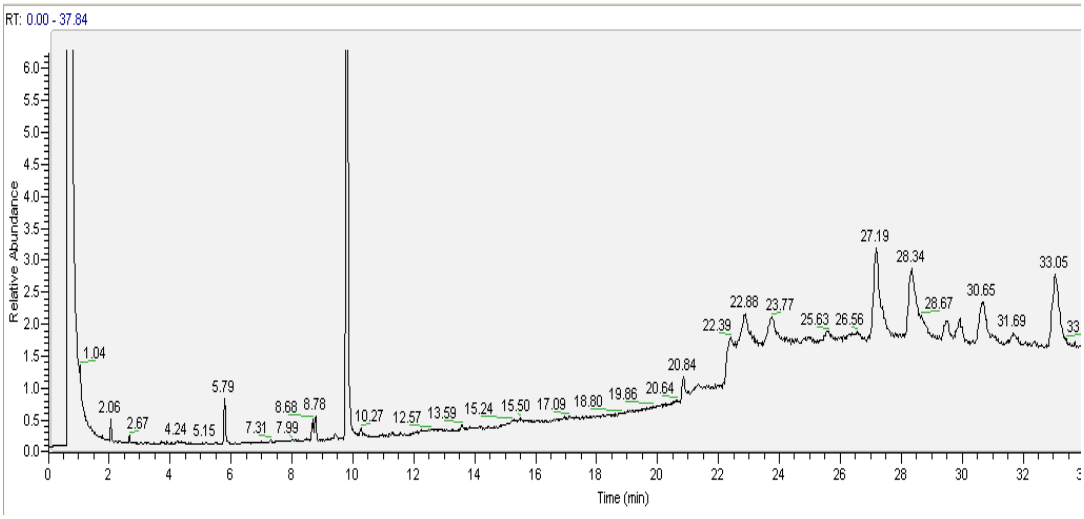

*Figure 24 Chromatogram of **sporophyll** extract on sterol profile by GC-MS*

It is worth noting that identifications using mass spectral libraries can at times be confusing. For example, stigmasta-5.24(28)-dien-3-ol is also called fucosterol, while 24-methylenecholest-5-en-3b-ol is known as 24-methylenecholesterol (Newburger, Uebel, Ikawa, Andersen, & Gagosian, 1979). Once this confusion was clarified, the positive identification of fucosterol and 24-methylenecholesterol proved that fucosterol is the major sterol in *Undaria* samples and the concentration 0.323 mg/g is similar compared to Boulom's project (338 µg/g). It was also found

that the concentration of 24-methylenecholesterol ranged from 8 – 48 µg/g from small size algae to big size algae according to the previous research.

5.9 LC-MS analysis of sample extracts.

Total lipid extracts (fresh) from *Undaria* were analysed by triple quad LC- MS

The triple quad instrument uses a soft electrospray ionisation technique that produces mostly molecular ions with one or more hydrogen ions and at times sodium, potassium and ammonium ions on the charged species (Mazumdar, 2012). These ions are accelerated into the first quadrupole and separated into various "fractions", these fractions pass into a drift cell which contains a low pressure of nitrogen gas. The ions either decompose into smaller fragments on their own account or collide with the gas in the drift cell and transfer extra energy. The products of these interactions then pass into a second analyser and the masses of the products are determined (Volmer, Published online in Wiley InterScience (www.interscience.wiley.com)./2004).

The triple quad can be operated in a number of modes depending on how each analyser is scanned *(Figure 25).*

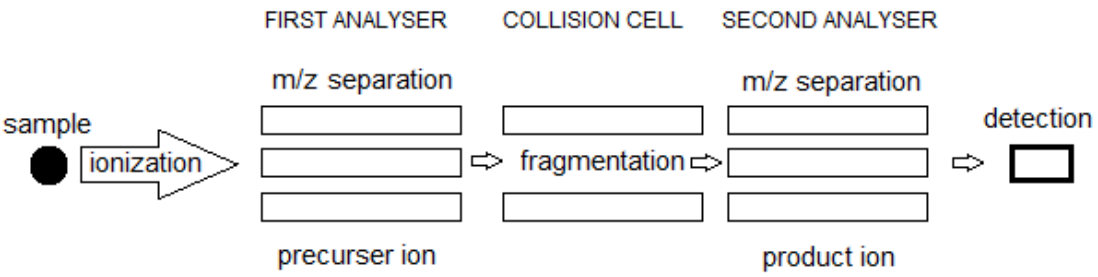

Figure 25 an Agilent 6420 series LC-Triple quad MS coupled to 1200 series LC system

