# Biochemical and spectral characterisation of micro and macroalgae

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## Abstract

Algae contain many compounds which are of interest to the pharmaceutical, agricultural and food industries, among others. Despite the potential for many applications, a quick, accurate and inexpensive method for characterising algae is lacking. The objective of this thesis was to investigate the ability to determine protein, carbohydrate and fatty acid contents in algae, using infrared spectroscopy. Algal samples were analysed using some of the most accurate chemical methods, by hydrolysing proteins and polysaccharides and quantifying the resulting amino acid and monosaccharide contents using chromatography techniques. Fatty acid contents were similarly quantified by extraction followed by gas chromatography. This data was subsequently used to calibrate spectral prediction models, using different infrared spectroscopies. The accuracy of protein prediction by diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS) and near-infrared spectroscopy (NIR) using partial least squares regression (PLSR) was compared to traditional methods like N-protein ratios and colorimetric assays. DRIFTS, attenuated total reflectance (ATR) Fourier transform infrared spectroscopy (ATR) and NIR were also comparatively used to characterise seaweed carbohydrates. Fatty acid contents were studied using DRIFTS in both microalgae and seaweeds. Infrared spectroscopy coupled with PLSR was shown to be highly accurate in characterising algal biomass, provided a sufficiently robust library of calibration samples. These methods require little or no chemicals and are rapid and easy to use, making them both environmentally and economically attractive.



Keywords: Algae, seaweed, spectroscopy, FTIR, NIR, characterisation, PLSR, multivariate

# Biokemisk och spektral karaktärisering av mikro- och makroalger


## Sammanfattning

Alger innehåller många ämnen som är av intresse för läkemedels-, jordbruks- och livsmedelsindustrin, bland andra. Algbiomassa har många olika användningsområden, men en snabb, precis och billig metod för att karaktärisera denna biomassa saknas. Målet med denna avhandling var att utforska möjligheten att mäta protein, kolhydrater och fettsyror i alger med hjälp av infraröd spektroskopi. Algprover analyserades med några av de mest exakta kemiska metoderna, genom hydrolys av proteiner och polysackarider följt av kvantifiering av de resulterande aminosyrorna och monosackariderna med kromatografiska tekniker. Fettsyremängder kvantifierades genom extraktion följt av gaskromatografi. Mätvärdena användes för att kalibrera spektrala prediktionsmodeller, med olika spektroskopiska tekniker. Noggrannheten i proteinprediktion med diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS) och near-infrared spectroscopy (NIR) med partial least squares regression (PLSR) jämfördes med traditionella metoder som N-protein-kvoter och kolorimetriska analyser. DRIFTS, attenuated total reflectance Fourier transform infrared spectroscopy (ATR) och NIR användes också för att karaktärisera kolhydrater i mikroalger och tång. Infraröd spektroskopi i kombination med PLSR visade sig vara mycket exakt vid karakterisering av algbiomassa, förutsatt ett tillräckligt robust bibliotek av kalibreringsprover. Dessa metoder kräver få eller inga kemikalier och är snabba och enkla att använda, vilket gör dem både miljömässigt och ekonomiskt värdefulla.



Keywords: Algae, seaweed, spectroscopy, FTIR, NIR, characterisation, PLSR, multivariate

# Contents

# List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Calle Niemi, Agnes Mols Mortensen, Ralf Rautenberger, Sanna Matsson, András Gorzsás, Francesco G. Gentili (2023). Rapid and accurate determination of protein content in North Atlantic seaweed by NIR and FTIR spectroscopies. Food Chem., 404, Part B, https://doi.org/10.1016/j.foodchem.2022.134700

II. Calle Niemi, Junko Takahashi, András Gorzsás, Francesco G. Gentili (2024). Quantitative and qualitative saccharide analysis of North Atlantic brown seaweed by gas chromatography/mass spectrometry and infrared spectroscopy. Int. J. Biol. Macromol., 254, https://doi.org/10.1016/j.ijbiomac.2023.127870

III. Calle Niemi, Francesco G. Gentili. Fatty acid analysis in microalgal mono- and polycultures using diffuse reflectance infrared Fourier transform spectroscopy coupled with partial least squares analysis. Heliyon (submitted)

IV. Calle Niemi, Francesco G. Gentili. Fatty acid profiling and spectral analysis of North Atlantic seaweed. (manuscript)

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# 1. Introduction

## 1.1 Algae as industrial feedstock

Microalgae and seaweed both produce a host of compounds having industrial potential as feedstock for biorefining but they are currently underutilised with most produced seaweed being used as food. In recent decades, the seaweed market has been expanding rapidly and global production reached 30 million tonnes in 2016 (van den Burg et al. 2021). Algae have garnered a great deal of research interest, particularly with rising demands for environmentally sustainable chemicals and materials (Polat et al. 2023).Advantages of algal crops over terrestrial ones

In addition to the vast, unexplored array of biological molecules produced by algae, they have several advantages over terrestrial crops for the production of large-scale commodities such as biofuel. Firstly, they do not require arable land and thus do not compete with common food crops. Secondly, they accumulate biomass at a more rapid rate than even the most optimised industrial crop (Chisti 2008a), enabling higher degrees of productivity. Thirdly, they do not necessarily require potable water for cultivation, depending on the species and the intended product. Marine algae can be cultivated in seawater, and freshwater algae have been shown to proliferate in a wide variety of wastewater types (Gouveia & Oliveira 2009).

## 1.2 Potential uses for algae and algal compounds

### 1.2.1 Algae as food

Many microalgae and seaweeds are rich in proteins, ranging from 5 to 30 % by dry weight (%DW) (Angell et al. 2015) with up to 50 %DW observed in select microalgae (Becker 2007). A large proportion of algae biomass, up to 60-70 %DW in seaweeds, consists of dietary fibres, making them highly nutritious food additives for humans and livestock (Jiménez-Escrig et al. 2001). Microalgae also accumulate large proportions of fatty acids – often up to 20-30 %DW and potentially more – depending on the species and culture conditions (Griffiths et al. 2011). Seaweeds tend to contain lower quantities of fatty acids, with very few exceptions accumulating between 5 and 10 %DW (Ganesan et al. 2019; Penalver et al. 2020; Veliz et al. 2023). Microalgae food products are still uncommon, but seaweeds have been consumed by humans and fed to livestock for thousands of years, particularly in Asia (Buschmann et al. 2017). This section thus focuses mainly on the nutritional properties of seaweed.

Seaweeds which are commonly consumed by humans contain vitamins B-12 (Watanabe et al. 1999; Nielsen et al. 2021), vitamin C (Bekah et al. 2023), and pro-vitamin A (Yamada et al. 1996), contributing to the recommended daily intakes for maintaining human health. Inorganic compounds are also abundant in algae, due to their absorption of dissolved nutrients from the water column. For example, ash contents in algae range from 10 to 30%DW, with some seaweeds containing up to 40 %DW (Ruperez 2002). These minerals usually include combinations of most essential minerals K, Ca, Na, P, Cu, Fe, Se, Mn, Zn, Mg, Cr and I in higher concentrations than other mineral-rich foods (Lozano Munoz & Diaz 2022). Even one gram of dry seaweed fulfils a substantial amount of the daily requirement. Some seaweeds can also contain toxic elements including As, Pb and Cd, and in some species the amount of I, Se and Cr can be high enough to pose a health risk (Lozano Munoz & Diaz 2022; Bekah et al. 2023; Guo et al. 2023). It is important then that edible seaweeds be subject to food safety assessments and the consumption of specific species must be limited. Preparation methods like soaking or blanching (Devesa et al. 2008; Nielsen et al. 2021) can significantly reduce the amount of toxic constituents, or even render them less bioaccessible (Clemente et al. 2016).

*Protein*

The amino acid profile of seaweed usually consists of most of the essential amino acids (EAA), with the exception of tryptophan (Kolb et al. 2004; Pirian et al. 2018). Seaweed can thus supply protein alongside dietary fibres, omega-3 fatty acids, minerals and vitamins, making them a highly nutritious protein source when combined with other foods like legumes and vegetables (Willcox et al. 2009). Seaweed protein does exhibit a low degree of digestibility, however (MacArtain et al. 2007), an issue that can be remedied through fermentation or enzymatic treatments (Bleakley & Hayes 2017).

*Carbohydrates*

The carbohydrate composition of algae is highly distinct from land plants and many algal phyla produce unique carbohydrate polymers, which cannot be found elsewhere in the plant kingdom (Rioux & Turgeon 2015).

For dietary purposes, polysaccharides are classified either as digestible or as dietary fibres. The only polysaccharide considered to be digestible by humans is the β(1→4) glucan starch, which forms a large proportion of human food intake through plant consumption (Gul et al. 2021). All other polysaccharides, and consequently cell wall polysaccharides, are dietary fibres. Starch contents of 55-60 %DW have been observed in some microalgae through limitation of phosphorous or sulphur nutrients during growth (Branyikova et al. 2011), but seaweeds generally contain far less or even no starch, depending on species. Green seaweeds of the Ulva genus can contain up to 30 %DW starch (Prabhu et al. 2019; Kazir et al. 2021), and red seaweeds contain a heavily branched form of starch called Floridean starch, but in lower quantities (Yu et al. 2002). Brown seaweeds on the other hand are quite unique in that they do not store carbon in starch at all, but in an alternative β(1→3) glucan called laminarin, which is not digestible to humans. Thus, the vast majority of seaweed carbohydrates and indeed sometimes even the majority of their total DW consists of dietary fibres (Rioux & Turgeon 2015).

*Lipids*

Like terrestrial plants, algae usually have higher proportions of unsaturated fatty acids than do animal-derived food, making them beneficial for cardiovascular health (Hu 2003). Certain phyla also contain large quantities of omega-3 fatty acids which are known to have many beneficial effects on overall health (Arterburn et al. 2006). While most algae contain the medium-

chain 18:3 omega-3 fatty acid alpha-linolenic acid (ALA), many diatoms and seaweed species also contain long-chain omega-3 fats like eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These are essential fatty acids needed for several physiological functions such as neurological development and cardiovascular health (Kim 2007; Ohnishi & Saito 2013). Humans can convert ALA to long-chain omega-3 fatty acids, but the conversion rate is extremely low so direct consumption of EPA and DHA is considered necessary (Baker et al. 2016). ALA has many health benefits on its own, however, and some algae contain large quantities of the 18:4 omega-3 fatty acid stearidonic acid (SDA) (Miyashita et al. 2013), an intermediate in the conversion of ALA to EPA with a higher conversion rate (Baker et al. 2016). Consumption of algae containing SDA can thus supplement the need for EPA and DHA to some degree, albeit not completely.

### 1.2.2 Pharmaceuticals

Seaweeds are of medical and pharmaceutical interest due to the antiviral, antibacterial, antioxidant, anticancer, wound healing and gelling properties of several of their constituents (Lomartire & Goncalves 2022). Both polysaccharides, polyphenols, proteins and pigments, among others, are being investigated for their medicinal properties (Smit 2004). The antiviral properties of seaweed metabolites has been shown to be effective against herpes, influenza and coronavirus (Lomartire & Goncalves 2022), and the epiphytic microbiota attached to seaweeds produce antibacterial compounds that can target antibiotic-resistant bacteria (Asharaf et al. 2022). This will be a particularly important field of study in the coming decades in response to increasing antibiotic resistance. Phenolic compounds and polysaccharides from seaweed can also be used to target human breast cancer cell lines (Vaikundamoorthy et al. 2018), but more research is needed before these medicines can be tested in humans.

These are only a handful of examples of the many medical uses of seaweeds which are currently being investigated, and this is a relatively new area of research which is likely to be expanded in the coming years.

### 1.2.3 Biostimulants, biofertilisers and crop protection

Phytohormones and other bioactive compounds secreted by or extracted from algae are effective biostimulants in agriculture (Dmytryk & Chojnacka

2018). Research into plant biostimulants is expanding rapidly due to their potential as a remedy for climate-derived threats to crop productivity. They can be defined as follows:

> "Plant biostimulants, […], are a diverse classification of substances that can be added to the environment around a plant and have positive effects on plant growth and nutrition, but also on abiotic and biotic stress tolerance." (Van Oosten et al. 2017).

Several studies have investigated the use of both microalgal cultures and algal extracts to enhance the growth, quality or resilience of food crops or trees (Ammaturo et al. 2023). Application of microalgae to seeds and foliage (Grzesik & Romanowska-Duda 2014; Grzesik et al. 2017; Alling et al. 2023) can improve qualities such as plant height and shoot development. Further, microalgae in consortium with cyanobacteria have been shown to function as effective biofertilisers and can reduce chemical fertiliser use in wheat cultivation by 75% (Ramírez-López et al. 2019). Algal and cyanobacterial extracts can also prevent crop damage from parasites such as nematodes (Sithole et al. 2023), offering a sustainable pest management solution. These agricultural uses of algal products improve food security in the face of climate change, which is set to decrease crop productivity through extensive redistribution and decline in biodiversity (Pecl et al. 2017) and more volatile and unpredictable weather (Wheeler & von Braun 2013).

Seaweed extracts also contain biostimulating substances capable of enhancing the productivity of terrestrial crops. Foliar application or soil-drenching with seaweed extracts has been shown to improve the yield of bean crops through the biochemical alleviation of drought stress (Ziaei & Pazoki 2022; El Boukhari et al. 2023). Polysaccharide extracts from seaweeds can protect land crops from pathogens both through their inherent antimicrobial properties and by stimulating the production of proteins for defence against pathogens, among other protective benefits (Hossain et al. 2024).

