Queen Margaret University
EDINBURGH

School of Health Sciences

Division of Dietetics, Nutrition & Biological Sciences, Physiotherapy, Podiatry and Radiography

Subject Area: Dietetics, Nutrition & Biological Sciences

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D4154 - Honours Project Declaration Form

I certify that this work is original in its entirety and has never before been submitted for any form of assessment.

The practical work, data analysis, presentation, and written work presented are all my own work unless otherwise stated

Student name: [redacted]

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[redacted]

Date: 24th April 2020

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(i) Acknowledgements

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(ii) Abbreviations

ROS-Relative Oxidative Stress

FSE- Ferrous Sulphate Equivalent

GAE- Gallic Acid Equivalent

TPC- Total Phenolic Content

AC- Antioxidant Capacity

CVD- Cardiovascular Disease

QMU- Queen Margaret University

SOP- Standard Operating Procedure

1. Introduction

Reactive oxygen species (ROS), including hydrogen peroxide (H2O2), hydroxyl radical (.OH), singlet oxygen and superoxide anion radical (O-.2), are reactive molecules found naturally within the body and are produced from oxygen metabolism (Machu et al. 2015 and O'Sullivan et al. 2011). ROS are useful molecules within the body when regulated as they can act as intracellular signalling molecules (Zhang et al. 2016). Internal stores of ROS are formed as a by-product from oxidative phosphorylation reactions from the mitochondria (Machu et al. 2015). External sources of ROS are found from UV radiation (Mutton 2012) and exists within an equilibrium with an antioxidant defence system which can reduce the oxidative damages caused by ROS (Waris and Ahsan 2006). When the availability of antioxidants is overrun by excess ROS, a depletion of antioxidants or a combination of both, can disrupt the balance, leading to the body experiencing oxidative stress (Waris and Ahsan 2006). This can cause oxidative damage to tissues by reacting with DNA nucleotides, lipids of cell membranes and sulphydryl groups of proteins (Waris and Ahsan 2006 and Machu et al 2015) and can lead to cell death (Matanjun et al. 2007). This damage can result in the development of over 200 diseases including atherosclerosis, cardiovascular disease, diabetes mellitus, hypertension, cancer, ageing and neurological diseases such as Parkinson's and Alzheimer's disease (Machu et al 2015, O'Sullivan et al 2011, Zhang et al 2006 and Matanjun et al 2007).

Cellular defence systems are found to counteract the effects of ROS, consisting of enzymes that scavenge and neutralise unstable ROS, terminating further oxidation and damage it may cause (Saez et al. 2015). Antioxidants are one of many enzymes involved in the primary defence system. Secondary defence systems are composed of non-enzymatic antioxidants which can accumulate from the diet including carotenoids, glutathione, phenols (O'Sullivan et al. 2011), vitamin C and E (Eufic 2015).

Phenolic compounds include phenolic acids, polyphenols and flavonoids including flavanols, anthocyanins, isoflavones and tannins (Machu et al. 2015). These compounds are characterised by having at least one aromatic ring and one or more hydroxyl group attached. There are over 8000 known phenolic structures, ranging from simple single aromatic ringed compounds to large high molecularly weighed complex polyphenols. These can be categorised by the arrangement and number of carbon atoms (Crozier et al. 2007).

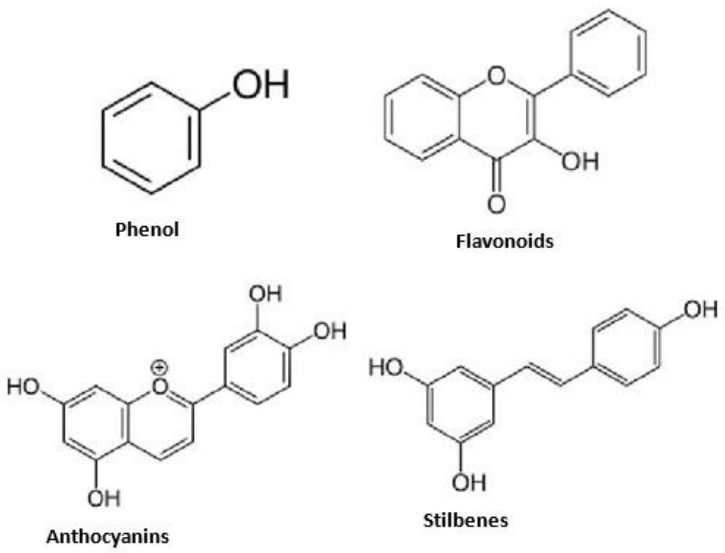


Figure 1. Phenol Compound Chemical Structures (Leite De Souza et al. 2018)

Phenolic compounds have been shown to have strong antioxidant activity (Deighton et al. 2000) and are classified as natural secondary metabolites. These are specialised compounds that do not contribute to growth and development but are needed to act as a protection mechanism to aid survival within a given environment. Secondary metabolites can have roles as signal molecules and UV protectants (Crozier et al. 2007).

Recently in human nutrition research, the protective properties of secondary metabolites have been further investigated. Evidence suggests a long-term intake of antioxidants is associated with a decreased risk of chronic diseases (Crozier et al. 2007 and Deighton et al. 2000). This is due to the protecting properties that antioxidants have, to defend cells against oxidative damages, therefore reducing the risk of degenerative diseases. This is achieved by phenolic compounds reacting with free oxidative radicals through hydrogen bonding to form phenoxy ions. Their electrons are delocalised from the aromatic carbon where this negative charge is transferred along the ring and released to be donated to the free radical which then stabilises it, terminating the oxidation chain reactions (Pandey and Rizvi 2009).

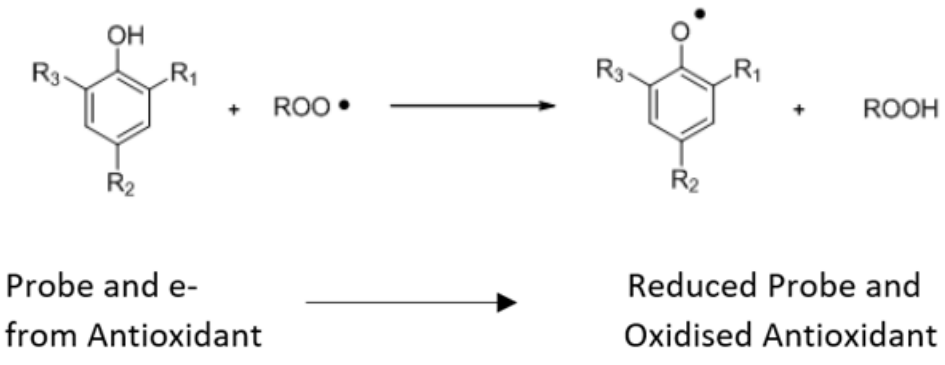


Figure 2. Reduction of ROS (Easchem 2013 and Petit 2016)

Food manufactures currently use antioxidants as a marketing tool due to their health-related benefits as consumers are more health conscious (Machu et al. 2015). However, there is yet to be an official health claim and dietary requirement for antioxidants, hence no legislation has been constructed regarding the advertisement of antioxidant levels on food labelling as it has not been authorised and listed on the EU registration (ASA and CAP 2016).

