Protein content and amino acid profile in New Zealand *Undaria pinnatifida*

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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.

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Abbreviations

HPLC: High performance liquid chromatograph

dw: Dry weight

v: Volume

w: Weight

L: Liter

m: Milli

µ: Micro

g: Gram

U: *Undaria*

ANOVA: Analysis of variance

PITC: Phenylisothiocyanate

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

PVPP: Polyvinylpolypyrrolidone

PVP: Polyvinylpyrrolidone

BL: Blade

SP: Sporophyll

PTC-amino acid: Phenylthiocarbamyl amino acid

mM: Millimoles per litre

EDTA: Ethylenediaminetetraacetic acid

Tris: Tris (hydroxymethyl) aminomethane

Tris•Cl: 2-Amino-2-hydroxymethyl-1, 3-propanediol hydrochloride

BSA: Bovine serum albumin

AR: Analytical reagent

AU: Area unit

ASP: Aspartic Acid

GLU: Glutamic Acid

4-OH-PRO: trans-4-Hydroxyl-L-Proline

SER: Serine

GLY: Glysine

ASN: Asparagine

GLN: Glutamine

THR: Threonine

ALA: Alanine

HIS: Histidine

PRO: Proline

ARG: Arginine

VAL: Valine

TYR: Tyrosine

MET: Methionine

ILE: Isoleucine

LEU: Leucine

CYS+CYT: Cysteine+Cystine

PHE: Phenylalanine

TRP: Tryptophan

LYS: Lysine

hr: Hour

UV: Ultraviolet

VIS: Visible

RSD: Relative standard deviation

AUT: Auckland University of Technology

MAF: Ministry of Agriculture and Forestry

Abstract

Undaria pinnatifida was collected from four farms near Nelson, New Zealand on a monthly basis from June to September in 2011. The total nitrogen content and amino acid profile of the samples were determined. Seasonal and geographical variations in the total nitrogen and amino acid composition of the samples were evaluated in the blade and sporophyll.

The total nitrogen content determined by the Kjeldahl method ranged between 12.99 mg/g to 30.00 mg/g of dry weight with an average content of 21.02 mg/g. Total nitrogen content was significantly higher ($p < 0.05$) in June and July for both exposed site in Port Underwood. Higher value of the total nitrogen was observed in exposed farm 327 in Port Underwood and sheltered farm 233 in Pelorus Sound in the blade, and farms 327 and sheltered farm 122 in Pelorus Sound in the sporophyll at the harvest month September. The content in blade was significantly higher than the one in sporophyll in farm 327 between four months.

High performance liquid chromatography was used to determine the composition amino acids in *Undaria pinnatifida*. A total of 15 amino acids acids including 7 essential amino acids, 2 semi-essential amino acids and 6 non-essential amino acids were detected. Similar to total nitrogen, seasonal, geographical and plant parts affected amino acid composition. The proportion of essential amino acids to total amino acid was close to the recommended WHO/FAO standard except for sulphur containing amino acids. Glutamic acid, aspartic acid and alanine that can contribute to flavour were present at high concentrations in *Undaria*.

The average nitrogen-to-protein conversion factor based on nitrogen determination by Kjeldahl method and amino acid analyses was calculated to be 4.44. Hence the traditional conversion factor of 6.25 may overestimate the true protein content of *Undaria* in New Zealand. Further protein characterization of the *Undaria* species by SDS-PAGE revealed seven protein bands having molecular weight of 77 kDa, 55 kDa, 37 kDa, 26 kDa, 20 kDa, 17 kDa and 14 kDa, which has not been reported in other algae species.

Chapter 1 Introduction

1.1 The objectives of this study

Undaria Pinnatifida is endemic to South East Asia and Japan. It is a traditional food which is now cultured extensively. *Undaria* was discovered in New Zealand in the 1980's and was initially treated as an exotic pest. As the nutritional value of *Undaria pinnatifida* is now recognised, it can be legally grown, harvested, sold and exported from New Zealand. While some studies on Asian *Undaria* have been done, there has been no research on the endemic New Zealand product. While it was not expected that the nutritional composition should vary significantly from *Undaria* grown in Asian, it was reasonable to expect some local differences.

This study was carried out as a part of a larger study by the *Undaria* group at AUT, which investigated nutritional composition and distribution of these seaweeds in the Marlborough Sounds, New Zealand.

The overall objective of this thesis was to investigate the protein and amino acid composition of New Zealand *Undaria* from two locations in the Marlborough Sounds.

1.2 Seaweed and *Undaria pinnatifida*

1.2.1 **Algae and Seaweeds**

Algae are simple marine organisms, widely distributed in the ocean, and an important marine resource. Because it grows in seawater, the chemical composition of algae is somewhat different to that of land plants. Marine algae are often the initial part of a marine food chain and play an important role in marine taxonomy, ecology and physiology (Wikipedia, 2011a).

1.2.2 **Classification of seaweed**

Marine macro-algae or seaweeds are classified into three groups: brown algae (*Phaophyceae*), red algae (*Rhodophyceae*), and green algae (*Clorophyceae*) (Jiménez-Escrig & Sánchez-Muniz, 2000). Brown algae are a more multicellular algae group, found in cold waters along the mainland. Their colour depends on the ratio of the brown and green pigments, and their sizes vary widely from a few microns to tens of meters long (*Brown Seaweeds* ; Wikipedia, 2011b). Examples of common brown algae include kelp, wakame and *sargassum*.

1.2.3 *Undaria pinnatifida*

Undaria pinnatifida is temperate brown seaweed. It is called wakame or Japanese kelp. It can tolerant a wide range of seawater temperatures. Wakame is also referred to as a "sea vegetable" as it contained a variety of essential nutrients such as: iodine, calcium, iron, dietary fibre and protein. In addition, it also contained vitamin A, vitamin B1, vitamin cable B2, vitamin C, folic acid, magnesium, and alginates.

1.2.4 **Nutritional value of *Undaria pinnatifida***

The main nutritional compositions of algae are protein, lipid, carbohydrate and minerals (Carvalho et al., 2009). *Undaria pinnatifida* is a reasonable healthy low-fat food and was described as having high ash contents, appreciable protein contents, low total lipid contents, and relatively high levels of polyunsaturated fatty acids (Sánchez-Machado, López-Cervantes, López-Hernández, & Paseiro-Losada, 2004).

The protein content of *Undaria pinnatifida* was reported to be between 11-24% dry weight(J. Fleurence, 1999). Most protein value of *Undaria pinnatifida* was reported fall within this range. The total lipid level was approximately 1 g/100 g dry weight, with the single most abundant fatty acid being C18:4 ω3 (linolenic acid), and polyunsaturated fatty acids constituting 69.11±9.01% of the total fatty acids (Sánchez-Machado et al., 2004) (Cofrades, López-Lopez, et al., 2010).

1.2.5 ***Undaria pinnatifida* consumption in the world**

The use of seaweed as food has been traced back to the fourth century in Japan and the sixth century in China (McHugh, 2003). In Japan there was a long history of the cultivation of wakame as one of major edible seaweed. Japan is recognized as a country with longevity, and some believed that one of the main reasons for this was daily consumption of *Undaria* by most Japanese families. *Undaria* an essential ingredient in dishes for children and students in Japan and South Korea (Dharmananda, 2002).

1.2.6 **Consumption of alginate and other products from brown seaweed**

Alginate is a polysaccharide extracted from brown seaweed and used as a thickener, emulsifier, stabilizer, binder, sizing agent. It is widely used in foods, pharmaceuticals, textiles, printing and dyeing, etc. Since the 1980s, the application of sodium alginate in the food industry has expanded. Sodium alginate is not only as a safe food additive but also a substrate for functional food. It behaves as a fiber with the additional characteristics of water-holding capacity, viscosity, absorptive capacity and ferment ability in the alimentary canal. It can reduce the absorption of fat, sugar and bile salts, blood triglycerides and blood sugar (A. F. U. Carvalho et al.,

2009; Wijesekara & Kim, 2010). Alginates are claimed to decrease the risk of coronary heart disease,(Jiménez-Escrig & Sánchez-Muniz, 2000), and prevent hypertension, diabetes, obesity and other modern diseases (Aisa et al., 2005; Cherbut, 2002). Furthermore, alginate has an extensive history of use in medicine, pharmacy and basic sciences as reported recently (D'Ayala, Malinconico, & Laurienzo, 2008).

1.3 Status of the introduced brown seaweed *Undaria* in New Zealand

New Zealand is a maritime nation, surrounded by the world's largest ocean - the Pacific Ocean, and its exclusive economic zone with an area of 4.2 million square kilometres is the fifth largest in the world, only after United States, Australia, Russian Federation, and French Polynesia. It has long coastline estimated to be between 15,000 to 18,000 km, and across the 13^{o} latitude, from subtropical at 34.5^{o}S to cool temperate at 47.5^{o}S. This provides a favourable geographical and climatic conditions for the growth of algae (Gordon, Beaumont, MacDiarmid, Robertson, & Ahyong, 2010).

1.3.1 **History**

Undaria pinnatifida was first recorded in New Zealand in the Wellington Harbor in 1987. Since then, it has expanded its geographic range to include all of the major ports throughout New Zealand and a number of secondary ports (Figure **1**). A study focused on southern New Zealand demonstrated that this species had an ability to grow in a broad range of environments, enabling it to expand to almost all rocky coastline in temperate locations of New Zealand (Russell, Hepburn, Hurd, & Stuart, 2008). As result of fast spreading, *Undaria* has ever been found in sites along most of New Zealand coast.

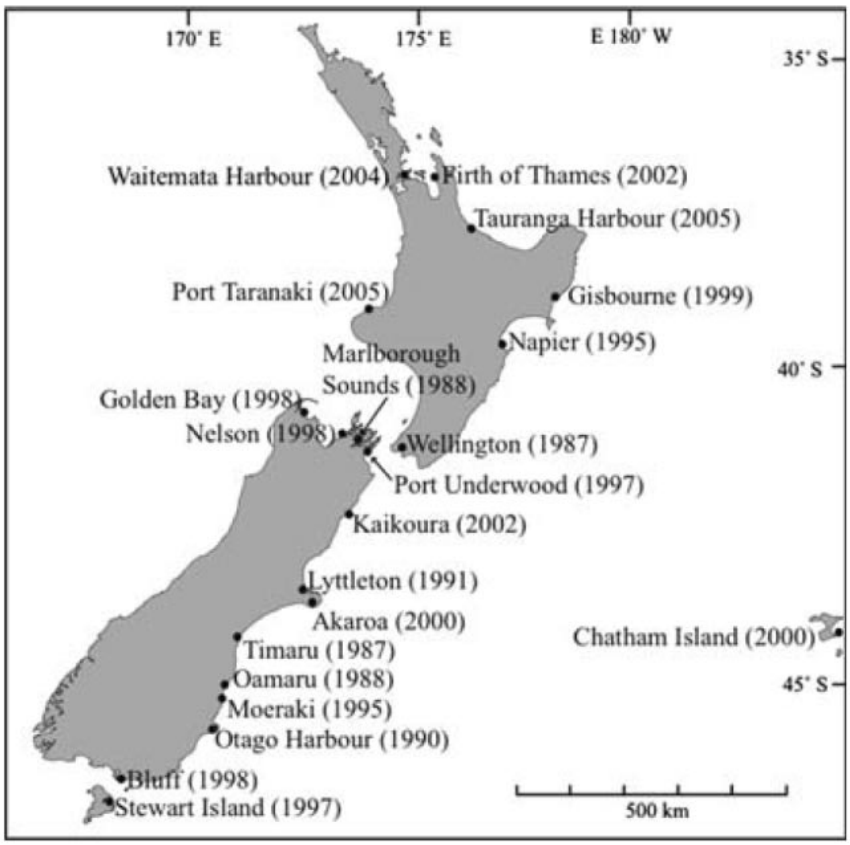

Figure 1: Locations of *Undaria pinnatifida* populations in major and secondary ports in New Zealand as of July 2006. The year *U. pinnatifida* was first observed in each port is included in brackets (Russell et al., 2008).

1.3.2 **Control of *Undaria***

The fast spread and dense formation of *Undaria* can easily create a forest underwater that will choke light and space of native sea creatures, and invade native communities. The current range of *U. pinnatifida* is much greater than expected and appears to be expanding (Russell et al., 2008).

While the natural spread of *Undaria* is generally very slow, it is easily spread by the hulls of vessels, aquaculture and other marine equipment. As a result it has the potential to become a nuisance for marine farms by increasing both labor and harvesting costs due to fouling problems.

For controlling this pest, the New Zealand government has invested huge fund and labor to all kinds of controlling program around the coastal area. For example, a new mode of tackling the invasive *Undaria* was carried out in the Sunday Cove in Break Sea Sound by the Department of Conservation, MAF and Environment Southland (McKNIGHT, 2011).

1.3.3 International demand for wakame and the potential commercial use of *Undaria* in New Zealand

Combined with other aquatic plants, the production of seaweed reached 16.8 million tons in 2008 for the whole world (FAO, 2010). The third highest production of cultured seaweed, Wakame (*Undaria Pinnatifida*), was 1.8 million tons. Countries in East and Southeast Asia dominated seaweed culture production 99.8 percent by quantity and 99.5 percent by value in 2008. At the same year, the production of seaweed in New Zealand was 196 tones, only 0.02% of total world production (FAO, 2010).

With an average annual growth rate of 7.7 percent from 1970, the potential economic benefit of seaweed industry cannot be ignored. Because of heavy metal pollution and radioactive waste at the Fukushima power plant which was destroyed by an earthquake and tsunami in 2011, the image of products from traditional Asia producers has tarnished, accompanied by an urgent demand of more safe suppliers of seaweed products in the international seaweed market.

Considering this potential export market and failure of *Undaria Pinnatifida* eradication at several locations (Louise Hunt, 2009), the government permitted farming and harvesting of *Undaria* at established areas in May 2010 (HOWIE, 2010).

Because *Undaria* grows on any hard surface including shells, reefs, ropes, wharf piles, vessel hulls, moorings and other artificial structures, it can be harvested as a by-product of marine farms that could have a potential commercial value.

1.4 Protein determination in food

Protein content in food can be determined by total nitrogen content using the Kjeldahl method or elemental analysis. Soluble and insoluble protein content can be determined using various extraction protocols that employ techniques such as colorimetry. Amino acid analysis however is the most accurate method protein content determination, but involves a more complicated analysis procedure. The requirement for expensive equipment and a skilled analyst limits its use in the food industry. Compared to amino acid analysis, colorimetric methods are fast and cheap. Numerous researchers have successfully determined protein content by using the colorimetric method combined with various protein extract protocols, in different kinds of algae, especially microalgae (López et al., 2010; Malea, Rijstenbil, &Haritonidis, 2006). However there a practical difficulty exists because different structure and chemical compositions of cell walls and components of the extracted solution introduce can interfere with analysis. Incomplete protein extraction and an un-calibrated negative effect can affect the final results.

1.4.1 Crude protein content and the nitrogen-to-protein conversion factor

The reference method for protein in foods is the Kjeldahl method. First described in 1883, the Kjeldahl method has gained wide acceptance and is now used for a variety of applications because of its simplicity and low cost. In essence, a sample is digested in boiling sulphuric acid and organic nitrogen containing compounds are converted to ammonium sulphate. The sample is then made alkaline, and the ammonia steam distilled from the mixture was determined titrimetrically. A conversion factor was then used to convert total nitrogen to protein.

If this conversion factor has been derived by a correct and complete identification of the protein content of that particular food, then the Kjeldahl method gives a reliable measure of the protein. Unfortunately each type food has slightly different protein content, so these conversion factors would have to be determined for each food type. The conversion factor ranges from roughly 4.5 and 6.5 for plant food, and if the correct conversion factor is not used, the protein content estimation will be wrong.

1.4.1.1 The effect of non-proteinaceous nitrogen on protein determination

The nitrogen compounds in plant tissues include the proteinaceous nitrogen and non-proteinaceous nitrogen, such as nucleic acids, amines, urea, ammonia, nitrates, nitrites, phospholipids, nitrogenous glycosides, etc. (Figure 2). (Lourenço, Barbarino, Lavín, Lanfer Marquez, & Aidar, 2004).

Figure 2: The contribution of nitrogenous compounds in food stuffs.

Customarily, a conversion factor of 6.25 is often applied, but this was based on an assumption of an insignificant amount of non-protein nitrogen. Different values however need to be applied to arrive at the correct value of the protein content. For example, 6.25 for meat and eggs, 6.38 for milk products, 5.83 for most grains and

5.70 for wheat. Considering that algae have significant amounts of non-protein nitrogen, a conversion factor of 6.25 was undoubtedly too high.

1.4.1.2 Protein content and nitrogen-to-protein conversion factor of some plant foods

Table 1 summarizes the net protein content and calibrated nitrogen-to-protein conversion factor of some plant foods. All the calibrated conversion factor was less than traditional conversion factor of 6.25. This proved the 6.25 conversion factor significantly over estimated the actual protein level of most plant foods.

Table 1: Comparison of the difference between traditional and calibrated conversion factor of some plant foods

Plant food	Total amino acid (g/100 g dry mass)	Calibrated nitrogen-to-protein conversion factor
Mushroom		
P. ostreatus	1.97*	4.97[1]
***A. bisporus*, brown**	2.07*	4.55[1]
Pleurotus ostreatus	1.97*	4.08[2]
Agaricus bisporus	2.08*	3.45[2]
Cereal		
corn	87.99	5.68[3]
Brown rice	6.39	5.25[4]
Purple-black rice	9.32	5.35[4]
Red rice	8.85	5.17[4]
Hard durum wheat	15.86	5.55[4]
Hard flour	15.03	5.45[4]
Medium flour	11.04	5.51[4]
Soft flour	6.76	5.37[4]
Whole wheat flour	16.29	4.74[4]
Corn flour	6.14	5.77[4]
Corn grits	9.00	5.94[4]
Vegetable		
Cabbage	17.82	4.54[5]
Carrot	6.16	4.93[5]
Cucumber	19.58	4.65[5]
Edible burdock	9.57	3.49[5]
Eggplant	13.72	4.53[5]

*: The data was expressed as g/100 g fresh mass.
[1]: Data is from (Mattila, Salo-Väänänen, Könkö, Aro, & Jalava, 2002).
[2]: Data is from (Jaworska & Berna, 2011).
[3]: Data is from (Sriperm, Pesti, & Tillman, 2011).
[4]: Data is from (Fujihara, Sasaki, Aoyagi, & Sugahara, 2008).
[5]: Data is from (Fujihara, Kasuga, & Aoyagi, 2001).

1.4.2 **Protein content of seaweed**

The total protein content in the macro algae spanned a wide range depending on species, harvest season, location and varieties (Patarra, Paiva, Neto, Lima, & Baptista, 2010).

The protein content of seaweeds differs according to species. Generally, the protein fraction of brown seaweeds was lower compared to the green or red seaweeds. For example, a comprehensive review published in 2010 compared protein contents of nine genera or species of seaweed, located in European temperate Atlantic waters (Holdt & Kraan, 2011). Protein content of brown seaweed was found to be normally lower than the other kinds of seaweeds. This is supported by a large number of other studies (MacArtain, Gill, Brooks, Campbell, & Rowland, 2007; Rupérez&Saura-Calixto, 2001), with only one study showing otherwise (Ortiz et al., 2009). The protein levels reported for individual seaweed species are summarized in Table 2.

There is large variation of protein levels in the algae between species and location, especially for green and red seaweeds. The protein levels can be as high as 44.0% and 26.62% of dry weight (Table 2), for green (*Porphyra sp.*) and red algae (*Ulva compressa*) respectively (Hwang et al., 2008). At the same time, the protein level can be as low as 0.94% and 2.9% dry weight for *G. verrucosa* (a red seaweed) and *U. rigida* (a green seaweed) respectively (Dere, Dalkiran, Karacaoğlu, Yildiz, & Dere, 2003).

Generally, most brown seaweed, with the exception of *Undaria pinnatifida (*wakame) and *Alaria esculenta*, which contain protein levels in the range 11%–24% and 9%–20% (w⁄w), respectively, have maximum protein contents of about 15% (w ⁄w) (Morrissey et al. 2001, Burtin 2003, Fleurence 2004); and lowest protein contents of 6.81% dry weight (Patarra et al., 2010).

It should be noted, there is obvious difference of same species from different studies, for example, the protein level of *Porphyra sp* from three locations varied from 25.80% to 44.0% dry weight (Table 2).

The protein content in *Undaria pinnatifida* varied between 11 to 24% of dry weight, the variation depending on location and harvest season (J. Fleurence, 1999), it was reported as 15.47% of dry weight in Spanish *Undaria pinnatifida* (Gómez-Ordóñez, Jiménez-Escrig, & Rupérez, 2010); the highest protein content reported was up to 24% dry weight (Holdt and Kraan, 2011).

Table 2: Protein levels in some seaweeds and seaweed products

Species	Crude protein (% dw)	Collected location	Harvest season
Red algae			
Gelidium microdon	15.18(1)	Portugal	during January and February (winter)
Osmundea pinnatifida	20.64(1)	Portugal	during January and February (winter)
Porphyra sp.	25.80(1)	Portugal	during January and February (winter)
Pterocladiella capillacea	20.52(1)	Portugal	during January and February (winter)
Sphaerococcus coronopifolius	19.56(1)	Portugal	during January and February (winter)
Gracilaria chilensis	13.7(2)	Chile	in January (winter)
Laurencia filiformis	11.3(3)	Brazil	not mentioned
L. intricata	6.7(3)	Brazil	not mentioned
Gracilaria domingensis	7.6(3)	Brazil	not mentioned
G. birdiae	9.10(3)	Brazil	not mentioned
Porphyra sp.	31.4(4)	Japan and Korea	not mentioned
Porphyra sp.	30.9(4)	China	not mentioned
Porphyra sp.	44.0(5)	United Kingdom	not mentioned

Brown algae			
Cystoseiraabies-marina	6.81(1)	Portugal	during January and February (winter)
Fucus spiralis	10.77(1)	Portugal	during January and February (winter)
Macro cystispyrifera	13.2(2)	Chile	in January (winter)
Undaria pinnatifida	19.8(4)	China, Japan and Korea	not mentioned
Laminaria sp.	7.5(4)	China, Japan and Korea	not mentioned
Hizikia fusiforme	11.6(4)	China, Japan and Korea	not mentioned
Green algae			
Ulva compressa	26.62(1)	Portugal	during January and February (winter)
Codium fragile ssp. tomentosoides	10.8(2)	Chile	in January (winter)
Capsosiphon fulvescens	29.5(4)	Korea	in December (winter)

(1) Values from (Patarra et al., 2010)
(2) Values from (Ortiz et al., 2009)
(3) Values from (Hwang, Amano, & Park, 2008)
(4) Values from (Dawczynski, Schubert, & Jahreis, 2007).
(5) Values from (Marsham, Scott, & Tobin, 2007)

1.5 The nitrogen-to-protein conversion factor of algae

Conversion factors for various s macro-algae and microalgae vary widely. A conversion factor value of 5.95 for nitrogen as measured by Kjeldahl was used for the dry biomass of microalgae, *Porphyridium cruentum, Scenedesmus almeriensis*, and *Muriellopsis sp. (López et al., 2010).*

A conversion factor of 4.92 was proposed as the overall mean nitrogen- protein factor (Loureno, Barbarino, De-Paula, Pereira, & Lanfer Marquez, 2002). This was calculated based on the sum of amino acid residues in six green, four brown and nine red marine algae, with the highest nitrogen- protein factor of 5.38 from the brown algae group, and the lowest nitrogen- protein factor of 4.59 is calculated from the red algae group.

1.6 Amino acid composition of seaweeds

The nutritional quality of food can be determined by the content, proportion and availability of its amino acids, particularly for evaluation of a new protein resource (Gressler et al., 2009). Table 3 summarizes the amino acid composition of some common edible green seaweed. Although a total of eighteen amino acids were detected in some studies (Hwang et al., 2008; Vinoj Kumar, 2007), it is more common to be reported seventeen amino acids in macro-algae. Aspartic acid, glutamic acid, valine, leucine and alanine contents were high in green macro-algae. Acidic residues of aspartic and glutamic acid that ranged from 19.12 mg/g dry weight up to 94.02 mg/g dry weight were the two most abundant amino acids in four out of six species listed in Table 3. These two amino acids constituted 21% to 36% of the total amino acids. Cystine, methionine and histidine were found in low concentrations in green macro-algae. However there was an abundance of essential amino acids, such as, phenylalanine (up to 21.54 mg/g dry weight), valine (up to 23.22 mg/g dry weight) and leucine (up to 33.70 mg/g dry weight), that increases the nutritional value of green seaweed.