### 1.2.4 Biomaterials

Of particular interest for industrial utilisation of algal biomass is the wide array of polymers that algae contain in their cytoplasm, cell wall, and those secreted or attached to the exterior of the cell, known as extracellular

polymeric substances (EPS). EPS are a gelatinous matrix of proteins, fatty acids and, primarily, exopolysaccharides (Xiao & Zheng 2016). EPS play a key role in the ability of microalgae to adsorb heavy metals from wastewater, likely due to the abundance of functional groups in secreted exopolysaccharides (Mehta & Gaur 2005), and may be useful for several industrial purposes. The microalgal cell wall is, itself, a source of myriad polysaccharides, exhibiting immense diversity between species (Arad & Levy-Ontman 2010; Baudelet et al. 2017). Compared to terrestrial plants and seaweeds, microalgae are not as commonly exploited for their polysaccharides, although research is advancing (Colusse et al. 2022).

Seaweed have long been cultivated or foraged for their polysaccharides, particularly in Asia (Armisén & Gaiatas 2009). Agar and carrageenan from red seaweeds are sulphated polygalactans commonly used in foods for their gelling and preservative properties (Liao et al. 2021; Udo et al. 2023). Alginate, a polyuronic acid polysaccharide that constitutes most of the cell wall in brown seaweeds, also has many uses in the food industry and a history of use in dentistry for diagnostic purposes as well as for making molds for prosthetic implants (Cervino et al. 2018). Brown seaweed also contain fucoidan, a fucose-based polysaccharide which can contain highly varied quantities of other monosaccharides like galactose, mannose, glucuronic acid and others (Anisha et al. 2022). All these polysaccharides have intriguing physicochemical properties, which have prompted research into their use for production of sustainable bioplastics (Kanagesan et al. 2022), 3-D printing polymers (Mandal et al. 2023) and even for use as matrices for bioprinting of replacement organs (Markstedt et al. 2015). Alginate is a particularly abundant macromolecule, constituting 30-45 % of dry brown seaweed biomass (Rioux & Turgeon 2015), making it easy to produce in large quantities. Its molecular structure is relatively simple, consisting of only two different monosaccharide subunits: β-D-mannuronic acid (M) and α-L-guluronic acid (G). The ratio and distribution of these monomers change the properties of the polymer however, and gels and biofilms made from alginate differ in their rigidity based on what metal ion it is binding (Costa et al. 2018). The many –OH groups on the M and G subunits are also readily modified with other functional groups, enabling tailor-made polymer properties (Yang et al. 2011).

### 1.2.5 Biofuels

In order to reduce reliance on fossil fuels, algae have been widely investigated as feedstock for biofuels. The high lipid contents of microalgae and their proficient productivity makes them good candidates for the production of biodiesels (Chisti 2008b; Gouveia & Oliveira 2009). The fatty acid profile of microalgal oil is also deemed to be of good quality for biodiesel, enabling fuels with decent properties (Deshmukh et al. 2019). Seaweeds are less suitable for biodiesel due to their low lipid yield. But their extremely high polysaccharide contents make them candidates for bioethanol production (Borines et al. 2013; Kim et al. 2015; Sharmila et al. 2021).

## 1.3 Characterisation of algal biomass

Before any given biomass can be used for anything, it is necessary to know what it consists of. Broadly speaking, one could group characterisation methods into one of two different categories depending on the purpose of the characterisation: methods for research purposes, and applied methods. The goal of techniques used in research is to gain a deeper fundamental understanding of a material or organism. In contrast, applied methods are those used for quality assurance in industries or nutrition labelling of foodstuffs for instance. The reason for these two separate categories is that they often differ in the methods used, and the expected degree of accuracy can differ considerably. National and international food administrations such as the US Food and Drug Administration (FDA) and EU regulations both set the acceptable margin of error in food labelling at 20%, which might be acceptable to consumers since that is unlikely to cause any substantial harm, but in research one typically sets the bar for expected accuracy higher. There is of course overlap, however, as some methods used in industrial settings are routinely used in the research lab as well.

### 1.3.1 Traditional chemical methods

*Protein analysis*

In the food industry, total protein contents are typically determined by N-conversion factors; in other words, simple multiplication of nitrogen contents by a predetermined factor. Specific factors have been established for specific foodstuffs such as wheat flour, milk, beef, etc., (Jones 2018) but for grains,

legumes and other plant-based foods the commonly recognised 'universal' N-factor of 6.25 is often used. This leads to a crude approximation of actual protein contents since N-ratios of different grain or legume species can vary substantially (Mariotti et al. 2008), but it is considered accurate enough for food labelling nonetheless. The 6.25 ratio has been consistently shown to lead to overestimation of protein in microalgae (Lourenço et al. 2004) and seaweed (Biancarosa et al. 2016), as the actual N-ratio of certain species of algae can be close to or occasionally even lower than half of this 'universal' ratio. A factor of 5 has been proposed as a general conversion factor for seaweeds (Angell et al. 2015), but while it is closer to the ratio of the average seaweed species, it is still likely best to use species-specific ratios. In research, N-ratios are also quite commonly used but when higher accuracy is demanded amino acid quantification is utilised. Colorimetric protein estimation assays like the Bradford (Bradford 1976), Lowry (Lowry et al. 1951) or Bicinchoninic acid (BCA) (Smith et al. 1985) assays are also commonly used due to their relative ease of use and low cost. Their popularity partially stems from the development of standardised kits by various chemical manufacturers, making them very accessible.

The drawback with colorimetric assays is their sensitivity to interference from compounds, which are commonly present in plants and algae. Polyphenolic compounds like flavonoids (Singh et al. 2020), as well as pigments are some of the substances known to interfere with these assays either through interaction with assay reactants or light absorbance at wavelengths that overlap with those used in the assays. Seaweeds contain phlorotannins and phenolic terpenoids (Barbarino & Lourenço 2005; Cotas et al. 2020), as well as high quantities of salt which can potentially affect the results of such assays (Lucarini & Kilikian 1999).

*Lipid analysis*

Lipids are typically defined as esterified fatty acids, particularly triglycerides, but the definition is often expanded to include other lipid substances like mono- and diglycerides, phospholipids, sterols etc. Both in research and in the food industry, fats are often quantified by extraction using one of several organic solvent protocols, followed by transmethylation into fatty acid methyl esters (FAMEs) for detection using gas chromatography (GC) (ISO protocol: 12966-2:2017). This allows for quantification of all fatty acid species in the sample, and subsequent determination of e.g. the ratio of saturated to unsaturated fats, relevant to nutritional information.

There are also fluorescent dye-based assays like the Nile Red and BODIPY™ assays which selectively bind neutral lipids (Rumin et al. 2015). This is highly useful for screening of lipid contents since the vast majority of fats in plants and algae are neutral lipids, but could still result in some underestimation. A more approximate measurement of fat contents relies on gravimetric estimation, where lipids are extracted using an extraction protocol of choice, all solvents are evaporated, and the mass of the resulting oil is used as an estimate of fat content. This is a rather crude method, however, since it accounts for all lipid-soluble substances present in the sample, usually leading to considerable over-estimation (Rumin et al. 2015).

*Carbohydrate analysis*

Carbohydrates are arguably the most difficult to quantify of the main biomass constituents. In biochemistry, any saccharide, whether a mono- di- oligo- or polysaccharide, is considered a carbohydrate. What is harder to define is what constitutes a 'dietary fibre' in food science. This is not a strict term, but generally refers to any polysaccharides which cannot be digested by humans; any polysaccharide which is not starch in addition to lignin (Dhingra et al. 2012). A major reason why carbohydrate quantification is challenging is that they lack unifying characteristics that would allow them to be estimated using a universal method. Proteins can be relatively rapidly estimated by N-factors, as mentioned above, and quantification of amino acids gives an accurate measurement of protein contents. Total 'lipids' can also be quantified rapidly by gravimetric methods, using fluorescent dyes, or more accurately through GC analysis of fatty acids. For carbohydrates, however, there is no reliable rapid assay. This is because smaller saccharides like glucose and fructose are orders of magnitude smaller than polysaccharides, and different polysaccharides like cellulose, hemicellulose, pectin etc. have radically different chemical properties and molecular weights and react differently to common colorimetric reactants (Mecozzi 2005). They can be quantified in a similar way to amino acids and fatty acids, through hydrolysis and derivatisation followed by GC or HPLC analysis, but this is generally a far more laborious process and is mostly done in research labs. For food labelling purposes, carbohydrates are thus often not directly quantified at all, but rather assumed to be whatever remains after determining protein, fat, ethanol, moisture and ash contents (McCleary & McLoughlin 2021). To determine dietary fibre content, one simply measures starch, usually with enzymatic assays, and assumes the remaining

carbohydrates are fibres (a reasonable assumption since starch is the only digestible polysaccharide).

In summation, the characterisation of proteins, lipids and carbohydrates generally requires extensive use of chemicals, some of which are severely hazardous to human and environmental health. They are also time-consuming. Many of them require highly specialised instrumentation, and the most accurate methods, such as GC or HPLC analysis of amino acids, monosaccharides or fatty acids, often entail lengthy extraction and derivatisation procedures as well as interpretation of chromatographic data. The less labour-intensive protocols on the other hand, such as gravimetric fat estimation or determination of protein content through N-ratios, suffer from a high degree of unreliability. For this reason, there is a need for more rapid, environmentally friendly, non-hazardous and less laborious methods that do not sacrifice accuracy for convenience.

### 1.3.2 Spectral characterisation methods

As an alternative, or complement, to chemical characterisation methods, spectroscopic techniques are frequently used. For example, infrared (IR) spectroscopy is common used for quality assurance in the food industry (Biancolillo et al. 2020; Kucharska-Ambrozej & Karpinska 2020; Tirado-Kulieva et al. 2022). IR spectroscopy techniques include near-infrared spectroscopy (NIR), as well as Fourier transform infrared spectroscopy (FTIR). FTIR can be applied to both the NIR and mid-IR spectrum but is most common for the mid-IR region. When FTIR spectroscopy is mentioned in this thesis, it refers solely to the mid-IR region.

The NIR spectrum consists of overtones and composite vibrations, and contains absorbance bands that are broad and undefined. This is a drawback of the technique, but the benefits of NIR are the small footprint of its instrumentation (often being portable), its ease of use and its low cost due to its tolerance of inexpensive glass and quartz sampling containers compared to generally more expensive FTIR equipment. NIR is also non-destructive, as the sample is not consumed or mixed with other chemicals in the process and this contributes to its popularity.

In this thesis two types of mid-IR FTIR techniques were used: diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS) and attenuated total reflectance (ATR). The mid-IR spectrum contains well-defined spectral bands that are strongly correlated to specific functional

groups. Different biological compounds exhibit characteristic spectral signatures due to these groups, rendering them quantifiable based on the intensity of signature peaks, via the Beer-Lamber law (Mackie et al. 2016). FTIR spectroscopic techniques also benefit from high accuracy and spectral resolution. However, the DRIFTS technique requires solid dilution, which is achieved by mixing the sample with a salt of minimum spectral footprint (typically KBr, as the optical components of the spectrometer are often made of KBr as well) and grinding it to a fine, homogenous powder. This considerably increases the sample preparation and makes the technique destructive and somewhat invasive. For example, the grinding affects the degree of polymerisation, and the generated heat can alter the composition of sensitive samples. The ATR technique does not require mixing with other chemicals and can be used with liquid samples (DRIFTS cannot). However, in the case of solid samples, applied pressure is needed to ensure good contact with the internal reflection element (a crystal made of a material with a very high refractive index, such as ZnSe, Ge or diamond). This pressure can also affect sensitive samples. Additionally, the ATR technique is surface sensitive, as the penetration depth of the evanescent wave is only a few micrometres at most. Moreover, this penetration depth is wavelength-dependent, which results in non-uniform signal strength across the mid-IR spectral range. Since ATR spectra has higher a reflection character, while DRIFTS spectra are more transmission-like, band positions can differ slightly. Taken together, this means that DRIFTS and ATR spectra are not interchangeable or directly comparable in every aspect.

FTIR techniques have been used to accurately identify and quantify contaminants and adulterants in food commodities like olive oil (Rohman & Che Man 2012) and various dairy products (Limm et al. 2018). It has also been used to monitor the presence of fungal contamination and mycotoxins in cereal grains to prevent the spread of disease (Levasseur-Garcia 2018). NIR spectroscopy can be used to characterise paper pulp with similar accuracy to traditional methods (Moral et al. 2015), with a fraction of the preparation time and without destroying the sample.

As with NIR instruments, there are portable FTIR spectrometers available (both in DRIFTS and ATR modes). These have lower resolution than their high-performance desktop counterparts, and handheld DRIFTS devices suffer from distortions caused by specular reflectance, including reststrahlen (residual rays) effects which nullify certain absorbance bands (Arrizabalaga

et al. 2014; Steger et al. 2018). This means that analysis involving these devices requires special considerations when interpreting spectra. However, recent comparative studies have shown handheld ATR devices to perform similarly well for analysis of food items like milk (Gorla et al. 2020) and vegetable oils (Allendorf et al. 2012) as desktop instruments (Fomina et al. 2023). Improvements in handheld FTIR spectrophotometers are likely to make them more prevalent in the future, due to their portability and ease-of-use.