Plants and plant-based products have a reputation of having a high abundance for antioxidants (red wine (340 mg GAE/g) green tea (165 mg GAE/g), cocoa (611 mg GAE/g) and black tea (124mg GAE/g)) (Machu et al. 2015 and Schiener et al. 2014). Plant phenolics are found within the cell walls and can absorb UV radiation and are involved in protecting against oxidative harm. Machu et al (2014) found phenolic levels in Icelandic seaweed ranged from 0.7-192.6mgGAE/g using FCR assay. Irish seaweed's phenolic content ranged from 0.13-4 151.33 (Cox 2012 and O'Sullien et al. 2011).

Seaweed is classified as a marine plant or microalgae which can be found on rocks (National Ocean Service, nd) and is a key ingredient within Asian diets. Recently, seaweed has become more prevalent in the western diet as a functional ingredient and food product (Pereira 2011).

Recently, the nutritional value of seaweed has been discovered including dietary fibre, essential fatty acids, proteins, minerals, vitamins (Pereira 2011) and is considered a source of natural antioxidants including ascorbate, glutathione and phenolic compounds, such as carotenoids, and catechins and phlorotannin (Schiener et al. 2014). Due to the high levels of nutrients, seaweed also possess many health benefits; antimicrobial, anti-inflammatory (Schiener et al. 2014) anti-obesity, antidiabetic, antiallergenic, which is why researchers have been interested in the characterisation of seaweed. Seaweed is used in seasoning, sushi and condiments and can be consumed on its own (Pereira 2011).

Seaweed is usually used in food supplements rather than whole foods and are marketed as tablets and pills (Pereira 2011). However, Mara Seaweed have produced a line of seaweed which can be consumed as a flavour enhancer rather than a supplement. This makes it easier to introduce seaweed into a traditional western diet as the individual's diet is not majorly disrupted. (Schiener et al 2014).

It is interesting to determine the levels of antioxidant activity within seaweed as each species are exposed to different levels of harsh, oxidative environmental conditions from nutrient availability, salinity, temperature, high oxygen concentrations and UV radiation diversity, enhancing the formation of free radical and other strong oxidizing agents (Mekinic et al. 2019). Interestingly, seaweed rarely suffers from major cellular damage both photodynamically and structurally from these stressful oxidative habitats, implying that seaweed is highly protected by high levels of metabolites, such as phenolics, as they have a high capacity to scavenge and stabilise free radicals (Mekinic et al. 2019, Matanjun et al 2007 and Cox 2008). As seaweed is highly protected by the high abundance of antioxidants, it is thought they could have similar effects on the human body in delaying the onset of multiple chronic illnesses (Schiener et al. 2014).

There is yet to be clarification of the ideal extraction method for antioxidants from seaweed due to the complexity of this topic (Diem do et al. 2014). The use of unreliable methods to determine this leads to the questioning of the level of accuracy and validity of the results obtained by these assays, making it a necessity to dissect the methods used (Diem do et al. 2014). This study aims to determine the optimal extraction method using variations of the method using different solvents, sample size and different volumes of solvent.

The secondary objective was to quantify TPC through the FCR assay and the antioxidant capacity through the FRAP assay of four different Scottish and Irish seaweed species *Laminaria digitata, Palmaria palmata, Alaria esculenta and Saccharina latissima* used by Mara Seaweed.

2. Method and Materials

Colorimetric assays modified from QMU SOP (Petit 2016).

2.1Sample collection

Dried seaweed samples *Palmaria palmata, Laminaria digitate, Saccharina Latissima, Alaria esculenta* were sourced from Mara Seaweed (Edinburgh, Scotland).

Table 1. Characterisation of Seaweed Samples

Species	Type	Country of Harvest
Laminaria digitata	Brown	Scotland
Palmaria palmata	Red	Scotland/ Ireland
Saccharina latissima	Brown	Faroe Island
Alaria esculenta	Brown	Faroe Island

2.2 Sample Preparation

1g ± 0.1 of dried seaweed sample was weighed into a universal tube using a balance (Sauter CP2245, UK). For seaweed flakes PP and LD 1g ± 0.1 of dried seaweed sample and 1g ±0.1 of acid washed sea sand were weighed onto a weighting boat. A motor and pestle were used to grind the sand and seaweed into a fine powder. The powder was transferred to a tared universal tube and weights were recorded. The tubes were labelled and repeated in triplicate for each sample.

A Fisher brand elite pipette 0.5-5ml was used to transfer solvent into universal tubes containing the seaweed samples and heated in a water bath for thirty minutes at 70°C. Then the tubes were shaken in an alpha laboratories V400 multitube vortxer at speed 1.5 for thirty minutes. Samples were then vacuum filtered using Khf lab laboport filter pump attached to a 1L conical flask with a büchner funnel on the top.

Samples were poured onto 70mm Whatman filter paper in the funnel. Liquid sample found in the flask was then poured into a new universal tube where the instruments used were rinsed out with the same solvent used in the seaweed extract and repeated for each sample.

2.3 Extraction methods

Six variations of the extraction method were used with alterations made for the type and volume of solvent used and weight of the raw seaweed flake/powder (Table 1).