Geographic factors seemed to have a significant effect on the amino acid composition of green macro-algae. For example, *Ulva lactuca*, a common consumable sea vegetable, was evaluated from different locationsuch as, India, Chile, and Spain etc (Ortiz et al., 2006; Taboada, Milln, & Mguez, 2009; Vinoj Kumar, 2007). Although the trend of amino acids was the same, there was a difference in specific amino acid, such as, proline, phenylalanine and methionine (Table 3).

Table 3: Summary of amino acid composition of some edible green macro-algae

Amino acid	*C. Fragile*	*Capsosiphon fulvescens*	*C. lentillifera*	*Ulva lactuca*	*Ulva lactuca*	*Ulva lactuca*
ASP	8.23±0.11	43.58±0.42	8.33±0.11	15.9	14.87 ±0.08	12.90 ± 0.11
GLU	10.89±0.27	51.44±0.22	13.47±0.23	14	15.08 ±0.09	12.94 ± 0.10
SER	5.11±0.09	19.19±0.09	5.49±0.20	9.4	8.33 ± 0.06	6.94 ± 0.02
GLY	5.37±0.14	25.51±0.08	5.14±0.03	7.1	8.16 ± 0.06	6.65 ± 0.05
***THR**	5.86±0.10	19.22±0.10	5.84±0.22	9.9	7.98 ± 0.07	6.31 ± 0.07
ALA	6.31±0.13	39.54±0.16	6.88±0.19	8.5	10.96 ± 0.10	9.16 ± 0.13
***HIS**	1.00±0.04	6.78±0.05	1.44±0.13	3.1	1.34 ± 0.01	1.39 ± 0.04
PRO	0.01±0.00	31.61±0.11	4.29±0.11	4.1	0.01 ± 0.00	4.12 ± 0.14
ARG	4.20±0.08	23.68±0.20	5.71±0.22	8.9	4.87 ± 0.03	6.19 ± 0.04
***VAL**	14.18±0.18	23.22±0.16	6.18±0.02	6.6	3.39 ± 0.04	9.15 ± 0.26
TYR	3.89±0.08	12.20±0.07	3.33±0.08	3.9	4.35 ± 0.01	5.99 ± 0.16
***MET**	9.47±0.10	ND	1.58±0.08	1.9	6.71 ± 0.08	2.38 ± 0.07
***ILE**	4.02±0.10	17.84±0.14	5.06±0.12	3.8	5.50 ± 0.07	4.76 ± 0.17
***LEU**	7.30±0.15	33.70±0.18	7.79±0.19	7.2	10.34 ± 0.09	8.25 ± 0.12
CYS	1.60±0.03	21.63±0.10	ND	1	0.55± 0.06	1.94 ± 0.06
***PHE**	4.76±0.11	21.54±0.13	19.95±1.41	6	12.45 ± 0.12	2.70 ± 0.32
***TRP**	ND	1.87±0.02	ND	1.3	ND	ND
***LYS**	5.45±0.09	15.14±0.10	1.22±0.05	4.6	7.23 ± 0.08	6.50 ± 0.09
TOTAL	53.59±0.88	417.55±2.54	101.63±2.92	117.2	122.12±0.07	108.27±0.12
collecting location	Chile	Korea	Malaysia	India	Chile	Tunisia
collecting season	January 2007 (winter)	December 2000 (winter)	Not mentioned	July (summer)	November 2003 (summer)	July 2007(summer)
Data resource	Ortiz et al. (2009)	Hwang et al. (2008)	Matanjun et al. (2009)	Vinoj Kumar (2007)	Matanjun et al. (2009)	(Yaich et al., 2011)

All values were expressed as the mean ± standard deviation, except data of *Ulva lactuac* collected from India, which was expressed as the mean value, and all unit of value is mg/g dry weight.
ND: not detected.
*: it is essential amino acid.

The amino acid profile of some edible red macro-algae is summarized in Table 4. Similar to green macro-algae, acidic residues of aspartic and glutamic acids that ranged from 15% w/w to 33% w/w were rich in red macro-algae, cysteine and histidine were the lowest concentration amino acids in red seaweed. There were also big variations in the individual amino acid between species of red macro-algae. For example, proline was 0.01% w/w of total amino acid in *G. chilensis,* but higher as 5% w/w in *L. filiformis*; and histidine was low as 1.3% w/w in *G. domingensis*, higher as 14% w/w in *G. chilensis* (Table 4).

Table 4: Summary of amino acid profile of four edible red macro-algae concentrations are in mg/100mg dry weight

Amino acid	*G. Chilensis*	*G. domingensis*	*L. filiformis*	*E. cottonii*
ASP	11.02±0.10	1.0 ±0.0	1.5 ±0.0	2.65±0.15
GLU	15.47±0.13	0.9 ±0.0	1.4 ±0.0	5.17±0.13
SER	7.48±0.10	0.4 ±0.0	0.6 ±0.0	1.92±0.04
GLY	4.11±0.05	0.5±0.0	0.7 ±0.0	2.27±0.32
***THR**	6.44±0.05	0.4 ±0.0	0.6 ±0.0	2.09±0.01
ALA	6.64±0.09	0.6 ±0.0	0.7 ±0.0	3.14±0.11
***HIS**	11.25±0.11	0.1 ±0.0	0.2 ±0.0	0.25±0.10
PRO	0.01±0.00	0.4 ±0.0	0.5 ±0.0	2.02±0.09
ARG	5.96±0.04	0.4 ±0.0	0.6 ±0.0	2.60±0.14
***VAL**	7.66±0.06	0.4 ±0.0	0.5 ±0.0	2.61±0.07
TYR	3.89±0.05	0.2 ±0.0	0.6 ±0.0	1.01±0.12
***MET**	18.80±0.12	0.2 ±0.0	0.3 ±0.0	0.83±0.17
***ILE**	8.03±0.05	0.4 ±0.0	0.5 ±0.0	2.41±0.04
***LEU**	4.59±0.05	0.7 ±0.0	0.8 ±0.0	3.37±0.06
CYS	7.56±.06	0.03 ±0.0	0.1 ±0.0	ND
***PHE**	10.88±0.10	0.4 ±0.0	0.5 ±0.0	19.07±2.48
***TRP**	ND	0.2 ±0.0	0.2 ±0.0	ND
***LYS**	6.59±0.08	0.4 ±0.0	1.0 ±0.0	1.45±0.48
TOTAL	81.78±0.68	7.6 ±0.1	11.3 ±0.0	52.86±3.37
Location	Chile	Brazil	Brazil	Malaysia
Collecting season	January 2007 (winter)	Not mentioned	Not mentioned	Not mentioned
Reference	Ortiz et al. (2009)	Gressler et al. (2009)	Gressler et al. (2009)	Matanjun et al. (2009)

All values were expressed as the mean ± standard deviation, and except specific labeled, all unit of value is mg/g dry weight.
ND: not detected.
*: it is essential amino acid.

In green and red seaweed, aspartic and glutamic acids were significantly higher than brown seaweed. The total content of these two amino acids was approximately 20% of the total amino acids in most brown edible seaweed listed in Table 5, and an exception of *M. pyrifera* in which was up to 62% of total amino acid. The high content of these two acidic amino acids in all edible seaweed are typically associated

with umami flavor in seaweed. Alanine, a sweet amino acid, ranged from 5% to 16% of the total amino acid and was high in brown seaweed, which was also consistently rich in the glycine, valine, leucine and lysine. Cysteine and histidine were present at low concentration in most of the brown seaweed listed in Table 5.

Table 5: Summary of amino acid composition of some brown edible macro-algae

Amino acid	*M. pyrifera*	*Himanthalia elongata*	*Laminaria ochroleuca*	*S. polycystum*	Thick stem of kelp	Leaves of kelp	*H. elongata*
ASP	13.39±0.23	1.6 ± 0.5	3.8 ± 0.6	4.47±0.87	2.99	1.39	5.2
GLU	18.27±0.15	3.3 ± 0.8	4.7 ± 0.6	8.08±1.08	5.82	2.78	6.8
SER	8.31±0.10	1.0 ± 0.2	2.0 ± 0.5	2.58±0.16	4.16	2.22	2.9
GLY	6.65±0.10	0.6 ± 0.3	2.1 ± 0.7	3.19±0.35	4.08	1.95	2.7
***THR**	7.35±0.07	0.8 ± 0.2	1.6 ± 0.3	2.60±0.16	2.53	1.14	2.6
ALA	6.44±0.14	3.6 ± 1.2	4.9 ± 0.9	4.25±0.15	3.88	1.94	3.4
***HIS**	1.62±0.06	1.7 ± 0.4	4.1 ± 1.1	0.26±0.11	0.424	0.138	1.0
PRO	0.01±0.00	2.0 ± 0.7	2.5 ± 0.4	2.55±0.14	2.49	1.38	1.8
ARG	9.45±0.10	1.5 ± 0.4	5.0 ± 0.7	2.88±0.17	4.05	2.12	2.6
***VAL**	11.40±0.12	0.7 ± 0.1	1.9 ± 0.3	3.13±0.14	3.77	1.48	3.1
TYR	4.26±0.09	0.6 ± 0.2	1.3 ± 0.5	1.26±0.06	1.08	0.787	2.7
***MET**	11.12±0.11	0.1 ± 0.03	0.7 ± 0.2	1.25±0.04	2.08	1.31	1.3
***ILE**	5.07±0.10	0.8 ± 0.2	1.6 ± 0.3	2.94±0.16	1.08	0.542	2.1
***LEU**	3.39±0.13	0.6 ± 0.3	1.8 ± 0.5	4.67±0.25	3.11	1.61	3.8
CYS	2.28±0.08	ND	ND	ND	2.08	1.31	0.31
***PHE**	5.90±0.07	2.4 ± 0.5	5.7 ± 0.8	3.42±4.43	0.943	0.386	2.8
***TRP**	ND	ND	ND	ND	2.23	1.25	ND
***LYS**	3.21±0.09	1.1 ± 0.3	2.2 ± 0.5	2.11±0.77	1.66	0.624	2.9
TOTAL	51.34±0.83	22.4 ± 1.9	45.9 ± 2.4	76.38±2.31	48.46	24.36	48.01
collecting location	Chile	Spain	Spain	Malaysia	NM	NM	Spain
collecting season	January 2007 (winter)	July 2001 (summer)	July 2001 (summer)	NM	NM	NM	NM

All values were expressed as the mean ± standard deviation, except data of kelp and *H. elongata* collected from Spain, which was expressed as the mean value, and all the unit of value is expressed as mg/g dry weight;
ND: not detected;
NM: not mentioned.

1.7 Amino acid profile of *Undaria pinnatifida*

Same as other algae, aspartic and glutamic acids were the most abundant amino acids in *Undaria pinnatifida*. As shown in Table 6, glutamic acid content was the highest in mixed speices and higher in Korean samples compared to the Spanish samples. Cystine content was the lowest in all *U pinnatifida* samples, followed by methionine. The content of essential amino acid in *U. pinnatifida* was relative high, and the ratio of essential amino acid/non-essential amino acid (EAA/NEAA) of *Undaria pinnatifida* ranged from 0.66 to 0.92.

Table 6: Amino acid profile of *Undaria pinnatifida*

Amino acid	Spanish	Korean	Spanish	Mixed species
ASP	6.0 ± 0.7	13.31	12.2	16.44± 2.08
GLU	11.5 ± 1.5	20.75	13.5	27.40 ±6.05
SER	2.9 ± 0.4	5.36	6.2	7.56 ±0.76
GLY	3.9 ± 0.5	6.04	6.4	9.64 ±1.32
***THR**	0.8 ± 0.2	5.98	0.6	8.32 ±1.13
ALA	7.8 ± 1.1	13.29	8.6	8.88 ± 1.13
***HIS**	5.3 ± 0.5	13.09	2.4	4.72 ±0.57
PRO	4.8 ± 0.5	5.81	4.8	6.80 ±3.02
ARG	13.9 ± 3.0	5.27	6.1	9.83± 0.38
***VAL**	3.5 ± 0.5	6.25	7.3	9.83 ±0.94
TYR	2.0 ± 0.3	3.77	5.5	5.48 ±0.94
***MET**	1.9 ± 0.3	3.24	3.5	3.21 ± 0.94
***ILE**	3.0 ± 0.4	4.91	5.3	7.75 ± 0.57
***LEU**	4.8 ± 0.7	8.92	10.0	13.99 ± 0.76
CYS	ND	1.16	0.82	1.70 ± 0.38
***PHE**	16.6 ± 2.0	5.67	6.8	8.88 ± 0.57
***TRP**	ND	1.90	ND	1.32 ± 0.19
***LYS**	5.7 ± 1.0	6.18	6.5	10.58 ±0.76
TOTAL	94.4 ± 4.4	130.9	111.9	165.00 ± 14.93
EAA	48.8	56.14	42.4	68.6
NEAA	52.8	74.76	64.12	93.73
Ratio of EAA/NEAA	0.92	0.75	0.66	0.73
Collecting location	Spain	Korea	Spain	China, Japan and Korea
Collecting season	April 2002 (spring)	Not mentioned	Not mentioned	Not mentioned
Data resource	Snchez-Machado et al. (2003)	Je et al (2009)	Cofrades et al. (2010)	Dawczynski et al. (2007)

All values were expressed as the mean ± standard deviation, except data of sample collected from Spain and Korea, which was expressed as the mean value, and all unit of value is mg/g dry weight.
ND: not detected

1.8 Free amino acid composition

Free amino acids composition affects the taste of edible seaweed (Niwa, Furuita, & Aruga, 2003). Aspartic acid, glutamic acid, alanine and taurine are predominant in the total free amino acid in the blade of red algae (*Porphyra yezoensis*); asparagine, aspartic acid, glutamine, glutamic acid, glycine, serine, and D-cysteinolic acid were major components of the total free amino acid in the green algae (*Ulva pertusa*) (Kakinuma, Shibahara, Ikeda, Maegawa, & Amano, 2001).

Glutamic acid was the abundant free amino acid found in the brown seaweeds, *Fucus evanescens* and *Laminaria cichorioides* and ranged from 26.0% to 31.8% of total free amino acid. Alanine was another main free amino acid in the brown algae, *C. costata*. These amino acids constituted more than 50% of the total free amino acid in brown seaweed, and are major contributors to umami flavour and sweet taste of seaweed (Imbs et al., 2009). Large amounts of glycine and proline are also found in *Undaria*.

1.9 Nutritional value and amino acid score

The nutritional value of food protein is mainly determined by the type, amount and proportion of essential amino acids. The amino acid score is the most popular method to evaluate the nutritional value of protein. Initially, essential amino acids from the proteins in eggs or human milk protein were selected as the reference. However this has changed slightly and the most authoritative reference protein is an ideal protein model which was set up by FAO / WHO.

The amino acid score usually refers to the first limiting amino acid score, with a perfect score being 100. The closer the amino acids score is to 100, the higher its nutritional value is. The amino acids score of the proteins in marine algae ranged from 60 to 100, with values higher than that of the proteins in cereal and vegetables. The amino acid score of proteins in *Porphyra* and *Undaria* was 91 and 100, respectively, the same as that of animal foods (Table 7) (Murata & Nakazoe, 2001).

Table 7: First limited amino acids and amino acid score in proteins of various foods (Murata & Nakazoe, 2001)

	First limiting amino acid	Amino acid score[a)] 1973 FAO/WHO
Porphyra complex (Amanori)	Lysine	91
Laminaria saccharina (Konbu)	Lysine	82
Hizikia fusiformis (Hiziki)	Lysine	62
Undaria pinnatifida (Wakame)	–	100
Short neck shellfish	Valine	81
Sardine	–	100
Squid	Valine	71
Beef	–	100
Milk	–	100
Rice	Lysine	65

a): Amino acid score was calculated from amino acid requirements by FAO in 1973.

1.10 Extraction and characterization of protein from *Undaria Pinnatifida*

1.10.1 **SDS-PAGE**

To date, application of SDS-PAGE as an analytic tool for the identification of algae species is the method chosen by most researchers. Because most common metabolic processes in seaweeds are moderate and the seaweed proteins suffer little damage in preparation, the protein signature obtained by SDS-PAGE can be used for their identification (Antoine & Fleurence, 2003). It has been successfully used for different macro-algae species, such as, green seaweeds (*Ulva* and *Enteromorpha*)(C. Rouxel et al., 2001); red algae (*Palmaria palmata*, *Chondrus crispus, Porphyra umbilicalis, Gracilaria verrucosa* (Table 8).

Table 8: Molecular weight distribution of bands composing the reference pattern of red algae and green algae using SDS PAGE (expressed as unit kDa)(C. Rouxel et al., 2001; Rouxel, Daniel, Jérôme, Etienne, & Fleurence, 2001)

Band	Green algae				Red algae			
	Ulva rigida	***Ulva rotundata***	***Enteromorpha compressa***	***Enteromorpha intestinalis***	***Gracilaria verrucosa***	***Palmaria palmata***	***Porphyra umbilicalis***	***Chondrus crispus***
Band 1	68.5	69.9	65.8	66.4	62.1	59.6	73.1	49.3
Band 2	56.4	54.2	57.4	56.2	49.1	48.3	49.6	46.2
Band 3	44.7	29.5	26.2	25.9	45.9	32.7	32.1	43.2
Band 4	–	26.3	23.9	23.9	31.9	25.9	26.2	19.8
Band 5	–	22.9	23.1	22.5	25.5	20.3	22	17.2
Band 6	–	17.6	19.8	19.4	20.6	15.2	17.8	16.4
Band 7	–	15.5	–	–	16.8	–	15.9	15.2

1.10.2 **The interference of phenolic compounds**

The major problem in the characterisation of *U pinnatifida* using SDS-PAGE, especially for brown macro-algae was believed to be caused by phenolic compounds, polysaccharides, or other interfering secondary metabolites in the plant tissues (W. Wang, Tai, & Chen, 2008).

Phlorotannins are widely present in brown algae. They are a class of plant polyphenols made up entirely by polymerization of phloroglucinol or phloroglucinol units further substituted by halogens. They can form strong complexes with proteins either reversibly by hydrogen bonding through peptide or amide linkages, or irreversibly by covalent condensations (Waterman, 1994). To overcome this, insoluble polyvinylpolypyrrolidone (PVPP) that contained groups similar to the peptide bonds was an effective way of removing phlorotannin. Phlorotannin content can be reduced from 100% to 89% by adding given amount of PVPP at different PH values (Toth & Pavia, 2001).

1.10.3 **The interference of polysaccharides**

The high content of neutral polysaccharides, such as fucoidan and alginate in some brown seaweed can also limit the protein accessibility. These anionic and neutral polysaccharides can form very viscous solutions when cross linked with oxidized polyphenols. This decreases protein solubility in the extraction buffer during the extraction and purification of seaweed protein (G. Wang, Li, Xia, & Duan, 2005; Zhao et al., 2010).

An effective protein extract protocol from algae was reported by (Barbarino & Lourenço, 2005). Water was used to extract protein instead of high salt lysis solution. Protein content in fifteen marine algae was determined. Two important pre-treatments that improved protein extraction from lyophilized algae sample were incubation period and homogenisation. Sample incubated for 12h incubation and homogenized at 4℃ yielded the highest protein concentration.

Loretto Contreras and colleagues developed a powerful protein extracting protocol, which utilized phenol as an extra extracting reagent. It was found that phenol combined with the tris-lysis buffer overcame the common protein extraction problem caused by anionic polysaccharides, polyphenols, salts, and pigments that are rich in brown algae. The main role of phenol was not only to extract protein in the aqueous phase but also to disrupt membranes. As a result, a high-quality and high-yield protein was extracted with highly resolved bands with few contamination by SDS-PAGE(Contreras et al., 2008).

1.11 Monthly variations in the chemical composition of seaweeds

The chemical composition of natural algal populations is further influenced by spatial and temporal changes in environmental parameters including light, temperature, nutrients and salinity, as well as biotic interactions (Stengel, Connan, & Popper, 2011). The seasonal variations in the chemical composition of marine algae, such as, crude protein, total lipid, carbohydrate and element, etc., are well documented (Banerjee, Ghosh, Homechaudhuri, & Mitra, 2009; Gerasimenko, Busarova, & Moiseenko, 2010; Hernández-Carmona et al., 2009; Renaud & Luong-Van, 2006). A monthly variation in protein structure of *U. armoricana* was found in response to the digestive action of human enzymes (Joël Fleurence, Chenard, & Luçcon, 1999). In addition, variations associated with depth at which algae are found can affect the chemical composition of marine algae (Dere et al., 2003; Greenwell, Bird, & McLachlan, 1984).

The seasonal variations of some biochemical components of marine algae including lipids, carbohydrate and crude protein are summarized in Table 9.

Table 9: Seasonal variation in the chemical composition of marine algae

Species	Collecting location	Protein		Carbohydrate/Total fibre		Lipid		Data resource
		Highest value& season	Lowest value& season	Highest value& season	Lowest value& season	Highest value& season	Lowest value& season	
Flabellia petiolata	Turkey	24.72 spring&24.51 autumn	17.5 winter	54.2 summer	29.63 spring	1.83 winter	0.61 spring	Bilgin & Ertan (2002)
Halimeda tuna	Turkey	15.38 winter	12.79 summer	23.82 summer	10.04 winter	2.1 spring	0.68 winter	Bilgin & Ertan (2002)
Eisenia arborea	Mexico	11.68 later spring (May)&winter (December)	5.54 spring (March)	54.30 autumn (September)	43.32 winter (December)	0.45 later spring (May)	0.60-0.66 no significant difference from March to December, except May&June	Hernández-Carmona et al. (2009)
Ulva pertusa	Thailand	16.1 rainy season	14.6 summer	59.0 summer	52.2 rainy season	7.4 rainy season	2.1 summer	Benjama & Masniyom (2011)
Ulva intestinalis	Thailand	19.5 summer	16.4 rainy season	62.2 rainy season	51.3 summer	8.7 rainy season	7.3 summer	Benjama & Masniyom (2011)
Caulerpa racemosa	Australia	6.9 NS summer	6.8 NS winter	16.6 summer	14.7 winter	4.4 NS winter	3.8 NS summer	Renaud & Luong-Van (2006)
H. opunta	Australia	3.2 in summer and winter		2.7 summer NS	2.5 winter NS	2.9 winter	2.3 summer	Renaud & Luong-Van (2006)

All value was expressed as mean value, and presented as % dry weight, all value with different seasonal variable were significant different, except the value with superscripts-NS**,** which indicated no significant difference between seasons.

1.11.1 **Seasonal effects on the protein content of the seaweeds.**

Protein content is affected by season and other variables including nutrient supply (Stengel et al., 2011). Generally, the highest nitrogen content in seaweed was found in winter, that then start to decrease in spring, and reaching a bottom point in summer. It then starts to increase again in autumn. This is the typical annual pattern for most marine algae summarized in Table 9, Table 10 and Table 11.

In a study on tropical Australian marine macro-algae (Table 9), the protein content of *chlorophytes* and *rhodophytes* was the reverse of the usual pattern. The protein content of the *phaeophytes D. ciliolata, P. boryana and R. nhatrangensis* increased in the winter (Renaud & Luong-Van, 2006). The cause of these exceptions is unknown but might depend on the reproductive development of these species, or different nitrate availability in seawater during growth phases (Rico & Fernández, 1996; Stengel et al., 2011).

Table 10:Protein content of the four green algae sampled at 2-month intervals in 1997 (C. Rouxel et al., 2001)

month	*Ulva Rigida*	*Ulva Rotundata*	*Enteromorpha Compress*	*Enteromorpha Intestinlis*
February	17.3 ± 1.3	18.7 ± 1.4	13.9 ± 0.6	12.7 ± 1.4
April	11.3 ± 0.9	16.9 ± 1.2	14.1 ± 0.9	12.4 ± 1.3
June	16.2 ± 1.1	13.8 ± 1.1	16.4 ± 1.2	13.3 ± 1.0
August	19.6 ± 1.5	17.6 ± 0.8	12.7 ± 1.3	19.6 ± 0.8
October	21.9 ± 1.2	16.7 ± 0.5	9.6 ± 0.7	19.9 ± 0.6
December	23.6 ± 1.0	18.9 ± 1.3	7.2 ± 0.5	11.3 ± 1.1

All data was expressed as mean±SD, and the unit was % dry weight.