*Spectroscopic characterisation of algae*

Interest in adapting spectroscopic characterisation techniques to algal science has been increasing in the last two decades. Most advances have been made in microalgae, with several studies investigating different methods for using both NIR and FTIR for prediction of lipids (Dean et al. 2010; Laurens & Wolfrum 2010; Feng et al. 2013), protein (Strachan et al. 2007), and even overall biomass composition (Wagner et al. 2010; Mayers et al. 2013). These studies have accomplished this using protein-N ratios, the Lowry assay (or other colirimetric protein assays), gravimetric lipid estimation, and colorimetric carbohydrate estimation for model calibration, and usually in a single species, however.

In seaweed, FTIR was used to determine protein contents by Campbell et al. (2022), and some studies have characterised carbohydrates in seaweed extracts (Chandía 2001; Robic et al. 2008). For the most part, spectroscopic characterisation has not been done directly in seaweed biomass, and in those instances where it has, it mostly involved the use of individual band intensities. The M/G ratio of brown seaweed alginate was previously estimated using the ratios of ATR bands associated with M and G residues (Sakugawa et al. 2004; Gómez-Ordóñez & Rupérez 2011), specifically at 808/787 $cm^{-1}$ and 1030/1080 $cm^{-1}$. Therefore, there is much room for advancement in the field of spectroscopic characterisation of algal biomass.

## 1.4 Objectives

The advances within the algal sciences during recent decades and the expansion of the algal market has improved the methodology of characterisation. Spectral characterisation methods do away with many of the pitfalls of traditional ones that are sensitive to the complex chemical

composition of algal biomass, and that require extensive chemical use and labour to perform. The objective of this thesis is to advance the field of spectral characterisation of algae, both for research purposes and industrial application. The aims of this research can be summarised as:

- Accurately quantifying protein and carbohydrate contents of seaweeds using multivariate spectral analysis.
- Comparing the accuracy of different infrared spectroscopic techniques in characterising seaweed.
- Determining the M/G ratio of alginate directly in brown seaweed using infrared spectroscopy.
- Quantifying fatty acid contents in algae by diffuse reflectance infrared Fourier transform spectroscopy

# 2. Materials and methods

## 2.1 Algal samples

### 2.1.1 Seaweed sampling and pre-processing

Seaweed samples were acquired from the North Atlantic region, primarily from the Norwegian coast and the Faroe Islands, with some additional samples being provided from Ireland and Greenland. Most samples were in a form typical for commercial use, i.e. processed whole thalli hot-air-dried at 30 °C, while others were freeze-dried. Some samples were harvested from natural populations while others were cultivated either directly in the Atlantic Ocean or in cultivation tanks. The sample set contained a variety of seaweed species, primarily *Alaria esculenta*, *Saccharina latissima*, *Laminaria digitata*, and *Palmaria palmata*, with a few individual samples of *Himanthalia elongata*, *Porphyra umbilicalis*, *Mastocarpus stellatus*, *Fucus vesiculosus* and *Pelvetia canaliculata*. The cell walls of seaweeds are extremely recalcitrant, and to ensure thorough extraction of proteins, carbohydrates and fatty acids, all samples were submerged in liquid $N_2$ and milled using a Mixer Mill MM 400 (Retsch GmbH, Haan, Germany) in rounds of 2 min. at 25 Hz, until the whole sample could be passed through a screen with a mesh size of 250 µm. A detailed description of the seaweed samples used in Papers I, II, and IV is provided in Paper I.

### 2.1.2 Microalgal culturing and pre-processing

The samples used in Paper III consisted of microalgal cultures grown under a variety of different culture conditions. Some were cultivated in open raceway ponds using untreated municipal wastewater, while others were

cultivated in closed photobioreactors using either synthetic growth media or sterilised, filtered wastewater. The samples were freeze-dried and milled using a modified protocol developed by Alling et al. (2023) to obtain efficient cell disruption.

## 2.2 Protein quantification

Several common protein determination methods were tested on seaweed samples in Paper I, including colorimetric methods, protein determination by N-ratios, and total amino acid quantification by LC-MS/MS. These were compared to novel spectroscopic methods, described in Section 2.5.

### 2.2.1 Colorimetric assays

Two colorimetric protein determination methods, which are commonly used in algal science, were assayed. These include the Lowry protein assay, and the bicinchoninic acid (BCA). The Bradford assay is another popular colorimetric protein assay but it has been shown to be highly inaccurate in algal samples and was thus not included (Lucarini & Kilikian 1999).

Prior to determination, proteins were precipitated from algal samples using either a trichloroacetic (TCA) protocol (Koontz 2014) or a more advanced protocol using TCA, acetone and 2-Mercaptoethanol (2ME) (Mechin et al. 2007) designed to remove interfering compounds from plant and algal samples. Proteins were resolubilised in the Lowry D reagent since it is compatible with both the Lowry and BCA assays. As a control, a third set of samples was solubilised directly in the Lowry D reagent without undergoing precipitation.

### 2.2.2 Protein estimation by N-ratios

The total N content of seaweed samples was determined by elemental analyser isotope ratio mass spectrometry (EA-IRMS). The ratio of total amino acids quantified by GC-MS to total N was calculated both for individual species and universally for all assayed species. The accuracy of determining protein contents using these ratios was compared to spectroscopic methods.

### 2.2.3 Amino acid quantification

The total amino acid contents were quantified by the Swedish Metabolomics Centre (Umeå, Sweden) using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The sum of amino acids was used to determine protein contents, and this was used as the benchmark for comparing the other protein estimation methods.

## 2.3 Carbohydrate characterisation

### 2.3.1 Crystalline cellulose

While the main constituent of the cell walls of brown seaweed is alginate, crystalline cellulose is also usually present, albeit in lower quantities than higher plants (Kaur et al. 2018). The crystalline cellulose contents of brown seaweeds were quantified in Paper II by use of the Updegraff method (Updegraff 1969) and anthrone assay (Scott & Melvin 1953). The Updegraff reagent was used to remove amorphous polymers and soluble sugars under heat. Crystalline cellulose was hydrolysed to glucose monomers through Saeman hydrolysis using 72 % $H_2SO_4$ and sonication (Saeman 1945). The anthrone assay was then used to quantify the resulting glucose by the addition of the anthrone reagent and heating. The glucose concentration was used to infer crystalline cellulose.

### 2.3.2 Trimethylsilyl-derivatisation and GC/MS

In Paper II, the total carbohydrate contents as well as the quantity of alginate and M/G ratio of alginate were determined through trimethylsilyl (TMS) derivatisation and GC/MS. Complete polysaccharide hydrolysis was performed using 72% $H_2SO_4$, aided by sonication and heating at 100°C for 150 minutes. After neutralisation with $CaCO_3$, undissolved particles were removed from the hydrolysates through two rounds of centrifugation at 18700 ×g for 10 minutes, and the solvent was sparged using $N_2$ gas at 60°C. Methanolysis was performed through the addition of 2 M of HCl/MeOH. The solvent was removed by sparging with $N_2$ at 40°C, and saccharides were washed twice with methanol. TMS-derivatisation was performed using a silylating reagent (85,431; Merck KGaA, Darmstadt, Germany) at 80°C for 20 minutes. The majority of the solvent was sparged with $N_2$, and the derivatised saccharides were dissolved in hexane. After centrifugation at

18700 ×g for 5 minutes to remove large particles, the samples were filtered through glass wool. The filtrate was concentrated down to approximately 100-200 µL through sparging with $N_2$. For quantification of silylated monosaccharides, 0.5 µL was injected into a 7890A/5975C GC (Agilent Technologies, Santa Clara, US). Monosaccharides were separated using a J&W DB-5MS column (30 m length, 0.25 mm ID, 0.25 µm film thickness; Agilent Technologies, Santa Clara, U.S.). MS data was captured using Chemstation Data Analysis v. E.02.00.493 software (Agilent), converted to NetCDF format and exported to RDA v. 2016.09 (Swedish Metabolomics Centre) for processing.
Identification and quantification was carried out using a standard mixture of arabinose, fucose, xylose, glucuronic acid, galacturonic acid, mannose, glucose and galactose (Merck KGaA). These are common monosaccharide species found in both land plants and algae, and to these were added the alginate uronic acid residues mannuronic acid (Merck KGaA) and guluronic acid (MCE, Princeton, NJ, US). Inositol was used as an internal standard.

## 2.4 Fatty acid quantification

### 2.4.1 Extraction, purification and transmethylation of fatty acids

In Papers III and IV, lipids were extracted from microalgae and seaweed, respectively. A modified Folch extraction protocol (Folch *et al.* 1957) was used, developed by Axelsson and Gentili (2014), using a chloroform/methanol solvent system. Due to the considerably lower fatty acid contents expected in seaweed, 40 mg of dry sample was used for extraction from seaweeds, while 20 mg was used for extraction from microalgae. As the extraction method produces a crude lipid extract containing many lipid-soluble compounds aside from fatty acids, extracts were purified using solid-phase extraction (SPE) according to a protocol described by Lage and Gentili (2018). Crude lipids were bound to Hypersep SI SPE columns (Thermo Scientific, Waltham, Massachusetts, US) which had been equilibrated with hexane. Elution of neutral lipids was carried out through vacuum suction using 80:20:1 hexane:diethyl ether:acetic acid (v/v/v) as a solvent mixture, followed by elution of polar lipids by 2:2:1 MeOH:acetone:hexane (v/v/v). Purified fatty acids were transmethylated to fatty acid methyl esters (FAMEs), for analysis using gas chromatography

flame ionisation detection (GC-FID). Transmethylation was performed through incubation for 2 h at 80°C in the presence of an excess of MeOH used as methylation reagent, and 1% $H_2SO_4$ used as transmethylating catalyst.

### 2.4.2 Gas chromatography flame ionisation detection of fatty acids

FAME samples were injected at a volume of 1 µL in 1:10 split mode into a Trace 1310 gas chromatograph (Thermo Scientific), and FAMEs were separated using a FAMEWAX column (30 m, 0.32 mm ID, 0.25 µm Crossbond polyethylene glycol stationary phase, Restek, Bellefonte, Pennsylvania, US). FAMEs were identified and quantified using a marine FAME standard mixture (35066, Restek). Data capture and processing were done using Chromeleon 7 software (Thermo Scientific).

## 2.5 Spectroscopic analysis

### 2.5.1 Spectroscopic methods

Three different infrared spectroscopic techniques are featured in this thesis: near-infrared (NIR), attenuated total reflectance Fourier transform spectroscopy (ATR) and diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS). All three techniques have their advantages and disadvantages, and the accuracy of NIR and DRIFS for seaweed protein quantification is compared in Paper I, while all three are compared during the characterisation of seaweed carbohydrates in Paper II.

*Near-infrared spectroscopy*

In Paper I, NIR spectroscopy was performed on finely milled seaweed powders using a LabSpec ASD NIR spectrophotometer using a contact probe (Portable Analytical Solutions, Copacabana, NSW, Australia). Standard normal variate (SNV) normalisation was performed on a spectral range of 1000-2500 nm, and averaged diffuse reflectance spectra from three measurements were used for further analysis. Spectral processing was performed with Evince (Prediktera AB, Umeå, Sweden).

*Diffuse reflectance infrared Fourier transform spectroscopy*

The DRIFTS method was used throughout the thesis, in Papers I-IV, as it has the highest resolution and sensitivity of the techniques and was expected to produce the best results. Analysis was performed as described by Gorzsas and Sundberg (2014). Seaweed samples were ground with FTIR spectroscopy grade KBr in an approximate 1:10 ratio (seaweed:KBr) by volume. An IFS 66 v/S vacuum spectrometer (Bruker Optik GmbH, Ettlingen, Germany) was used to record spectra in the 400-4000 $cm^{-1}$ region at a resolution of 4 $cm^{-1}$. 128 scans were co-added per sample and pure KBr was used as blank, which was subtracted from the sample spectra as background. Data collection was performed using OPUS (version 5, Bruker Optik GmbH), and the spectra were further processed using the MCR-ALS GUI available at the Vibrational Spectroscopy Core Facility, Department of Chemistry, Umeå University (v4c, https://www.umu.se/en/research/infrastructure/visp/downloads/) with MATLAB (version R2017b, MathWorks, Natick, MA, USA). The spectral regions outside the $800^{-1}800$ $cm^{-1}$ fingerprint region were cut, due to the fingerprint region's numerous absorbance bands associated with biological components like proteins, carbohydrates and fatty acids (Schmitt & Flemming 1998). Baseline correction was performed using asymmetric least squares (AsLS), followed by total area normalisation to render the spectra comparable.

*Attenuated total reflectance spectroscopy*

Since ATR spectra suffer from decreased spectral intensity at higher wavenumbers, lower wavenumbers are emphasised. Thus, ATR was used in Paper II for carbohydrate analysis, since the primary bands associated with saccharides are in the 900-1200 $cm^{-1}$ region, as opposed to proteins and fatty acids which have strong absorbance bands between 1400 and 1800 $cm^{-1}$ (Schmitt & Flemming 1998). ATR spectra were recorded using a Vertex 80v FTIR vacuum spectrometer (Bruker Optik GmbH) at the same resolution as the DRIFTS spectra and baseline correction was performed in OPUS (version 7, Bruker Optik GmbH) using a 64-point rubberband, excluding $CO_2$ bands. As with the DIFTS spectra, the spectral regions beyond the 800-1800 $cm^{-1}$ fingerprint region were omitted using the MCR-ALS GUI, with subsequent total area normalisation.