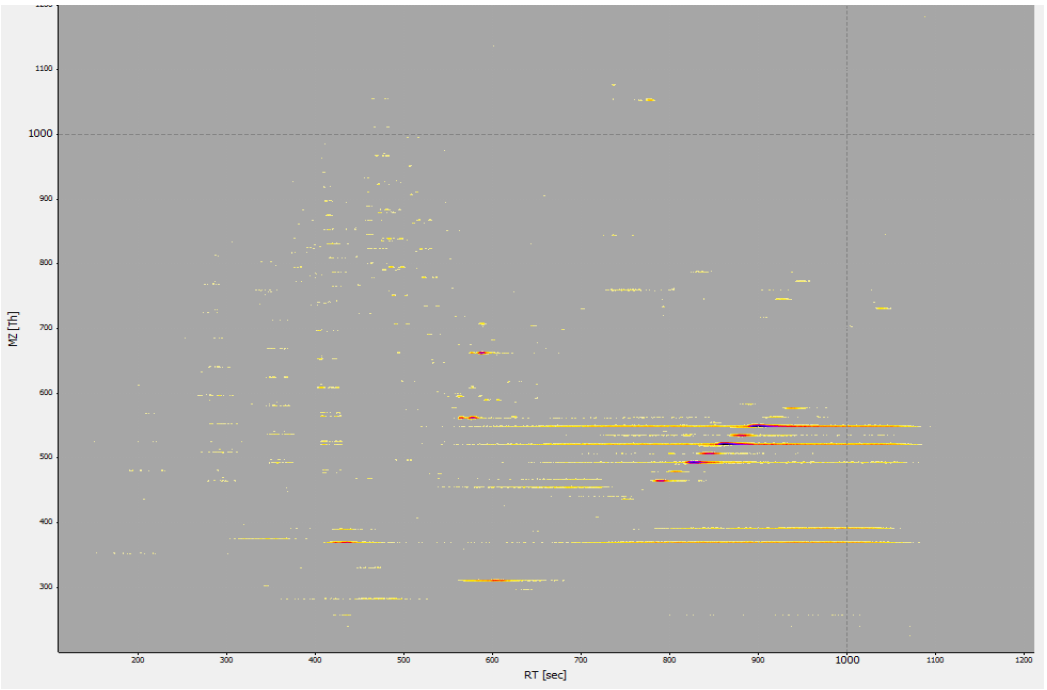

Figure 26 LC-MS result of blank sample on positive mode

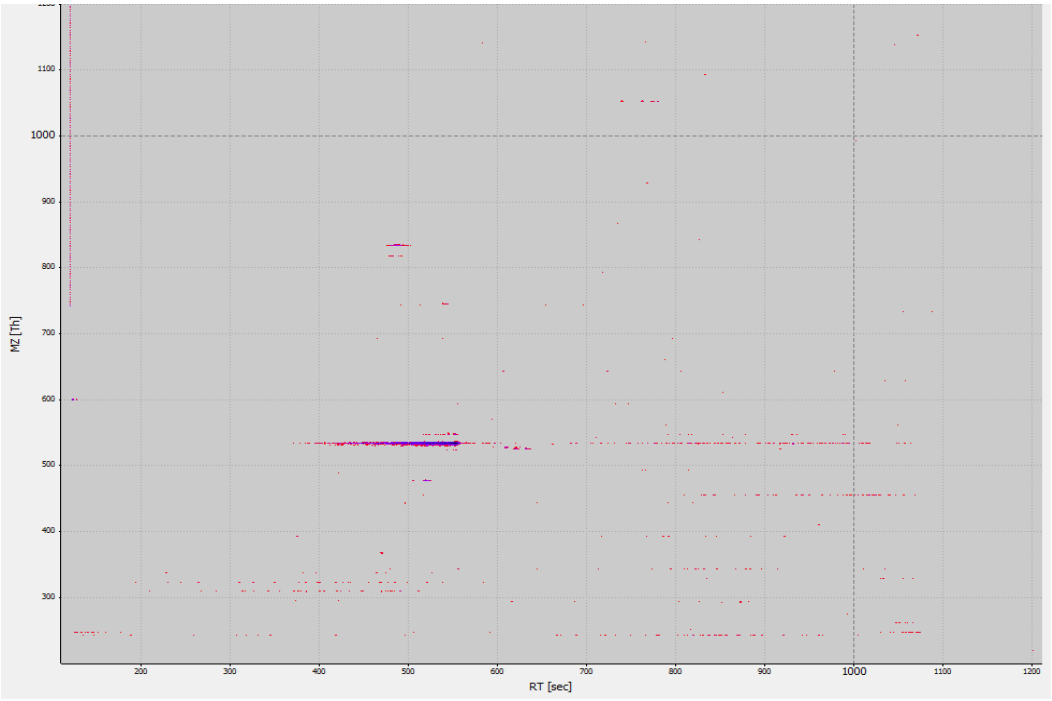

Figure 27 LC-MS result of blank-sample on negative mode

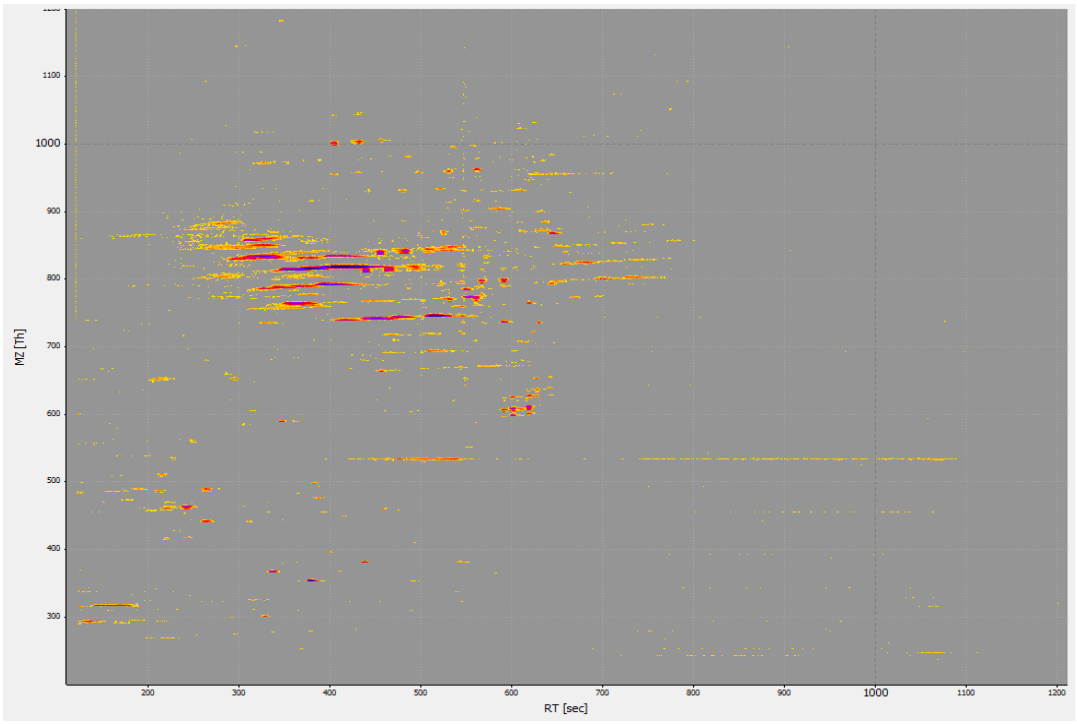

Figure 28 LC-MS result of blade on negative mode

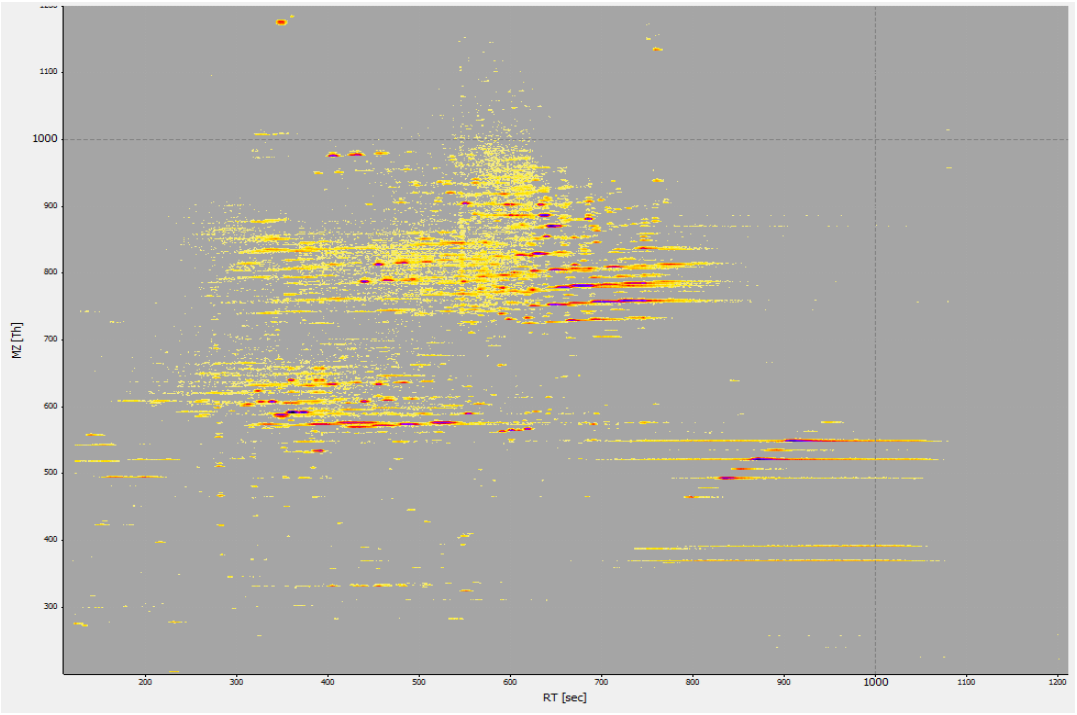

Figure 29 LC-MS result of blade on positive mode

Figure 30 LC-MS result of sporophyll on positive mode

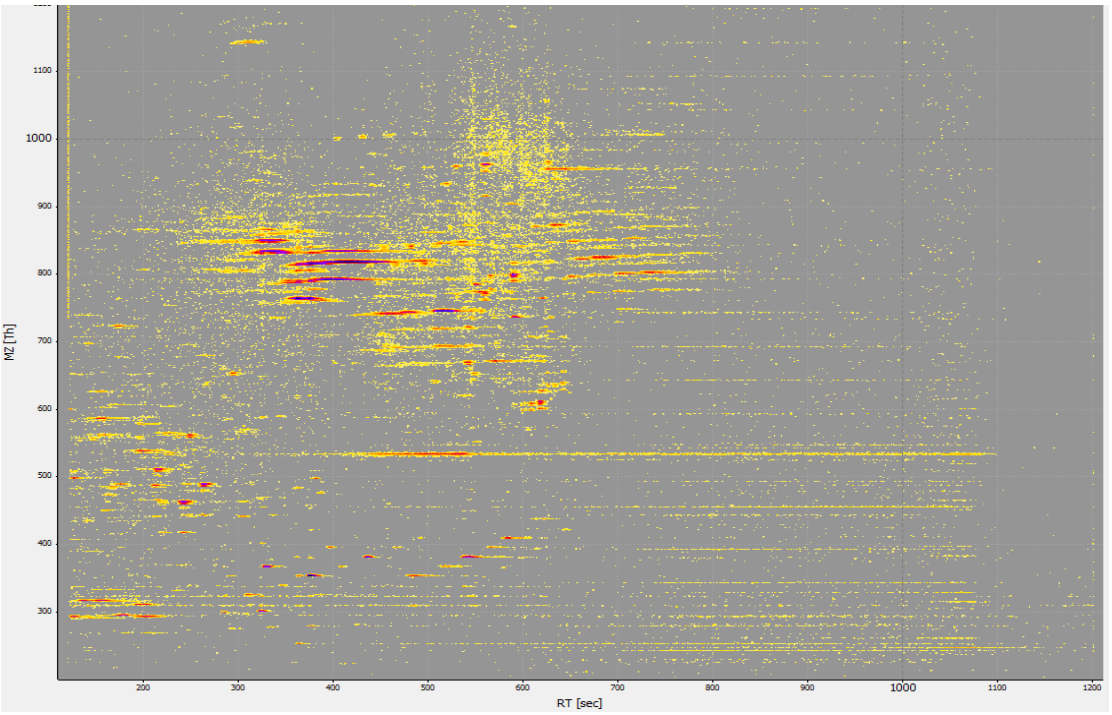

Figure 31 LC-MS result of sporophyll on negative mode

Table 18 LC-MS results of blade on positive mode

M/Z	Name	Formula	CAS	Metlin ID	Similar compounds		Desprition
593.3	avermectin B1b aglycone	C33H46O8		63686			
871.5	PS(20:5(5Z,8Z,11Z,14Z,17Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	C48H72NO10P		78520			
782.5	PG(14:1(9Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	C42H69O10P		78963	79264	79207	Glycerophospholipids
593.2	decuroside III	C26H34O14	96638-81-2				Phytochemical compounds
887.5							
830.6	PE(P-20:0/22:4(7Z,10Z,13Z,16Z))	C47H86NO7P		77669	79130	79620	Glycerophospholipids
758.6	DGTS(16:0/18:2(9Z,12Z))	C44H81NO7		46618			Glycerolipids
976.6	PI(22:4(7Z,10Z,13Z,16Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	C53H83O13P		80974			Glycerophospholipids
760.6	PA(21:0/18:2(9Z,12Z))	C42H79O8P		81851	81525	82186	Glycerophospholipids
609.2							
882.7	PC(23:0/18:0)	C49H98NO8P		39871	60964	76272	Glycerophospholipids
814.5	saponin E	C42H68O14	85191-73-7	91144			
780.5	Unknown						
855.7	Unknown						