Table 2- Extraction Methods used

Extraction method	*Abbreviation*
10ml Ethanol, 1g of Seaweed Sample	10Eth1g
10ml Ethanol, 1g of Seaweed Sample	10Eth1g
10ml Ethanol, 2g of seaweed Sample (0.5g AE)	10Eth2g
10ml Acetone, 2g of seaweed Sample (0.5g AE)	10A2g
20ml Acetone, 2g of seaweed Sample (0.5g AE)	20A2g
10ml Methanol, 2g of seaweed Sample (0.5g AE)	10Mth2g
10ml distilled water, 2g of seaweed Sample (0.5g AE)	10W2g

2.4 Measuring Antioxidant capacity- FRAP assay

This assay can directly measure the reducing ability of antioxidants where it is assumed that reducing capacity is equivalent to the antioxidant capacity. The FRAP assay undergoes a redox reaction, reducing yellow ferric-TPTZ complex ions to blue ferrous ions- TPTZ complex via an electron transfer reaction within acidic conditions. FRAP value is proportional to antioxidant potential of the extracts, where the sample is capable of electron transfer if the value is positive (Matanjun et al 2007).

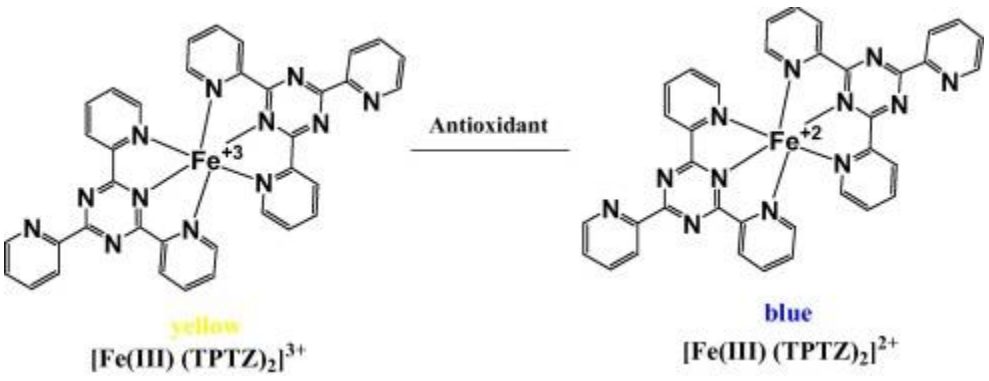


Figure 3. Reduction of Fe^{3+} (Pérez-Cruz et al 2018)

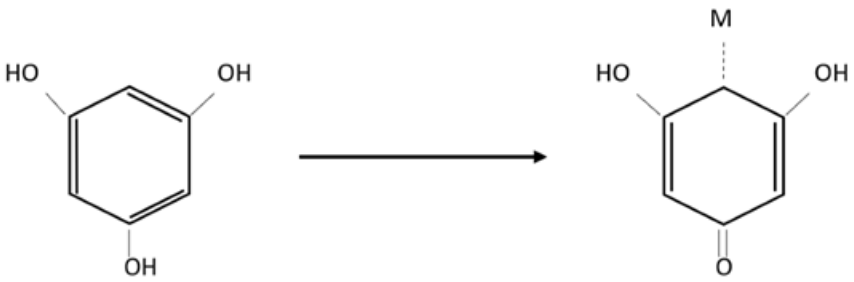
The antioxidant capacity for seaweeds was determined colourmetrically using the FRAP assay. Samples were diluted 1 in 10 with 1ml of sample and 9ml of distilled water. 10µl of ferrous sulphate standards ranging from 0-1mM FSE to provide a standard curve, diluted and undiluted sample and 250µl of working FRAP solution were pipetted using a Fisher brand 5-50microgram pipette into a nunclcn delta surface 96 well plate. The plate was then incubated for four minutes at 37°C and placed into a Dynamica halo MPR-96 visible microplate reader at a wavelength of 590nm. Results expressed as mM FSE/g

2.5 Measuring Total Phenolic Content - FCR assay

FCR assay is based on the generic reducing properties of phenols, making it unspecific as oxidants reduced by nonphenolic compounds such as vitamin C (Huang et al. 2005). Even though this assay is unable to identify individual chemical variations of each compounds present, it can quantify the TPC as they share similar structures, polarities and reactivities (Nollet and Gutierrez-Uribe 2018).

The FCR chemical nature is unknown, however it is theorised that the yellow heteropoly-phosphotunstate-molybdate ions is reduced by antioxidants to produce a blue chromophore thought to be a phosophotungstic phosphomolybdic complex with an absorbance of 760nm, proportional to the phenolic content of the sample (Petit 2016). The reaction must be in a basic environment (pH 10) for phenolic compounds to react with the reagent, as the proton dissociation of phenolic compounds enhances the samples reducing capacity, hence the addition of sodium carbonate (Huang et al 2005).

The basis of the FCR reaction occurs when phenolate anions, formed by the dissociation of a phenolic proton, reduce FCR (Huang et al 2005).



Mo (IV) + e^- Mo(V)

Figure 4 Reduction of FCR by the Oxidation of Phenols (Ford et al 2019)

The TPC was determined colorimetrically using the Folin-Ciocalteu assay.100µl of each diluted and undiluted sample and gallic acid standards ranging from 0-500mM GAE (Table 4) were added to a new

universal tube. 100µl of 200mM and 0mM of gallic acid was added to a universal tube for every six samples analysed. 5ml of Folin reagent and 0.9ml of distilled water was added to each universal tube and left for five minutes. After five minutes 3.2ml of sodium carbonate was added to each universal tube and left to develop for two hours. The samples were then transferred into cuvettes and placed into a UV-spect-Shimadzu UV-1280 UV-vis spectrometer calibrated at 765nm using water as a blank. The absorbance was measured and recorded for the seaweed samples and known concentrations of gallic acid standard to produce a standard curve. TPC results were expressed as mg GAE/g.

2.6 Statistical Analysis

Raw data was expressed as the mean and analysed using IBM Statistical Package for Social Sciences, software version 23 and Microsoft Excel 2020. A one-way ANOVA was used with a Turkey post-hoc analysis to determine the statistical difference between extraction method and species of seaweed for TPC and AC levels. $P<0.05$ was considered statistically significant.

3. Results

Total polyphenol content and antioxidant capacity was determined using spectrophotometric assays, after using six variations of the extraction method (Table 1). The figures below illustrate the TPC and antioxidant capacity of four seaweed samples.