### 2.5.2 Multivariate analysis

While FTIR spectra benefit from quite distinct absorbance peaks strongly correlating to specific substances, simply quantifying the intensity of at a given position in the spectra is not necessarily a reliable way to quantify the corresponding analyte. Peaks can overlap, and shift in position and alter shape depending on the chemical environment in which the analyte is present, or whether it is forming ionic bonds with other compounds or metal ions (Sakugawa et al. 2004). The use of multivariate statistical methods can overcome this issue by taking into account the whole spectrum. A multivariate prediction model can account for contributions from irrelevant but overlapping absorbance bands through negative correlation, and even shifts in band position by not relying on one specific absorbance maximum.

When deciding what multivariate prediction technique to use for spectral analysis in this thesis, a few factors had to be considered. The complexity of the sample being analysed, in terms of the number of constituting chemical components, was one of the main factors. Another is the fact that it is not possible to know all expected components in the samples and a lack of access to pure spectra of these components (Beebe et al. 1998). When predicting specific compounds in a highly complex mixture like algal biomass, it is necessary to use inverse least squares (ILS) techniques rather than classical least squares (CLS), as ILS allows for the construction of quantitative models without forehand knowledge of components in the sample (Strachan et al. 2007). Common ILS techniques include multiple linear regression (MLR), principal component regression (PCR) and partial least squares regression (PLSR). In samples with a small number of variables, MLR can be used, but it becomes unreliable when a large number of wavenumbers are used for calibration, especially when the wavenumbers are correlated to each other. For complex samples, PCR and PLSR are more appropriate, since these can effectively reduce the number of variables to a small number of so-called ‘components’ or ‘latent variables’ which are combinations of many wavenumbers (Strachan et al. 2007). PCR and PLSR calculate these components in a similar fashion, but PCR only takes into account the maximum spectral variation whereas PLSR takes into account the covariance between spectral data and the concentration of the analyte in question. ILS techniques like PCR and PLSR have the strong advantage of diminishing the contribution from spectral regions, which are not relevant to the predicted

analyte. Provided that the calibration set contains the same redundant compounds as future samples, their spectral signature will be ignored.

PLSR was deemed to be the most fitting technique for the prediction of biochemical components in algal biomass, due to the complexity of the samples and the ability of PLSR to correlate spectral and compositional variance.

*Partial least squares regression*

Multivariate statistical analysis by PLSR was used to predict the protein quantities of red and brown seaweeds in paper I, carbohydrate and alginate quantities as well as the alginate M/G ratio of brown seaweed in paper II and total fatty acid quantities in microalgae in paper III. Prediction models were calibrated approximately 75% of the available samples, with the remaining 25% being used as validation samples to test the models. RStudio Desktop software (RStudio, Boston, Massachusetts, US) was used for PLSR analysis, using scripts from the PLS package (v. 2.8–0, https://CRAN.R-project.org/package=pls). Leave-one-out cross-validation was used to estimate the optimal number of components, based on the lowest root mean square error (RMSE) of cross-validation. For the evaluation of predictive error, the root mean square error of prediction (RMSEP) and relative error (RE) were used, and the determination coefficient $R^2$ of predicted samples was used as a measure of predictive reliability.

*Principal component analysis*

Principal component analysis (PCA) was used in Paper III to assess whether variables like culture duration, temperature, growth medium or species would affect the spectral profile of microalgae, and subsequent fatty acid prediction using spectroscopy. PCA was performed with RStudio Desktop software (RStudio), with scripts from the FactoMineR package (v. 2.9, https://cran.r-project.org/web/packages/FactoMineR/index.html), and scatter plots were visualised using factoextra (v. 1.0.7, https://cran.r-project.org/web/packages/factoextra/index.html).

# 3. Results and discussion

## 3.1 Comparison of common protein quantification methods to infrared spectroscopic techniques – Paper I

### 3.1.1 Discrepancies in colorimetric protein assays

Both the Lowry and BCA assays severely overestimated proteins in brown seaweed, by a factor of 1.2-2.1 times depending on species and method (Figure 1). The exception was *S. latissima* which was slightly underestimated by the BCA assay. Protein estimation of the two red seaweed species was more accurate, however. The two precipitation methods did not reliably improve assay accuracy, although the TCA-acetone-2ME method did improve estimation in the red seaweeds somewhat when using the Lowry assay. Both precipitation protocols caused underestimation in the red seaweeds when using the BCA assay. These assays were thus highly unreliable for protein estimation in brown seaweed, and the accuracy could not be improved by common precipitation protocols. Red seaweeds appeared more amenable to colorimetric protein assays, but further experiments with more species would be needed to confirm. The tendency to over- or underestimate protein contents in a wide variety of sample types has been documented before (Sapan et al. 1999), and while precipitation protocols have been developed in an attempt to remedy this problem (Berges et al. 1993; Slocombe et al. 2013; Singh et al. 2020), this study further demonstrates this issue.

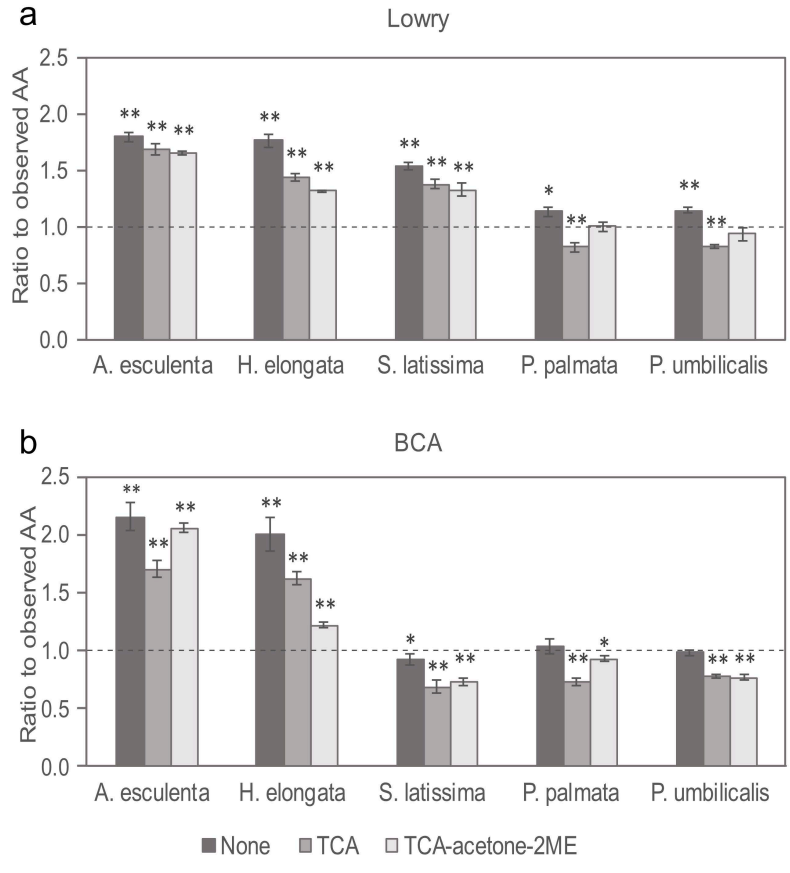


Figure 1. Ratios of total protein estimated by colorimetric assays compared to total amino acids (AA). Protein contents that were estimated by the a) Lowry and b) BCA assays, using different precipitation methods in five different seaweed species. The Y-axis indicates the ratio of estimated protein to AA content, and thedashed line indicates a 1:1 ratio. Error bars indicate the standard deviation of triplicate analyses, Asterisks show significant deviation from AA contents. * = $p > 0.05$, ** = $p > 0.01$.

### 3.1.2 Protein prediction by nitrogen ratios

Nitrogen-ratios for protein estimation in five seaweed species was determined (Table 1), ranging from 3.46 (±0.42) in *H. elongata* to 4.49 (±0.23) in *A. esculenta*. Through one-way ANOVA it was shown that there was significant statistical difference between at least two of the species ($F = 10.8394$, $p = 6.45 \cdot 10^{-6}$), and pairwise t-tests showed significant difference between *A. esculenta* and *S. latissima*, and also *A. esculenta* and *L. digitata*. Use of a singular N-conversion factor to estimate protein in all these species would thus likely be inaccurate, but a 'universal' ratio was calculated to be 4.14. This is considerably lower than the ratio of 5 suggested by Angell et al. (2015), and indeed none of the seaweeds included in Paper I had an N-ratio ≥5.

Table 1. Species-specific and universal N-conversion factors

| **Species** | **N-factor** |
|---|---|
| *A. esculenta* | 4.49 ± 0.23 |
| *H. elongata* | 3.46 ± 0.42 |
| *L. digitata* | 3.77 ± 0.46 |
| *S. latissima* | 3.99 ± 0.27 |
| *P. palmata* | 4.25 ± 0.30 |
| Universal | 4.14 ± 0.43 |

### 3.1.3 Univariate and multivariate spectral protein prediction

All spectroscopic prediction methods were able to predict protein contents in seaweeds, with the multivariate ones being considerably more accurate (Table 2). Univariate spectral prediction of protein contents, with use of the height of the Amide II peak (1540 $cm^{-1}$) resulting in decent predictive accuracy (RMSEP = 1.5095, RE = 0.0261, $R^2 = 0.8631$), and the integral of the same performing slightly better (RMSEP = 1.2376, RE = 0.0244, $R^2 = 0.8752$). This shows that simple linear regression of the Amide II peak is capable of producing at least a rough estimation of protein contents in seaweed in a very rapid manner. The two multivariate methods, NIR and DRIFTS spectroscopy coupled with PLSR, exhibited comparable performance. NIR had slightly lower predictive error (RMSEP = 1.1939, RE = 0.0095) than DRIFTS (RMSEP = 1.2376, RE = 0.0100), but DRIFTS had higher $R^2$ (0.9540) than NIR (0.9258). It must be noted that the DRIFTS

model used 7 components while the NIR one used 4, which is a significant difference. These were determined to be optimal component numbers for the respective models through leave-one-out CV, but it is possible that the DRIFTS model needed more components due to extensive overlap between the Amide I peak and the nearby alginate -C=O peak, an issue that was not apparent in the NIR spectrum. It is highly likely that better accuracy could be obtained with DRIFTS if separate prediction models were made using brown and red seaweed, since alginate is only present in brown seaweed. Regardless, it was clear that the PLSR methods outperformed univariate spectral methods, as well as protein prediction by N-ratios, showing the predictive power of multivariate spectral analysis.

Table 2. Linear and partial least squares regression prediction results

| | RMSEC | RMSEP | RE | $R^2$ |
|---|---|---|---|---|
| **DRIFTS** | | | | |
| Amide II Height | 1.4448 | 1.5095 | 0.0261 | 0.8631 |
| Amide II Integral | 1.3792 | 1.4912 | 0.0244 | 0.8752 |
| PLSR 7 comp. | 0.9220 | 1.2376 | 0.0100 | 0.9540 |
| **NIR** | | | | |
| PLSR 5 comp. | 1.2614 | 1.1939 | 0.0095 | 0.9258 |
| **N-ratio** | | | | |
| 4.14 ratio | N/A | 1.2733 | 0.0211 | 0.9176 |

## 3.2 Spectral characterisation of brown seaweed saccharides – Paper II

### 3.2.1 Carbohydrate composition of North Atlantic brown seaweed

All brown seaweeds contained a small amount of crystalline cellulose, between 2.1 and 4.7 %DW (Table 3). This is negligible compared to plants that are industrially exploited for cellulose, and use of this seaweed cellulose would be hard to motivate economically (Kaur et al. 2018). The seaweeds contained between 19.1 and 60.3 % carbohydrates by DW as determined by GC/MS analysis of hydrolysed monosaccharides. The vast majority of carbohydrates consisted of alginate, since the alginate residues M and G accounted for 73.8 % of total monosaccharides on average (Figure 2). M was

by far the most abundant monosaccharide, totalling 56 % of total monosaccharides on average.

Alginate contents were inferred from summing up of M and G residues, showing that the seaweeds contained between 12.2 to 45.1 %DW. Curiously, the lowest contents were in *S. latissima* from the Faroe Islands, and the highest were in the same species from Tromsø, showing a remarkable difference within this species. These samples also contained the lowest and highest M/G ratios at 2.5 and 7.0, respectively. Overall, M/G ratios were high, as most recorded ratios are between 0.8 and 2.5, but similarly high ratios do also occur (Jensen et al. 2015; Jiao et al. 2019).

These seaweeds contained abundant alginate, making them a potential feedstock for alginate production. The high M/G means that a gel produced from this alginate would be flexible, and not useful for products requiring structural rigidity. Soft biofilms such as those used in food preservation (Tavassoli-Kafrani et al. 2016) would be a more appropriate use for these alginates, as well as for use as a general gelling agent.