730.5	OH-Chlorobactene glucoside ester	C46H64O6		41422			Prenol Lipids
754.05	Unknown						
806.6	PG(20:2(11Z,14Z)/17:0)	C43H81O10P		79391	79555	78367	Glycerophospholipids
568.5	all-trans-retinyl oleate	C38H62O2		41510			Prenol Lipids
905.6	PI(22:1(11Z)/17:1(9Z))	C48H89O13P		80725	80565	80460	Glycerophospholipids
575.5	montecristin	C37H66O4	185336-15-6	86502			
816.5	thalicoside A	C42H70O14	93208-45-8	67328			Phytochemical compounds
559.5	1,2-Epoxy-1,2,7,7',8,8',11,12-octahydro-psi,psi-carotene	C40H62O	51598-36-8	86547			

564.5	diepomuricanin A	C35H62O4	142733-57-1	95370			
978.6	negalomicin C1	C48H84N2O17		40992			Polyketides
838.6	PI(O-16:0/18:2(9Z,12Z))	C43H81O12P		81064	78814	81166	Glycerophospholipids
903.5	Asparagoside D	C45H74O18	60267-24-5	87958			
732.05	UNKNOWN						
1177.5	UNKNOWN						
776.5	PC(18:3(9Z,12Z,15Z)/18:4(6Z,9Z,12Z,15Z))	C44H74NO8P		75965	59375	75936	Glycerophospholipids
520.3	ixocarpalactone B	C28H38O8	71801-44-0	89859			
919.6	PI(18:1(9Z)/22:1(11Z))	C49H91O13P		80327	80701	80851	Glycerophospholipids
779.7	TG(12:0/16:0/18:0)	C49H94O6		4706	40223	61719	Triradylglycerols
496.3	antibiotic JI-20B	C20H41N5O9	51846-98-1	71868			
913.8	TG(18:0/18:0/18:0)	C57H110O6		4701	4895	4851	Triradylglycerols
921.6	PI(P-18:0/22:4(7Z,10Z,13Z,16Z))	C49H87O12P		81133	81157	81049	Glycerophospholipids
911.8	TG(18:0/18:0/18:1(9Z))[iso3]	C57H108O6		4884	4898	4901	Triradylglycerols
575.5	Montecristin	C37H66O4	185336-15-6	86502			
908.8	UNKNOWN						
981.7	DAT(18:0/22:0(2Me[S],4Me[S]))	C54H102O13		82440	82435		Acyltrehaloses