Table 11:Protein levels of red algae according to the season (Catherine Rouxel, André Daniel, et al., 2001)

Month	*Palmaria palmata*	*Porphyra umbilicalis*	*Chondrus crispus*	*Gracilaria verrucosa*
February	22.0±1.3	25.8±1.5	25.5±1.1	22.5±1.1
April	20.4±1.1	26.0±1.1	25.4±1.2	20.4±0.8
June	10.1±0.6	19.8±0.9	7.9±0.4	7.3±0.5
August	9.5±0.5	15.5±1.0	7.7±0.5	15.8±0.6
October	13.7±0.9	15.9±0.9	11.9±0.8	14.9±0.3

All data was expressed as mean±SD, and the unit was % dry weight.

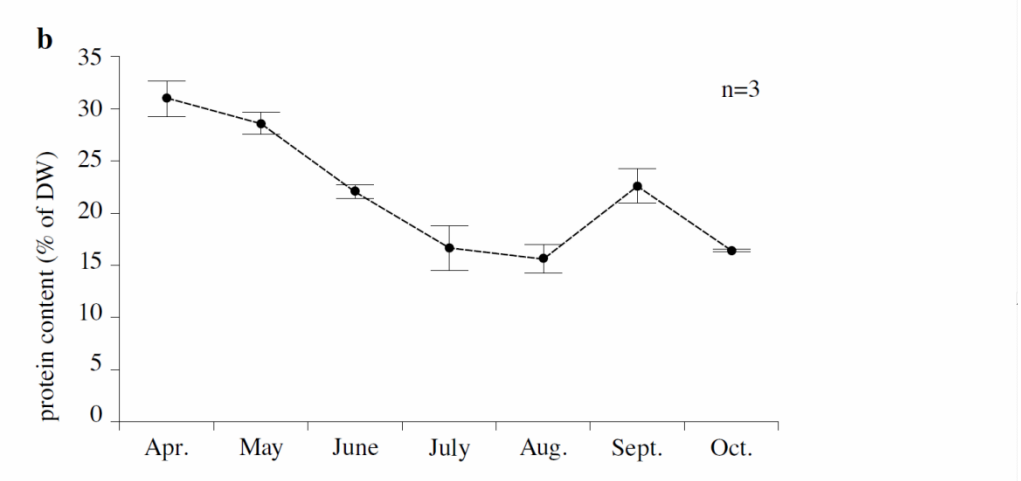

Figure 3: Seasonal changes in the protein content (% of dw) of *Enteromorpha spp*. from Jurata. Values are given as a mean with standard deviation (Haroon, Szaniawska, Normant, & Janas, 2000).

1.11.2 Seasonal effects on the amino acid profile of the seaweeds

A number of studies were evaluated the seasonal variation of individual amino acid component in a range of marine algae, a significant seasonal effect on amino acid composition was conducted by researchers.

Gustavo Hernández-Carmona and his colleagues conducted over ten months of collection and comparison on the *Eisenia arborealis* which was brown algae rich along the west coast of the California peninsula. Dry algal chemical composition was analysis, protein content was 9.44%, and 17 amino acids were determined as well, the most abundant three were glutamic acid, aspartic acid and leucine (Hernández-Carmona et al., 2009). Same trend with significant monthly variation of protein content, except three amino acids, isoleucine, leucine, and threonine, the other amino acid content significantly varied along months changing, these differences were presented by Table 12 (Hernández-Carmona et al., 2009).

A number of variations on amino acid profile were observed in *Palmaria palmate* through 10 month study, glutamic acid, serine, and alanine were present in a relatively high amount during late winter, spring, September, and October, and were absent the rest of the year. Arginine, lysine, hydroxyproline disappeared in spring, proline appeared only in February, September and October (Galland-Irmouli et al., 1999).

A few studies demonstrated this seasonal variation of amino acid profile reflected seasonal alterations in the types of proteins or enzymes present in algal tissues. A

specific 54 kDa protein band was observed at February in *Ulva armoricana* at three collecting month, November, December and February with mainly variation of glutamic and aspartic acid levels (J. Fleurence, Chenard, & Luçon, 1999), and similar seasonal variation of protein pattern was observed in *Gracilaria verrucosa* as well (Catherine Rouxel et al., 2001).

Table 12: Monthly variation in the amino acids in *Eisenia arbore* a(Hernández-Carmona et al., 2009)

Month	Glutamic acid[1]	Aspartic acid[2]	Leucine[3]	Arginine[4]	Alanine[5]	Valine[6]	Lysine[7]	Isoleucine[7]
MAR	9.93 ± 0.04[1]	7.15 ± 0.01[1]	5.19 ± 0.03[1]	4.75 ± 0.09[1]	3.95 ± 0.04[1]	4.02 ± 0.01[1]	3.71 ± 0.01[1]	3.17 ± 0.07[1]
APR	9.90 ± 0.01[1]	5.85 ± 0.00[2]	5.23 ± 0.02[1]	4.78 ± 0.01[1]	4.06 ± 0.01[2]	4.06 ± 0.01[1]	3.78 ± 0.01[2]	3.24 ± 0.02[1]
MAY	10.12 ± 0.01[2]	7.84 ± 0.00[3]	5.53 ± 0.01[1]	5.32 ± 0.01[2]	4.58 ± 0.00[3]	4.03 ± 0.00[1]	3.89 ± 0.00[3]	3.43 ± 0.01[1]
JUN	10.26 ± 0.01[3]	6.26 ± 0.03[4]	5.55 ± 0.01[1]	4.88 ± 0.01[3]	4.86 ± 0.00[4]	4.21 ± 0.00[2]	3.87 ± 0.03[3]	3.32 ± 0.01[1]
JUL	10.52 ± 0.01[4]	6.35 ± 0.03[5]	5.89 ± 0.00[1]	4.88 ± 0.01[3]	4.87 ± 0.03[4]	4.25 ± 0.00[3]	3.84 ± 0.02[3]	3.33 ± 0.00[1]
AUG	10.20 ± 0.01[3]	6.19 ± 0.02[4]	5.79 ± 0.00[1]	4.92 ± 0.01[3]	4.73 ± 0.05[5]	4.26 ± 0.00[3]	3.88 ± 0.01[3]	3.32 ± 0.00[1]
SEP	9.88 ± 0.01[1]	6.04 ± 0.02[6]	5.69 ± 0.01[1]	4.97 ± 0.01[3]	4.59 ± 0.07[3]	4.27 ± 0.01[3]	3.92 ± 0.00[3]	3.30 ± 0.01[1]
OCT	10.12 ± 0.01[2]	7.21 ± 0.02[7]	5.20 ± 0.02[1]	4.94 ± 0.01[3]	4.21 ± 0.00[6]	4.04 ± 0.01[1]	3.58 ± 0.02[4]	3.20 ± 0.02[1]
NOV	9.97 ± 0.01[1]	6.23 ± 0.014	5.74 ± 0.02[1]	5.26 ± 0.01[2]	4.88 ± 0.01[4]	2.88 ± 0.02[4]	3.88 ± 0.00[3]	3.37 ± 0.03[1]
DEC	10.22 ± 0.01[3]	7.88 ± 0.01[3o]	5.23 ± 0.01[1]	5.38 ± 0.00[2]	4.86 ± 0.01[4]	4.28 ± 0.00[3]	3.97 ± 0.01[5]	3.04 ± 0.71[1]

Values are means (g per 100 g of protein) ±SD, n=3. Different superscripts indicate significant differences among amino acids, and months

1.12 Effect of location on protein content of marine algae

Discussed at above, because of their commercial importance, seasonal variability in marine algae composition has long been of interest. The evidence proves that geographical location affects the chemical composition of algae, especially the protein content (Dere et al., 2003; Haroon et al., 2000).

One study focused on macro-algae collected from Gemlik-Karacaali (Bursa) and Erdek-Ormanlı (Balıkesir) in the Sea of Marmara of Turkey was determined the relationship between the total protein, location and the depth, remarkable variations were observed by this study. Total protein content varied depending on the species, location and depth. There were some noticeable specific differences in total protein when macro-algae from the Karacaali and Ormanl stations were compared (Figure 4), it supported that environmental properties of seawater is one of the most important factors affecting algae growing (Dere et al., 2003). And this noticeable geographical variation of protein was observed on different species from different location, such as, *Enteromorpha spp*. from the Gulf of Gdańsk, Poland (Haroon et al., 2000).

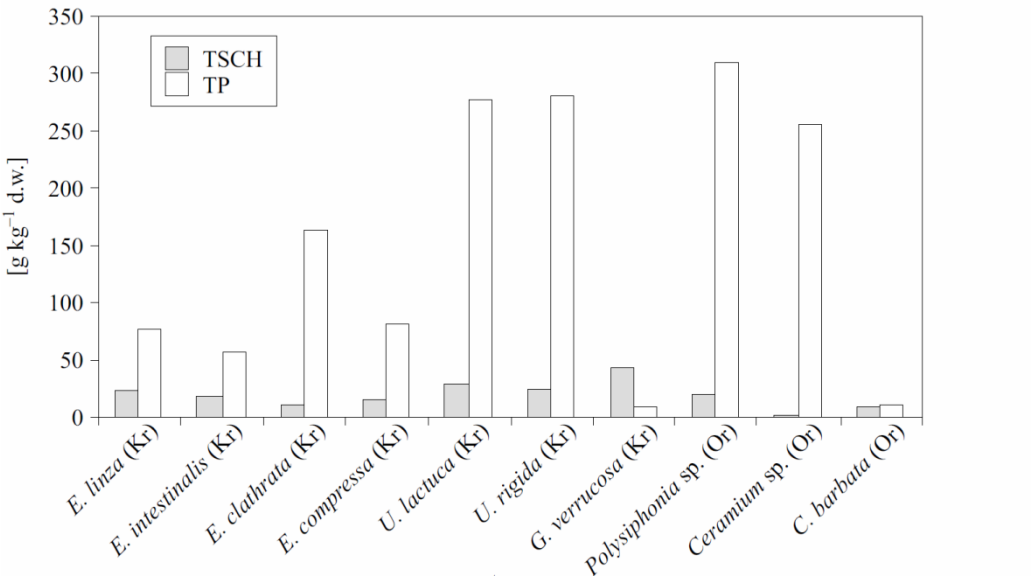

Figure 4: The change in total soluble carbohydrate and total protein levels in a number of taxa collected from surface waters at Ormanlı and Karacaali stations. Kr = Karacaali, Or = Ormanli (Dere et al., 2003)

Chapter 2Methodology

2.1 Sample collection

The *Undaria pinnatifida* samples were collected at monthly intervals from June to December, 2011 from four mussel farms located at the Port Underwood and Pelorus Sound coastal area around Nelson in New Zealand (Figure **5**). Seaweed was removed from the mussel lines by hand. The holdfast was completely removed from the line, preventing them from growing back at the same area. Twenty replicate samples were collected from the same farm for each month.

For each sample, the blades were separated from the sporophyll on the boat before being placed in labelled bags. These samples were then frozen overnight prior to being air freighted to Vitaco Limited, a freeze-drying plant in Auckland, to be lyophilized in bulk within 48 hours of frozen storage. Dry weights of the freeze-dried samples were recorded prior to the samples being milled using a coffee grinder (Breville CG2B Coffee'n Spice Grinder) and sieved using a 600 micron sieve. The powdered samples were then stored in 200 mL PET bottles and kept in a cupboard at room temperature prior to total nitrogen and amino acid analysis.

Another sample comprised of the complete plant that was placed in individually labeled plastic bag and air freighted to the laboratory as soon as possible. Fresh plants were thoroughly removed of their epiphytes, and rinsed with de-ionized water. These samples were evenly divided into three portions. One portion was immediately used for protein extraction. The second portion was oven-dried at 60 ℃ for 48 hours, and the third portion was freeze-dried for 72 hours. These two dried samples were milled using a coffee grinder (Breville CG2B Coffee'n Spice Grinder) and sieved using a 600 micron sieve. The powdered samples were then stored in 200 mL PET bottles and kept in a cupboard at room temperature prior to protein extraction and gel electrophoresis.

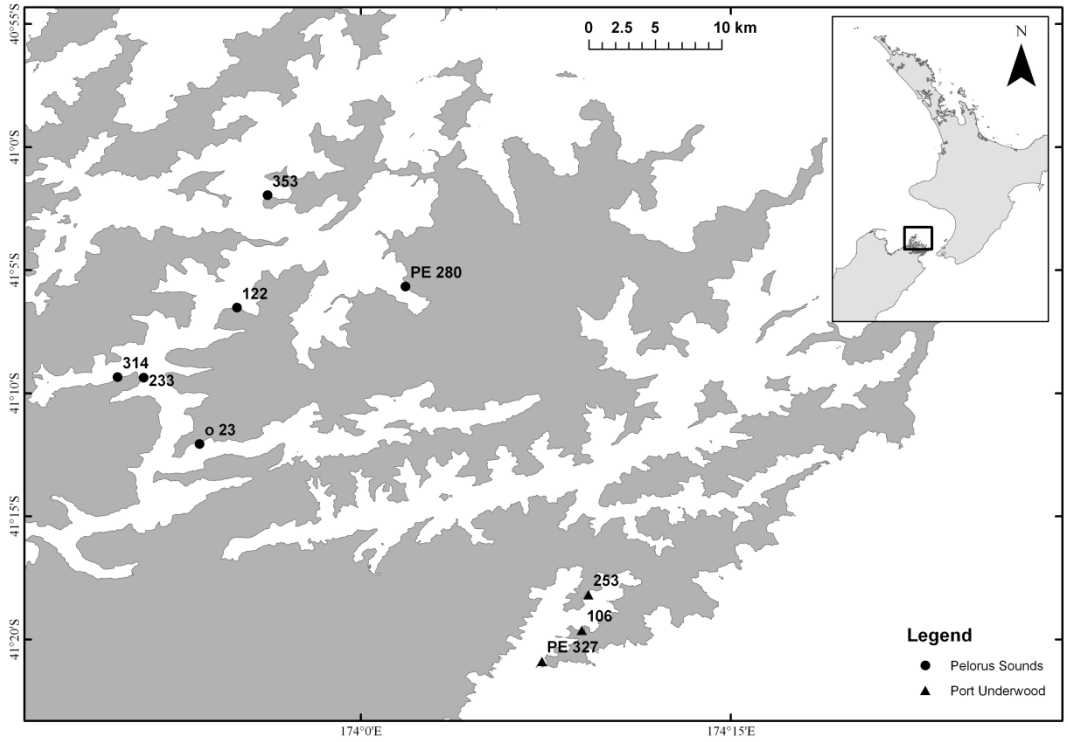

Figure 5: Sampling farms from the Port Underwood and Pelorus Sound coastal areas around Nelson in New Zealand.

2.2 Determination of nitrogen content in *Undaria pinnatifida*

Apparatus

A VelpUDK 139 distillation unit equipped with a Velp DK 20 heating block (VELP Scientifica)

Reagent

All reagents are AR grade, supplied by AUT applied science laboratory.

Procedure

The ground sample (500 mg) was accurately weighed, wrapped in nitrogen-free paper, and then placed in a 250 ml digestion tube. Concentrated sulphuric acid (15mL) was added and the tube was carefully shaken to begin the digestion. The sample was then digested at 420℃ for 60 minutes using a Velp DK 20 heating block. The level of liquid was monitored during the digestion, and if the level dropped significantly, an extra 5-10 mL of concentrated sulphuric acid was added. After 60 minutes, the tube was allowed to cool for 5 minutes and 3 mL of cold 30% hydrogen peroxide was added. The liquid in the test tube now should be clear and colourless. If the liquid was cloudy or retained a colour, a further 3 mL of 30% peroxide was

added and the mixture was digested for another 20 minutes. This was repeated as necessary until the solution remained colourless and clear.

After digestion, the sample was distilled using a VelpUDK 139 distillation unit. The digestion tube and sample were attached, and the automatic distillation process started. The digest was made alkaline with 50 mL of 35% sodium hydroxide solution, and the released ammonia was steam distilled into a receiver filled with 20 mL of 4% boric acid.

When distillation finished, 10 drops of mixed Kjeldahl indicator was added to the receiver flask and the contents titrated with 0.1mol L^{-1} standard hydrochloric acid. Nitrogen content was calculated according to the equation below:

$$\text{Nitrogen content (mg g}^{-1}\text{)} = \frac{(V_1 - V_2) \times C \times 14}{W}$$

V1 - titrated volume of standard acid for sample, mL;
V2 - titrated volume of standard acid for reagent blank, mL;
C- concentration of standard hydrochloric acid, mmol mL^{-1};
14 = Molar mass of N, mg $mmol^{-1}$
W - sample weight, g

2.3 High-performance liquid chromatographic analysis of amino acids with pre-column derivatization using phenyl isothiocyanate

2.3.1 Principles of method

2.3.1.1 Pre-column derivatization

To date, about 180 natural amino acids have been found, but only 20 amino acids are known to be involved in protein synthesis. As amino acid analysis plays an important role in protein chemistry, it has been given considerable attention. Instrumental methods for amino acid analysis have been extensively studied (Cohen & Strydom, 1988).

A quantitative analysis of amino acids using ion-exchange chromatography with post-column ninhydrin derivatization used to be the common method of amino acid analysis (Moore et al.). However the cost and difficulty of this traditional method are obvious.

2.3.1.2 Phenyl isothiocyanate as a derivatization reagent

Since the 1970s, the development of high-performance liquid chromatography, especially in combination with reversed-phase columns and pre-column derivatization, has to some extent simplified the analysis of amino acids.

Pre-column methodology was considered to be more sensitive than many of the post-column approaches. Several pre-column derivatization options included:

1. The o-phthalaldehyde (OPA) method (Betancort Rodríguez, García Reina, & Santana Rodríguez, 1997; Concha-Herrera, Torres-Lapasió, Vivó-Truyols, & García-Álvarez-Coque, 2007),

2. The 6-aminoquinolyl-*N*-hydrosysuccinimidyl carbamate (AQC) method (Bosch, Alegría, & Farré, 2006; Callejón et al., 2008; Li, Yang, Xie, Shan, & Dong, 2007), and 9-fluorenylmethyl chloroformate (FMOC)method (López-Cervantes, Sánchez-Machado, & Rosas-Rodríguez, 2006),

3. The phenylisothiocyanate (PITC) method (Inglis, Bartone, & Finlayson, 1988; Shang & Wang, 1996; Vasanits & Molnár-Perl, 1999) and dansyl method (André et al., 2007; Naval, Gómez-Serranillos, Carretero, & De Arce, 2006),

4. The 4-nitrophenylisothiocyanate (NPITC) method (Amini, Rouini, Asad-Paskeh, & Shafiee, 2010; Cohen, 1990),

Each of these methods had some advantages as well as disadvantages. Phenylisothiocyanate (PITC) however is by far the most common pre-column derivatization reagent. PITC reacts with both primary and secondary amines, whereas some other methods will not react with proline. Advantages of this method included sensitivity, UV detection (as opposed to fluorescence), and stability (Andersen, 1995).

2.3.1.3 The method chosen in this study

The PITC method of Bartolomeo and Maisano has been cited frequently and is popular for the determination of amino acid content in marine algae (Bidlingmeyer, Cohen, & Tarvin, 1984). Isocratic separation was not effective for amino acids, and even a single linear gradient does not supply good enough resolution. Hence multi-linear gradients of buffer and solvent are the best choice (Concha-Herrera et al., 2007). An adaption of this method was used in this study.

2.3.2 Procedure of individual amino acid standard qualification and mixed amino acids standard curve calculation

2.3.2.1 Apparatus

A Shimadzu high performance liquid chromatograph consisted of a Shimadzu controller (Model SCL-10A), quaternary pump (Model LC-10AT), Shimadzu auto sampler (Model SIL-10A), and a UV/VIS detector (ICI LC 1200). Data collection was acquired using Shimadzu LC work station software (version 2008).

2.3.2.2 Preparation of Reagents

Acetonitrile (HPLC grade), methanol (HPLC grade), phenylisothiocyanate (PITC), norvaline were purchased from Sigma-Aldrich new Zealand Ltd. HPLC grade water was prepared by using a Milli-Q system (Millipore, MA, USA). 22 amino acid standards were supplied by Selena McMillan, PhD student from university of Auckland. Other chemicals were supplied by AUT applied science laboratory. Reagents are AR grade, except as noted.

Hydrolysis and derivatization tubes with resealable enclosures were purchased from Thermo Fisher Scientific New Zealand Pty Ltd.

Redissolve solution

It is constituted 2:2:1 mixture by volume of ethanol: water: triethylamine.

Derivative solution

The derivative reagent consisted of 7:1:1:1 solution (by volume) of ethanol: triethylamine: water: phenylisothiocyanate (PITC).

Dilution solution

The dilute solution consisted of disodium hydrogen phosphate (Na2HPO4), acetonitrile and was prepared as follows:

710 mg of Na2HPO4 was added to 1 L of water and titrated to pH 7.40 with 10 % phosphoric acid (H3PO4). The resulting solution was mixed with acetonitrile so that acetonitrile was 5 % by volume.

Stock mixed amino acid solution and individual amino acid solution

The 22 mixed amino acid and individual amino acid standard solution were prepared according to Table 13, weight mentioned amount amino acid in 50mL beaker, and dissolved by Milli-Q water, completely transferred to 25mL volumetric flask, filled Milli-Q water to the mark.

Separated mixed and individual amino acid standard solution into 2.0 mL screw cap tubes, and stored at -20℃, until it is ready for use.

Table 13: Composition of individual amino acid standard solution

Amino acid	Molecule weight	Concentration (µmol/L)	Amino acid weight (g)
L-Alanine	89.09	250	5.5681±0.005
L-Arginine Hydrochloride	210.66	250	13.1662±0.005
L-Asparagine	132.12	250	8.2575±0.005
L-Aspartic Acid	133.1	250	8.3187±0.005
L-Cysteine Hydrochloride	157.62	250	9.8512±0.005

L-Cystine	240.3	125	7.5094±0.005
L-Glutamic Acid	147.13	250	9.1956±0.005
L-Glutamine	146.14	250	9.1337±0.005
Glycine	75.07	250	4.6919±0.005
L-histidine HCL monohydrate	209.63	250	13.1019±0.005
trans-4-Hydroxyl-L-Proline	131.13	250	8.1956±0.005
L-Isoleucine	131.17	250	8.1981±0.005
L-Leucine	131.17	250	8.1981±0.005
L-Lysine•HCl	182.65	250	11.4156±0.005
L-Methionine	149.21	250	9.3256±0.005
L-Phenylalanine	165.19	250	10.3244±0.005
L-Proline	115.13	250	7.1956±0.005
L-Serine	105.09	250	6.5681±0.005
L-Threonine	119.12	250	7.4450±0.005
L-Tryptophan	204.23	250	12.7644±0.005
L-Tyrosine	181.19	250	11.3244±0.005
L-Valine	117.15	250	7.3219±0.005

2.3.2.1 Chromatographic conditions

Mobile phase A: 0.14 M sodium acetate buffer pH=6.4

The method of preparation is given in detail because this is critical to reproducible separation:

Anhydrous sodium acetate (11.484 g) was weighed into 800 mL Milli-Q water in a 1 L beaker and stirred to completely dissolve. Triethylamine (0.50 mL) and 10 mM EDTA (1 mL) was added, and after stirring, the mixture was adjusted to pH = 6.4 ± 0.05 with acetic acid. The mixture was then introduced into a 1 L volumetric flask and made up to the mark with Milli-Q water. Before use, the phase was filtered through a 0.45 µm filter and degassed with helium.

Mobile phase B: 32% acetonitrile

HPLC grade acetonitrile (320 mL) with Milli-Q water (680 mL) were mixed together. Before use, the phase was filtered through a 0.45 µm filter and degassed with helium.

Mobile phase C: Pure acetonitrile (HPLC grade)

Column: Waters, Nova-Pak silica-based, reversed-phase C18 column, 4 µm, 3.9 x 300 mm. Operated at room temperature.

Injection volume: 20µL (Automatic injection mode)

Detection wavelength: 245 nm

2.3.2.2 Gradient elution programme

Table 14: Gradient elution programme

Time (minute)	Flow rate (mL/min)	Phase A (%)	Phase B (%)	Phase C (%)
0.01	1.00	96	4	0
2.00	1.00	96	4	0
50.00	1.00	0	100	0
51.00	1.00	0	0	100
55.00	1.00	0	0	100
57.00	1.00	96	4	0
62.00	1.00	96	4	0
62.00	1.00	96	4	0
68.00	stop			

2.3.2.3 Mixed amino acid standard curve preparation

The concentrations of 5 µmol/L, 10 µmol/L, 20 µmol/L, 40 µmol/L, and 60 µmol/L for each amino acid were prepared by diluting the stock mixed amino acid solutions with 0.1 mol L^{-1} HCL Milli-Q water. 100 µL of the mixed amino acid standard was transferred to a 1.5mL screw cap vial, and vacuum dried.