Table 3. Carbohydrate contents of analysed brown seaweeds

| **Species** | **Region** | **Total carbohydrates** | **Cellulose** | **Alginate** | **M/G ratio** |
|---|---|---|---|---|---|
| *A. esculenta* | Bodø | 33.4 ± 3.8 | 3.6 ± 0.1 | 27.3 ± 2.2 | 5.3 ± 0.8 |
| | Tromsø | 37.3 ± 2.5 | 2.1 ± 0.4 | 29.8 ± 1.8 | 3.9 ± 0.6 |
| | Faroes | 37.3 ± 8.5 | 2.8 ± 0.6 | 26.4 ± 5.4 | 2.4 ± 0.5 |
| | Ireland | 59.1 ± 2.2 | 4.6 ± 1.3 | 43.8 ± 5.5 | 3.9 ± 1.2 |
| | Greenland | 31.9 ± 4.9 | 4.7 ± 0.2 | 20.9 ± 3.8 | 3.8 ± 0.4 |
| *H. elongata* | Faroes | 30.6 ± 3.1 | 2.3 ± 0.2 | 21.7 ± 3.0 | 2.5 ± 0.1 |
| *L. digitata* | Bodø | 51.3 ± 5.8 | 4.5 ± 0.8 | 42.6 ± 5.5 | 3.0 ± 0.1 |
| | Tromsø | 40.3 ± 6.2 | 4.2 ± 0.3 | 30.1 ± 5.6 | 2.9 ± 0.5 |
| | Faroes | 48.0 ± 1.7 | 5.6 ± 1.0 | 34.0 ± 1.2 | 4.7 ± 0.3 |
| *S. latissima* | Bodø | 39.9 ± 0.7 | 3.5 ± 0.7 | 32.4 ± 0.1 | 4.9 ± 0.6 |
| | Tromsø | 60.3 ± 7.9 | 4.7 ± 0.9 | 45.1 ± 7.0 | 7.0 ± 1.7 |
| | Faroes | 19.1 ± 3.0 | 2.9 ± 2.3 | 12.2 ± 1.6 | 2.5 ± 0.5 |

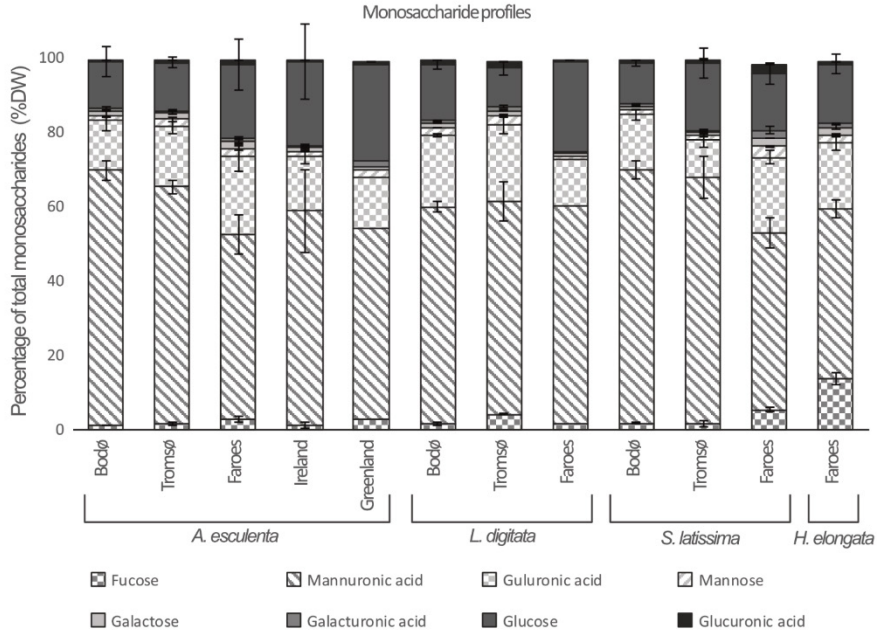


Figure 1. Distribution of major monosaccharide species in brown seaweed. Monosaccharide profiles were measured by GC/MS, and are presented in terms of percentage of total monosaccharides. Error bars indicate standard deviation.

### 3.2.2 Spectral characterisation of carbohydrates

Crystalline cellulose failed to be predicted by any of the three spectroscopic methods (Table 4). This is likely due to cellulose being one of several different glucose-based polymers, therefore lacking a distinct spectral signature differentiating it from the others. Another major reason is that the cellulose contents were very low, and thus the cellulose bands were obscured by the far more distinct alginate bands.

Total carbohydrates could be predicted, but not with a high degree of accuracy. Only DRIFTS resulted in an $R^2$ value <0.8, but this method also had the highest error (RMSEP = 5.72, RE = 0.11). This performance is passable however for estimating carbohydrate contents. Alginate contents were most accurately predicted using DRIFTS ($R^2$ = 0.82, RMSEP = 4.89, RE = 0.23) while ATR was the only method to succeed at predicting M/G ratios ($R^2$ = 0.86, RMSEP = 0.65, RE = 0.14). This is interesting, since it appears that ATR was better at correlating bands associated with different

M/G ratios while DRIFTS was better at correlating spectral bands to alginate contents. In Figure 3, it is demonstrated that ATR has a far more linear correlation between M/G ratios and specific peaks at 880-890, 930, 1025, 1060 and 1080 $cm^{-1}$ than DRIFTS (Figure 3b), as well as apparent negative correlation to a band at 1415 $cm^{-1}$ and, curiously, the alginate C=O band at 1615 $cm^{-1}$. Most of these bands featured in the PLS regression coefficients for ATR as well (Figure 4), meaning that the prediction model relied on the intensities and positions of these bands for predicting M/G ratio. The strong correlation to the double peak at 880-890 $cm^{-1}$ is a particularly good indication that the model is using relevant spectral data, since these peaks are associated with β-anomers like M, meaning that they should diminish in intensity with higher G-contents, since these are α-anomers (Hong et al. 2021). The ATR model also showed strong negative correlation to a band around 1415 $cm^{-1}$, known to correlate to metal-carboxylate interactions (Palacios et al. 2004). This is reasonable since G-blocks form ion-binding sites whereas M-blocks do not, meaning that this band should be weaker in samples with high M/G ratios.

Table 4. Prediction results from PLSR modelling of carbohydrate contents using different infrared spectroscopies

| **Predicted quantity** | **Spectra** | **Comp. number** | **RMSEP** | **RE** | **$R^2$** |
|---|---|---|---|---|---|
| Total carbohydrates | NIR | 5 | 5.20 | 0.11 | 0.79 |
| | DRIFTS | 5 | 5.72 | 0.11 | 0.81 |
| | ATR | 5 | 5.21 | 0.11 | 0.79 |
| Cellulose | NIR | 3 | 1.16 | 0.29 | 0.24 |
| | DRIFTS | 2 | 1.14 | 0.26 | 0.21 |
| | ATR | 3 | 1.16 | 0.26 | 0.26 |
| Alginate | NIR | 3 | 6.75 | 0.19 | 0.52 |
| | DRIFTS | 6 | 4.69 | 0.12 | 0.82 |
| | ATR | 6 | 5.16 | 0.14 | 0.70 |
| M/G ratio | NIR | 5 | 0.70 | 0.19 | 0.68 |
| | DRIFTS | 5 | 0.81 | 0.16 | 0.74 |
| | ATR | 5 | 0.65 | 0.14 | 0.86 |

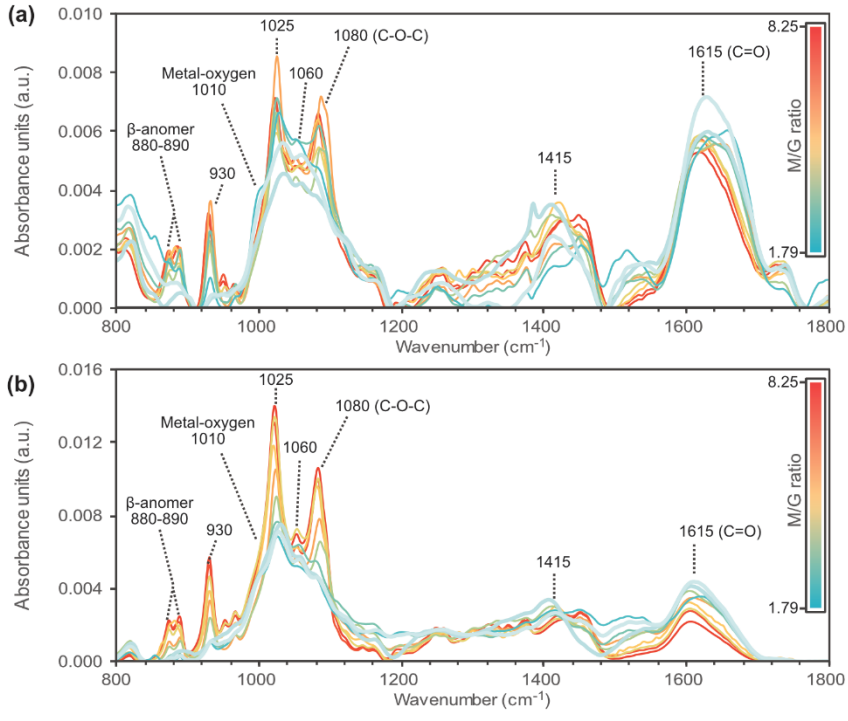


Figure 2. The effect of differing M/G ratios on spectral FTIR absorbance in brown seaweed. (a) DRIFTS spectra and (b) ATR spectra. Potential absorbance bands which are relevant to determining M/G ratio or that are known to be associated with alginate are labelled. The heatmap indicates the M/G ratio of the sample, with red being the highest and blue being the lowest.

This study showed that infrared spectroscopy coupled with PLSR analysis can be used to characterise alginate directly in dry seaweed biomass, omitting the need for alginate-extraction and NMR analysis, which is the most common method.

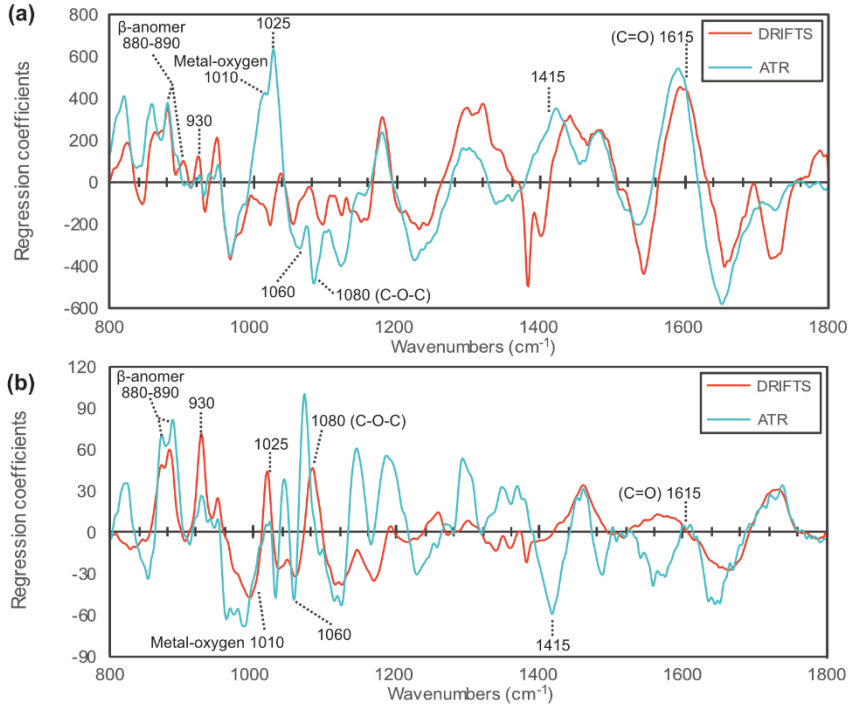


Figure 3. Regression coefficients from PLSR prediction modelling. (a) Total alginate and (b) M/G ratios. Positive values imply positive correlation and negative values imply negative correlation to alginate contents or M/G ratio.

## 3.3 Spectral analysis of algal fatty acids

### 3.3.1 Estimation of fatty acids in microalgal mono- and polycultures by DRIFTS – Paper III

*Principal component analysis*

PCAs was used to determine whether culture characteristics such as culture duration, temperature, medium, or species affected the spectral profile of the monoculture samples. The same was done for the polyculture samples, but the only variable that was investigated was the month and year of harvest.

The PCA indicated no noticeable influence from culture duration, temperature or growth medium on the spectra of the monocultures, while some clustering occurred in relation to the species (Figure 5), with

*Scenedesmus obliquus* and *Chlorella vulgaris* samples grouping almost entirely separately from each other. This can likely be explained by differences in fatty acid and carbohydrate contents, since the carbohydrate-associated bands between 900 and 1200 $cm^{-1}$ were of considerably higher intensity in *S. obliquus* samples than in *C. vulgaris*, while the fatty acid band at 1740 $cm^{-1}$ was of lower intensity.

The polyculture set showed no clustering depending on month of harvest, and the 2017 and 2019 seasons did not group separately from each other. Despite being inoculated with different species and being grown two years apart, their spectral profiles looked almost identical, showing a high degree of stability in the composition of microalgal cultures grown in open raceway ponds.

Table 5. Microalgal fatty acid prediction results using partial least squares regression

| Culture type | Comp. number | RMSEP | RE | $R^2$ |
|---|---|---|---|---|
| Monocultures | 5 | 1.26 | 0.02 | 0.94 |
| Polycultures | 5 | 0.54 | 0.25 | 0.05 |
| Combined | 6 | 1.38 | 0.14 | 0.92 |

*PLSR prediction of fatty acid contents*

PLSR was utilised to predict fatty acid contents in microalgal samples, with assessment of the monoculture and polyculture datasets being performed separately, as well as with a combined predictive model. The monoculture model performed well, with an RMSEP = 1.2615, RE = 0.0231 and $R^2$ >0.93 (Table 5). This is a low predictive error, given the large span of fatty acid contents in the validation set (1.91-15.23 %DW). The regression coefficient also indicated strong correlation to relevant spectral bands like the 1740 $cm^{-1}$ fatty acid carbonyl peak and other fatty acid-associated peaks at 1470 and 990 $cm^{-1}$ (Rohman & Che Man 2012).