Table 19 LC-MS results of sporophyll on positive mode

M/Z	Name	Formula	CAS	Metlin ID	Similar Compounds		Descrption
782.5	PS(15:1(9Z)/19:1(9Z))	C40H74NO10P		77843	78149	78420	Glycerophospholipids
830.5	PS(20:3(8Z,11Z,14Z)/18:3(6Z,9Z,12Z))	C44H74NO10P		78267	78542	78306	Glycerophospholipids
758.5	PS(16:0/16:0)	C38H74NO10P	3036-82-6	5587			Glycerophospholipids
496.3	PC(8:0/7:0)[U]	C23H46NO8P		39984	40682		Glycerophospholipids
754.5	PS(P-18:0/15:1(9Z))	C39H74NO9P		78773	78750	78656	Glycerophospholipids
591.5	DG(16:0/16:0/0:0)	C35H68O5	30334-71-5	4255			Glycerolipids
806.5	PS(18:3(6Z,9Z,12Z)/18:1(9Z))	C42H74NO10P		78042	78550	78538	Glycerophospholipids
577.5	Helianyl octanoate	C38H66O2	290305-83-8	93057			Found in oils
544.3	Withangulatin A	C30H38O8	120824-03-5	92302			Found in fruits
593.3	Avermectin B1b aglycone	C33H46O8		63686			
855.7	UNKNOWN						
756.6	PE(O-20:0/16:0)	C41H84NO7P		77570	76517	62187	Glycerophospholipids
940.8	UNKNOWN						
589.2	UNKNOWN						
575.4	UNKNOWN						
810.6	PC(20:1(11Z)/18:3(6Z,9Z,12Z))	C46H84NO8P		76099	59910	77245	Glycerophospholipids
575.5	montecristin	C37H66O4	185336-15-6	86502			Found in green vegetables
572.4	PG(21:0/0:0)	C27H55O9P		80017			Glycerophospholipids
615.5	DG(20:1(11Z)/14:1(9Z)/0:0)	C37H68O5		58912	58796	4277	Glycerophospholipids
828.5	goyaglycoside	C43H70O14	333333-14-5	93070			Found in fruits
776.5	PC(16:0/18:4(6Z,9Z,12Z,15Z))	C42H76NO8P		59425	39472	59334	Glycerophospholipids
730.5	PS(O-18:0/13:0)	C37H74NO9P		78666	78652	78751	Glycerophospholipids