3.1 Standard Curve

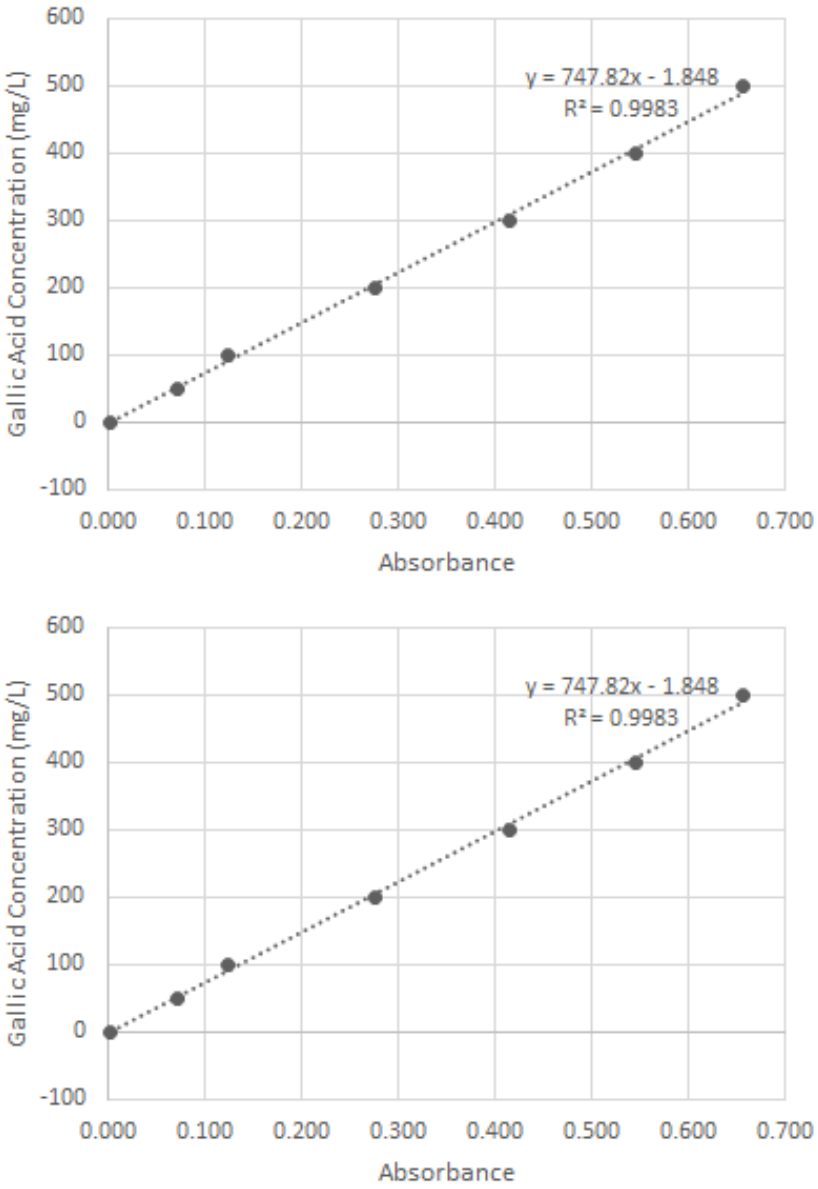


Figure 5. FCR Gallic Acid Standard Curve

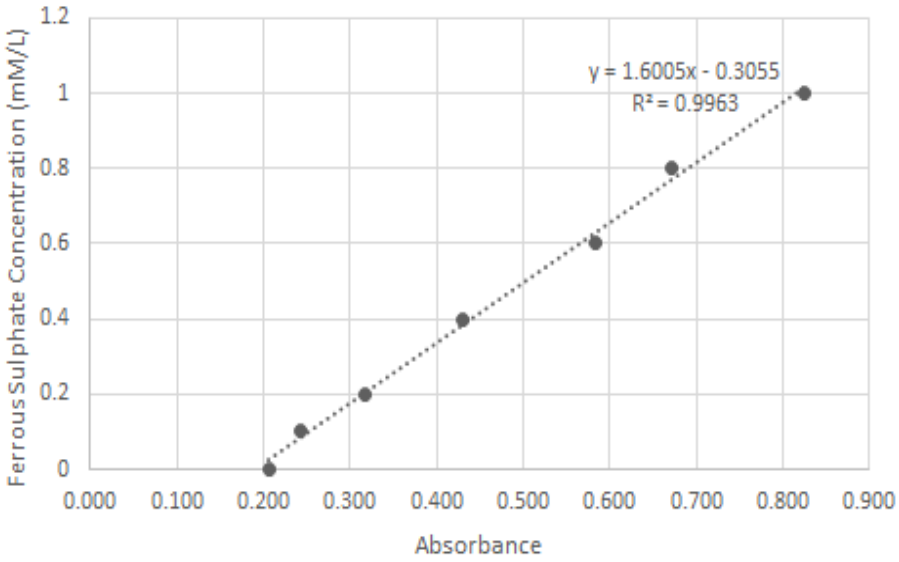
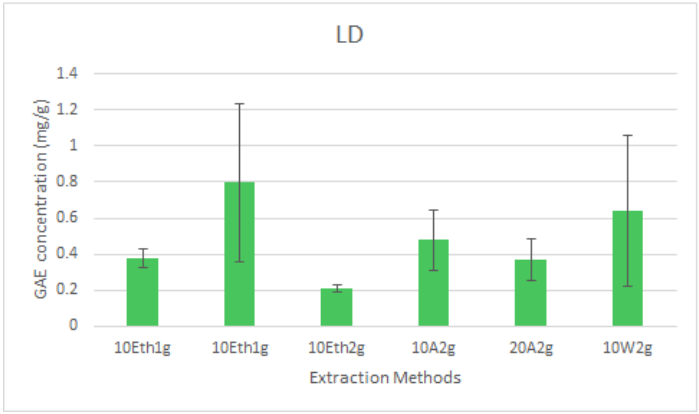


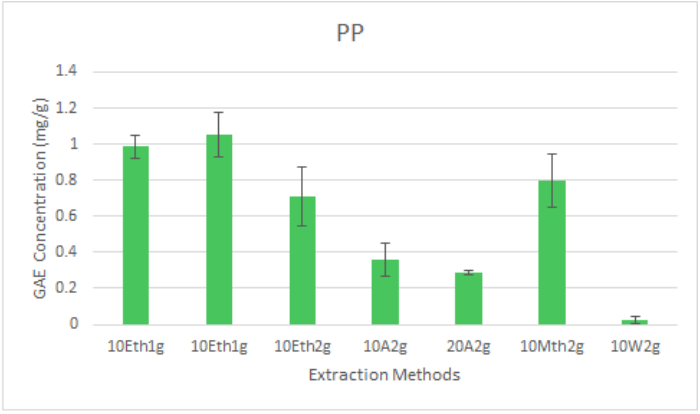
Figure 6. FRAP Ferrous Sulphate Standard curve

The R^2 value analyses the scatter of data around the line of regression. A R^2 value close to 1 is considered high, suggesting that both values within this study were high (0.9983 and 0.9963). This suggests the difference between the fixed values from the standard curve and data collected from the study was relatively low, suggesting there is a correlation between the average absorbance and the standard used. Relating this to the findings in the current study, suggests this is a suitable model. This illustrates that the variation of TPC and AC values can be justified by the extraction method and seaweed species (Frost 2020).