Conversely, fatty acids in the polyculture data set could not be predicted using the polyculture model, with considerable noise being incorporated in the regression coefficient even with only two components. This was caused by the narrow range of fatty acid concentrations in the polyculture data set (0.76-4.21 %DW), making the calibration of the model unreliable. The monoculture set contained a far broader variety of fatty acid concentrations (1.32-22.48 %DW), which allows for far more robust calibration.

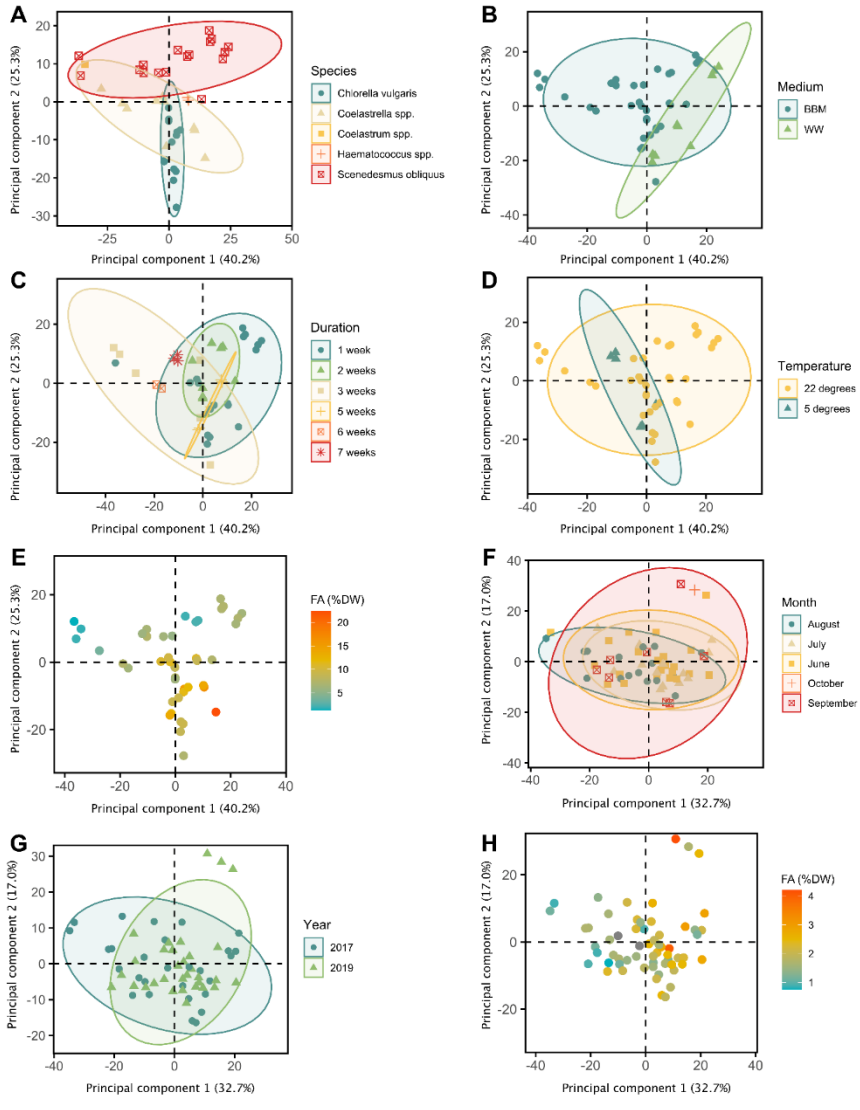


Figure 4. PCA scatterplots for spectral data of microalgal monocultures (A-E) and polycultures (F-H) datasets. The ellipses show the 90% confidence interval of the indicated category.

Both the monoculture and polyculture validation samples could be accurately predicted by the combined model, however, resulting in an RMSEP = 1.3772, RE = 0.13969, and $R^2$ = 0.9212. This performance was almost

comparable to that of the monoculture model on its own, showing that the spectral profile of green algal cultures is not markedly affected by species composition or culture medium, even though the polycultures were grown in raw, unfiltered municipal wastewater. It must be noted that only green microalgae were included in this study, and it is likely that the spectral profiles would differ noticeably in other major groupings of microalgae such as diatoms, a difference that could be seen in Paper I between the red and brown seaweeds. Due to fundamental differences in cell wall composition and the complex spectral features of polysaccharides, there would probably be large differences in the aforementioned 900-1200 $cm^{-1}$ region at least. The 1740 $cm^{-1}$ band associated with fatty acid esters does seem to not commonly overlap with bands from other compound classes in algae, however, so it is unclear if this would affect prediction. In Paper I, protein could be successfully predicted in both red and brown seaweeds despite considerable overlap between protein and alginate bands in the brown seaweeds, so it seems plausible that a sufficiently broad calibration model consisting of microalgae from diverse phyla would be able to perform well in all major microalgal groupings.

### 3.3.2 Fatty acid profiling and spectral analysis of North Atlantic seaweed – Paper IV

*Fatty acid profile of North Atlantic seaweed*

All the seaweed species investigated in Paper IV contained relatively low amounts of saturated fatty acids (SFA) (average = 41.22 % of total), and a large proportion of polyunsaturated fatty acids (PUFA) (average = 43.20 %). Of these, a decent quantity was the long-chain omega-3 fatty acid EPA (average = 9.98 %) (Figure 6), considered essential for human health (Simopoulos 2008). DHA was not detected in any of the samples. Further, the medium-chain omega-3 fatty acid SDA was present in some species (average = 11.68 %). SDA can be converted to EPA with greater efficiency than ALA can (Harris 2012), meaning that it also contributes to the dietary intake of long-chain omega-3 fatty acids. ALA was present too, in somewhat lower quantities (average = 5.99 %), as well as the long-chain arachidonic acid (ARA), taking the total proportion of omega-3 fatty acids in these seaweeds to 27.66 % of the total. The high levels of different omega-3 fatty acids in these seaweeds thus indicate that they could be a good omega-3 food supplement.

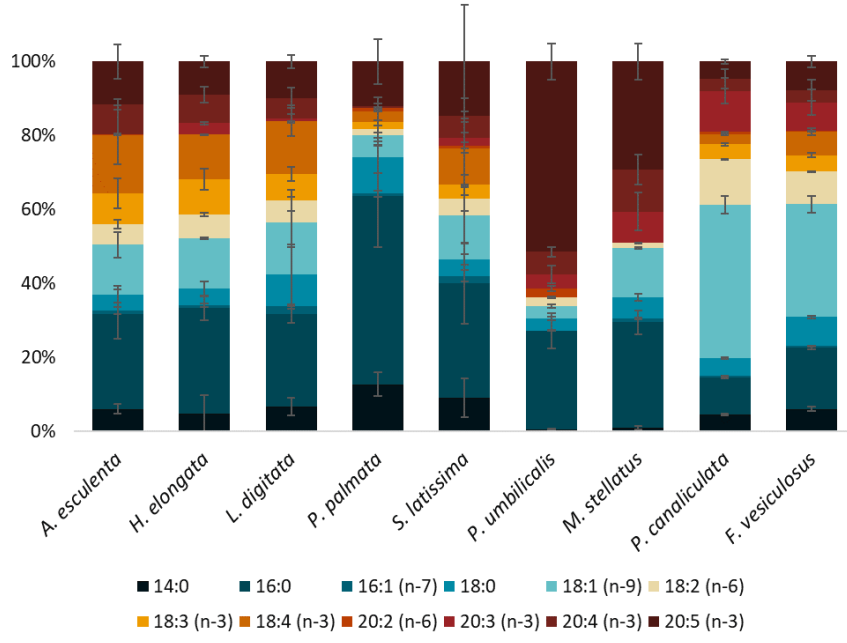


Figure 5. Fatty acid profiles of North Atlantic seaweeds, determined by gas chromatography flame ionisation detection. The y-axis indicates the percentage of total fatty acids. 3 to 12 biological replicates were analysed per species, with three technical replicates each. Error bars indicate standard deviation.

*Spectral analysis of North Atlantic seaweed*

DRIFTS in combination with PLSR was unable to predict fatty acid contents in a combined model consisting of brown and red seaweed samples (table 6), resulting in an RMSEP = 1.1502, RE = 1.1523 and $R^2$ = 0.6877. This was likely due to large spectral differences between the samples combined with the low amount of fatty acids in many seaweeds. In Paper I, protein could be determined accurately in both red and brown seaweeds using the same models, but that can be explained by the far greater diversity of protein concentrations.

Excluding red seaweeds and only using brown seaweed samples for calibration and validation, the model performed considerably better with an RMSEP = 0.7668, RE = 0.1591 and $R^2$ = 0.8871. It must be noted however that this largely depended on a few individual samples of *P. canaliculata* and *F. vesiculosus* with high fatty acid contents, making this model somewhat unreliable since the majority of samples were in the 0.14-3.04 %DW range

while the *P. canaliculata* and *F. vesiculosus* samples contained between 6.08 and 9.16 %DW. Including samples with more intermediate concentrations would likely improve the model, but with the current data that could not be definitely stated. Of note is however that the calibration model only required 4 components, and the regression coefficient clearly indicates the 1740 $cm^{-1}$ band as the most important, meaning that relevant spectral information was discerned by the PLS algorithm.

A tentative attempt at predicting more detailed information like the omega-3 and total PUFA content was also tested, but no model could be generated from this, likely due to such fine spectral details being lost in the biomass spectra (data not shown). Specific fatty acid types like omega-3 and trans fats do have characteristic bands associated with them (Hernández-Martínez et al. 2010), but these can more easily be discerned in a lipid extract rather than directly in biomass.

Judging by the difficulty in measuring fatty acids in seaweeds with low fatty acid contents, this method may be inappropriate for species which do not accumulate larger amounts of lipids. It might be best applied to more oleaginous species, like those in the aforementioned *Fucus* and *Pelvetia* genera.

# 4. Conclusions

This thesis demonstrated the accuracy of using infrared spectroscopy coupled with partial least squares regression to predict biomass composition in algae. Chief findings of this research include:

- Protein can be estimated more accurately and with lower time-investment using infrared spectroscopy, compared to using N-ratios or colorimetric assays like the Lowry and BCA assays.

- The alginate quantity as well as the M/G ratio in brown seaweed can be determined directly in dry seaweed biomass using FTIR techniques

- Infrared spectroscopy is insensitive to the growth temperature, medium, and growth cycle phase as well as species composition of green microalgal cultures when determining fatty acid contents in harvested biomass, making it potentially universally applicable on microalgal cultures.

The broad adoption of spectroscopic methods would considerably cut down on labour time required by most traditional methods. Additionally, it would remove the extensive need for chemicals like solvents, reagents, acids, etc., reducing both hazards to workers and environmental impact.

# 5. Future research

The next step in spectral characterisation of algae is to investigate the use of handheld or portable devices, to enable field studies and on-site quality determination. The development of methods for estimating the biomass composition of seaweed directly on the farm, or microalgal cultures in a pond or photobioreactor, would assist in determining the correct harvest timing or the suitability of algal biomass for further processing. With the numerous studies showing the efficacy of spectral analysis of grains and other agricultural commodities, it is only a matter of time before these methods are adapted to algae. For research purposes, infrared microspectroscopy should also be further developed, as this allows for detailed studies of carbon partitioning in cells and tissues by coupling microscopy and spectroscopy.

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# Popular science summary

Algae are a renewable source of a wide variety of different biomaterials, biofuels, food additives, pharmaceuticals, plant biostimulants and other societally and economically important substances. Interest in extracting these substances has been increasing over the years, especially with the desire to transition to a sustainable economy. Algae themselves are a potential food source that is presently underutilised, and algal plant biostimulants can be used to improve crop yields, contributing to countering rising food insecurity.

To determine the usefulness of algal biomass, it is necessary to characterise its contents. The complex chemical composition of algae can complicate characterisation, however, and a method that can identify and quantify these compounds in a rapid and accurate way would be greatly beneficial. Spectroscopic techniques have been developed for characterisation of other types of biological materials, based on the principle that specific compounds have characteristic spectral bands which can be used to quantify these compounds. This thesis focuses on advancing spectral characterisation of seaweed and microalgae.

To achieve this, seaweeds and microalgae were thoroughly analysed by some of the most accurate traditional chemical methods, through the breakdown of proteins and carbohydrates into amino acids and monosaccharides, as well as extraction of fatty acids. These compounds were analysed using different types of chromatography techniques, and the results were used to calibrate multivariate spectroscopic prediction models.

Results showed that seaweed proteins and microalgal fatty acids could be determined with high accuracy. Spectral protein prediction outperformed other common methods, while being quicker and requiring no or very little chemicals. Carbohydrates were more difficult to predict spectroscopically,

likely due to carbohydrates having more complex and overlapping spectral signatures, while proteins and fatty acids have a handful of highly distinct spectral bands, at least in FTIR. While microalgal fatty acids could be predicted with relative ease, the seaweeds used in the thesis contained too low fatty acid contents to accurately calibrate a prediction model.

Overall, the potential for using spectroscopic characterisation methods in algae was demonstrated, with these techniques being able to determine the quantities of the most common biochemical compounds present in algal biomass with high accuracy.