976.6	PI(22:4(7Z,10Z,13Z,16Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	C53H83O13P		80974	80836		Glycerophospholipids
804.5	UNKNOWN						
520.3	ixocarpalactone B	C28H38O8	71801-44-0	89859			Found in green vegetables
804.5	astragaloside III	C41H68O14	84687-42-3	67273			Phytochemical compounds
522.3	tauroursodeoxycholic acid	C26H45NO6S	14605-22-2	205			Sterol lipids
978.5	CDP-DG(16:0/18:2(9Z,12Z))	C46H81N3O15P2		58607			Diacylglycerol
798.6	PC(17:0/18:0)	C43H86NO8P		39450	39351	40529	Glycerophospholipids
882.7	PC(19:0/22:0)[U]	C49H98NO8P		39722	39403	40584	Glycerophospholipids
941.6	UNKNOWN						
617.4	PA(17:2(9Z,12Z)/13:0)	C33H61O8P		81443	81368	81239	Glycerophospholipids
535.1	UNKNOWN						
816.5	PS(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/15:0)	C43H72NO10P		78485	77964	77935	Glycerophospholipids
563.4	rhodoxanthin	C40H50O2		41497			Prenol Lipids
631.5	muricatenol	C37H68O6		91103			Found in fruits
568.5	all-trans-retinyl oleate	C38H62O2		41510			Prenol Lipids
836.6	UNKNOWN						
913.7	TG(17:2(9Z,12Z)/18:2(9Z,12Z)/20:3(8Z,11Z,14Z))[iso6]	C58H98O6		37049	37042	37059	Glycerolipids
828.5	PS(20:4(5Z,8Z,11Z,14Z)/18:3(9Z,12Z,15Z))	C44H72NO10P		78298	78119	78487	Glycerophospholipids

Table 18 and *Table 19* show the LC-MS results of both blade and sporophyll on the positive mode. Because of the space problem, the intensity and the retention time are not shown in the tables above (it is given in the appendix), and the results are ordered by the 40 most intense peaks. The "metlin" database (http://metlin.scripps.edu/metabolites_list.php) is used mainly for the identification of the unknown compounds by the mass with the three major parameters namely, M+H, $M+NH_4$, and M+Na, in the positive mode. It also should be pointed out that, a small chemical structure difference within the same molecule mass can result in lots of different compounds being identified, especially in lipids. Some similar compounds with their "metlin ID" are also listed in the table for the further analysis.

According to the results, many lipids including, sterol lipids, diacylglycerol glycerophospholipids and glycerolipids are found either in the blade or in the sporphyll. All these lipids are proved in seaweed either in this project or other projects. Especially the glycerophospholipids part, it accounts over half of the identified compounds. This result is correspond to Murata, whose research found phospholipids were the major lipids in seaweed (M. Murata, & Nakazoe, J, 2001). However, as mentioned above, this result is only a part of the total results. The yellow, red and purple parts are unknown compounds and it is also possible to get hundreds of other classes of lipids with the low intensity.

As a result, at current stage it is not sensible to conclude that "the main lipids in seaweed are phospholipids". A lot of further work remains to be done however, some interesting compounds were detected; most of them have been found already in green vegetables and fruits, or as compounds such as their glycocides. Unfortunately, because of the low resolution of the instrument used, these results are only suggestive.

Table 20 LC-MS results of blade on negative mode

M/Z	Name	Formula	CAS	Metlin ID	Description
355.1	(E)-2-O-Cinnamoyl-beta-D-glucopyranose	C15H18O7	94356-16-8	91086	Found in green vegetables
817.3	premithramycin A3'	C40H50O18		63782	Polyketide products
819.3	sialyl-Lewis X	C31H52N2O23	98603-84-0	58459	Found in human cancer tissues
747.3	taccalonolide A	C36H46O14	108885-68-3	67087	Phytochemical compounds
793.2	chalconaringenin 2'-O-glucoside 4'-O-gentobioside	C33H42O20		52059	Polyketides
765.2	UNKNOWN				
835.3	5-Methyl-5,6,7,8-tetrahydromethanopterin	C31H47N6O16P	92481-97-5	63901	Methane metabolism
745.3	nudicauline	C38H50N2O11	99815-83-5	67136	Phytochemical compounds
838.2	UNKNOWN				
743.3	scillipheosidin 3-[glucosyl-(1->2)-rhamnoside]	C36H52O14	261158-68-3	91934	Found in green vegetables
789.2	spinosin C	C37H38O17	77690-93-8	92356	Found in fruits
610.3	dihydroergocristine	C35H41N5O5		4041	
612.4	PA(12:0/13:0)	C28H58NO8P		40899	Glycerophospholipids
843.2	kaempferol 3-O-[2"-(4'"-acetyl-rhamnosyl)-6"-glucosyl]	C35H42O21		50379	Polyketides
369.1	styraxin	C20H18O7	69742-32-1	68657	Phytochemical compounds
465	vismodegib	C19H14Cl2N2O3S	879085-55-9	85191	
738.3	UNKNOWN				
801.3	glucoscilliroside	C38H54O16		57801	Sterol Lipids
851.2	UNKNOWN				
818.3	alatanin C	C38H40O20		46913	Polyketides