3.2 Total Phenolic Content



A



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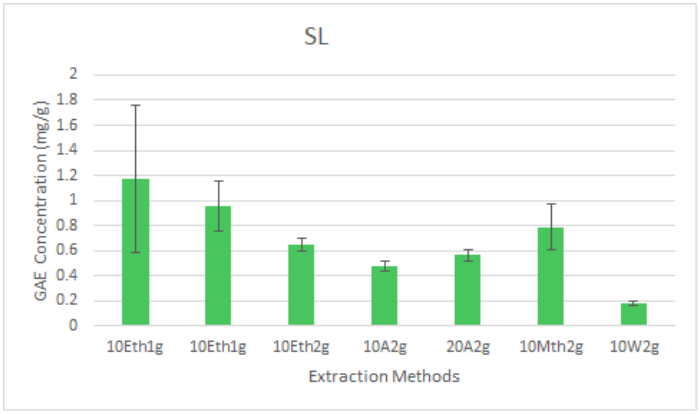
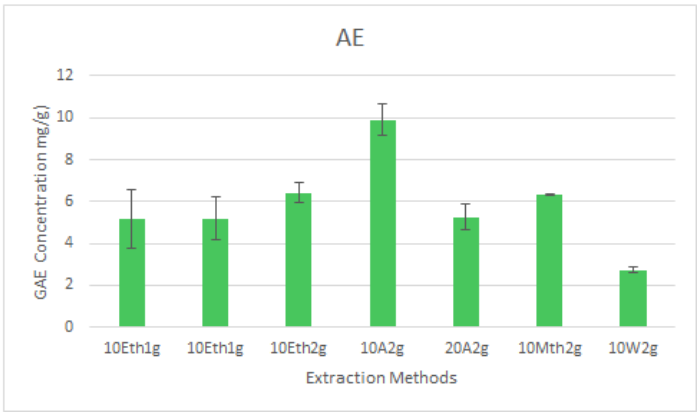


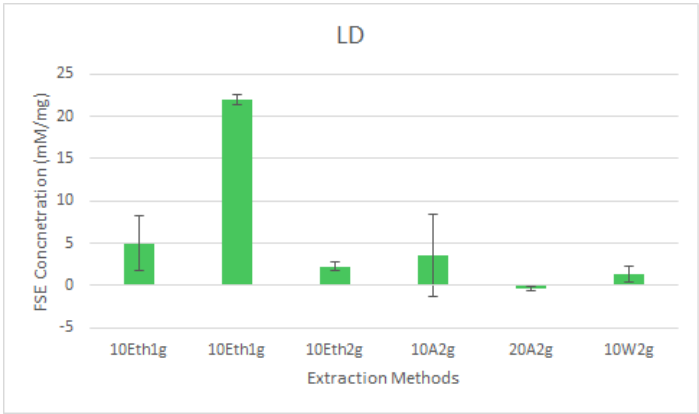


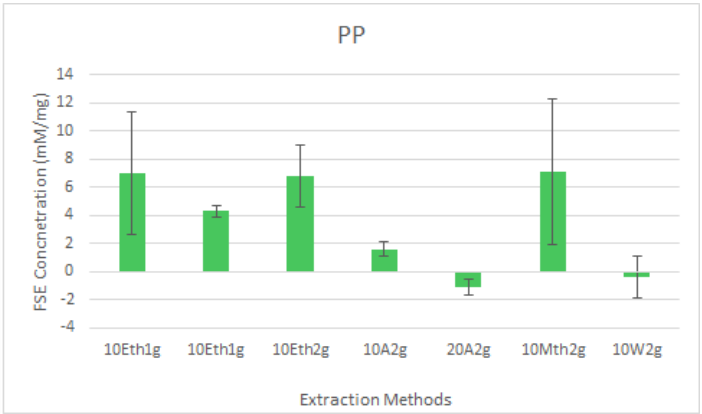
C D

Figure 7. Total Phenolic Content of Four Seaweed Species

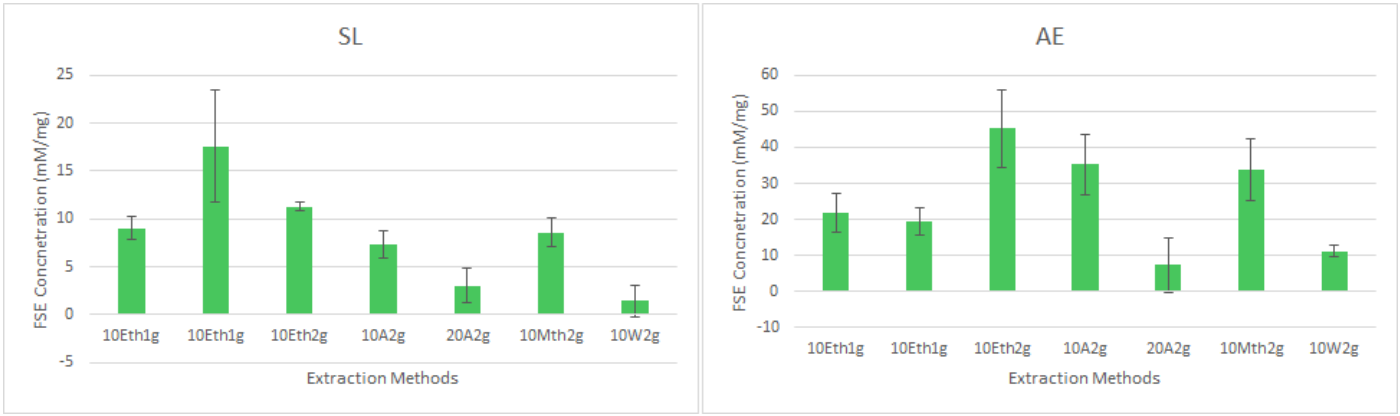
The mean TPC values were obtained from the calibration curve and is expressed as mg GAE/g dry seaweed extract using the FCR assay. The levels ranged from 0.027- 9.903mg GAE/g. Overall, AE had the highest TPC compared to the other species (2.74-9.90mg GAE/g), where the highest value was obtained through the extraction method 10A2g. The lowest TPC was found in species LD (0.214 -0.796mg GAE/g), using method 10Eth2g. The TPC range was noticeably large in PP (0.027-1.054mg GAE/g) and in SL (0.180 -1.176mg GAE/g). The extraction method which provided the lowest TPC value for all species with LD being the exception, was method 10W2. In contrast, the highest TPC value was obtained through the extraction using 10Eth1g with AE being the exception. Statistically, AE had higher phenolic levels compared to the other species ($P<0.05$). The other three samples were statistically similar as they did not show a statistical significance ($P>0.05$). There was no statistical significance found between extraction methods.