# Populärvetenskaplig sammanfattning

Alger är en förnybar källa till en mängd olika biomaterial, biobränslen, livsmedelstillsatser, läkemedel, biostimulanter för växter och andra samhälleligt och ekonomiskt betydelsefulla ämnen. Intresset för att utvinna dessa ämnen har ökat under årens lopp, särskilt med tanke på önskan att övergå till en hållbar ekonomi. Alger är i sig en potentiell livsmedelsråvara som för närvarande är underutnyttjad, och biostimulanter från alger kan användas för att förbättra jordbruksskördar, vilket bidrar till att motverka den alltmer osäkra livsmedelsförsörjningen.

För att kunna avgöra hur användbar algbiomassan är, är det nödvändigt att karaktärisera dess innehåll. Algernas komplexa kemiska sammansättning kan dock försvåra karakteriseringen, och en metod som kan identifiera och kvantifiera dessa ämnen på ett snabbt och precist sätt skulle vara till stor nytta. Spektroskopiska tekniker har utvecklats för karakterisering av andra typer av biologiska material, baserat på principen att specifika substanser har karaktäristiska spektrala band som kan användas för att kvantifiera dessa substanser. Denna avhandling fokuserar på att utveckla spektral karaktärisering av tång och mikroalger.

För att uppnå detta analyserades tång och mikroalger grundligt med några av de mest exakta traditionella kemiska metoderna, genom nedbrytning av proteiner och kolhydrater till aminosyror och monosackarider, samt extraktion av fettsyror. Dessa ämnen analyserades med olika typer av kromatografitekniker, och resultaten användes för att kalibrera multivariata spektroskopiska prediktionsmodeller.

Resultaten visade att tångproteiner och fettsyror från mikroalger kunde bestämmas med hög noggrannhet. Spektral proteinprediktion överträffade andra vanliga metoder, samtidigt som den var snabbare och inte krävde några eller mycket få kemikalier. Kolhydrater var svårare att förutsäga

spektroskopiskt, vilket sannolikt beror på att kolhydrater har mer komplexa och överlappande spektrala signaturer, medan proteiner och fettsyror har en handfull mycket distinkta spektrala band, åtminstone i FTIR. Medan fettsyror från mikroalger kunde förutsägas relativt enkelt, innehöll de tångarter som användes i avhandlingen för låga fettsyrahalter för att kalibrera en pålitlig prediktionsmodell.

Sammantaget demonstrerades potentialen för att använda spektroskopiska karaktäriseringsmetoder i alger, eftersom dessa tekniker med hög noggrannhet kan kvantifiera de vanligaste biokemiska beståndsdelarna som finns i algbiomassa.

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# I

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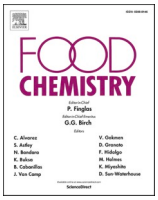

# Rapid and accurate determination of protein content in North Atlantic seaweed by NIR and FTIR spectroscopies

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NIR

ABSTRACT

Seaweed is considered a potentially sustainable source of protein for human consumption, and rapid, accurate methods for determining seaweed protein contents are needed. Seaweeds contain substances which interfere with common protein estimation methods however. The present study compares the Lowry and BCA protein assays and protein determination by N-ratios to more novel spectroscopic methods. Linear regression of the height or the integrated area under the Amide II band of diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS) was used to predict seaweed protein with good prediction performance. Partial least squares regression (PLSR) was performed on both DRIFTS and near-infrared (NIR) spectra, with even higher prediction accuracy. Spectroscopy performed similar to or better than the calculated N-ratio of 4.14 for protein prediction. These spectral prediction methods require minimal sample preparation and chemical use, and are easy to perform, making them environmentally sustainable and economically viable for rapid estimation of seaweed protein.

## 1. Introduction

### 1.1. Seaweed as a food source

Seaweed has gained much attention as an efficient producer of biomass with a wide array of applications, with particular interest aimed at edible varieties for use as a sustainable food source (Mohamed, Hashim, & Rahman, 2012). The characterisation of seaweed biomass, however, requires rapid and accurate quantification methods. For nutritional purposes, the overall quantities of proteins, carbohydrates, fats and minerals are of particular interest. The percentage of total protein content tends to range between 5 and 30 % of dry weight (Angell, Mata, de Nys, & Paul, 2015). In most seaweed, the carbohydrate content usually makes up about 30–50 %, while many brown macroalgae, especially kelp, contain as much as 60 % or even 70 % carbohydrates (Rioux & Turgeon, 2015). Although the ash content is species dependent, it is also controlled by environmental conditions. It typically ranges between 10 and 30 %, with 40 % in extreme cases (Ruperez, 2002). The overall distribution of these macronutrient compounds, as well as the relative presence of essential minerals and vitamins, determine the suitability of seaweed for human consumption.

To meet the growing need for sustainable food products on a global scale, seaweed is considered a food source with high potential. Seaweed, as a marine organism, does not compete with land crops for arable land and does not require freshwater. Seaweed is known to contain numerous bioactive compounds of nutritional interest, including pigments, polyphenolic compounds with high antioxidant and radical scavenging properties, considerable quantities of A, B, C and E vitamins ~~and vitamins~~, as well as minerals like magnesium, potassium, calcium, iron, copper and iodine, which have many potential health benefits (MacArtain, Gill, Brooks, Campbell, & Rowland, 2007; Mohamed, Hashim, & Rahman, 2012). Naturally, excessive consumption of minerals like sodium and iodine can have negative health impacts, and so the type and quantity of seaweed consumed should be taken into consideration (MacArtain, Gill, Brooks, Campbell, & Rowland, 2007). For centuries, seaweed has been cultivated as a food source in enormous quantities in

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Asia, with the largest present-day production taking place in China, Indonesia, the Philippines, Korea and Japan (Nayar, 2014). In contrast, the adoption of seaweed as a staple food has remained relatively uncommon in Western countries.

For nutritional purposes, proteins are of particular interest. A high protein content, with amino acids being critical for many physiological and metabolic functions, is seen as a sign of high nutritional value. Therefore, protein deficiency leads to detrimental effects for human health, such as the loss of muscle mass, stunted growth in children and impairment of immune system responses (Arnold, Vladutiu, Kirby, Blakely, & Deluca, 2002; Li, Yin, Li, Kim, & Wu, 2007; Paddon-Jones & Rasmussen, 2009). Red meat, as one of the most common protein sources, however, is associated with an increase in the probability of cardiovascular disease and type-2 diabetes (Bernstein, Sun, Hu, Stampfer, Manson, & Willett, 2010; van Dam, Willett, Rimm, Stampfer, & Hu, 2002). Therefore, the replacement of protein from red meat with, for instance, plant-based sources, such as legumes and seaweed, is desirable to reduce the risk of these common diseases (Hu, 2003; Song, Fung, Hu, Willett, Longo, Chan, et al., 2016). Moreover, the carbon footprint of seaweed cultivation is considerably lower than that of animal farming, making it a more sustainable protein source (Rawiwan, Peng, Paramayuda, & Quek, 2022).

### 1.2. Use of seaweed as a health additive

The quality of seaweed proteins used as human health food has been the subject of extensive research in the last several decades (Fleurence, 1999; Rawiwan, Peng, Paramayuda, & Quek, 2022). The bioavailability of proteins from seaweed varies from species to species and it is often relatively low due to the strong association of proteins with fibres (MacArtain, Gill, Brooks, Campbell, & Rowland, 2007). Seaweed protein bioavailability has been shown to be drastically improved by fermentation or enzymatic treatments (Bleakley & Hayes, 2017). Moreover, studies showed that patients on diets such as the Okinawan diet, which incorporate considerable quantities of seaweed, legume and vegetable consumption in conjunction with low meat and dairy consumption, tend to have very low incidence of cardiovascular disease, cancer, diabetes and many other diseases (Willcox, Willcox, Todoriki, & Suzuki, 2009). Recent research also indicates a reduction in the glycaemic potential of refined grain foodstuffs, such as rice and bread, in diets supplemented with seaweed (Lu & Chen, 2022). This provides evidence of the health benefits of the regular consumption of seaweed, although further research into the exact health effects of seaweed consumption is still needed (Murai, Yamagishi, Kishida, & Iso, 2021).

### 1.3. Protein content estimation

Accurate protein content estimation in both plant and algal samples can be difficult due to the presence of numerous bioactive substances that interfere with the measurements and are co-extracted along with proteins (Barbarino & Lourenço, 2005; Lucarini & Kilikian, 1999). Furthermore, many algae species accumulate large quantities of salt, which may also interfere with protein solubility and reduction reactions with copper cations (Lucarini & Kilikian, 1999). There is also a potential problem of inefficient extraction from algae due to recalcitrant cell walls requiring extensive cell disruption methods to break and the presence of phycocolloidal substances that hinder protein solubility (Barbarino & Lourenço, 2005). Precipitation protocols are typically used to concentrate proteins while removing unwanted substances, rendering the sample more suitable for protein quantification. The choice of precipitation method affects how well proteins are precipitated and what non-proteinaceous compounds will be removed from the extracts (Koontz, 2014; Mechin, Damerval, & Zivy, 2007).

There are a wide variety of methods for the quantification of proteins that rely on a great variety of mechanisms for detection. Although all available methods have advantages and disadvantages, colorimetric methods still tend to be commonly used due to low costs and ease of use. Despite the estimation by nitrogen (N) ratios being arguably-one of the quickest methods, it is unreliable due to the well-known differences in the protein-to-N ratio among species and even between different growth phases within one species (Forbord, Matsson, Brodahl, Bluhm, Broch, Handå, et al., 2020). Attempts have been made to define a relatively universal *N*-conversion factor for all algae (Finkel, Follows, Liefer, Brown, Benner, & Irwin, 2016), with a commonly accepted seaweed N ratio of 5 (Angell, Mata, de Nys, & Paul, 2015). For rapid estimation, a relatively accurate N ratio is highly useful if one has access to the necessary instrumentation for N analysis. Another rapid method of protein estimation is to measure the UV absorbance of a crude protein extract. This method, however, is also heavily prone to biases because the measurements at 280 nm are significantly influenced by the presence of nucleic acid residues, while the peptide absorbance band at 200–210 nm can be affected both by the buffer and impurities in the extract (Goldring, 2012). The issue of nucleic acid interference can be compensated for using the 280/260 nm absorbance ratios (Groves, Davis, & Sells, 1968). The $UV_{280}$ absorbance of proteins depends on the presence of tyrosine, tryptophan and, to a lesser extent, phenylalanine (Goldring, 2012). This means that the absorbance differs substantially from protein to protein, making an unknown mixture of protein hard to estimate. In seaweed, where tyrosine and tryptophan tend to be lacking and phenylalanine would be the main contributor to $UV_{280}$ protein absorbance (Lourenço, Barbarino, Lavín, Lanfer Marquez, & Aidar, 2004), this method is particularly unreliable. Acid hydrolysis and quantification of total amino acids by mass spectrometry are considered one of the most reliable methods for protein quantification and are typically used as the basis for calculating N-ratios (Angell, Mata, de Nys, & Paul, 2015; Finkel, Follows, Liefer, Brown, Benner, & Irwin, 2016; Forbord, et al., 2020; Lourenço, Barbarino, Lavín, Lanfer Marquez, & Aidar, 2004). The total quantity of bound amino acids corresponds to the total amount of proteins, but this method requires access to costly equipment, considerable labour time and chemicals, as well as technical knowledge of how to perform the analysis.

### 1.4. Spectroscopy for quantitative analysis

Various types of spectroscopy have also been used for the quantification of biochemical compounds, including proteins, fatty acids and carbohydrates. The infrared spectrum is particularly useful for this purpose because it contains numerous distinct signatures of functional groups associated with these compounds (Schmitt & Flemming, 1998). Spectroscopic techniques can also be used for the quantitative analysis of individual substances, such as pharmaceuticals and specific polysaccharide species, as opposed to being useful only for broader compound classes, such as total protein (Strachan, Rades, Gordon, & Rantanen, 2007). The use of these techniques in microalgae has also been widely explored within the past decade (Feng, Zhang, Cheng, Xu, Zhang, & Chen, 2013; Ferro, Gojkovic, Gorzsas, & Funk, 2019; Horton, Duranty, McConico, & Vogt, 2011), while less work has focused on the quantification of proteins in seaweed biomass. Studies have involved the use of nuclear magnetic resonance (NMR) spectroscopy techniques for measuring value-added compounds like pigments in seaweed extracts (Rajauria, Foley, & Abu-Ghannam, 2017). In contrast, only a few studies have used near-infrared (NIR) and Fourier-transform infrared (FTIR) spectroscopies for the analysis of seaweed samples. FTIR spectroscopic techniques have been applied to the quantification of the primary components of ulvan polysaccharides in extracts from *Ulva* spp., including rhamnose, xylose, glucuronic acid and sulphate (Robic, Bertrand, Sassi, Lerat, & Lahaye, 2008). Recently, the use of these techniques for protein estimation has been demonstrated directly in seaweed samples (Campbell, Ortuño, Koidis, & Theodoridou, 2022), providing support for a more reliable method for protein measurements in seaweed with minimal sample preparation and chemical use.

The present study evaluates the drawbacks of commonly used

colorimetric protein assays for protein content determination in five common, edible seaweed species harvested for human food. We investigated the use of N ratios for the estimation of protein content and compared it to spectroscopic prediction methods. Univariate, simple linear regression was used to predict the protein content based on the signal intensity of the Amide II band of the FTIR spectra, and multivariate partial least squares regression (PLSR) was used to create protein prediction models using both FTIR and NIR spectroscopic data. This study therefore presents one of the most comprehensive works to date on methods for the quantification of protein content in seaweed. We also evaluate the use of *in situ* (i.e., extraction free), non-destructive, fast, environmentally sustainable, easy-to-perform spectroscopic methods for high-throughput, low cost and accurate protein estimation as potential replacements for unreliable traditional methods.