799.1	UNKNOWN				
869.2	iresinin I	C36H42N2O23	78413-55-5	67058	Phytochemical compounds
319	UNKNOWN				
1003.4	PIP2(16:0/16:1(9Z))	C41H79O19P3		61411	Phosphatidylinositol bisphosphate
741.3	physagulin E	C36H50O14	148054-13-1	94248	Found in fruits
319.1	dikegulac	C12H18O7	18467-77-1	72590	
591	UNKNOWN				
771.3	methyl nomilinate 17-glucoside	C35H50O16		94431	Found in fruits
1005.2	salviadelphin	C42H40O26		47069	Polyketides
383	UNKNOWN				
491.1	paederoside	C18H22O11S	20547-45-9	67939	Phytochemical compounds
443.1	nodakenin	C20H24O9	495-31-8	67571	Phytochemical compounds
665.3	lanceotoxin A	C32H44O12	93771-82-5	67237	Steroids
303	coumestrol	C15H8O5	479-13-0	48332	Polyketides
883.2	sennoside D	C42H40O19	37271-17-3	71350	Phytochemical compounds
961.2	pelargonidin 3-(6-(malonyl)glucoside)-7-(6-(4-(glucosyl)-p-hydroxybenzoyl)glucoside)	C43H46O25		46853	
463.1	UNKNOWN				

Table 21 LC-MS Results of sporophyll on negative mode

M/Z	Name	Formula	CAS	Metlin ID	Description
819.3	sialyl-Lewis X	C31H52N2O23	98603-84-0	58459	Found in various human cancer tissues
355.2	ibogaine	C20H26N2O	83-74-9	67523	Phytochemical compounds
765.3	ritonavir	C37H48N6O5S2		43377	Phytochemical compounds
793.3	methanofuran	C34H44N4O15	89873-36-9	63295	Compounds with biological roles
817.3	premithramycin A3'	C40H50O18		63782	Biosynthesis of type II polyketide products
835.3	n,n-Diacetylchitobiosyldiphosphodolichol	C31H56N2O17P2		6072	an intermediate in the N-glycan biosynthesis
747.2	ramontoside	C34H38O16	133882-75-4	93541	Found in fruits
383.2	11β,21-Dihydroxy-5β-pregnane-3,20-dione	C21H32O4	566-01-8	63342	Steroids
851.3	UTILIN	C41H52O17	31218-22-1	43671	Entandrophragma utile
520.3	ixocarpalactone B	C28H38O8	71801-44-0	89859	Found in green vegetable
801.2	wyomin	C33H40O20		48977	Polyketides
963.3	epimedin K	C45H56O23		50444	Polyketides
490.9	UNKNOWN				
303.1	UNKNOWN				
297	UNKNOWN				
612.2	neoacrimarine H	C33H29NO8	217199-06-9	88923	Found in citrus
291.5	UNKNOWN				
786.2	malvidin 3-(6-malonylglucoside) 5-glucoside	C32H37O20		93459	Found in green vegetable
773.3	licoagrodin	C45H44O9		90669	Found in herbs and spices
319.1	dikegulac	C12H18O7	18467-77-1	72590	Plant groth
874.3	UNKNOWN				

313.1	4-methoxyhomopterocarpin	C18H18O5		48164	Polyketides
804.2	delphinidin 3-lathyroside 5-glucoside	C32H39O21		92066	Found in green vegetable
500	azorhodine 2G	C18H15N3O8S2		91810	
673.2	premithramycin A2'	C33H38O15		63781	Biosynthesis of type II polyketide products
411.1	loganate	C16H24O10	22255-40-9	64047	Phytochemical compounds
355.2	spirasine I	C22H29NO3	106777-13-3	67144	Phytochemical compounds
723.2	linoside A	C32H38O16		49487	Polyketides
419.1	UNKNOWN				
957.3	epimedokoreanoside I	C43H54O22		50411	Polyketides
629.2	(R)-Rutaretin 1'-(6"-sinapoylglucoside)	C31H34O14		93647	Found in green vegetable
397	5-Chloro-6-methoxy-2(3H)-benzoxazolone	C16H12Cl2N2O6		88766	Found in cereals and cereal product
301	ellagic acid	C14H6O8	476-66-4	3430	Phytochemical compounds
327.1	anisatin	C15H20O8	5230-87-5	67580	Phytochemical compounds

The same principle is used as in *Table 18* for the selection of peaks and results. Peaks over 2000 intensity are listed in *Table 21* and *Table 20* with their possible identified compounds, followed by CAS number, metlin ID and description.

Internet reaches on the compounds were used was to identify compounds that could reasonably be expected to be “found in fruits or found in vegetables”.

In contrast to positive mode results, the negative results of both samples do not show many lipids. This is reasonable because negative ion mode would only emphasise free fatty acids or acidic lipids and these samples should not have undergone much hydrolysis.

Generally, many phytochemical compounds, polyketides, and some steroids are found in both samples, and previous researchers have indicated that this result is typical of algae containing polyketides and steroids (Julia Kubanek, 2002; Teas, 1981).

Chapter.6 Conclusion

Undaria analysis has been actively undertaken at AUT for the past two years. To some extent, this project repeats part of previous work to confirm earlier results, but considerably extends that work to include a much wider range of lipids and lipid fractions. There are some differences between this and previous work both quantitatively and qualitatively.

It was found that the main lipid class in *Undaria* is non-polar lipids, which is approximately 60% in each sample, followed by phospholipids (15% - 20%) and glycolipids (10%-15%). The total lipid concentration of different parts in *Undaria* was also determined. The sporophyll contains 2.34% lipids by weight while the blade contains 1.53% lipids.

In terms of the fatty acid analysis, 7 different fatty acids were detected in this project compared to previous work. They are caprylic, heneicosylic, docosadienoic, tricosanoic, nervonic, cis-11-eicosenoic and erucic acid. Generally, it was found that polyunsaturated fatty acids and saturated fatty acids are the two major fatty acid classes found in *Undaria*, of which palmitic and henicosanoic are the major saturated fatty acids, and gamma-Linolenic and dihomo-g-linolenic are the principal polyunsaturated fatty acids. It was also proceeded a FAME analysis between blade and sporophyll. The result also showed that sporophyll contains more fatty acids than blade does.