50µL of diluting solution was added to the dried standards for neutralization of HCL. The mixture was vortexed to re-solubilise amino acids and then vacuum-dried (Bidlingmeyer et al., 1984; Campanella, Crescentini, & Avino, 1999; Fermin, Radinsky, Kratochvil, Hall, & O, 2003). The re-dried standards were derivatised with 100 µL of the derivative solution (Bidlingmeyer et al., 1984; Campanella et al., 1999; Fermin et al., 2003) and allowed to stand at room temperature for 30 minutes.

After derivatization, the standards were vacuum-dried. PITC had to be adequately removed to avoid chromatography problems and early column degradation (Fermin et al., 2003). This was followed by re-suspension using 1mL of diluting solution. The amino acid extract is now ready for HPLC analysis. Concentration of each amino acid and related peak area was plotted and formed the standard calibration curve.

2.3.2.4 Aspartic acid and glutamic acid standards preparation

The loss of asparagine and glutamine was of concern in this study, especially if storage of the mixed amino acid standard was extended. It was found that these PTC-derivatised amino acids decreased with storage time.

Amino acid concentrations of 5 µmol/L, 12.5 µmol/L, 10 µmol/L, 20 µmol/L, 25 µmol/L, 37.5 µmol/L, 40 µmol/L, 50 µmol/L, 60 µmol/L and 62.5 µmol/L of aspartic acid and glutamic acid were prepared by diluting a stock solution of aspartic acid and glutamic acid solution with 0.1 mol/L HCL. 100µL each of the individual aspartic acid and glutamic acid standards were transferred into a 1.5mL screw cap

vial. The amino acid extract is now ready for HPLC analysis. Concentration of each amino acid after HPLC analysis was determined by plotting the standard calibration curve.

2.3.3 Mixed amino acid standard calibration curve using an internal standard

2.3.3.1 Choice of an internal standard

The basic principles of choosing an internal standard are as follows. Firstly, it should not be contained in the original sample; secondly, it should have similar properties with the test sample; next, it should be miscible with the sample; and lastly, the retention times of the internal standard and sample should be as close as possible with baseline separation.

Based on above recommendations, norleucine and norvaline, which are common internal standards frequently selected other researchers (Avino, Campanella, & Russo, 2003; Bailey-Shaw, Golden, Pearson, & Porter, 2009; Cigić, Vodošek, Košmerl, & Strlič, 2008; Frank & Powers, 2007; Loureno et al., 2002; Yi et al., 2011) were considered in this study. However norleucine was not chosen due to budget limitation. Norvaline, which was used initially and performed well when using the Phenomonex Luna column, did not perform well when a replacement Nova-Pak C18 column was used. Norvaline and valine could not be separated well using the newer column. Tryptophan was substituted as an internal standard to monitor derivatization and the drying steps that followed it. But for unknown reason, the quantitative analysis of sample using tryptophan was unsuccessful. Hence quantitative analysis of the *Undaria* samples was only based on the amino acid standard curves without the calibration using an internal standard. And accurate inject volume by auto sampler should assure repeatability and accuracy of this method.

2.3.4 Repeatability and accurate test

2.3.4.1 Stability test for PTC-amino acid

100 µL of 40 mM the mixed amino acid standards were derivatised according to the above method, and kept at 4^{o}C after dilution and run HPLC every five hours.

2.3.4.2 Precision (repeatability) test via multiple injection of the same sample

Forty millimolar of the mixed amino acid standard (100µL) was derivatised and consecutively injected six times. This was used to calculate the relative standard deviation.

2.3.4.3 Intermediate precision (repeatability) test via multiple determination for *Undaria* samples at different day

Accurately weighed (accuracy to 0.0001 g) *Undaria* blade sample powder (0.04 g) was placed in a 2mL screw cap vial with a PTFE lined silicone cap liner. Samples from the same farm and on the same month were analysed. Six determinations were made for each sample, and all determinations were carried out using the same HPLC conditions over two days.

One millilitre of HCl solution (1:1 v/v) was added to the sample in an ampoule flushed with nitrogen gas, and sealed. The ampoule was placed in an oven at 110 ℃ and digested for 22 hours. When digestion was finished, the ampoule was cooled down to room temperature, filtered through a 0.45μm membrane filter, and then diluted to 5 ml using Milli-Q water.

One hundred microliters of the hydrolysed sample was transferred to a 2 mL screw cap vial. The sample was derivatised and analysed as previously described. Standard deviations of peak areas for the six determinations were calculated.

2.3.4.4 Accuracy test via multiple determinations of reference protein samples

Approximately 0.004 g of bovine albumin was weighed to an accuracy of 0.0001 g and analysed according to the above procedures. Six determinations were carried out using the same conditions on different days. Standard deviations of the peak areas for the six determinations were calculated.

2.3.5 Sample hydrolysis and amino acid content determination

0.04 g of the sample powder (accurate weighed to 0.0001 g) was placed in a 2mL screw vial with silicone white/PTFE red cap. The acid hydrolysis procedure was the same as the reference amino acid standards procedure. The derivatization procedure and HPLC analysis was the same as that carried out for the mixed amino acid standards.

2.3.6 Calculation of the nitrogen-to-protein factor and essential amino acid ratio

The calculation of nitrogen-to-protein factor used the equation reported (Loureno et al., 2002) which was determined by the ratio of amino acid residues to total nitrogen of the sample.

2.4 Protein extraction

2.4.1 **Protein extraction and characterization**

2.4.1.1 Principle

Composition of the extraction buffer used for protein extraction from *Undaria* was a modification of the method by Coyer et al. (1994). New components were either added or concentrations of the components were varied.

2.4.1.2 Preliminary soluble and insoluble protein tests

Most studies determined the soluble protein content of algae samples using the Bradford colorimetric method (Gressler et al., 2009; Hong, Zhou, Lan, & Liang, 2010; Pisani, Munzi, Paoli, Bačkor, & Loppi, 2009). The advantages of the Bradford method are high sensitivity, fast determination and the presence of less interfering substances compared to the Lowry method. Frequently utilized reagents such as $K^{+,}$ Na^{+}, Mg^{2+} ions, Tris buffer, glucose, sucrose, glycerol, β-mercaptoethanol, and EDTA will not cause interference with this assay (Gotham, Fryer, & Paterson, 1988). For these reasons, the Bradford method was chosen to determine the soluble and insoluble protein content in *Undaria pinnatifida*.

2.4.1.3 Efficiency of different protein extraction protocols

In order to overcome the interference of polysaccharides, it was necessary to remove polysaccharides on the surface of *Undaria* in this study. Homogenizing the sample at low temperature will avoid proteolysis and other modes of protein degradation during sample preparation. Phenol extraction followed by methanolic ammonium acetate precipitation was reported to give high yield protein extraction from protein-poor, recalcitrant plants tissues, such as *Hypericum perforatum* which are rich with phenolic compounds (Karppinen, Taulavuori, & Hohtola, 2010) and fruit tissues, such as apple and strawberry (Zheng, Song, Doncaster, Rowland, & Byers, 2007). This procedure involved selective extraction of proteins with phenol from the lysate that leaves polysaccharides and phenolics in the aqueous phase (Figure 6) (Berkelman, 2008; W. Wang et al., 2008). This method has been successfully used for the extraction of protein from various marine algae (Contreras et al., 2008; Yotsukura, Nagai, Kimura, & Morimoto, 2010).

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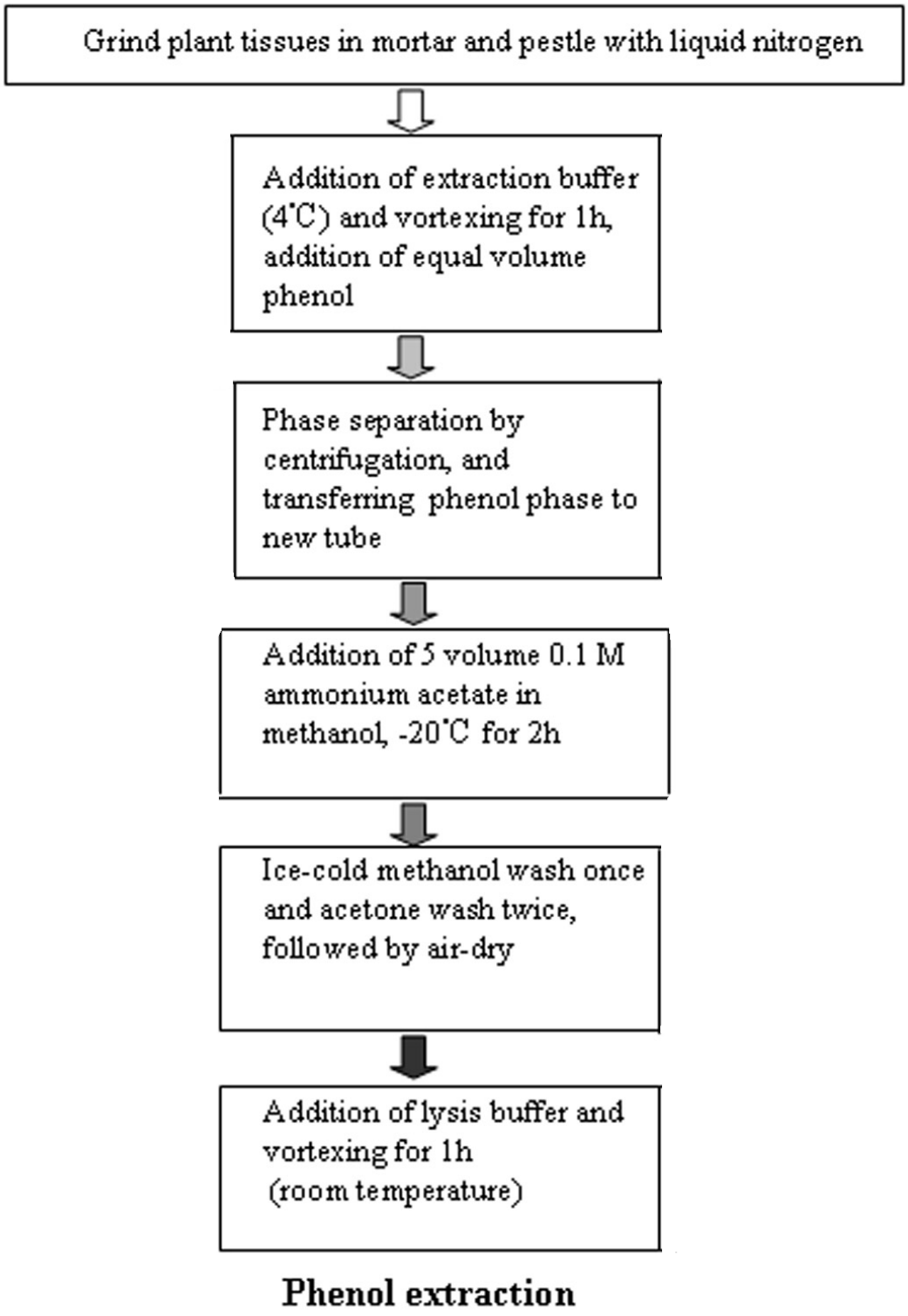

Figure 6: Schemematic flow diagram of phenol extraction followed by methanolic ammonium acetate precipitation protocol for protein extraction (W. Wang et al., 2008)

2.4.1.4 The effect of different components in the protein extraction solution

Protein sample preparation varies with the nature of sample, and efficiency of the components of the lysis buffer. The buffer may either contain a single protein solubilizer, or a complex mixture of a variety of detergents and reducing agents. The change in concentration of detergent, protein solubilizer, reducing reagent and electrolyte in lysis buffer would impact the results when using two-dimensional electrophoresis.

Utilization of a solubilizer can damage the hydrogen bonds of protein and increase protein solubility. Detergent is frequently chose as a protein solubilizer, because it can break hydrophobic interactions between protein, and enhance protein solubility at its isoelectric point as they have variable binding affinity to proteins (Tanford & Reynolds, 1976). The two most common surfactants used as are either anionic (sodium dodecyl sulphate, SDS) or non-ionic of the polyoxyethylene type (alkylphenol ethoxylates such as the Triton X series) (Helenius & Simons, 1975).

Introduction of a reducing agent can break the disulfide bonds between protein molecules to facilitate separation of the peptide chain, thereby increase protein solubility. The most popular reducing reagents are dithiothreitol (DDT) and 2-mercaptoethanol.

2.4.1.5 Soluble protein extraction and gel electrophoresis procedure

Apparatus

IKA® ULTRA-TURRAX® T-25 basic disperser (IKA Works, Inc)

Thermo Scientific Owl P9DS Dual Gel System equipped with PowerPac™ HC High-Current Power Supply (Thermo Fisher Scientific New Zealand Ltd)

Precise precast 12% Tris-HEPES Gels, 10 x 8.5cm, 10-well (Thermo Fisher Scientific New Zealand Ltd)

Reagent

All reagents are AR grade, except labelled specifically. Except Polyvinylpyrrolidone (PVP) and Triton x-100 were provided by DR. Mark Duxbury, the other reagents were supplied by AUT laboratory.

Extraction and gel electrophoresis procedure

Oven-dried and freeze-dried *Undaria* sample powders (500mg) or fresh sample (5g) were immersed in 5mL of extracted lysis (1.5% w／v Polyvinylpyrrolidone [PVP],

0.7M sucrose, 0.1M Potassium chloride [KCL], 0.5M Tris-HCL PH=8.0, 500mM Ethylenediaminetetraacetic acid [EDTA], 2% v/v β-Mercaptoethanol and 2% v/v Triton x-100) for 12 h (20 minutes for fresh samples) at 4℃. After incubation, immersed samples were homogenised by IKA® ULTRA-TURRAX® T-25 basic disperser for 4 min at 4℃ with speed of 13500L/min, then, an equal volume of Tris–HCl pH 7.5–saturated phenol was added, and the mixture was re-homogenized for 5 min at 4℃. The mixture was centrifuged at 10,000g for 20 min, and the upper phenol phase was removed. Lower phase was re-extracted using the same volume of phenol as above. Combined these two volumes upper phenol phase, then added five volumes of 0.1 M ammonium acetate methanol solution which was pre-cooled in freezer to combined phenol phase and incubated at -20℃ overnight. The extract was centrifuged at 10,000g for 20 minutes at 4℃, the supernatant was discarded, and the protein pellet was rinsed in ammonium acetate (0.1 M in methanol) for 20 minutes at -20℃ for three times, subsequently, the protein pellet was rinsed two times in four volumes of cold acetone and air dried.

Next dissolved fresh protein pullet by 100µL sample buffer (5% w/v sodium dodecyl sulphate [SDS], 10% v/v glycerol, 0.005% w/v Bromophenol blue, 6.25% v/v 1M Tris-HCL PH=6.8 and 1.5% v/v 2-β-mercaptoethanol), heated 1 minute in boiling water, cooled down, centrifuged at 10000g for 1 minute, upper clear supernatant was ready for loading SDS-PAGE.

SDS-PAGE was performed using a Thermo Scientific Owl P9DS Dual Gel System (Thermo Fisher Scientific New Zealand Ltd) with Precise precast 12% Tris-HEPES Gels (Thermo Fisher Scientific New Zealand Ltd) in 0.1M Tris, 0.1M HEPES, 3mM SDS, pH 8 running buffer. The separation was carried out at 100 mA/gel for 45 minutes. Each lane was loaded with 10µL of upper clear supernatant. After electrophoresis, gel was washed by 200 ml de-ionized water for 10 minutes, repeated this wash step for another two times, ensure sodium dodecyl sulphate (SDS) was completely removed. The polypeptides used as molecular mass markers (Sigma-Aldrich Co. LLC**.)** were: myosin (200 kDa), β-galactosidase from *E.coli* (116 kDa) phosphorylase b from rabbit muscle (97 kDa), bovine albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase from bovine erythrocytes (29kDa). After migration, protein bands of algal protein extracts were detected by coomassie staining.

Proteins were stained with 50% v/v methanol, 10% v/v glacial acetic acid and 0.25% w/v coomassie brilliant blue R-250 for 1 h. And de-stained by 16.5% v/v ethanol and 5% v/v glacial acetic acid overnight. The gel was stored in clear plastic zip bag, kept moisture by 5% acetic acid solution, and scanned by normal official scanner.

2.5 Statistics analysis

A one-way analysis of variance (ANOVA) was used to test significant differences between monthly means of total nitrogen and amino acid content to examine variations in month of harvest and geographical location using Minitab (version 16).

Two-way ANOVA was used to test significant differences between blade and sporophyll plant parts means of total nitrogen and amino acid content from *Undaria* harvested from four farms from two locations in September.

All data was checked for normality and homoscedasticity. Tukey HSD post hoc test was used to identify differences between means where the specific significant differences occurred ($p<0.05$). All data displayed a normal distribution. The results were given as a mean with standard deviation (±SD).

Chapter 3Results and discussion

3.1 Total nitrogen content of *Undaria pinnatifida* and variation of month, location and plant part

3.1.1 Total nitrogen content of *Undaria pinnatifida*

The total nitrogen content varied with months, farms and plant parts. The highest average value of total nitrogen was in blade samples from the sheltered farm 122 in Pelorus Sound at August (Table 15). As for the exposed site in Port Underwood location, the highest nitrogen content was determined in the blade sample from farm 106 in July (Table 16)). Total nitrogen content ranged from 14.28 to 30.00 mg/g dw at the sheltered location. At the exposed location, protein content ranged from 14.90mg/g to 25.54 mg/g. The total nitrogen content from both locations was slightly lower than the range of 17.6 to 38.4 mg/g reported by other researcher for same species (J. Fleurence, 1999). The wide range of differences could be due to be different harvest seasons. For example, the lowest value of 14.28 mg/g dw nitrogen content in September coincided with the reproductive season in spring for marine algal. In this period, there is a high demand of nutrients for growth that can contribute to a limitation in storage nitrogen (Martínez, Pato, & Rico, 2012; Martínez & Rico, 2002).

Table 15: The changes in total nitrogen content of *Undaria* on a monthly basis harvested from a sheltered location in the Pelorus Sound

Farm	Part	June	July	August	September
Sheltered farm 122 in Pelorus Sound	**Sporophyll**	NF	NF	NF	20.43±2.18
	Blade	NF	NF	30.00±5.59	18.24±2.12
Sheltered farm 122 in Pelorus Sound	**Sporophyll**	NF	NF	NF	NF
	Blade	NF	NF	NF	NF
Sheltered farm 122 in Pelorus Sound	**Sporophyll**	NF	NF	NF	14.28±1.10
	Blade	NF	NF	24.67±2.80	20.05±1.75

NF: No sample was found.

3.1.2 **Seasonal variation of total nitrogen content**

As sample collection was not consistent due to environmental conditions, no *Undaria* sample was found in some farms on specific month. Hence only the monthly variation in total nitrogen content of samples from exposed farms 327 and 106 in Port Underwood location was compared.

The total nitrogen content varied depending on plant parts and months (Table 15,

Table **16** and Figure 7). A big monthly variation was shown in both farms, and the maximum difference over four months was 10.64 mg/g from blade sample of farm 106.

Table 16: Total nitrogen content according month variable (expressed in mg/g of dry weight, n=5)

Month	Exposed			
	Farm 106		Farm 327	
	Blade	Sporophyll	Blade	Sporophyll
June	$21.26\pm0.55_{1,2}$	$20.60\pm1.54_{1,2}$	$25.30\pm2.10_{3}$	$23.21\pm3.07_{1}$
July	$25.54\pm 2.39_{1}$	$22.34\pm 3.22_{1}$	$26.22\pm0.81_{1}$	$22.04\pm 1.11_{1}$
August	$20.73\pm3.62_{2}$	$19.46\pm1.91_{1,2}$	$25.30\pm2.10_{1,2}$	$21.28\pm3.43_{1}$
September	$14.90\pm2.94_{3}$	$15.47\pm4.31_{2}$	$23.26\pm1.07_{2,3}$	$19.89\pm1.06_{1}$

$_{1, 2, 3}$: Different superscripts indicate significant differences among total nitrogen and months in the same column.

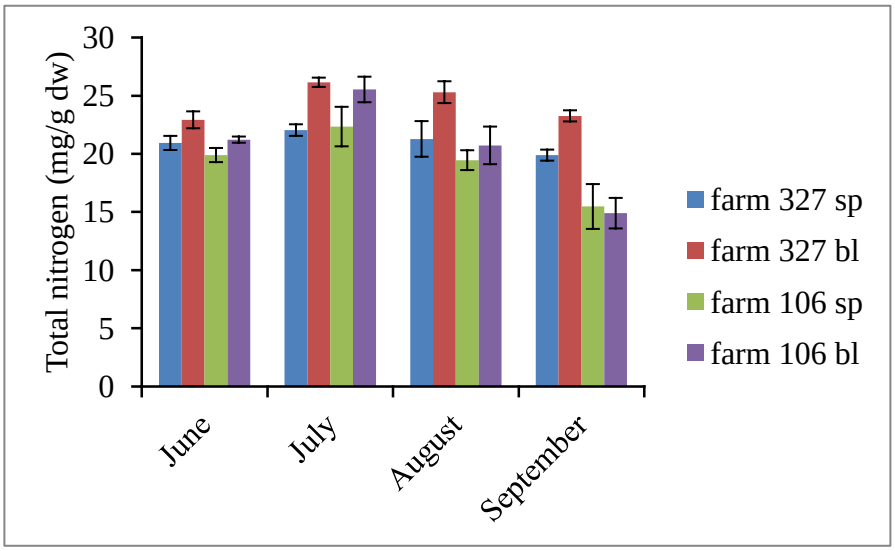

Figure 7: Monthly changes in the total nitrogen content levels of *Undaria pinnatifida* from different farms

Except for the sporophyll sample from farm 327, the total nitrogen content was higher in June or July, decreased from August, and had the lowest content in September (Figure 8, Figure 9 and Figure 10). Although, there was no significant difference in the monthly changes of total nitrogen content from the sporophyll sample from farm 327, the highest content occurred in July and the lowest in September (Figure 11).

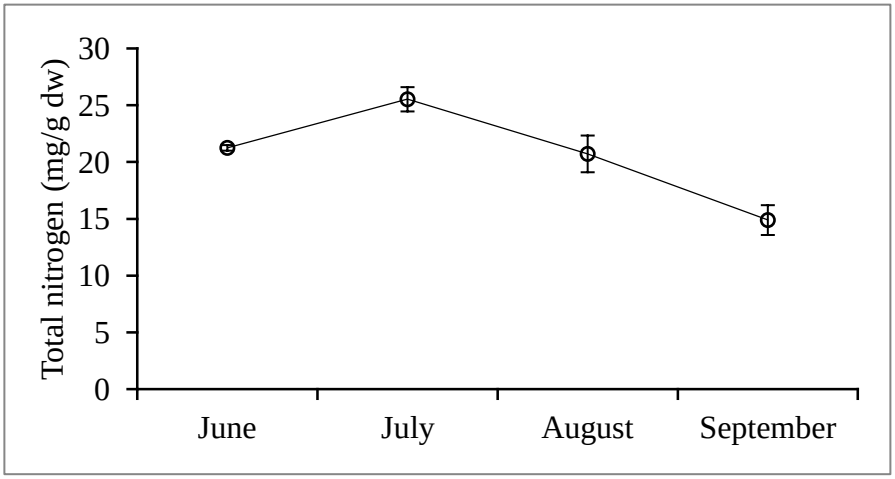

Figure 8: Monthly changes in the total nitrogen content (mg/g dry weight) of *Undaria pinnatifida* blade from exposed farm 106 in Port Underwood. Values are given as a mean with standard error.

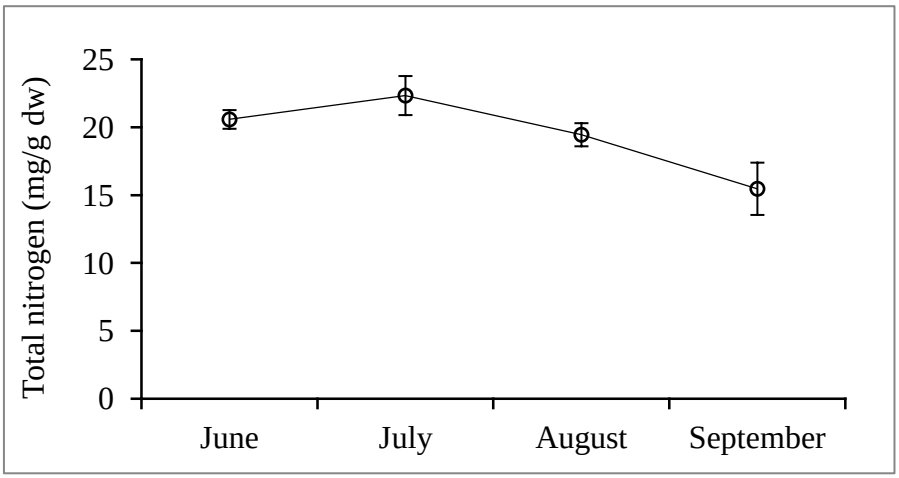

Figure 9: Monthly changes in the total nitrogen content (mg/g dry weight) of *Undaria pinnatifida* sporophyll from the exposed farm 106 in Port Underwood. Values are given as a mean with standard error.