3.3 Antioxidant Capacity





A B



C D

Figure 8- The Antioxidant capacity of Four Seaweed Samples

The mean AC values from FRAP assay obtained from the calibration curve and is expressed as mM/g dry seaweed extract.

The highest antioxidant capacity using FRAP assay was AE (0.045mg FSE/g). In contrast the lowest values were seen in species PP (-0.0004-0.007mM FSE/g) suggesting that there was no antioxidant activity. Similar, to the TPC results, extraction using ethanol yielded the highest antioxidant capacity results, except for PP. Method 20A2g and 10W2g provided the lowest value. Much like TPC, AE had a significantly higher reducing capacity than the other samples ($p<0.05$), where the other species were similar as their AC values were not statistically different from each other. There was statistically no extraction method that was significantly better than another.

4. Discussion

4.1 Method Optimisation

It is crucial to identify the optimal extraction method for the antioxidants in seaweed as there is a significant difference between the levels of phenols in seaweed and the extraction processes used (Machu et al. 2015).

There are many parameters which can contribute to the phenolic content of seaweed. Within the extraction methods alone there are many variables which can influence the final phenol levels of a sample. These include solvent type, extraction time, temperature volume and concentration of solvent, weight of sample and the type of extraction technique (Mekinic et al. 2019).

4.2 The Influence of Solvents on Antioxidant Levels

Optimising extraction conditions for antioxidants could be beneficial to obtain accurate results (Złotek et al. 2016). It is crucial to question the types of solvents used during extraction as well as the types of extraction

method used (Diem do et al. 2014), as it can impact the TPC and AC value of a sample (Diem do et al 2014). The chemical nature of phenols and the solvent must be considered as they contain different chemical structures, influencing their polarities which can either increase or decrease the levels of phenols extracted (Mekinic et al. 2019). As each species contains a different number of phenols and other polar molecules, the overall polarity of that sample can be affected (Matanjun et al. 2007), impacting the solubility when emerged in the solvents. This was demonstrated in this study as each extraction solvent provided a different TPC and AC for each seaweed extract (See section 3).

It is important to utilise a variety of extraction solvents as each has its own benefits. Ethanol and methanol have been known for their efficiency of extracting polyphenols with a high polarity, more specifically with a lower molecular weight for methanol, acetone for the extraction of flavanols with a higher polarity molecular weight and aqueous solvents for the extraction of bioactive compounds with strong polarity (Diem do et al 2014).

Statistically there was not an extraction method which performed better than another ($p>0.05$) as solvent extraction is dependent on solvent type and the solubility of phenol compounds. This makes it difficult to report which solvent was the best for extraction of phenolic compounds as the effectiveness of each solvent can be influenced by the species of seaweed due to the different variation of phenolic compounds within (Mekinic et al 2019).

4.2.1 Ethanol

Out of the four extraction solvents used there was not consistent data to prove that one method was superior. However, a pattern formed which heavily suggests the best extracting solvent was ethanol. Each species found that in at least one of the three extraction methods using ethanol yielded the highest TPC and AC findings, with AE being the exception (Figure 5-6).

Poole et al (2019) had come to a similar outcome to the current study, discovering that using 50% ethanol had similar TPC as 50% methanol.

Previous literature has also demonstrated that ethanol is the most effective solvent in extracting phenolic compounds (Matajun et al 2014). Chemically, this is due to the strong polarity that ethanol possesses, making it ideal to extract many phenolic compounds within a sample. This could indicate a higher abundance of phenolic compounds with high polarity as they are hydrophilic and can form hydrogen bonds with the aqueous solvents, increasing their solubility and therefore TPC (Tsao 2010).

4.2.2 Methanol

Methanol provided relatively high TPC and AC results for all species which agrees with other studies (Matanjun et al. 2007 and Barchan et al. 2014). It provided the highest AC value for PP (0.0071mM FSE/g). Theoretically, based on methanol having one of the highest polarities seen in this study, it should have yielded the highest TPC and AC value (Barchan et al 2014). Multiple studies agree with this discovery

(Vijayabaskar and Shiyamala 2012). This is due to methanol having a small chemical structure meaning that the distribution of charges is greater.

4.2.3 Acetone

The use of 70% acetone had shown to provide low values for the AC of LD, PP and AE, yet TPC for AE contained the highest value from the extraction of acetone (9.903mg GAE/g). This could be due to acetone having the lowest polarity compared to the other solvents used (Murov 2010), suggesting a large percentage of phenolics within AE are non/low-polar compounds.

On the contrary, previous studies have discovered that the use of 70%/75% acetone yielded the highest TPC results compared to other solvents used (Wang et al. 2009, Rodriguez et al. 2010 and Diem do et al. 2014).

4.2.4 Water

The use of distilled water provided the majority of the lowest TPC and (0.027-2.735mg GAE/g) and AC (-0.0004-0.011mM FSE/g) values for all seaweed samples, with LD being the exception. Diem Do et al (2014) and Matanjun et al (2014) had come to similar conclusions.

Solubility of the extracts could explain these low values, as solubility is dictated by polymerisation degree, solvent type used, and the formation of interactions made between other complexes which can lead to insoluble complexes (Mekinic et al. 2019). The formation of complexes was visible during the extracting method, due to high polarity that water has compared to other solvents, as water has a shorter hydrocarbon skeleton compared to alcohols and a higher ratio of unequal electron sharing. This allows water to form more hydrogen bonds between phenolic complexes via hydroxyl groups in phenols providing its polarity. In this study, water had activated gelling properties within the sample, reducing sample extraction yield. This indicates a high abundance of polar compounds found within the seaweed samples, explaining the variation seen in TPC results (Matanjun et al 2007). In theory, this reduced TPC and the AC values as these compounds could have been entrapped in the gel network. Due to strong polar components and increased levels of alginate present within SL and PP (Wang et al. 2009) causing the sample to be viscous and providing great difficulty during filtration. This outcome has also been documented by Wang et al. (2009).