Three sterols were identified in *undaria.* Fucosterol (cholest-5-en-3-ol.24-propylidene, stigmasta-5.24(28)-dien-3-ol and 24-methylenecholest-5-en-3b-ol) were identified and quantitated. It was determined that fucosterol is the major sterol followed by 24-methylenecholesterol in *Undaria* samples and the concentration of fucosterol (0.323 mg/g) is similar compared to Boulom's project (0.338 mg/g).

Interestingly, α-tocopherol (Vitamin E) found in previous thesis is not confirmed in this project

Unfortunately, the LC-MS analyses were only available at the end of the project and there was no time to further investigate these results. They suggested a huge number of compounds including lipids, pigments, polyketides, steroids and others. These results are intriguing and suggest a fruitful area of further study. For example, glycerophospholipids are found to be the major lipids, but the low resolution of the instrument means the individual compounds cannot be reliably identified.

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Appendices:

Table.I LC-MS results of blade on positive mode

RT	M/Z	Int	Name
361.23	593.3	1157579	Avermectin B1b aglycone
644.58	871.5	670590.5	PS(20:5(5Z,8Z,11Z,14Z,17Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))
676.84	782.5	450786.3	PG(14:1(9Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))
371.25	593.2	435120.4	Decuroside III
637.14	887.5	403073.2	
630.19	830.6	394885.9	PE(P-20:0/22:4(7Z,10Z,13Z,16Z))
698.67	758.6	394005.6	DGTS(16:0/18:2(9Z,12Z))
404.9	976.6	298247.3	PI(22:4(7Z,10Z,13Z,16Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))
731.92	760.6	297409.1	PA(21:0/18:2(9Z,12Z))
337.91	609.2	274339	
684.28	882.7	265263.5	PC(23:0/18:0)
455.52	814.5	265051.8	Saponin E
660.46	780.5	262207.9	Unknown
639.12	855.7	245022.5	Unknown
666.91	730.5	237777.7	OH-Chlorobactene glucoside ester
649.05	754.05	226807.4	Unknown
649.54	806.6	219489.4	PG(20:2(11Z,14Z)/17:0)
618.78	568.5	211429	all-trans-retinyl oleate
550.3	905.6	207706.5	PI(22:1(11Z)/17:1(9Z))
487.77	575.5	207256.8	Montecristin
483.31	816.5	188255.9	Thalicoside A
553.28	559.5	168509.9	1,2-Epoxy-1,2,7,7',8,8',11,12-octahydro-psi,psi-carotene
590.49	564.5	156204.2	Diepomuricanin A
430.71	978.6	151271.3	Megalomicin C1
744.33	838.6	123411.7	PI(O-16:0/18:2(9Z,12Z))
594.96	903.5	117823.3	Asparagoside D
597.94	732.05	88112.58	UNKNOWN
348.33	1177.5	84710.91	UNKNOWN
623.74	776.5	78369.8	PC(18:3(9Z,12Z,15Z)/18:4(6Z,9Z,12Z,15Z))
506.63	520.3	64073.27	Ixocarpalactone B
592.98	919.6	59214.82	PI(18:1(9Z)/22:1(11Z))
668.9	779.7	57527.79	TG(12:0/16:0/18:0)

198.47	496.3	54390.4	Antibiotic JI-20B
572.13	913.8	53954.36	TG(18:0/18:0/18:0)
534.42	921.6	53777.36	PI(P-18:0/22:4(7Z,10Z,13Z,16Z))
653.02	911.8	53204.86	TG(18:0/18:0/18:1(9Z))[iso3]
690.73	575.5	52496.46	Montecristin
685.77	908.8	50746.29	UNKNOWN
671.7	981.7	50312.13	DAT(18:0/22:0(2Me[S],4Me[S]))

RT: Retention time; M/z: Mass; Int: Intensity

Table.II LC-MS results of blade on negative mode

RT	M/Z	Int	Name
378.06	355.1	35464.7	(E)-2-O-Cinnamoyl-beta-D-glucopyranose
377.07	817.3	29638.72	Premithramycin A3'
436.11	819.3	26798.2	Sialyl-Lewis X
515.5	747.3	25464.48	Taccalonolide A
390.96	793.2	17164.02	Chalconaringenin 2'-O-glucoside 4'-O-gentobioside
365.16	765.2	16353.12	UNKNOWN
329.43	835.3	13542.74	5-Methyl-5,6,7,8-tetrahydromethanopterin
477.79	745.3	11922.12	Nudicauline
454.47	838.2	10637.88	UNKNOWN
451.99	743.3	9937.34	Scillipheosidin 3-[glucosyl-(1->2)-rhamnoside]
342.33	789.2	9174.14	Spinosin C
600.84	610.3	8924.86	dihydroergocristine
618.21	612.4	8804.7	PA(12:0/13:0)
482.26	843.2	8208.94	Kaempferol 3-O-[2"-(4'"-acetyl-rhamnosyl)-6"-glucosyl] glucoside
335.89	369.1	7647.04	Styraxin1111
242.1	465	7178.8	Vismodegib
591.42	738.3	6652.62	UNKNOWN
590.43	801.3	6592.08	Glucoscilliroside
322.49	851.2	6238.24	UNKNOWN
465.88	818.3	5719.42	Alatanin C
566.61	799.1	5700.48	Adenosine thiamine triphosphate
644.51	869.2	5381.32	Iresinin I
146.84	319	5192.24	UNKNOWN
404.36	1003.4	5095.78	PIP2(16:0/16:1(9Z))
416.76	741.3	4921.72	Physagulin E