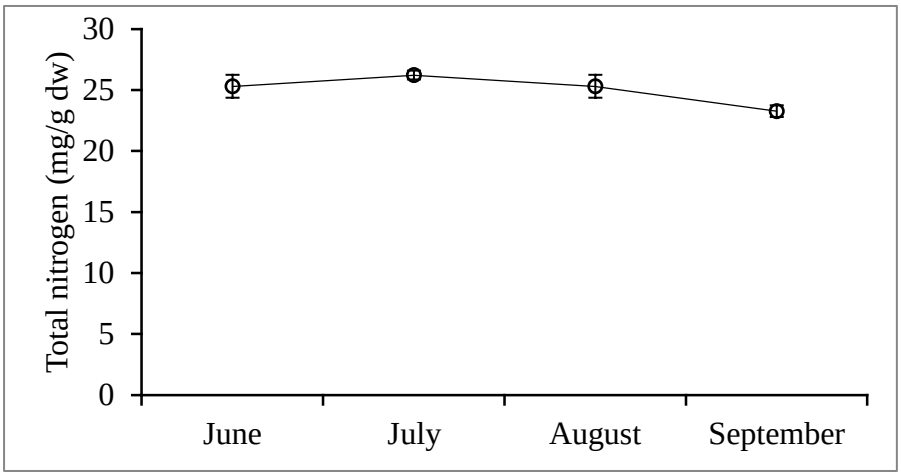

Figure 10: Monthly changes in the total nitrogen content (mg/g dry weight) of *Undaria pinnatifida* blade from the exposed farm 327 in Port Underwood. Values are given as a mean with standard error.

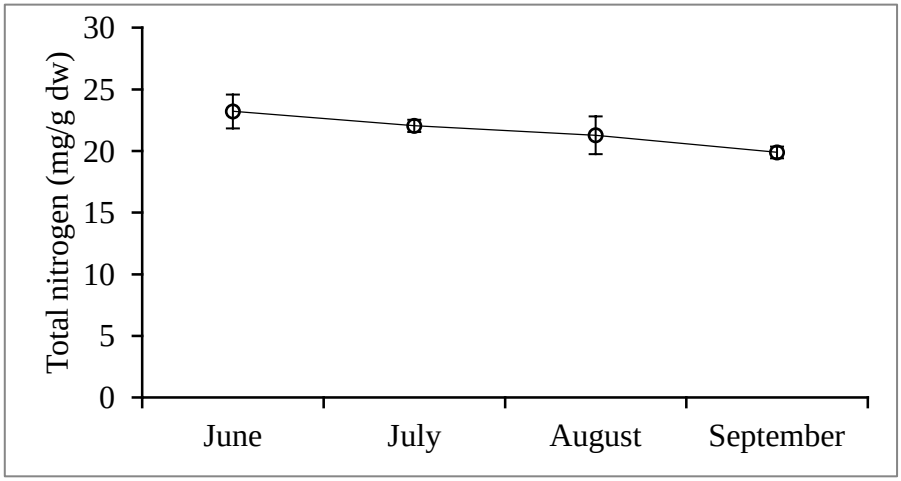

Figure 11: Monthly changes in the total nitrogen content (mg/g dry weight) of *Undaria pinnatifida* sporophyll from the exposed farm 327 in Port Underwood. Values are given as a mean with standard error

From the above results, the total nitrogen content of the *Undaria* was highly influenced by the harvest month, especially in the blade. This is supported in a variety of similar studies on different marine algae (Benjama & Masniyom, 2011; J. Fleurence, Chenard, & Luçon, 1999; Hernández-Carmona et al., 2009). The trend was a higher nitrogen content in late winter and a lower content in the summer, which implied that a lower nitrogen concentration might be caused by higher nutrient demands for growth in summer (Martínez & Rico, 2002).

The results from this study further demonstrated higher total nitrogen content from farm 327 than the other farms over the four collecting months. Multiple factors that can possibly contribute to this phenomenon , include factors like different concentrations of nitrogenous nutrients in the sea, water temperature or salinity of seawater (Banerjee et al., 2009; Rico & Fernández, 1996).

3.1.3 Geographical variation of total nitrogen content

As sample collection was not consistent due to limited *Undaria pinnatifida* growth in some farms and adverse weather conditions experienced during collection of samples, the total nitrogen content for both blade and sporophyll samples collected in September were compared in terms of different farms.

The total nitrogen content in *Undaria pinnatifida* displayed considerable individual differences between farms. For blade samples, the highest value of total nitrogen content was from exposed farm 327 in Port Underwood and the lowest content from exposed farm 106 in same location in September. As for the sporophyll samples, the value of total nitrogen content from sheltered farms 233 in Pelorus Sound and exposed farm 106 in Port Underwood were lower than exposed farms 327 in Port Underwood and sheltered farm 122 in Pelorus Sound (Table 16 and Table 17).

The results indicated important farm-dependent variations of the different mussel farms. The environmental factor could be responsible for this as similar findings

have been reported for a variety of marine algae by other studies (Banerjee et al., 2009; Dere et al., 2003).

Noticeably, there were no significant differences in the total nitrogen content between the sheltered Pelorus Sound farm 233 and the exposed Port Underwood farm 327 for blade. However for blade, there were significant differences between exposed farms 327 and 106 from Port Underwood. As for sporophyll there were significant differences between sheltered farms 233 and 122 in Pelorus Sound location, and exposed farms 106 and 327 from Port Underwood location (Table 17).

Table 17: Total nitrogen content according in blade and sporophyll (expressed in mg/g of dry weight, n=5)

Farm	Sheltered farm 122	Sheltered farm 233	Exposed farm 106	Exposed farm 327
Blade	$18.24\pm2.12_{2}$	$20.06\pm1.75_{1,2}$	$14.90\pm2.77_{3}$	$23.19\pm1.04_{1}$
Sporophyll	$20.43\pm2.18_{1}$	$14.28\pm1.10_{2}$	$15.47\pm4.31_{2}$	$19.89\pm1.06_{1}$

$_{1, 2, 3}$: Different superscripts indicate significant differences among total nitrogen and plant part in the same row.

Although sunlight has been identified as one of the main factors that influenced the nitrogen content in seaweed, nutrient storage responses have been suggested as another factor with more active ability to store N and P in autumn and winter due to less nitrogen metabolism in the algae tissue with less sunlight, and lower nitrogen content due to degradation to fulfil the N demands of the cell with more sunlight exposure (Martínez & Rico, 2008). The monthly variation results of this study support this conclusion, moreover, there may well be other environmental factors, which play more important roles in driving *Undaria* nutrient concentrations, rather than sunlight, which led difference of total nitrogen content between farms. The factors may include nitrate content in seawater, seasonal vegetative and reproductive development and salinity (Banerjee et al., 2009), this conjecture was proved by variety research (Banerjee et al., 2009; Martínez et al., 2012; Rico & Fernández, 1996).

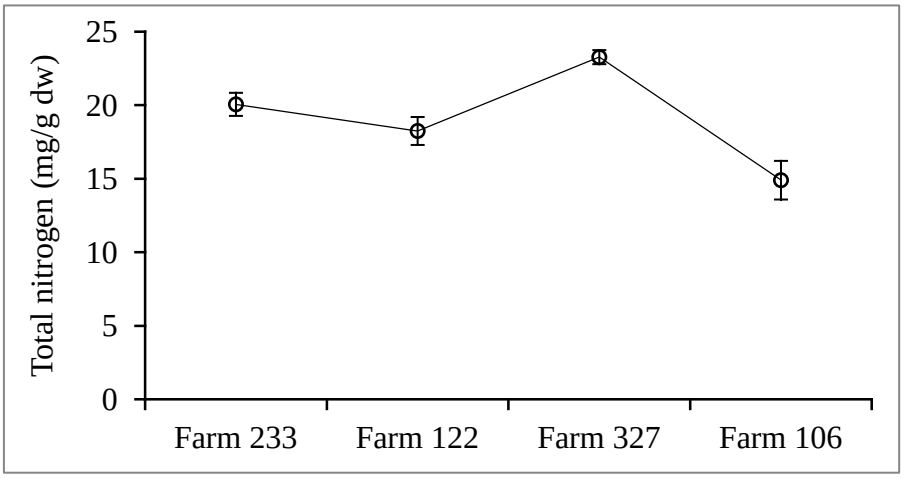

Figure 12: The variation in total nitrogen of four farms based on data from the blade sample harvested in September. Values are given as a mean with standard error.

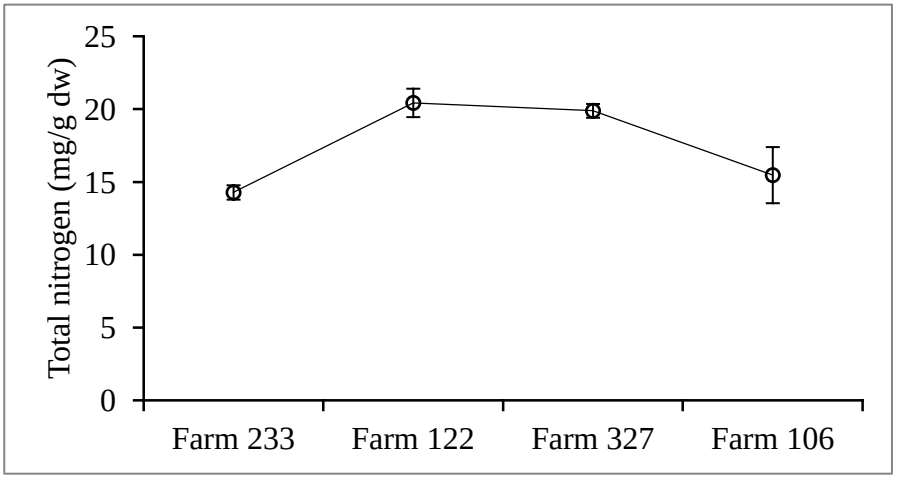

Figure 13: The variation in total nitrogen content of four farms based on data from the sporophyll sample harvested in September. Values are given as a mean with standard error.

3.1.4 Variation in the total nitrogen content of blade and sporophyll parts of *Undaria pinnatifida*

The monthly variations over the four months were significantly different (P-value=0.026) between the blade and sporophyll parts in exposed Port Underwood farm 327. Except for June, total nitrogen content in blade was significantly higher than that in sporophyll for the other three months (Figure 14). This might be due to blades absorbing mainly nitrogen from the environment and then transporting it to the sporophyll. It was also observed that the size of thallus from farm 327 was bigger than farm 106 throughout the four months, it can be assumed the big thallus was mature, and that rich nutrient in seawater may account for the bigger thallus (M. C. Carvalho, Hayashizaki, & Ogawa, 2008).

Compared to farm 106 from Port Underwood, nitrogen content in the sporophyll was higher although not significantly different than the blade in September (Figure 15).

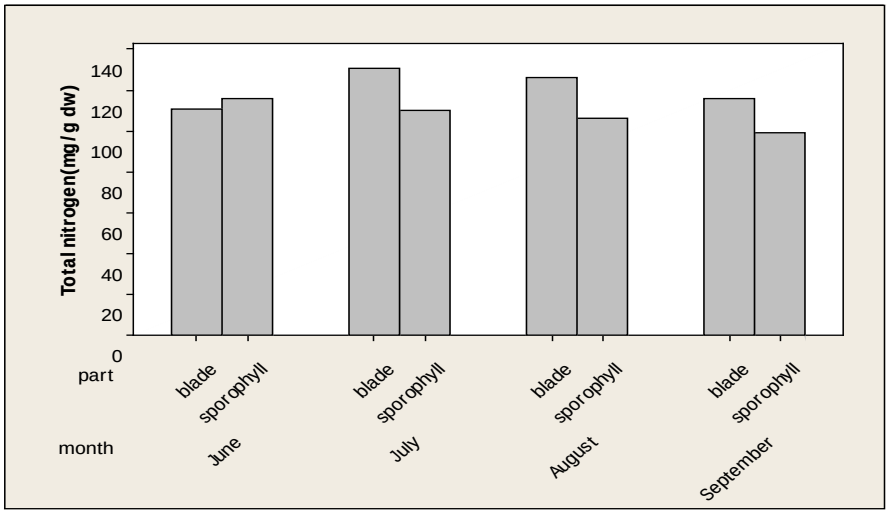

Figure 14: Mean value of total nitrogen content at different month and plant parts from *Undaria pinnatifida* harvested in the exposed farm 327 in Port Underwood

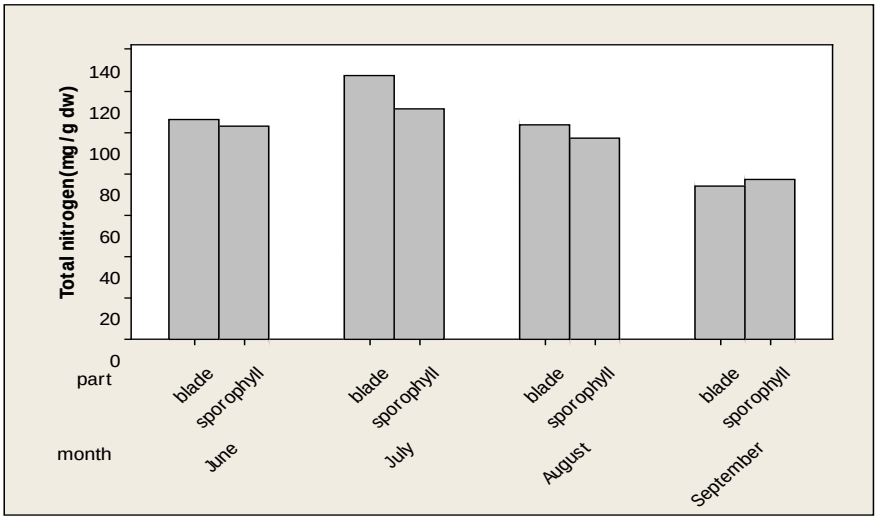

Figure 15: Mean value of total nitrogen content at different months and plant parts from *Undaria pinnatifida* harvested in the exposed farm 106 in Port Underwood

There was a significant interaction (P-value=0.002) between farm and plant part, in terms of the total nitrogen content in *Undaria* harvested in September. The total nitrogen content of blade from sheltered farm 233 in Pelorus Sound and exposed farm 327 in Port Underwood was significantly higher than sporophyll, but significant lower for sheltered farm 122 in Pelorus Sound and exposed farm 106 in Port Underwood (Figure 16).

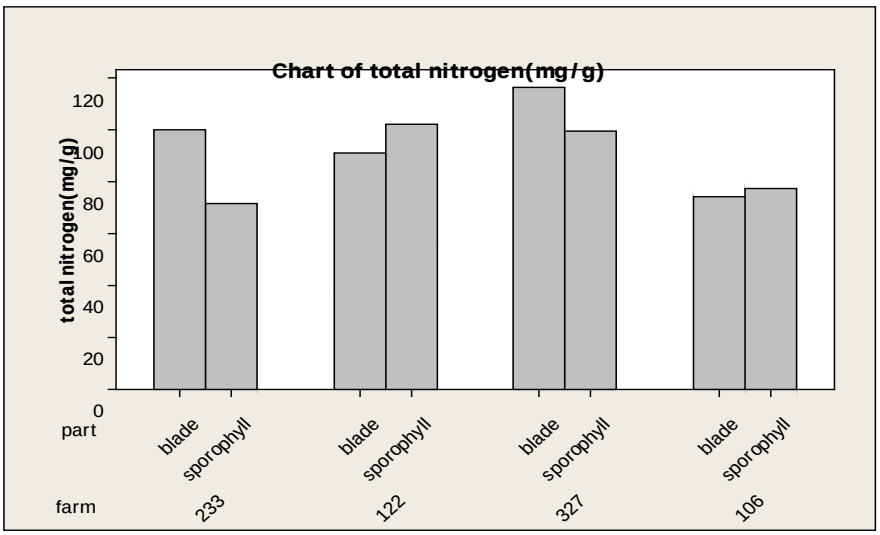

Figure 16: Mean value of total nitrogen content from different farms and plant parts of *Undaria pinnatifida* harvested in September.

Similar results have been reported with different collecting stations and plant parts by a previous study (M. C. Carvalho et al., 2008). The differences might be influenced by multiple factors, such as different nitrogen uptake rate from environment, or different growing rate, or even different nitrogen resource available etc.

Results from this study showed that plant parts was an important factor that affected total nitrogen content of *Undaria*, but this may in turn depend on other seasonal and farm variables.

3.2 Reliable test for determination of amino acid composition using pre-column PITC derivatization

3.2.1 Identification of individual PTC-amino acid in a mix 22 amino acid standard chromatogram

Identification of each amino acid peak was confirmed by comparison with the retention time of individual amino acids. The retention time for each individual PTC-amino acid is listed in Table 18.

Table 18: The retention time of individual amino acid

Name of amino acid	Retention time(minute)
L-Aspartic Acid	3.54
L-Glutamic Acid	4.17
trans-4-Hydroxyl-L-Proline	5.28
L-Serine	7.10
Glycine	8.24
L-Asparagine	8.38
L-Glutamine	9.63
L-Threonine	11.70
L-Alanine	12.62
L-Histidine	13.07
L- Proline	14.09
L-Arginine	14.60
L-Valine	21.79
L-Tyrosine	22.93
L-Methionine	23.81
L-Isoleucine	25.38
L-Leucine	26.28
L-Cysteine+ L-Cystine*	28.00
L-Phenylalanine	30.37
L-Tryptophan	32.14
L-Lysine	33.29

* Since cysteine easily oxidizes to cysteine (Campanella et al., 1999), and pre-treatment will introduce supplemental problems, so cysteine / cystine are represented as cystine+cysteine in this study.

The elution order of each the 22 mixed amino acids was confirmed by comparison of with a reference eluted pattern reported by (Woo, 2000) that was based on similar chromatographic conditions and column. The reference chromatogram of mixed 22 amino acid standard is shown in 17. Chromatogram obtained in this study is shown in 18, with the elution order slightly different from that of the reference chromatogram (Figure 17). The eluted PTC-amino acid was separated as four groups in both chromatogram, and the elution order of 11 mixed amino acids was the same. The differences in elution time could have been caused by the different mobile phase used.

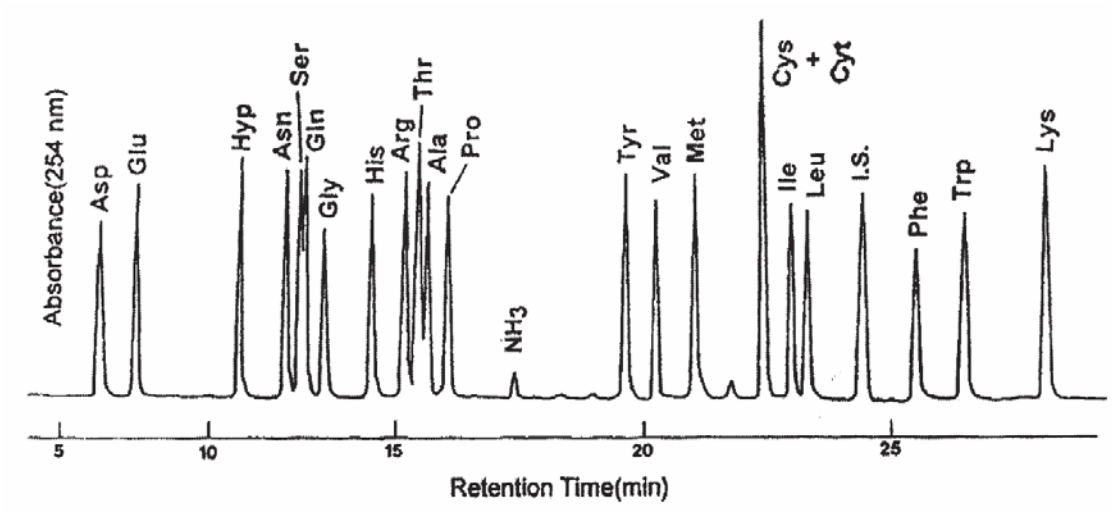

Figure 17: Example of a reported Chromatogram of standard amino acid derivatives resolved on a Nova-Pak (30 cm x 3.9 mm) C18 column. I.S. = norleucine, injected amount 0.625nmol Cyt = cystine; Cys = cysteine. (Woo, 2000)

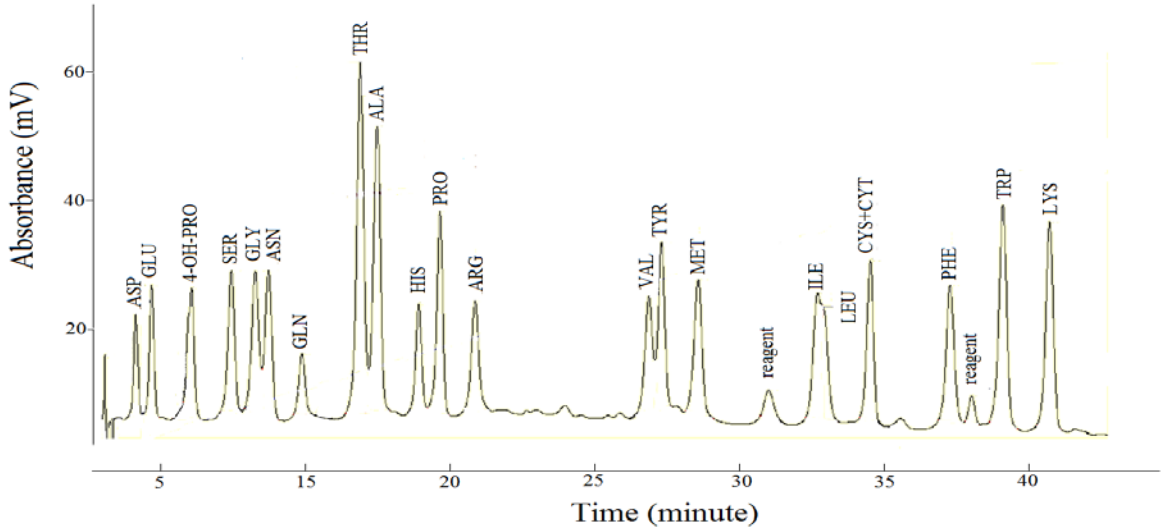

Figure 18: Chromatogram of 21 standard amino acids from this study

The reagent peak that eluted at around 32 minutes was close to the elution time of methionine. Another reagent peak that eluted at around 39 minutes was close to the elution time of phenylalanine. However the peak that comes after phenylalanine later overlapped with phenylalanine in the acid hydrolysed sample. The qualitative and quantitative analysis of mixed amino acid standards was not affected. Therefore the use of a reagent blank was important for calibrating the quantification of phenylalanine in this study. The two peaks increased with increased exposure time to the derivatization reagent, and extended storage of re-dissolving solution. The use of high purity reagent and fresh preparation of the re-dissolving solution are important to avoid spurious peaks occurring at these two retention times.

3.2.2 **Resolution and response linearity**

Under a set chromatographic condition, all 21 derivative amino acids were well separated, with most peaks relatively sharp, and peak shapes basically symmetry.

This meets the basic requirements of qualitative and quantitative analysis using HPLC.

Accuracy of quantitative HPLC analysis was decided by determination of the response linearity. This is the linear function between detection signal generated by sample and the amount of the sample. A good linear relationship between sample amount and output signal, indicates a more accurate quantitative determination.

The 19 figures showing response linearity are shown in the appendix. Results were based on three determinations for five concentration of each amino acid between 5mM to 60mM. The response linearity of aspartic acid was plotted based on three determinations of six concentrations between 5mM to 62.5mM. The response linearity of glutamic acid was plotted based on three determinations of five concentrations between 12.5mM to 62.5mM.

3.2.3 **Regression equation for mixed 22 amino acid standard curve**

The regression equations of 21 amino acids are summarized in Table 19. The R square of four amino acids equations were less than 0.98 but more than 0.96. The R square of seven amino acids was 0.99. Therefore the linearity of all 21 amino acids was satisfactory for further sample quantitative analysis within the specified concentration ranges.