4.3 The influence of Sample weight on Antioxidant Levels

Results showed use of 2g and 0.5g for AE provided a range of TPC and AC values, however the highest results were seen in 1g extraction methods. However, as AE was weighed out in 0.5g the results were higher than the 1g samples in both TPC and AC.

Initially the sample size remained at 1g for each sample, however it was discovered for AE that TPC and AC levels of were too high and did not appear on the standard graph (Figures1 and 2) and the remaining

samples results were too low. However, after the calculations it was still made evident that AE remained to contain the highest AE and TPC values. Hence, distribution of the samples remained the same.

It is theorised larger sample sizes would yield a higher TPC result with more reliable values which is representable for the species. This was demonstrated by Yuan and Bone et al (2004) as they used a smaller sample size of 1mg for PP and yielded a lower TPC value (0.010mgGAE/g) compared to this study (1mgGAE/g). Yuan et al (2006) used samples size of 250g and yielded a TPC result 12-fold increase compared to the results obtained from this study.

4.4 The Influence of Solvent Volume on the Antioxidant Levels

Results show that the use of 20ml of solvent provided lower AC results and midrange results for TPC. There was a statistical difference shown between method 10Eth2g compared to 20A2g ($p<0.05$). Evaluating the results, it was seen that use of 10ml of solvents yielded higher results compared to 20ml.

Rodriguez et al. (2010) found 20ml to be optimal as 10ml was too low to extract 100mg of seaweed sample. They found that SL had yielded a TPC of 1.4mg PGE/g, which is a 2-fold increase compared to the SL result obtained using 20ml solvent with the current study (0.56mgGAE/g). Hwang and Do Thi (2014) had also yielded a high TPC value of 22.2GAE/g using 20ml of solvent.

4.5 Total Phenolic Content and Antioxidant Capacity Values

There was a large variation of TPC results between the samples analysed, ranging from 0.027-9.90 mg GAE/1g of dried sample. Other studies have seen a large variation of results using FCR method (Matanjun et al. 2007 and Roleda et al. 2019). AE contained significantly higher TPC compared to the other samples ($p<0.05$). On the contrary, LD possessed the lowest TPC value. With experimental parameters not considered, other studies have found similar patterns. Icelandic seaweed analysed by Wang et al (2009) found LD contained the lowest TPC, with PP containing slightly more. SL and AE both contained similar values. Interestingly, the same samples used in this study were analysed, however the TPC obtained from the Icelandic samples were much greater than the results from the current study. This could indicate that aquatic environment can play a role in antioxidant activity of seaweed (Wang et al 2009). Roleda et al (2019) have shown that Norwegian and French AE (3.7mg PGE/g), SL (0.8 mg PGE/g) and Icelandic and Norwegian PP (0.5 mg PGE/g) samples had similar TPC results to the values obtained from this study. Other studies have also found a similar pattern in TPC values of AE, SL, PP and LD (Schiener et al 2014 and Rajauria et al 2010).

AE had significantly higher AC levels ($p<0.05$) compared to the other extracts. Irish seaweed analysed by O'Sullian et al (2011) extracted 100-fold higher AC compared to the samples in this study. This could be due to the variation of phenolic compounds present within different seaweeds and therefore the AC levels. However, PP values obtained from the present study were higher than Machu et al (2014) (0.00056mM/g).

Similar trends were discovered in Scottish seaweed. However, TPC values differ as AE, SL and LD were higher than the samples analysed by the current study, (AE 8.7mg/g, SL 4.2mg/g and LD 1.4mg/g) (Schiener et al. 2015). Bak (2019) analysed AE and SL harvested from the Faroe Islands and discovered TPC values for AE and SL were similar to the values obtained from the present study AE (3.8GAEmg/g) SL (0.8gGAE/g) and were classified as low having antioxidant activity.

4.6 The Influence of the Environment on Antioxidant Levels

When quantifying and understanding the TPC and AC of seaweed, external factors must be considered as each species naturally contains diverse levels, due to the vast external variants (Pandey and Rizvi 2009). These include, water depth and temperature, season of harvest, intertidal zone, solar light intensity, location, level of emersion and availability of nutrients (Connan et al 2007).

Sunlight and climate have a direct effect on the variation of phenols in seaweed. Thus, TPC of seaweed will vary from each geographical location it is harvested from, as each species used in the study are all found in different intertidal locations (Connan et al. 2007; O'Sullivan et al. 2011). These zones are characterised by a wide variety of environmental traits as phases of emersion and immersion vary. Due to these changes' seaweed experiences a stressful and dynamic frequent change in the environment, suggesting that each species has evolved to adapt to certain short-term temperamental environmental conditions (Flores-Molina et al 2014). Different species experience different stress tolerance which can been seen in their antioxidant metabolism (Guiry 2020). When seaweed is exposed to stressful environments, the phenols become free molecules within the cell walls, which are used to protect the plant against stressors (Guiry 2020), resulting in a higher tolerance to ROS and oxidative stresses. This is regulated by enhancing antioxidant metabolism, therefore increasing the production of antioxidants (Flores-Molina and Thomas et al 2014). This explains the high antioxidant activity of AE as it can be found in the upper sublittoral fringe of shallow cold-water shore areas and is one of the most exposed seaweed species used in this study (Pereira 2011).

4.7 Seaweed Industry

This study has shown that all four seaweed samples had antioxidant abilities, especially AE which contributes to dietary intake of antioxidants.

Seaweed is sold either whole or as extracts added to other food products (Cherry 2019), as a functional ingredient in condiments, seasonings, and sushi. The functional properties of seaweed are based on the levels of insoluble and soluble fibre and polysaccharides content such as carrageenan which can form gums, ideal for thickening and stabilising food systems (Yuan et al 2006).