169.66	319.1	4905.04	Dikegulac
346.8	591	4794.7	UNKNOWN
531.38	771.3	4655.76	Methyl nomilinate 17-glucoside
431.65	1005.2	4561.44	Salviadelphin
437.1	383	4501.14	UNKNOWN
263.44	491.1	3283.98	Paederoside
261.95	443.1	3025.98	Nodakenin
454.97	665.3	2916.52	Lanceotoxin A
327.94	303	2736.94	Coumestrol
276.84	883.2	2658.92	Sennoside D
530.39	961.2	2609.2	Pelargonidin 3-(6-(malonyl)glucoside)-7-(6-(4-(glucosyl)-p-hydroxybenzoyl)glucoside)
219.78	463.1	2555.72	UNKNOWN

RT: Retention time; M/z: Mass; Int: Intensity

Table.III LC-MS results of sporophyll on positive mode

RT (s)	M/Z	Intensity	Name
677.83	782.5	468442.6	PS(15:1(9Z)/19:1(9Z))
632.18	830.5	461933	PS(20:3(8Z,11Z,14Z)/18:3(6Z,9Z,12Z))
702.64	758.5	300428	PS(16:0/16:0)
196.98	496.3	300159.3	PC(8:0/7:0)[U]
650.04	754.5	300123.7	PS(P-18:0/15:1(9Z))
555.26	591.5	253117.3	DG(16:0/16:0/0:0)
651.53	806.5	246296.8	PS(18:3(6Z,9Z,12Z)/18:1(9Z))
443.61	577.5	243640.2	Helianyl octanoate
154.8	544.3	217205.7	Withangulatin A
357.76	593.3	201143.6	Avermectin B1b aglycone
639.62	855.7	158194.2	UNKNOWN
674.36	756.6	144644.1	PE(O-20:0/16:0)
627.21	940.8	143213.6	UNKNOWN
336.42	589.2	142658.7	UNKNOWN
345.36	575.4	135687	UNKNOWN
716.04	810.6	135568	PC(20:1(11Z)/18:3(6Z,9Z,12Z))
493.73	575.5	133004.8	Montecristin
414.33	572.4	126613.5	PG(21:0/0:0)
466.44	615.5	125334.3	DG(20:1(11Z)/14:1(9Z)/0:0)
610.84	828.5	123383	Goyaglycoside g
625.73	776.5	119090.4	PC(16:0/18:4(6Z,9Z,12Z,15Z))
669.39	730.5	112911	PS(O-18:0/13:0)

404.41	976.6	106871.5	PI(22:4(7Z,10Z,13Z,16Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z))
627.71	804.5	102435	UNKNOWN
156.29	520.3	100770.6	Ixocarpalactone B
686.76	804.5	99208.95	Astragaloside III
228.24	522.3	98637.48	Tauroursodeoxycholic acid
431.2	978.5	97389.01	CDP-DG(16:0/18:2(9Z,12Z))
594.96	798.6	85688	PC(17:0/18:0)
684.28	882.7	85677.77	PC(19:0/22:0)[U]
561.22	941.6	79642.24	UNKNOWN
455.02	617.4	79053.02	PA(17:2(9Z,12Z)/13:0)
385.05	535.1	77150.06	UNKNOWN
482.81	816.5	75644.83	PS(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/15:0)
521.52	563.4	71323.48	Rhodoxanthin
552.78	631.5	66434.33	Muricatenol
619.27	568.5	60121.4	all-trans-retinyl oleate
356.27	836.6	57776.29	UNKNOWN
640.21	913.7	54816.74	TG(17:2(9Z,12Z)/18:2(9Z,12Z)/20:3(8Z,11Z,14Z))[iso6]
365.2	828.5	50847.34	PS(20:4(5Z,8Z,11Z,14Z)/18:3(9Z,12Z,15Z))

RT: Retention time; M/z: Mass; Int: Intensity

Table.IV LC-MS results of sporophyll on negative mode

RT	M/Z	Int	Name
125.5	291.5	5194	UNKNOWN
135.92	319.1	3800	Dikegulac
180	297	5766.02	UNKNOWN
195.46	313.1	3454.22	4-Methoxyhomopterocarpin
126.99	500	3152.82	Azorhodine 2G
284.78	301	2204.7	Ellagic acid
309.58	327.1	2178.7	Anisatin
323.48	303.1	6259.06	UNKNOWN
395.92	397	2249.08	5-Chloro-6-methoxy-2(3H)-benzoxazolone
376.57	355.2	37274	Ibogaine
436.11	383.2	10849.34	11β,21-Dihydroxy-5β-pregnane-3,20-dione
486.72	355.2	2704.14	Spirasine I
585.46	411.1	2804.62	Loganate
242.1	419.1	2551.98	UNKNOWN
263.94	490.9	7618.92	UNKNOWN
332.91	851.3	10676.16	UTILIN
334.39	835.3	18723.08	N,N-Diacetylchitobiosyldiphosphodolichol

407.33	520.3	10408.92	Ixocarpalactone B
413.29	819.3	42573.14	Sialyl-Lewis X
376.57	817.3	22721.42	Premithramycin A3'
391.95	793.3	23446.08	Methanofuran
363.17	765.3	30976.32	Ritonavir
513.52	747.2	16652.66	Ramontoside
589.94	801.2	10210.72	Wyomin
560.65	963.3	9223.3	Epimedin K
617.71	612.2	5670.24	Neoacrimarine H
549.74	786.2	4639.1	Malvidin 3-(6-malonylglucoside) 5-glucoside
634.09	874.3	3528.06	UNKNOWN
735.18	804.2	3247.84	Delphinidin 3-lathyroside 5-glucoside
570.58	673.2	2827.34	Premithramycin A2'
540.81	723.2	2685.26	Linoside A
640.54	957.3	2362.26	Epimedokoreanoside I
619.2	629.2	2340.1	(R)-Rutaretin 1'-(6"-sinapoylglucoside)
560.56	773.3	3802.02	Licoagrodin

RT: Retention time; M/z: Mass; Int: Intensity