Table 19: Regression equation for 21 amino acids

Amino acid	Regression Equation	R square
ASP	y = 6654.x + 6198.*	R^2 = 0.971*
GLU	y = 7430.x – 5210*.	R^2 = 0.983*
4-OH-PRO	y = 5769.x - 12206	R^2 = 0.968
SER	y = 5818.x - 6690.	R^2 = 0.990
GLY	y = 6335.x - 6447.	R^2 = 0.988
ASN	y = 6273.x - 5015.	R^2 = 0.982
GLN	y = 2390x - 4617	R^2 = 0.989
THR	y = 7957x + 3854	R^2 = 0.991
ALA	y = 14505x - 3096.	R^2 = 0.985
HIS	y = 3993.x - 3656.	R^2 = 0.988
ARG	y = 7300x - 4036.	R^2 = 0.994
PRO	y = 6243x - 6640	R^2 = 0.973
VAL	y = 5642x - 3733.	R^2 = 0.985
TYR	y = 7342x - 9105.	R^2 = 0.969
MET	y = 8805x - 18898	R^2 = 0.991
ILE	y = 6481.x - 1638.	R^2 = 0.990
LEU	y = 8343x - 10034.	R^2 = 0.995
CYS+CYT	y = 9514.x - 51760	R^2 = 0.979
PHE	y = 9467.x - 22100	R^2 = 0.989
TRP	y = 12698x - 21309	R^2 = 0.981
LYS	y = 13997x - 30405	R^2 = 0.984

*It was result of individual Aspartic acid and Glutamic acid standard curve

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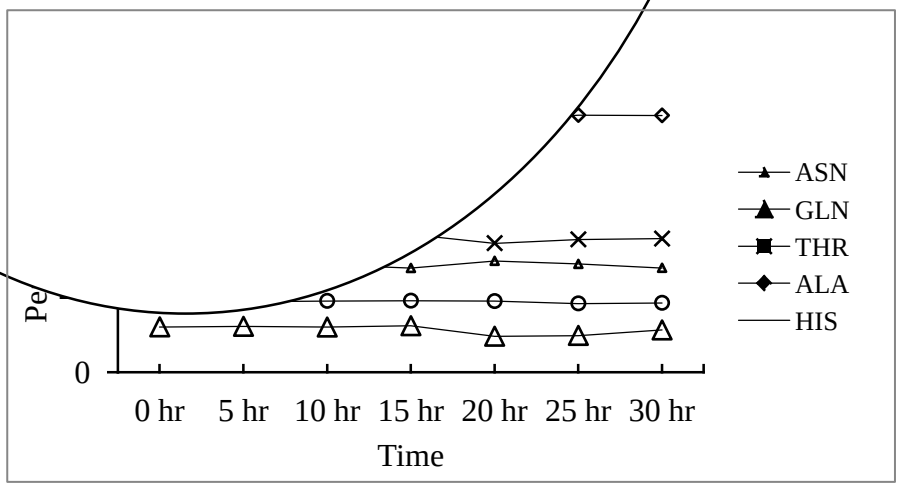
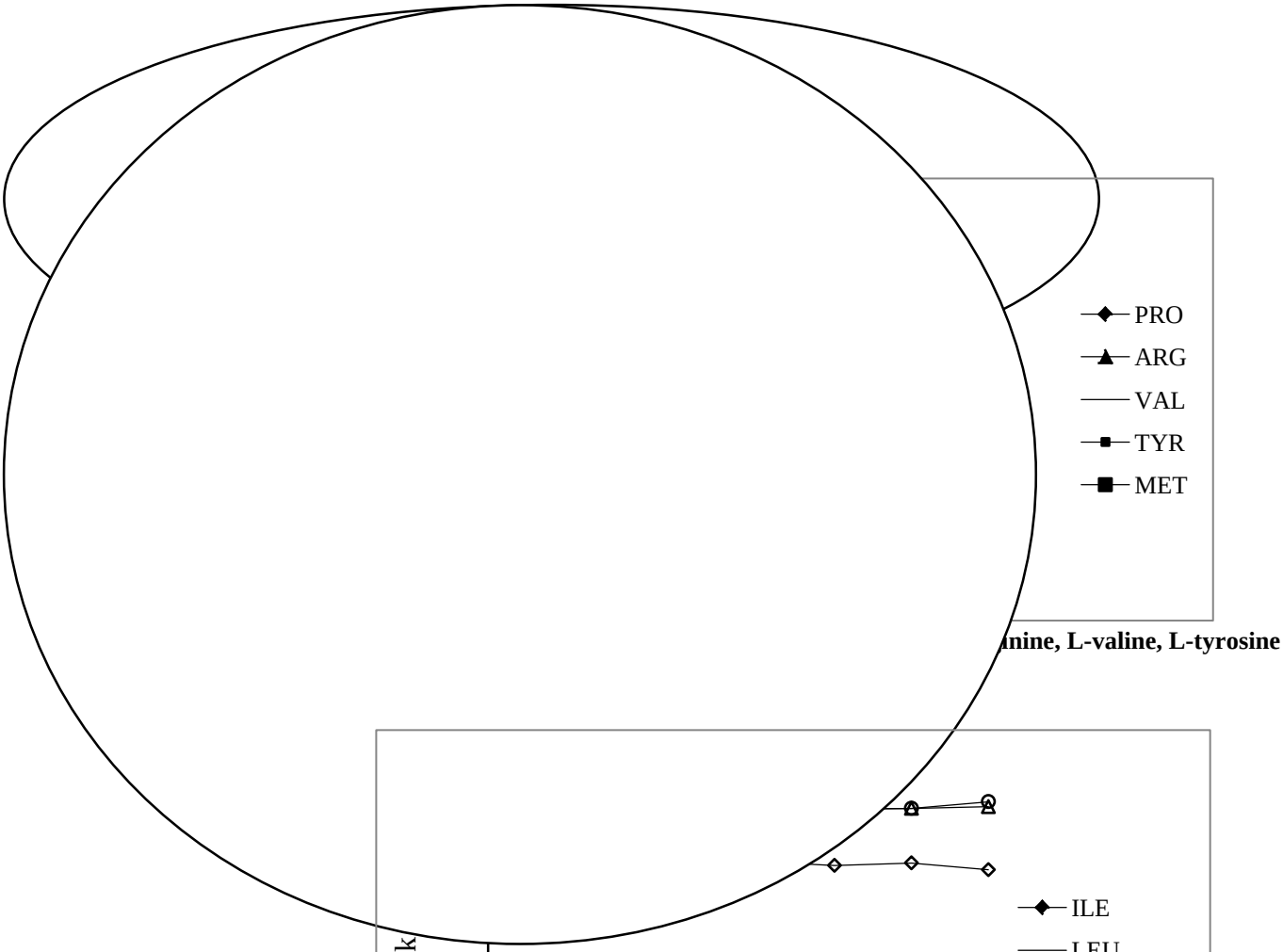


Figure 20: Stability of PITC derivatives of L-asparagine, L-glutamine, L-threonine, L-alanine and L-histidine



…inine, L-valine, L-tyrosine

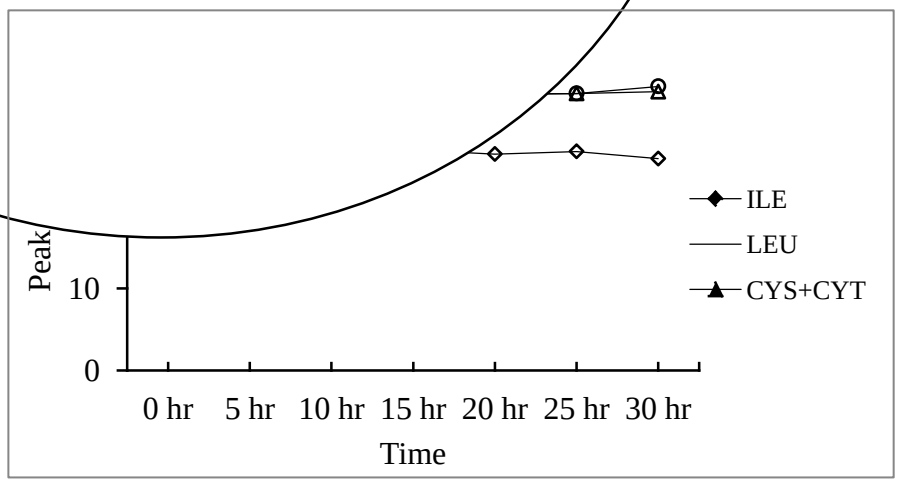


Figure 22: Stability of PITC derivatives of L-isoleucine, L-leucine and L-Cysteine+ L-Cystine

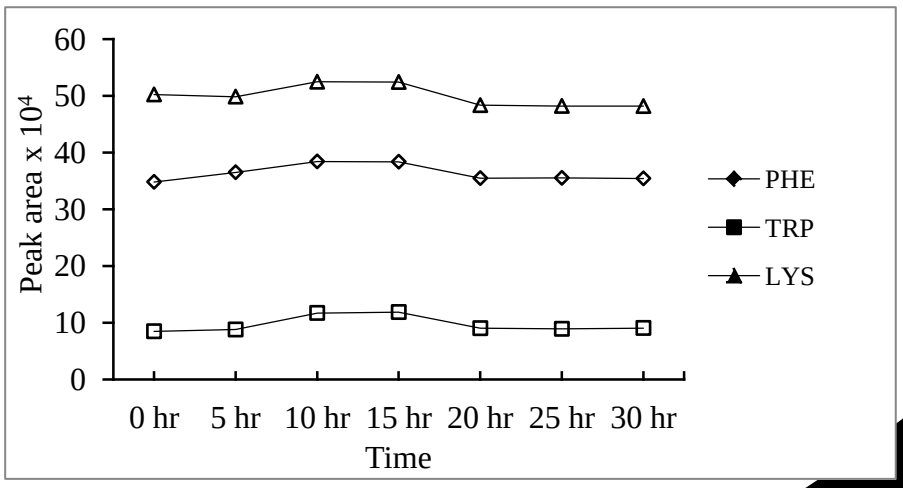


Figure 23: Stability of PITC derivatives of L-phenylalanine, L-tryptopha…

3.2.5 **Precision test for multiple injections of sam…**

…The precision of HPLC method in this study…
…f multiple injection…

specific controlled conditions. Generally precision was represented by value of the standard deviation (SD) or relative standard deviation (RSD). Small values indicated the closer the results, the better precision, and the higher accuracy of the methods.

The repeatability test of multiple injections for the same sample under the same HPLC conditions by the same analyst evaluates the consistency of analytical method.

As shown in Table 20, relative standard deviations for the retention times of 15 PITC-amino acids were less than 5%. The relative standard deviations for six other derivatised amino acids were less than 10%, because the auto-sampler broke down that month, and manual injection was carried out, introducing error from analyst's operation.

Table 20: Precision test for eight injections of the sample under the same HPLC condition (calculated by retention time)

Amino acid	Average (retention time)	Standard deviation (retention time)	RSD% (retention time)
Glu	4.84	0.37	7.7
4-OH PRO	6.97	0.67	9.56
Ser	8.50	0.65	7.62
Gly	9.39	0.63	6.66
Asn	10.13	0.63	6.25
Gln	11.19	0.56	4.98
Thr	12.99	0.46	3.51
Ala	13.57	0.42	3.13
His	14.67	0.42	2.86
Arg	15.51	0.26	1.66
Pro	16.74	0.27	1.63
Val	22.61	0.21	0.91
Tyr	23.05	0.25	1.08
Met	24.30	0.23	0.95
Ile	28.41	0.19	0.68
Leu	28.59	0.19	0.66
Cys+Cyt	30.31	0.21	0.69
Phe	33.02	0.22	0.67
Trp	34.88	0.25	0.73
Lys	36.51	0.29	0.81

Compared to retention time, consistency of peak area was more satisfactory, with relative standard deviations of 17 derivative amino acids of less or equal than 5%, except for aspartic acid, methionine, tyrosine and leucine, which were more than 5%, and less than 10%. The relative standard deviations of 21 derivative amino acids in this study were relatively consistent, indicative that the analytical HPLC method was satisfactory for analysing samples (The relative standard deviations of 21 derivative amino acids in this study were relatively consistent, indicative that the analytical HPLC method was satisfactory for analysing samples.

Table 21).

The relative standard deviations of 21 derivative amino acids in this study were relatively consistent, indicative that the analytical HPLC method was satisfactory for analysing samples.

Table 21: Precision test for eight injections of the sample under the same HPLC condition (calculated by peak area expressed as AU)

Amino acid	Average (peak area)	Standard Deviation (peak area)	RSD% (peak area)
Glu	136244.6	9420. 06	6.91
4-OH PRO	163688.0	8430.97	5.15
Ser	184136.1	7530.26	4.09
Gly	206957.8	5229.20	2.53
Asn	206377.0	6795.17	3.29
Gln	224111.6	7460.61	3.33
Thr	141043.8	5772.16	4.09
Ala	454817.3	8478.04	1.86
His	443820.9	11922.64	2.69
Arg	133956.0	2649.54	1.98
Pro	247303.6	3373.00	1.36
Val	126141.1	2815.35	2.23
Tyr	196446.6	9118.81	4.64
Met	244406.9	16764.00	6.86
Ile	249883.1	10463.51	4.19
Leu	182303.0	10665.28	5.85
Cys+Cyt	190401.6	5333.38	2.80
Phe	218827.8	17779.05	8.12
Trp	249373.9	13120.48	5.26
Lys	404140.3	20787.15	5.14

3.2.6 **Precision test for multiple determination**

Precision test for multiple injections can only evaluate how reliable of instrument is. It cannot determine the precision of the whole method. For example, there are several steps starting from hydrolysis of sample to sample injection. There exists a possibility to introduce error in every step. To overcome these shortcomings, a repeatability test of multiple determinations of the same sample was designed to determine the systematic error of the whole methodology.

Table 22 lists the relative standard deviations of 15 individual amino acids for six determinations. The relative standard deviation values of all amino acid derivatives were less than 5% except for methionine (10.94%), arginine (7.58%) and phenylalanine (6.23%). These results were better than those reported by Woo (2000), which had relative standard deviation values of glutamine, proline, alanine, and leucine that exceeded 5%, and for threonine, cystine+cysteine, and lysine, values

that exceeded 10%. Hence the precision of the HPLC method used in this study was satisfactory.

Table 22: Precision test for multiple determinations of the sample (n=6)

Amino acid	Average Content(mg/g)	Standard Deviation	RSD%
ASP	11.86	0.41	3.46
GLU	17.37	0.47	2.71
SER	9.15	0.20	2.19
GLY	12.36	0.37	2.97
THR	5.41	0.25	4.64
ALA	8.04	0.04	0.53
HIS	12.48	0.33	2.63
ARG	8.29	0.63	7.58
VAL	12.37	0.12	0.96
TYR	9.04	0.41	4.55
MET	4.50	0.49	10.94
ILE	9.00	0.18	2.01
LEU	16.59	0.74	4.49
PHE	7.77	0.48	6.23
LYS	9.74	0.23	2.37
Total amino acid	153.96	3.72	2.42

3.2.7 **Accuracy test for the reference protein, bbovine serum albumin**

Accuracy of the HPLC results in this study was evaluated by comparing the relative standard deviation of calculated values from six determinations and the true value.

The reference protein standard chosen was bovine serum albumin. The complete method for amino acid determination carried out on the *Undaria* sample used in this study was carried out on the reference protein. As the amount of bovine serum albumin was known, the total amino acid content was calculated. As tryptophan content in bovine serum albumin was only 0.34% (Woo, 2000), this calculated value should be close to true value. Hence the absolute standard deviation and relative standard deviation between the calculated total amino acid content and true protein content can be used to evaluate the accuracy of the results obtained from this study.

Except for proline, and cystine+cysteine, the % of the 17 amino acids content determined in this study was close to the reported values (Table 23). The total recoveries for pure bovine serum albumin ranged from 82% to 105% (Table 24), which was satisfactory. The failure to detect proline and its poor sensitivity for detecting proline in *Undaria* samples may be an obvious shortcoming of this method. This might have been caused by derivatization reagent degradation after storage or later contamination introduced by hydrolysis and neutralization that may interfere with the final analytical conditions. The overlap of proline and histidine was also a problem. However due to limitation of budget and time, the reason for this was not satisfactorily identified and needed to be studied further.

Table 23: Comparison of calculated amino acid composition with result reported by Woo (2000)

Amino acid	Calculated average content (%)	Reported content (%)(Woo, 2000)
ASP	10.54	9.68
GLU	13.32	13.07
SER	4.63	4.72
GLY	1.69	2.69
THR	3.79	5.61
ALA	4.24	6.87
HIS	6.23	3.75
ARG	4.61	5.59
PRO	0.17	5.27
VAL	5.87	6.64
TYR	2.28	3.73
MET	0.72	1.26
ILE	1.68	3.36
LEU	12.89	10.96
CYS+CYT	6.02	1.68
PHE	8.88	5.27
LYS	12.34	9.83

Table 24: Comparison of true protein amount and calculated total amino acid amount

Determination	Actual weight of BSA(mg)	Calculated total amino acid weight(mg)	Recovery (%)
First	4300	4552	105
Second	3200	2659	82
Third	4500	3996	89
Forth	4500	3713	82
Fifth	3200	3135	98
Sixth	4300	4507	105

3.3 Amino acid composition in *Undaria* and its monthly, location and plant part variations

3.3.1 Amino acid profile in *Undaria pinnatifida*

Two amino acids; cystine+cysteine pair and proline detected in the blade and sporophyll of *Undaria* samples were lower than most reported literature values (Cofrades, Lpez-Lopez, et al., 2010; Sánchez-Machado et al., 2004). However, these amino acids seemed to be quite variable in the literature. The cystine+cysteine pair was not detected in Spanish *Undaria* (Snchez-Machado et al., 2003). Insensitive detecting of proline might have been due to overlap with the adjacent histidine peaks

although there was no evidence from peak shape that this was true, or due to this specific amino acid being not present in significant amounts due to seasonal variations (Hagen Rødde, Vårum, Larsen, & Myklestad, 2004).

The composition of amino acids fluctuated at different months, farms and plant parts. Except for June, glutamic acid, aspartic acid and leucine were the three most abundant amino acids in blade harvested from exposed farm 106 in Port Underwood from June to September, instead of aspartic acid from July to September; tyrosine was one of most three abundant amino acids in June (Table 25).

Table 25: Monthly changes in the amino acid composition of blade from the exposed farm 106 in port Underwood (expressed in mg/g of dry weight, n=5)

Amino acid	June	July	August	September
ASP	$7.21\pm1.90_{2}$	$20.12\pm11.30_{1}$	$8.74\pm2.10_{2}$	$6.98\pm1.10_{2}$
GLU	$12.55\pm2.12_{1,2}$	$21.09\pm9.04_{1}$	$10.67\pm3.58_{2}$	$8.18\pm1.54_{2}$
SER	$6.14\pm0.94_{1,2}$	$7.23\pm2.79_{1}$	$4.98\pm1.19_{1,2}$	$3.30\pm0.37_{2}$
GLY	$7.48\pm0.88_{1}$	$7.45\pm1.44_{1}$	$6.59\pm1.50_{1}$	$4.12\pm0.59_{2}$
THR	$4.05\pm0.81_{1,2}$	$5.22\pm2.34_{1}$	$3.66\pm0.84_{1,2}$	$2.38\pm0.43_{2}$
ALA	$8.57\pm1.28_{1}$	$6.58\pm1.89_{1,2}$	$5.94\pm2.03_{1,2}$	$4.29\pm0.57_{2}$
HIS	$7.94\pm1.55_{1}$	$6.64\pm0.82_{1,2}$	$6.95\pm1.86_{1,2}$	$4.88\pm0.61_{2}$
ARG	$5.75\pm1.21_{1}$	$7.33\pm2.38_{1}$	$4.64\pm1.62_{1,2}$	$2.73\pm0.37_{2}$
VAL	$\mathbf{7.57\pm0.84_{1}}$	$\mathbf{4.20\pm2.81_{1}}$	$\mathbf{6.30\pm2.80_{1}}$	$\mathbf{5.26\pm0.55_{1}}$
TYR	$12.49\pm4.99_{1}$	$3.69\pm0.98_{2}$	$6.09\pm3.65_{2}$	$2.97\pm0.36_{2}$
MET	$\mathbf{3.53\pm0.61_{1}}$	$\mathbf{2.99\pm1.15_{1}}$	$\mathbf{3.27\pm0.63_{1}}$	$\mathbf{2.46\pm0.18_{1}}$
ILE	$\mathbf{5.23\pm0.56_{1}}$	$\mathbf{3.33\pm1.76_{1}}$	$\mathbf{5.01\pm1.05_{1}}$	$\mathbf{3.42\pm0.36_{1}}$
LEU	$9.10\pm1.55_{1}$	$6.67\pm1.22_{1,2}$	$8.60\pm2.13_{1}$	$5.57\pm0.66_{2}$
PHE	$\mathbf{4.52\pm1.13_{1}}$	$\mathbf{4.57\pm2.20_{1}}$	$\mathbf{3.77\pm2.38_{1}}$	$\mathbf{3.28\pm0.88_{1}}$
LYS	$7.85\pm0.75_{1}$	$4.75\pm2.58_{2}$	$7.70\pm1.85_{1,2}$	$5.27\pm0.62_{1,2}$

$_{1, 2}$: Different superscripts indicate significant differences among amino acid composition and months in the same row (P-value=0.05).

Table 26: Monthly changes in amino acid composition of sporophyll from exposed farm 106 in Port Underwood (expressed in mg/g of dry weight, n=5)

Amino acid	June	July	August	September
ASP	$8.33\pm1.40_{1,2}$	$10.35\pm1.60_{1,2}$	$11.93\pm1.27_{1}$	$7.71\pm3.54_{2}$
GLU	$\mathbf{13.77\pm2.93_{1}}$	$\mathbf{13.78\pm1.06_{1}}$	$\mathbf{12.97\pm1.64_{1}}$	$\mathbf{10.94\pm5.56_{1}}$
SER	$6.77\pm0.85_{1}$	$7.14\pm0.52_{1}$	$4.78\pm0.33_{2}$	$3.22\pm1.35_{3}$
GLY	$8.52\pm1.30_{2}$	$11.60\pm2.50_{1}$	$6.83\pm0.44_{2,3}$	$3.94\pm1.56_{3}$
THR	$4.62\pm1.16_{1}$	$4.54\pm0.91_{1}$	$3.45\pm0.30_{1,2}$	$2.47\pm1.04_{2}$
ALA	$14.03\pm1.53_{1,2}$	$17.81\pm5.12_{1}$	$9.70\pm1.94_{2,3}$	$6.19\pm3.09_{3}$
HIS	$8.18\pm1.44_{1}$	$7.95\pm1.55_{1}$	$5.05\pm0.44_{2}$	$4.00\pm1.53_{2}$
ARG	$5.23\pm0.65_{1}$	$5.48\pm0.56_{1}$	$3.55\pm0.30_{2}$	$2.50\pm0.92_{2}$
VAL	$7.38\pm1.00_{1}$	$7.91\pm1.38_{1}$	$6.52\pm0.42_{1}$	$4.25\pm1.71_{2}$
TYR	$9.85\pm5.09_{1}$	$5.93\pm1.02_{1,2}$	$3.78\pm0.07_{2}$	$2.90\pm0.98_{2}$
MET	$\mathbf{3.73\pm0.74_{1}}$	$\mathbf{3.08\pm0.73_{1}}$	$\mathbf{2.73\pm0.10_{1}}$	$\mathbf{3.03\pm0.98_{1}}$
ILE	$4.75\pm0.81_{1}$	$5.28\pm0.36_{1}$	$4.31\pm0.39_{1}$	$2.93\pm1.16_{2}$
LEU	$8.22\pm.092_{1,2}$	$10.43\pm3.89_{1}$	$5.77\pm0.22_{2}$	$4.34\pm1.56_{2}$
PHE	$\mathbf{4.37\pm1.02_{1}}$	$\mathbf{7.47\pm2.65_{1}}$	$\mathbf{5.43\pm4.55_{1}}$	$\mathbf{3.17\pm0.53_{1}}$
LYS	$3.37\pm0.671_{3}$	$7.92\pm0.21_{1}$	$5.70\pm0.38_{2}$	$4.13\pm1.44_{3}$

$_{1, 2, 3}$: Different superscripts indicate significant differences among amino acid composition and months in the same row (P-value=0.05).