The current market for natural foods had risen as consumers are becoming more health conscious and more aware of the effect's food produce can have on physiology. This has led to a peaked interest for food

manufacturers to determine sources and to understand characteristics of natural water and lipid soluble antioxidants (Pereira et al 2011)

4.7.1 Phenol Recommended Levels-

Regarding phenol daily intake requirements, there are varied responses from numerous sources. Eufic (2015) have stated there are no official dietary requirements published due to phenols not being an essential nutrient, meaning consumption is not crucial for maintaining life (Eufic 2015). Yet, a study by Grosso et al (2014) discovered the mean value of daily phenolic intake was 1662.5 mg/d, and Haleem et al (20) have shown that the Scottish populations intake of antioxidants was 680 mmol/ day, where the majority was sourced from plant-based products. The results in the current study found that even though phenolic content of 1g of seaweed is relatively low compared to the DRV, it can still contribute to the total phenolic intake when added to a balanced diet and consumed alongside food sources with high phenolic levels.

It is difficult to determine if the levels of phenols extracted from this study can contribute to health claims, as there is a lack of evidence through intervention studies associated with health claims for seaweed products (Cherry 2019).

Table 3- Food Products High in Antioxidants

Food product	**Antioxidant capacity (mM/100g)**
Dark chocolate	15
Pecans	10.6
Blueberries	9.2
Strawberries	5.4
Artichokes	4.7
AE seaweed	4.5
Goji berries	4.3
Raspberries	4
Kale	2.7
LD seaweed	2.2
Red cabbage	2.2
Beans	2
SL seaweed	1.7
Beets	1.7
Spinach	0.9
PP seaweed	0.7

List of food products which are naturally high in antioxidants with the highest values from the present study added. (adapted from Carlsen et al 2010)

Comparing to other food products, seaweed samples analysed have considerably less TPC than the likes of green tea or blue berries. Schiener et al (2014), The samples analysed in the present study have a high antioxidant profile, especially AE having a high/midrange antioxidant capacity. Thus, suggesting even though there is yet to be health claims and legislation made for seaweed published, these products can contribute to the daily antioxidant intake.

5. Limitations

The limiting factors identified in the extraction method involve particle size of the samples. Antioxidants are found in cell walls of seaweed, making it important for the dried seaweed sample particle size to be as small as possible. Species LD and PP were sourced in the form of flakes and even after being ground, using a motor and pestle, the particle sizes were still larger than the powdered samples. This led to a lower TPC and AC value in LD and PP samples.

The solvent concentration was not consistent for every solvent used and each alteration made to the extraction method was not repeated for all variants. This makes it difficult to identify which parameter was responsible for the change in results.

Due to the lack of resources and time constraints a larger sample was not used. If larger samples were used this could lead to more accurate and homogenous, representable data set. Sample LD did not undergo an extraction using methanol which could have altered the results and forming a new pattern.

When analysing antioxidant activity in seaweed, it is difficult to determine levels were considered high or low as there was no standard seaweed sample used which was known to be high in antioxidants, making it difficult to fully understand the samples antioxidant ability

It is difficult to compare each samples AC and TPC as the different extraction method conditions can influence the phenolic value of algae (Machu et al 2015).

Table 4- Overview of the total phenolic content of brown seaweed

Extraction Solvent	Species	Location	TPC
50% Ethanol	*Padina pavonica*	Tunisia	7.06mg PGE/g
	Cytoseria sedides	Tunisia	26.45mg PGE/g
	Cladostephus spongiosum	Tunisia	10.91mg PGE/g
	Sargassum muticum	France	76.62mg GAE/g
	Sargassum polycystum		0.37mg GAE/g

		Malaysia	
	Laminaria ochroleuca	Spain	173.65mg GAE/g
	Sargassum muticum	France	58.10mg GAE/g
50% Methanol	*Halopteris scoparia*	Spain	1.23mg GAE/g
	Padina antillarum	Malaysia	24.3mg GAE/g
	Fucus serratus	Ireland	240mg PGE/g
70% Acetone			
	Fucus vesiculosus	Iceland	24.2mg PGE/g
	Fucus vesiculosus	Portugal	39.1mg GAE/g
	Sargassum fusifarme	China/Japan	13.1 mg GAE/g
	Undaria pinnatifinda	Japan	5.7 mg GAE/g
	Saccharina latissima	japan	5.2 mg GAE/g
	Saccharina japomica	Japan	8.8 mg GAE/g
	Ecklonia bicyclis	France	84.1mg GAE/g
	Palmaria Palmata		0.010mg GAE/g

Shows how the location, species and extraction solvent can influence the levels of phenolics in algae. This demonstrates how it is important to explore all possible variants which can affect the levels of phenols within a sample and how this can be difficult to measure if all the parameters have not been considered. (Mekinic et al 2019)

The main limitations within this study were the scattered results providing a low validity as the standard deviation was high in all methods used. Low reproducibility and reliability results suggest antioxidants activities discovered may not be representable for the species analysed

This reduces the reliability due to the number of variants, both laboratory based (wavelength, concentration and volumes of reagents to types of solvents and their volumes and concentrations, time for reaction to occur and human manipulation) and confounding factors from the environment, including the species themselves.

6. Future Developments

For future developments of the optimisation of the extraction method, other variables could be investigated to identify their effects on the final antioxidant results. These may include; temperature of the water bath, time and speed of the vortex, the use of different volumes and concentrations of solvents, different masses of seaweed sample used.

Different experimental parameters regarding the assays used have the potential to be investigated to produce cohesive reliable results. These include; different wavelengths, volume and concentration of reagent used, the length of time for the reaction to occur and leaving it in the dark. which could remove interference of visible light on the final absorbance reading.

To understand antioxidant abilities, gain knowledge about the health effects of these products and to enforce the reliability and validity of other assay results, including the ROS scavenging capacity DPPH, metal chelating capacity, identifying specific phenolic compounds through HPLC and oxidative enzyme inhibition capacity, would need to be explored (Huang et al 2005). The use of only two assays does not show the full extent of the antioxidant abilities within a sample, therefore future studies should implement the methods above.

7.Conclusion

To summarise, this study demonstrated that no extraction method was optimal for antioxidant isolation in Scottish and Irish seaweed. However, the use of 10ml of ethanol with 2g of sample provided the highest TPC and AC values. Establishing a standard extraction method for all species is difficult, as each sample will contain a diverse level and type of antioxidant compounds based on their habitat and the levels of ROS they will encounter.

This study has verified that there are antioxidant properties within the four samples studied, with species AE containing a significantly higher antioxidant activity, concluding that it had better reducing abilities compared to other samples which could be beneficial in prevention of chronic diseases. As brown seaweed is a new and relatively unexplored source of biological compounds, further research into the diverse antioxidant ability that AE possess would be necessary for the discovery of the many applications it could possess within the food industry.

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