There was more variation in the most abundant amino acids in the sporophyll from June to September in the same farm. Alanine, tyrosine, glycine, aspartic acid and glutamic acid were the most abundant amino acids in sporophyll from exposed farm 106 in Port Underwood over the four months (Table 26).

There was a slight difference in the most abundant amino acids in blade from farm 327 over the four months. Glutamic acid (ranging from 14.16 to11.91 mg/g dw) and aspartic acid (ranging from 12.61 to 9.34 mg/g dw) the first and second abundant amino acids. The third abundant amino acid varied among isoleucine, tyrosine, leucine and valine as shown in Table 27.

Alanine, glutamic acid and aspartic acid were the three most abundant amino acids in both blade and sporophyll from the sheltered farm 233 in Pelorus Sound, and in sporophyll from sheltered farm 122 in same location. The most abundant amino acid in the blade from sheltered farm 122 was glutamic acid as well.

Results showed that glutamic acid, aspartic acid and alanine were most abundant amino acids in *Undaria* sample. This result was similar to that reported by other researchers (Dawczynski et al., 2007; Je et al., 2009). The high levels of aspartic and glutamic acids were responsible for the special flavour and taste of seaweeds (Taboada et al., 2009; Yaich et al., 2011).

3.3.2 **Monthly variation of amino acid composition**

Table 25 summarizes the content of individual amino acids from the blade of *Undaria pinnatifida* collected from the exposed farm 106 in Port Underwood at different months. Except for valine, methionine, isoleucine and phenylalanine, the levels of the remaining eleven amino acids were significantly different over the four months. The highest content was observed either in June or July, and lowest content was in September. This trend was similar to the monthly variation in total nitrogen content.

An interesting finding in Table 25 was that phenomenon four essential amino acids (valine, methionine, isoleucine and phenylalanine) were not influenced by monthly changes. This meant that the ratio of essential/non-essential amino acid was not influenced by monthly changes. However the total amino acid content decreased with increasing months, and protein quality (ratio of essential to non-essential amino acids) increased with increasing months from June to September in farm 106.

The profiles of amino acids from the sporophyll and blade of same plant were presented in Table 25 and Table 26 respectively. Except for glutamic acid, methionine and phenylalanine, there were significant differences in the other amino acids content with monthly variations.

Different from farm 106, there was no obvious monthly change in the amino acid profiles from both the blade and sporophyll from exposed farm 327 in Port Underwood. Only glycine content was significantly different with different months in blade (Table 27), and only histidine content significantly changed from June to September in sporophyll (Table 28). No significant differences were demonstrated for both protein quantity and quality in the blade and sporophyll from farm 327 between June to September.

Table 27: Monthly changes in amino acid composition of blade from exposed farm 327 in Port Underwood (expressed in mg/g of dry weight, n=5)

Amino acid	June	July	August	September
ASP	9.49±3.81$_1$	12.61±8.61$_1$	10.23±2.37$_1$	9.34±2.25$_1$
GLU	11.91±4.40$_1$	14.16±5.04$_1$	12.38±3.02$_1$	12.51±1.80$_1$
SER	4.26±1.33$_1$	6.05±1.69$_1$	6.09±0.70$_1$	5.45±0.60$_1$
GLY	**5.70±1.91$_1$**	**7.51±0.72$_{1,2}$**	**7.74±0.83$_2$**	**7.39±0.59$_{1,2}$**
THR	3.03±1.10$_1$	4.56±1.60$_1$	3.88±0.35$_1$	3.74±0.32$_1$
ALA	6.90±2.58$_1$	8.35±2.64$_1$	8.75±2.09$_1$	7.79±0.50$_1$
HIS	5.75±1.79$_1$	7.08±2.32$_1$	7.33±0.66$_1$	6.24±0.43$_1$
ARG	3.88±1.14$_1$	5.81±2.18$_1$	5.04±0.46$_1$	3.67±1.60$_1$
VAL	6.76±2.30$_1$	7.67±1.93$_1$	9.43±0.92$_1$	8.95±0.69$_1$
TYR	4.02±0.84$_1$	8.90±6.66$_1$	4.58±0.63$_1$	4.45±0.39$_1$
MET	3.45±0.97$_1$	3.35±1.19$_1$	3.00±0.55$_1$	3.00±0.27$_1$
ILE	4.57±1.66$_1$	5.37±2.04$_1$	6.19±0.57$_1$	5.87±0.53$_1$
LEU	6.92±2.32$_1$	7.62±3.26$_1$	9.87±1.01$_1$	8.79±0.72$_1$
PHE	3.79±1.59$_1$	7.39±5.56$_1$	4.02±0.77$_1$	3.63±0.54$_1$
LYS	6.82±2.31$_1$	7.21±2.64$_1$	8.94±1.15$_1$	8.64±0.56$_1$

$_1$, $_2$: Different superscripts indicate significant differences among amino acid composition and months in the same row (P-value=0.05).

Table 28: Amino acid composition of sporophyll according to month variable of exposed farm 327(expressed in mg/g of dry weight, n=5)

Amino acid	June	July	August	September
ASP	13.90±2.01$_1$	12.39±2.22$_1$	12.21±0.41$_1$	13.47±1.82$_1$
GLU	5.55±1.25$_1$	5.79±1.54$_1$	5.07±0.23$_1$	5.28±0.66$_1$
SER	10.87±3.71$_1$	10.61±3.78$_1$	7.52±1.33$_1$	7.92±1.13$_1$
GLY	4.80±1.18$_1$	4.20±1.61$_1$	3.34±0.47$_1$	3.39±0.30$_1$
THR	4.08±1.18$_1$	4.20±1.61$_1$	3.34±0.47$_1$	3.39±0.30$_1$
ALA	12.22±2.71$_1$	14.95±4.14$_1$	12.52±1.99$_1$	10.67±0.89$_1$
HIS	**7.88±1.72$_1$**	**5.68±0.41$_{1,2}$**	**6.44±1.82$_{1,2}$**	**5.48±0.59$_2$**
ARG	4.61±0.50$_1$	5.01±1.61$_1$	3.91±0.31$_1$	4.10±0.57$_1$
VAL	8.28±1.60$_1$	6.85±1.81$_1$	6.86±0.47$_1$	7.38±0.62$_1$
TYR	7.24±6.30$_1$	12.27±8.66$_1$	3.62±0.39$_1$	3.62±0.71$_1$
MET	2.75±0.66$_1$	3.43±1.39$_1$	2.44±0.39$_1$	2.04±0.79$_1$
ILE	5.24±1.08$_1$	5.23±1.53$_1$	3.89±0.43$_1$	4.60±0.57$_1$
LEU	7.76±1.52$_1$	6.32±1.67$_1$	6.30±0.47$_1$	6.22±0.56$_1$
PHE	3.49±1.95$_1$	5.63±2.22$_1$	2.76±0.86$_1$	3.08±0.95$_1$
LYS	7.46±1.20$_1$	7.22±2.17$_1$	6.31±0.52$_1$	6.21±0.78$_1$

$_1$, $_2$: Different superscripts indicate significant differences among amino acid composition and months in the same row (P-value=0.05).

There are many factors that might affect the amino acid profile (Galland-Irmouli et al., 1999). Season might be one of the main factors for changes in the amino acid composition in *Undaria* from farm 106. This variation was also reflected in the total nitrogen content in both blade and sporophyll (

Table **16**). There was no significant difference in the total nitrogen content with different months in the sporophyll from farm 327, and this was the same case with the related amino acid profile.

3.3.3 **Farm variation of amino acid composition of *Undaria pinnatifida***

Four farms were selected to analyse the effects of farm variations in the amino acid content of blade and sporophyll from *Undaria pinnatifida* collected in September. Except for histidine, phenylalanine and arginine, the other twelve amino acids content varied with farm in the blade sample. Among the four farms, the amino acid content from the exposed farm 327 was significantly higher than the other three farms (Table 29).

Table 29: Farm variation in the amino acid content of blade from *Undaria pinnatifida* harvested in September (expressed in mg/g of dry weight, n=5)

Amino acid	Sheltered farm 122	Sheltered farm 233	Exposed farm 106	Exposed farm 327
ASP	$4.79\pm1.03_{1}$	$7.70\pm2.74_{1,2}$	$6.98\pm1.10_{1,2}$	$9.34\pm2.25_{2}$
GLU	$7.27\pm1.28_{1}$	$9.04\pm1.85_{1}$	$8.19\pm1.54_{1}$	$12.51\pm1.80_{2}$
SER	$4.05\pm0.55_{1}$	$4.26\pm0.97_{1,2}$	$3.30\pm0.36_{1}$	$5.45\pm0.60_{2}$
GLY	$5.50\pm0.79_{1}$	$5.57\pm1.29_{1}$	$4.12\pm0.59_{1}$	$7.39\pm0.58_{2}$
THR	$3.33\pm0.37_{1}$	$3.34\pm0.73_{1}$	$2.38\pm0.43_{2}$	$3.73\pm0.32_{1}$
ALA	$5.47\pm1.05_{2,3}$	$6.41\pm1.81_{1,2}$	$4.29\pm0.57_{3}$	$7.79\pm0.50_{1}$
HIS	$\mathbf{5.68\pm0.78_{1}}$	$\mathbf{5.06\pm1.12_{1}}$	$\mathbf{4.88\pm0.61_{1}}$	$\mathbf{6.23\pm0.43_{1}}$
ARG	$\mathbf{3.22\pm0.38_{1}}$	$\mathbf{2.88\pm0.56_{1}}$	$\mathbf{2.73\pm0.37_{1}}$	$\mathbf{3.67\pm1.59_{1}}$
VAL	$5.94\pm0.81_{1}$	$5.26\pm1.49_{1}$	$5.26\pm0.55_{1}$	$8.95\pm0.69_{2}$
TYR	$3.61\pm0.41_{1}$	$3.16\pm0.40_{1}$	$2.96\pm0.36_{1}$	$4.45\pm0.39_{2}$
MET	$2.05\pm0.33_{2,3}$	$1.77\pm0.31_{3}$	$2.46\pm0.18_{2}$	$3.00\pm0.27_{1}$
ILE	$4.32\pm0.55_{1,2}$	$3.34\pm1.66_{2}$	$3.41\pm0.36_{2}$	$5.87\pm0.53_{1}$
LEU	$6.71\pm0.94_{1}$	$5.39\pm1.76_{1}$	$5.57\pm0.66_{1}$	$8.79\pm0.72_{2}$
PHE	$\mathbf{3.10\pm0.68_{1}}$	$\mathbf{2.93\pm0.70_{1}}$	$\mathbf{3.28\pm0.88_{1}}$	$\mathbf{3.63\pm0.54_{1}}$
LYS	$4.82\pm0.51_{1}$	$4.68\pm0.75_{1}$	$5.27\pm0.62_{1}$	$8.64\pm0.56_{2}$

$_{1}$, $_{2}$, $_{3}$: Different superscripts indicate significant differences among amino acids and farms in the same row (P-value=0.05).

Amino acid contents of tyrosine, phenylalanine, histidine, leucine and threonine in the sporophyll was not affected by farm variations in September. However the concentration of aspartic acid, glutamic acid, serine, glycine, alanine, Arginine, valine, methionine, isoleucine and lysine in farm 327 were significantly higher than the other three farms (Table 30). This corresponded to the farm variations in the total nitrogen content; where total nitrogen content was significantly higher in exposed farm 327 than other three farms in September.

Table 30: Farm variation in the amino acid content of sporophyll from *Undaria pinnatifida* harvested in September (expressed in mg/g of dry weight, n=5)

Amino acid	Sheltered farm 122	Sheltered farm 233	Exposed farm 106	Exposed farm 327
ASP	7.88±3.67$_1$	4.47±0.56$_1$	7.71±3.54$_1$	15.41±1.04$_2$
GLU	8.73±3.23$_{1,2}$	5.67±0.75$_1$	10.94±5.56$_{1,2}$	13.47±1.82$_2$
SER	3.58±1.19$_{1,2}$	2.96±0.50$_1$	3.22±1.35$_1$	5.28±0.66$_2$
GLY	4.50±3.04$_1$	3.95±0.77$_1$	3.94±1.55$_1$	7.92±1.13$_2$
THR	**3.71±1.87$_1$**	**2.59±0.40$_1$**	**2.47±1.04$_1$**	**3.39±0.30$_1$**
ALA	9.04±3.61$_{1,2}$	4.34±0.82$_3$	6.19±3.09$_{2,3}$	10.67±0.89$_1$
HIS	**4.72±1.83$_1$**	**3.81±0.60$_1$**	**4.00±1.53$_1$**	**5.48±0.59$_1$**
ARG	2.93±1.16$_{1,2}$	2.24±0.39$_1$	2.50±0.92$_1$	4.10±0.57$_2$
VAL	4.84±1.64$_{1,2}$	3.32±1.57$_1$	4.25±1.71$_1$	7.38±0.62$_2$
TYR	**3.06±0.98$_1$**	**3.01±0.74$_1$**	**2.90±0.98$_1$**	**3.62±0.70$_1$**
MET	1.88±0.66$_{1,2}$	1.59±0.36$_1$	3.03±0.98$_2$	2.04±0.79$_{1,2}$
ILE	3.17±1.19$_{1,2}$	2.67±0.35$_1$	2.93±1.16$_1$	4.60±0.57$_2$
LEU	**5.05±1.84$_1$**	**4.30±0.67$_1$**	**4.34±1.56$_1$**	**6.22±0.56$_1$**
PHE	**3.58±2.35$_1$**	**3.85±2.63$_1$**	**3.17±0.53$_1$**	**3.08±0.95$_1$**
LYS	4.04±1.42$_1$	3.81±0.51$_1$	4.13±1.44$_1$	6.21±0.78$_2$

$_1$, $_2$, $_3$: Different superscripts indicate significant differences among amino acids and farms in the same row (P-value=0.05).

3.3.4 **Plant part variation of amino acid composition of *Undaria pinnatifida***

Plant part variations were investigated in two ways. One was the comparison of individual amino acid from same farm over four months. The other was the comparison of individual amino acid from four farms in the same month

Plant parts did not affect amino acid composition over the four month in both farms 327 and 106. Only aspartic acid, glycine, threonine, alanine and leucine were significantly different between blade and sporophyll in exposed farms 327, alanine and arginine in farm 106 respectively, over the four months (Table 31). The changes of amino acids content in blade and sporophyll from both farms are random, so it can assume no difference in amino acid composition from both farms between blade and sporophyll over four month.

Within the same month, the amino acid content varied in the blade and sporophyll. Eight out of a total of fifteen amino acids in blade were significantly different to sporophyll (Table 32). This result corresponded to the effect of plant part variations on the total nitrogen content.

Table 31: The mean value of amino acid content of sporophyll and blade parts from June to September from exposed farm 327 and 106 in Port Underwood (expressed in mg/g of dry weight, n=20)

Amino	Exposed farm327		Exposed farm 106	
Acid	Blade	Sporophyll	Blade	Sporophyll
ASP	**10.77_1**	**13.60_2**	10.76_1	9.58_1
GLU	12.98_1	12.99_1	13.12_1	12.86_1
SER	5.42_1	5.42_1	5.41_1	5.48_1
GLY	**7.05_1**	**9.23_2**	6.41_1	7.72_1
THR	**3.79_1**	**3.94_2**	3.83_1	3.77_1
ALA	**7.88_1**	**12.59_2**	**6.34_1**	**11.93_2**
HIS	6.51_1	6.37_1	6.60_1	6.29_1
ARG	4.52_1	4.41_1	**5.11_1**	**4.26_2**
VAL	8.13_1	7.34_1	5.83_1	6.51_1
TYR	5.59_1	6.69_1	6.30_1	5.61_1
MET	3.17_1	2.67_1	3.09_1	3.14_1
ILE	5.46_1	4.74_1	4.25_1	4.32_1
LEU	**8.13_1**	**6.65_2**	7.49_1	7.19_1
PHE	4.76_1	3.74_1	4.03_1	5.11_1
LYS	7.81_1	6.80_1	6.39_1	6.28_1

$_1$, $_2$: Different superscripts indicate significant differences among amino acid composition and plant part in the same row (P-value=0.05).

Table 32: The mean value of amino acid composition of sporophyll and blade parts from four farms in September (expressed in mg/g of dry weight, n=20)

Amino acid	Blade	Sporophyll
ASP	**7.20_1**	**8.87_2**
GLU	9.25_1	9.70_1
SER	4.26_1	3.76_1
GLY	5.64_1	5.08_1
THR	3.20_1	3.04_1
ALA	**5.99_1**	**7.56_2**
HIS	**5.46_1**	**4.50_2**
ARG	3.12_1	2.94_1
VAL	**6.35_1**	**4.95_2**
TYR	3.55_1	3.15_1
MET	2.32_1	2.14_1
ILE	**4.24_1**	**3.34_2**
LEU	**6.61_1**	**4.98_2**
PHE	**3.24_1**	**3.42_2**
LYS	**5.85_1**	**4.55_2**

$_1$, $_2$: Different superscripts indicate significant differences among amino acid composition and plant part in the same row (P-value=0.05).

3.4 Nitrogen-to-protein conversion factor

For all the results collected from four farms over four months, a mean Kjeldahl nitrogen-to-protein conversion factor of 4.44 was established. This conversion factor varied on a monthly basis. The highest value of factor 4.93 observed in June, and then decreased steadily with increasing months, and reaching the lowest value of 3.69 in October (Table 33).

The average nitrogen-to-protein factor established by this study was lower than values reported in literature. For example, a conversion factor of 4.92 was determined for a total of nineteen tropical algae and 5.38 for brown algae (Loureno et al., 2002). This was reported to be attributed to multiple variables that included collecting season, location, and water flow.

Table 33: The mean value of nitrogen-to-protein conversion factor in *Undaria* over five months

Month	Nitrogen-to-protein conversion factor
June(n=21)	4.93
July(n=28)	4.70
August(n=20)	4.45
September(n=40)	4.06
October(n=20)	3.69
Average(n=129)	4.44

Table 34 gives the mean value of the nitrogen-to-protein conversion factor for *Undaria* from different locations and different plant parts. The conversion factor for *Undaria* at the exposed location was higher than the sheltered location. This might be caused by different growing seasons in the two locations as there was no or little sample growing in the sheltered location from June to August. There was also a slight difference of nitrogen-to-protein factor between blade and sporophyll.

Table 34: The mean value of nitrogen-to-protein factor according to location and plant part

		Nitrogen-to-protein conversion factor
Location	Exposed(n=89)	4.66
	Sheltered(n=40)	3.66
Plant part	Blade(n=65)	4.22
	Sporophyll (n=64)	4.71

3.5 Quality of protein of *Undaria* and the ratio of essential amino acid ratio and no-essential amino acid

The amount of essential amino acid is a factor to determine protein quality in food. This included histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine contents. All essential amino acids were detected in *Undaria* samples at relatively high amounts. The essential amino acids accounted for 48 % of the total amino acid content, which was compatible to that of the other seaweeds reported in earlier works, ranging from 45% to 49% for S.*wightii*, U.*lactuca*, K.*alvarezii*, H.*musciformis*, G.*corticata*, A.*spicifera* (Vinoj Kumar, 2007).

To evaluate protein quality, the ratio of essential and non-essential amino acid was determined in this study. The ratio was calculated based on the data obtained from

both blade and sporophyll parts of *Undaria* collected from four farms over five months. The total sample size was 129, and the ratio of essential and non-essential amino acid was calculated based on the following formula:

The ratio of essential and non-essential amino acid

= (average of total essential amino acid mg/g dry weight) / (average of total non-essential amino acid mg/g dry weight)

Based on this equation, the ratio of essential and non-essential amino acid was calculated to be 0.95 for the *Undaria* sample. Hence nearly half of the total amino acids in *Undaria* were essential amino acids.

Error! Not a valid bookmark self-reference. summarized the comparison of the ratio of essential amino acid and total nitrogen content in the present study with the FAO/WHO reference pattern (Vinoj Kumar, 2007). All essential amino acids fulfilled the FAO/WHO requirements except for the sulphur containing amino acids, cysteine and methionine. These two amino acids were the lowest in *Undaria*. In fact, sulphur amino acids are usually the limiting amino acids for algal proteins (Galland-Irmouli et al., 1999).

Table 35: The comparison of protein of *Undaria* and FAO/WHO pattern

Amino acid	Mean value of *Undaria*(mg/g dw) (n=129)	E/T ratio*	FAO/WHO pattern(Vinoj Kumar, 2007)
ASP	9.54	476	
GLU	11.24	560	
SER	4.70	235	
GLY	6.49	324	
THR	3.42	171	180
ALA	8.23	410	
HIS	6.12	305	
ARG	3.92	195	
PRO	1.30	65	
VAL	6.05	302	270
TYR	5.24	262	
MET	2.58	129	
ILE	4.20	210	270
LEU	6.76	337	306
PHE	4.18	208	180
LYS	5.86	292	270
MET+CYS	**2.58**	129	270
PHE+TYR	9.42	470	360
CYS	ND		
TRP	DH		90

ND: Not detected.
DH: Destroyed during hydrolysis.
*: The ratio of essential amino acid and total nitrogen which was expressed as mg amino acid per gram N.

3.6 Soluble protein quantitative analysis and SDS PAGE protein patterns

Quantitative analysis of soluble protein in *Undaria pinnatifida* by the Bradford method was unsuccessful due to abundant polysaccharides and glycoproteins in the algal cell wall that bind to the amino acids (Devillé, Damas, Forget, Dandrifosse, & Peulen, 2004; Díaz-Rubio et al., 2009; Gómez-Ordóñez et al., 2010). During the attempted extractions, the alginate formed an intractable gel in the presence of multivalent cations (Jørgensen, Sletmoen, Draget, & Stokke, 2007), and formed a blue precipitate which seriously interfered with the colorimetric Bradford assay.

A total of seven protein bands were identified in SDS-PAGE. The molecular weights of these seven bands are summarized in Table 36. Compared to other species, the protein pattern of *Undaria pinnatifida* was found to be different with unique protein band of molecular weights 77kDa, 37kDa, 20kDa and 14kDa (Table 36).

The effect of drying treatment and plant part variations were also evaluated. There was no difference observed with different drying treatments and algae parts (Figure 24). The same pattern for two drying treatments might reflect that no effect should be in amino acid profile between two drying treatments, it was reported no significant difference on amount of individual amino acid in brown algae, *Sargassum hemiphyllum, S. henslowianum* and *S. patens* by Wong & Cheung (Wong & Cheung, 2001).

Table 36: The comparison of protein pattern for *Undaria* and other algae species (expressed as kDa)

Band number	protein molecular weight of *U. Pinnatifida*	protein molecular weight of *Ulva rotundata*(1)	protein molecular weight of *Porphyra umbilicalis*(2)	protein molecular weight of *Chondrus crispus*(2)
Band 1	77	69.9	73.1	49.3
Band 2	55	54.2	49.6	46.2
Band 3	37	29.5	32.1	43.2
Band 4	26	26.3	26.2	19.8
Band 5	20	22.9	22	17.2
Band 6	17	17.6	17.8	16.4
Band 7	14	15.5	15.9	15.2

(1) : data was from (C. Rouxel et al., 2001)

(2) : data was from (Catherine Rouxel, André Daniel, et al., 2001)

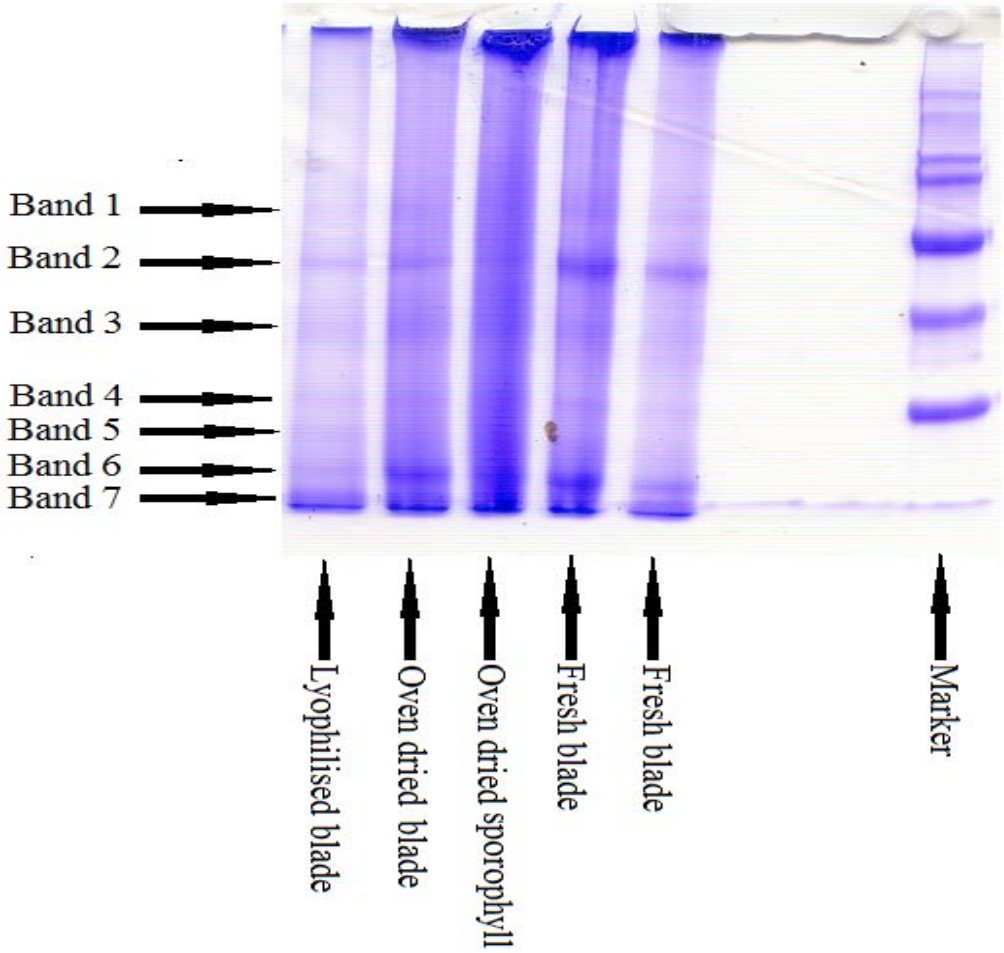


Figure 24: Protein patterns in SDS-PAGE of *Undaria pinnatifida*

Chapter 4Future study

4.1 Monthly variation on protein pattern

A number of other groups have shown a seasonal variation of protein patterns present in algal tissue (J. Fleurence et al., 1999; Catherine Rouxel, Eric Bonnabeze, et al., 2001). Accurate typical protein pattern in one-dimensional gel electrophoresis is key method for accurate characterization of *Undaria pinnatifida* and would be particularly useful when looking at seasonal variation, especially as a part of characterisation, species identification and quality control. Because of the limited time and budget of this study, this was only done on one May sample and this should be extended to all seasons and location types to provide a good reference set for future studies.

4.2 New methods for protein extraction

The extraction and purification protein from seaweed is difficult because of several issues. Thick cell walls, high content of polysaccharides, high concentration of ionic species etc. Although a variety of extraction methods to extract were tried none were entirely satisfactory. This is a recognised problem and definitely needs further improvement.

4.2.1 **enzymatic treatment**

Anionic polysaccharides are present in high levels in algae; viscous hydrocolloid formation will block protein solubility. Most work has focussed on trying to develop new extraction reagents or on exploring the suitable ratios of multiple reagents.

In contrast to these traditional methods, new methods using enzyme treatment are appearing. Numerous researches showed successful SDS-PAGE results and higher soluble protein yields on different samples using this microbial treatment technology (Joubert & Fleurence, 2008). High resolution SDS-PAGE results of different algae specie were reported (Denis, Le Jeune, Gaudin, & Fleurence, 2009; J. Fleurence, Le Coeur, Mabeau, Maurice, & Landrein, 1995; Joubert & Fleurence, 2008).

4.2.2 **Application of pulsed electric fields**

Electropermeabilization is a valuable application in molecular biology and biotechnology, which has opened new avenues in the approach of effective transformation and extraction strategies. Electric pulses with high field intensity can cause permanent cell membrane breakdown, this is a basis for numerous

applications on isolation and concentration of intracellular secondary metabolites, proteins and enzymes from plant and yeast cell with less protein denaturation than conventional methods (Suga, Goto, & Hatakeyama, 2007; Suga & Hatakeyama, 2009; Yang, Bayraktar, & Pu, 2003). It also seems to provide some selective release of intracellular proteins making it a powerful tool for protein extraction from cells (Tryfona & Bustard, 2006).

Chapter 5 Conclusion

The average total nitrogen content of *Undaria pinnatifida* is 21.02 mg/g dw from four farms located at Nelson, New Zealand from June to October in 2011, the average crude protein content is 13.13% dw using the frequently citied conversion factor 6.25, it fall in the range 11 to 24% dry weight documented by other study (J. Fleurence, 1999). In addition, the total nitrogen content fluctuate by seasonal, geographical and plant part alternation.

The amino acid profile of *Undaria pinnatifida* is balanced, essential amino acids (EAA) is 48% of the total amino acid (TAA), and the ratio of essential amino acids (EAA) / non-essential amino acids (NEAA) is 0.95, the most three abundant amino acids are three flavour amino acids-glutamic acid, aspartic acid and alanine. Same with total nitrogen content, variation of season, geography and plant part affect the amino acid profile in this study.

The nitrogen-to-protein conversion factor of *Undaria pinnatifida* established by this study is 4.44, lower than traditional conversion factor 6.25; it confirmed the traditional conversion factor is over estimated.

A clear SDS-PAGE patter of *Undaria pinnatifida* in New Zealand is obtained by this study, it supply an important reference for identification of this specific species.

In conclusion, *Undaria pinnatifida* in New Zealand can be a potential high quality protein resource for fish, poultry, pig and cattle etc., even further applications as natural functional food ingredients for human being. Furthermore, the best harvest season for better protein value should be at winter.

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Appendices

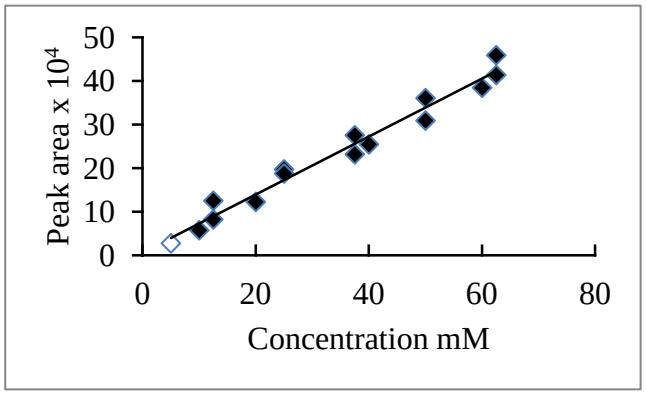

Figure 25: The standard curve of aspartic acid

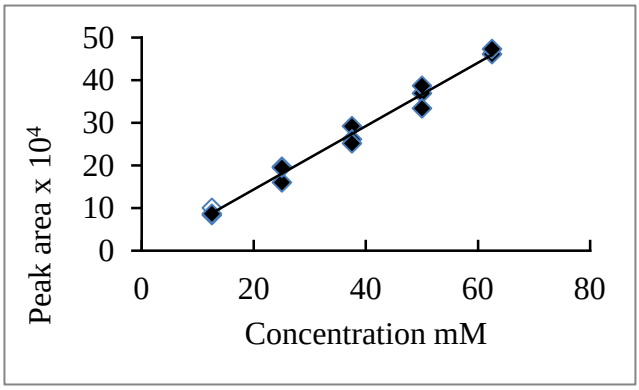

Figure 26: The standard curve of glutamic acid

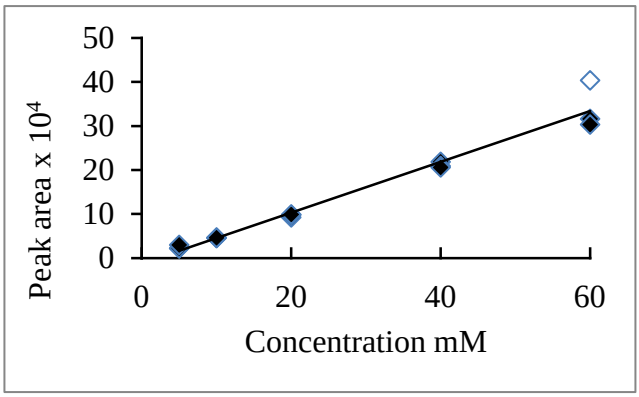

Figure 27: The standard curve of trans-4-Hydroxyl-L-Proline

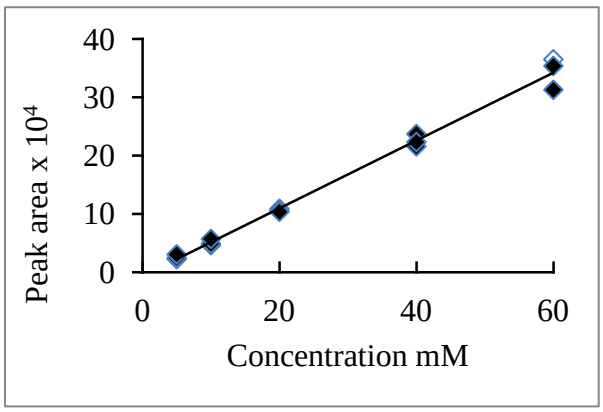

Figure 28: The standard curve of serine

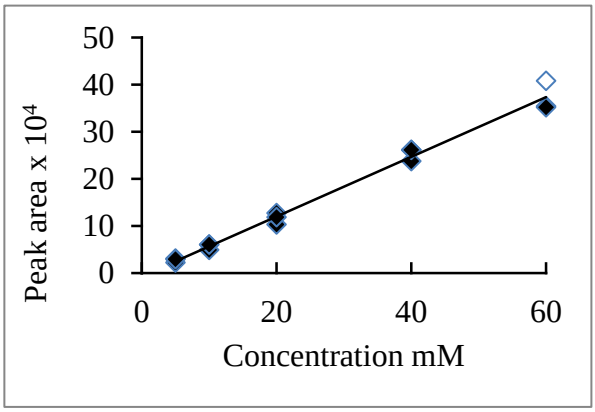

Figure 29: The standard curve of glycine

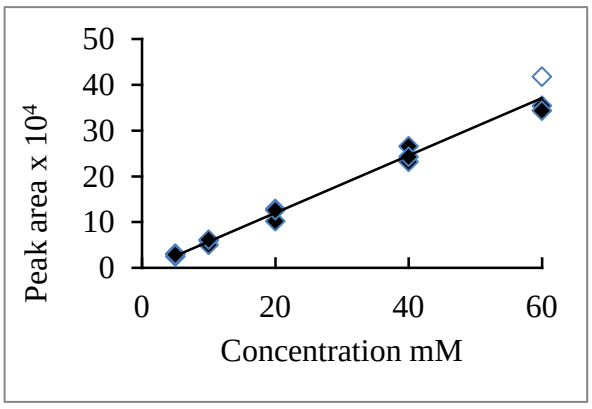

Figure 30: The standard curve of asparagine

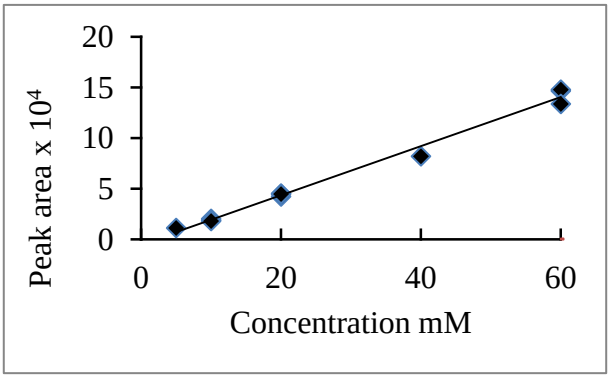

Figure 31: The standard curve of glutamine

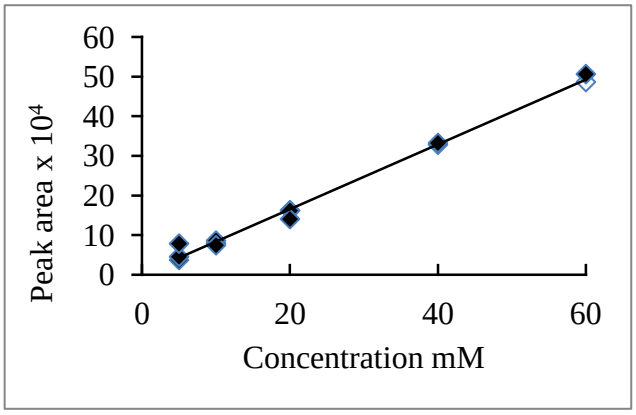

Figure 32: The standard curve of threonine

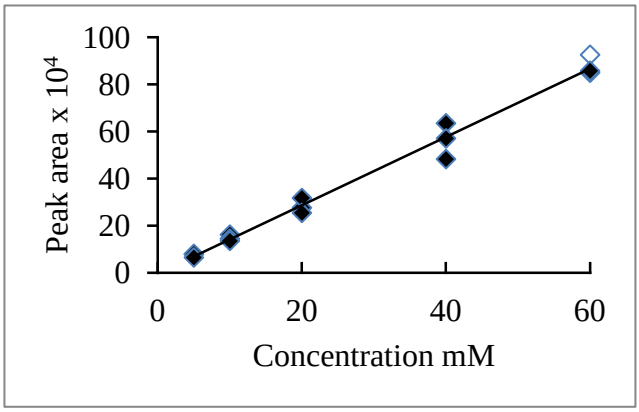

Figure 33: The standard curve of alanine

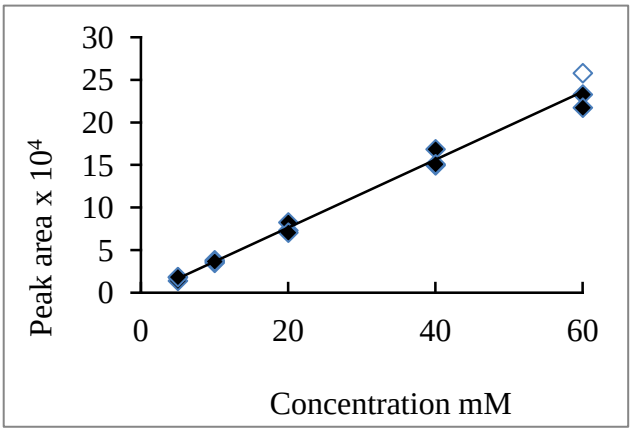

Figure 34: The standard curve of histidine

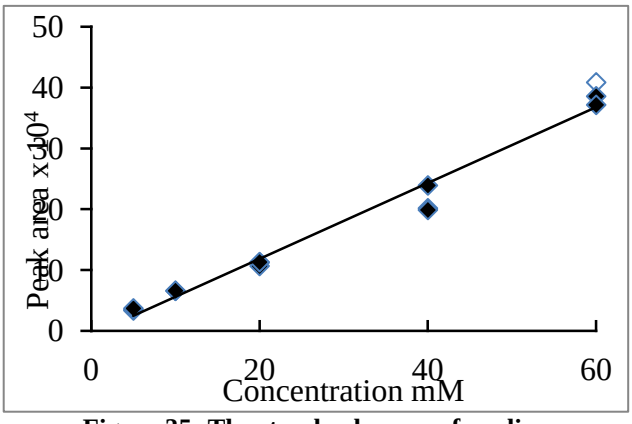

Figure 35: The standard curve of proline

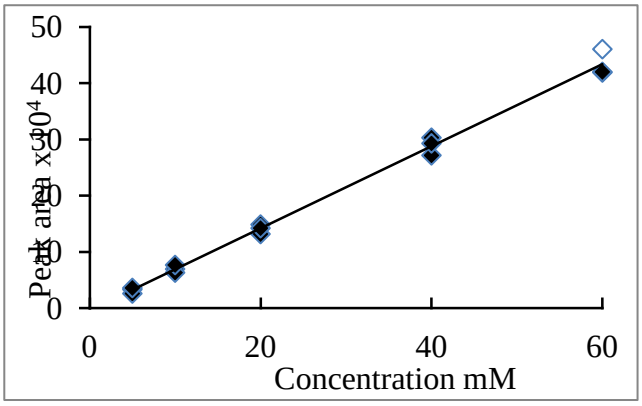

Figure 36: The standard curve of arginine

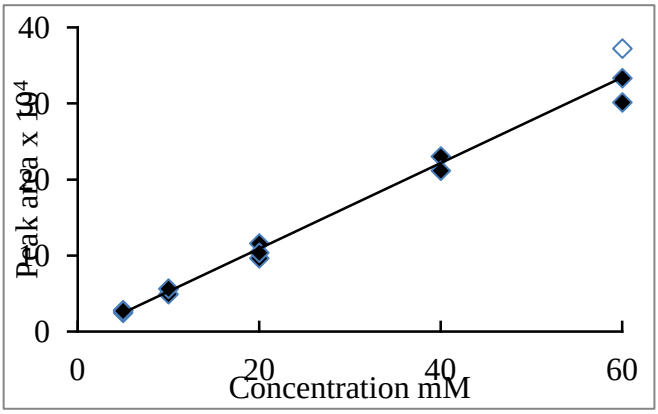

Figure 37: The standard curve of valine

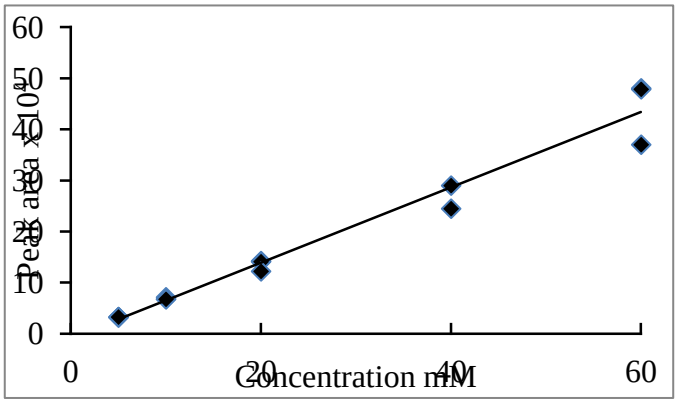

Figure 38: The standard curve of tyrosine

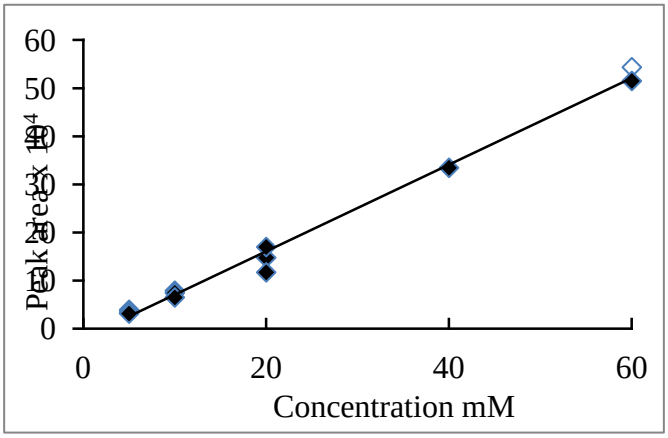

Figure 39: The standard curve of methionine

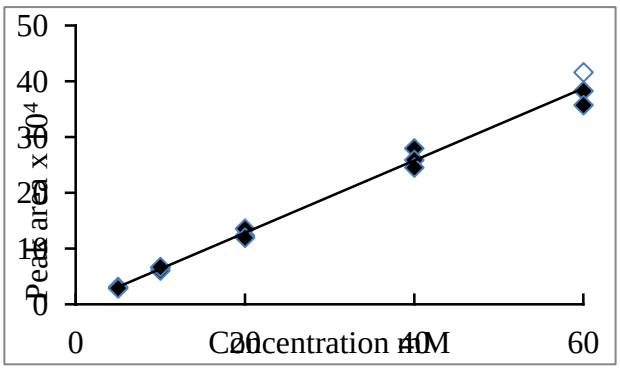

Figure 40: The standard curve of isoleucine

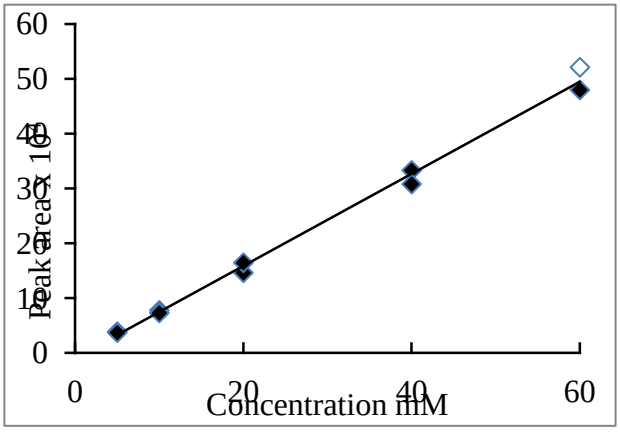

Figure 41: The standard curve of leucine

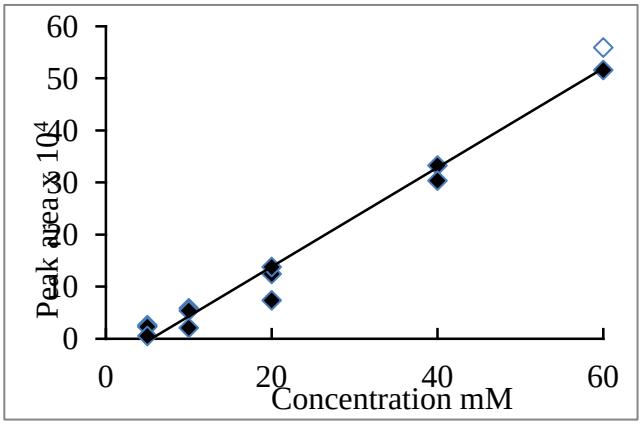

Figure 42: The standard curve of cysteine and cysteine

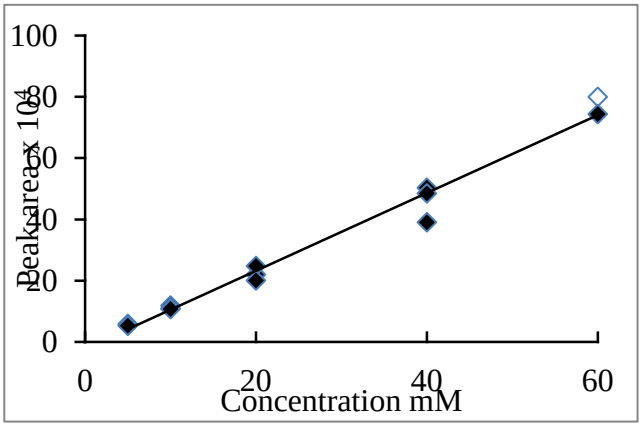

Figure 43: The standard curve of phenylalanine

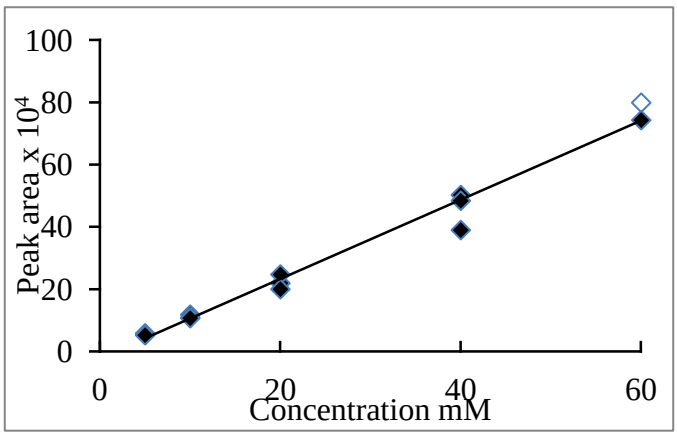

Figure 44: The standard curve of tryptophan

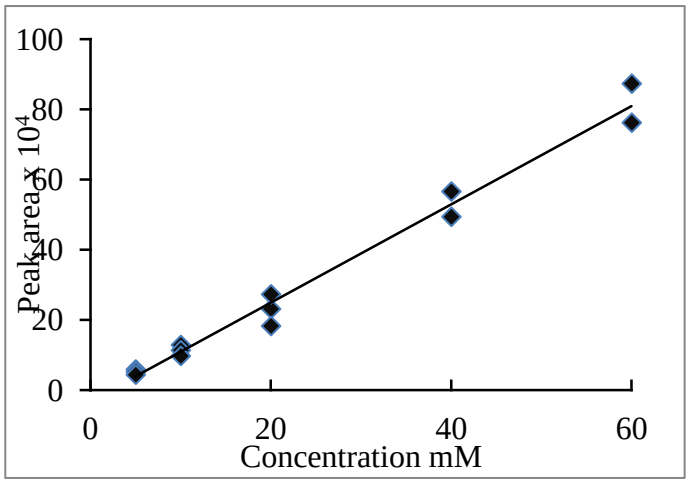

Figure 45: The standard curve of lysine