## 2. Materials and methods

### 2.1. Chemicals

Lowry reagent A (2 % (W/V) anhydrous $Na_2CO_3$ in 0.1 M NaOH), Lowry reagent B (1 % (W/V) NaK tartrate tetrahydrate) and Lowry reagent C (0.5 % (W/V) $CuSO_4$ pentahydrate) were prepared and stored at room temperature (RT). Lowry reagent D was prepared fresh for each experiment by mixing reagents A:B:C in a ratio of 48:1:1. Folin–Ciocalteu's phenol reagent was prepared fresh in a 1:1 dilution of a 2 N stock in MQ water. TCA water solutions were stored at 4 °C, while TCA acetone solutions were prepared fresh on the day of use to avoid acetone evaporation. When used, 2-mercaptoethanol (2ME) was added to precipitation solution stocks the same day as each experiment, as 2ME is unstable in the solution (Foroumadi & Saeedi, 2014). The acetone used for the assays was kept at − 20 °C whenever possible to ensure maximum precipitation efficiency. The bicinchoninic acid (BCA) assay was performed using the Pierce BCA Protein Assay Kit (Thermo Fisher, Waltham, MA, US). All chemicals were of analytical grade and were purchased from Merck Group (Darmstadt, Germany) unless otherwise specified.

### 2.2. Seaweed cultivation and harvest

Seaweed samples were collected primarily from Norway and the Faroe Islands, with some additional *Alaria esculenta* samples supplied from Connemara, Ireland. The sample set contained a mixture of wild and cultivated seaweed, dried by either freeze-drying or hot air drying.

#### 2.2.1. Norwegian seaweed sampling

Wild *Palmaria palmata*, *Laminaria digitata* and *A. esculenta* samples from Tromsø were collected on the island of Tromsøya (69°37′37″ N, 18°54′55″ E), Norway, in April 2021.. Cultivated *Saccharina latissima* samples were grown at Kvaløya (69°45′36″ N, 19°2′56″ E) and harvested in August 2017.. Precise conditions for cultivation and drying are described by Matsson et al. (2021). Dry samples were stored at room temperature until analysis.

Samples of *P. palmata*, *L. digitata*, *A. esculenta* and *S. latissima* were collected from Mørkvedbukta (67°16′32″ N, 14°34′10″ E) in Bodø, Norway. The seaweed was rinsed with running seawater to remove epibionts, followed by freezing at − 80 °C prior to freeze-drying at − 55 °C.

#### 2.2.2. Faroese seaweed sampling

*Alaria esculenta* and *S. latissima* were cultivated on Kaldbaksfjørður at TARI's cultivation location (62°03′33′'N, 6°49′05′'W). The *A. esculenta* samples were harvested on 14 June 2019, 27 May 2020, 12 June 2020 and 24 June 2020. *S. latissima* was harvested on 11 June 2020.

*Himanthalia elongata*, *L. digitata*, *P. palmata* and *Porphyra umbilicalis* were all harvested from natural populations. *H. elongata* was harvested from Tjørnuvík (62°17′49′'N, 7°08′40′'W) on 20 July 2018 and from Gøtugjógv (62°11′26′'N, 6°44′49′'W) on 15 July 2020. *L. digitata* was harvested from Oyragjógv (62°06′36′'N, 7°09′37′'W) on 15 August 2019. *P. palmata* and *P. umbilicalis* were harvested from Oyragjógv on 19 August 2020 and 15 July 2020, respectively.

All harvested biomass was transported in boxes to the commercial drying facility at Faroe Marine Products and dried in closed rooms with warm air blowing and temperatures below 30 °C for 48–72 h.

### 2.3. Cell disruption and sample homogenisation

To ensure sample homogeneity and effective cell disruption, the seaweed samples were submerged in liquid nitrogen ($N_2$) and ground into flakes using a mortar and pestle. The flakes were loaded into a liquid $N_2$-cooled, 50 mL stainless steel ball grinder chamber along with a steel ball with a diameter of 20 mm. The flakes were then milled using a Mixer Mill MM 400 (Retsch GmbH, Haan, Germany) at a frequency of 25 Hz for 2–3 min until the full sample had reached a particle size small enough to pass through a 200 μm sieve. Milled samples were then stored in a dark, dry atmosphere in the presence of desiccants until analysis to prevent photodegradation and to reduce the moisture content that may have been absorbed by condensation during liquid $N_2$-grinding.

### 2.4. Precipitation and purification of proteins

To evaluate the ability of common precipitation methods to reduce interference in the Lowry and BCA assays, two different protocols for protein precipitation from plants and algae were used. These two methods were also compared to the direct quantification of unprecipitated samples.

#### 2.4.1. TCA precipitation

A TCA precipitation protocol was adapted from Koontz (2014), with some alterations to the volumes of TCA and acetone, precipitation duration, and centrifugation parameters. Proteins were precipitated from 5 mg dried seaweed powder by suspension in 1.8 mL of a 6 % TCA solution in water (w/v), followed by incubation at 4 °C for 1 h. The samples were centrifuged at 14,000 × g for 20 min at 4 °C, and the supernatant was removed by vacuum aspiration through a thin needle, taking care not to disturb the pellet. The precipitated proteins were washed twice with 1.8 mL of ice-cold acetone to remove traces of TCA, which might affect later re-solubilisation. The acetone was vacuum-aspirated after centrifugation, and the remaining acetone traces were evaporated for 20 min.

#### 2.4.2. TCA–acetone–2ME precipitation

TCA and acetone are commonly used in conjunction for protein precipitation, as they precipitate proteins by different mechanisms and because acetone is easier to remove from the resulting protein pellet than TCA using evaporation (Mechin, Damerval, & Zivy, 2007). The addition of 2ME to the precipitation mixture can further aid precipitation by disrupting tertiary protein structures and exposing the hydrophobic amino acid residues that are normally unexposed to the polar solvent (Foroumadi & Saeedi, 2014). A TCA–acetone–2ME precipitation protocol was thus adapted from Méchin et al. (2007), with slight alterations to ensure that the same solubilisation protocol was applied to all methods. Briefly, 5 mg dried seaweed powder was covered in 1.8 mL TCA–acetone–2ME solution (10 % TCA (w/v), 0.07 % 2ME (w/v) in acetone) and stored at − 20 °C for 1 h. The samples were centrifuged as above, and the supernatant was removed by vacuum aspiration. Pellets were washed twice with 1.8 mL of rinsing solution (0.07 % 2ME in acetone) to remove trace TCA. After vacuum aspiration, the residual acetone was evaporated for 20 min.

#### 2.4.3. Re-solubilisation

For both the Lowry and BCA assays, the precipitated proteins and the non-precipitated seaweed powders were dissolved in 1 mL of the Lowry

D reagent. As this reagent is an alkaline suspension buffer that is largely identical to the one used in the BCA assay, albeit with differing $CuSO_4$ contents, it was determined to be a suitable initial solubilisation buffer prior to both assays. The samples were heated at 55 °C for 180 min with occasional vortexing to help disrupt clusters, as determined to be optimal by Slocombe et al. (2013) for full solubilisation without degradation. The samples were then allowed to cool to room temperature and centrifuged at 15,000 × g for 15 min at room temperature to pellet cell debris and other insoluble components.

### 2.5. Lowry protein assay

The Lowry assay used in this study was based on a modified protocol by Slocombe et al. (2013). Briefly, protein precipitates were re-solubilised in 1 mL Lowry reagent D. Twenty microliters of the protein extract was transferred to a fresh tube and 980 μL Lowry reagent D was added to reach a volume of 1 mL. After 10 min of incubation at RT, 100 μL Folin–Ciocalteu reagent (diluted from 2 N in water, 1:1) was added, and the samples were vortexed immediately. This mixture was incubated at RT for 30 min; 200 μL was transferred to a 96-well microplate and the absorbance was measured at 750 nm using an Epoch 2 microplate reader (Biotek, Winooski, VT, USA). A calibration curve consisting of bovine serum albumin (BSA) with a concentration range from 250 to 1500 μg $mL^{-1}$ was used to determine the protein concentration of the samples. This is a range that is likely sufficient for macroalgal samples, as it corresponds approximately to a protein content range of 5–30 % by DW, assuming a sample amount of 5 mg is used. This range needs to be adjusted to obtain higher amounts of protein or to use a larger sample. The calibration curve began to lose linearity at concentrations above 2000 μg $mL^{-1}$.

### 2.6. Bicinchoninic acid protein assay

The BCA assay was performed using a Pierce BCA Protein Assay Kit (Thermo Fisher), according to the manufacturer's instructions, with some minor adaptations. As previously mentioned, the proteins were initially solubilised in the alkaline Lowry D reagent prior to the assay. In brief, 100 μL of this protein solution was transferred to a 2 mL microcentrifuge tube, and 2 mL of the BCA Working Reagent was added. The reaction was carried out at 37 °C for 30 min, as recommended by the manufacturer. Samples were cooled to room temperature for 30 min prior to the transfer of 200 μL to a 96-well microplate. Absorbance was measured at 562 nm using an Epoch 2 microplate reader (Biotek).

### 2.7. Nitrogen analysis

Nitrogen analysis of all seaweed samples was undertaken using Elemental Analyser Isotope Ratio Mass Spectrometry (EA-IRMS). The linear relationship between nitrogen and protein in seaweed species was verified by simple linear regression. An average N-ratio was calculated from all samples and used to estimate the error of using this ratio for protein estimation. Species-specific N-ratios were also determined and analysed by ANOVA to determine whether there were statistically significant differences in N-ratios between species. N-ratios were only calculated for species with three or more individual samples, and as such, *P. umbilicalis* was excluded, as these were from a single, bulk harvest.

### 2.8. Amino acid analysis

Bound and free amino acids were quantified by liquid chromatography tandem mass spectrometry (LC-MS/MS) at the Swedish Metabolomics Centre, Umeå, Sweden. The sum of free and bound amino acids was used as an estimation of total protein, and this was used as a benchmark for comparing the other methods. For comparison to the colorimetric assays and for infrared spectroscopic modelling however, only bound amino acids were used. This was done as the colorimetric assays in principle should react primarily with peptide bonds, and the spectral signatures also depend on chemical structures specific to polypeptides rather than free amino acids.

### 2.9. Diffuse reflectance Fourier transform infrared spectroscopy (DRIFTS)

Fourier transform infrared (FTIR) spectroscopy was evaluated as a potential method for estimating protein content in dried macroalgae. Measurements were carried out using a previously described protocol (Gorzsas & Sundberg, 2014). The dried algae were mixed with potassium bromide (KBr) to a ratio of approximately 1:10 algae:KBr and ground to a homogenous powder using an agate mortar and pestle. DRIFTS measurements were performed using an IFS 66 v/S vacuum spectrometer (Bruker Optik GmbH, Ettlingen, Germany), covering the 4000–400 $cm^{-1}$ spectral region at a resolution of 4 $cm^{-1}$. A total of 128 scans were co-added, and pure KBr was used as a background and automatically subtracted by the software operating the instrument (OPUS, version 5, Bruker Optik GmbH).

The recorded spectra were exported as.mat files and processed using MCR-ALS GUI, available at the Vibrational Spectroscopy Core Facility, Department of Chemistry, Umeå University (v4c, https://www.umu.se/en/research/infrastructure/visp/downloads/) in MATLAB (version R2017b, MathWorks, Natick, MA, USA). All spectra were cut to the 800–1800 $cm^{-1}$ range (fingerprint region) to focus on specific bands strongly related to fatty acids, carbohydrates and proteins (the primary biochemical components of algal biomass) and to minimise potential baseline correction and normalisation difficulties over the unspecific -O—H vibrations in the high wavenumber region of the spectra. The cut spectra were baseline corrected using asymmetric least squares (AsLS) (lambda = 20 000, p = 0.001). Baseline-corrected spectra were normalised for the total area in the cut spectral range. Processed DRIFTS spectra were used for both simple linear regression and PLSR modelling.

FTIR spectra contain spectral bands that are more or less diagnostic of specific classes of compounds due to the unique vibrational signatures of their functional groups in a matrix of substances (e.g., amide functions of proteins and carboxylic acid moieties of fatty acids). Typically, the strongest band for the quantification of protein using FTIR spectra in the fingerprint region is the Amide I band centred around 1650 $cm^{-1}$ (largely due to the contribution from the -C═O stretching vibrations in the peptide bonds (Schmitt & Flemming, 1998)). In brown seaweed, however, this band is less suitable for rapid quantification purposes, as it heavily overlaps with large bands from alginate (Taha, Aiedeh, Al-Hiari, & Al-Khatib, 2005) (Fig. 1), a very abundant uronic acid polysaccharide typical of the cell walls of brown seaweed (Rioux & Turgeon, 2015). As such, univariate linear regression using Amide I band intensity was not possible in these seaweed samples. However, the Amide II band between approximately 1485 $cm^{-1}$ and 1565 $cm^{-1}$ (largely stemming from N—H and C—N bending vibrations within peptide bonds (Schmitt & Flemming, 1998)) appeared to be relatively free from interference from alginate. Thus, while generally weaker and at times broader than the Amide I band, the Amide II band was chosen for univariate protein estimation for these samples.

### 2.10. Near-infrared spectroscopy (NIR)

Near-infrared (NIR) spectroscopy was also investigated as a rapid method of protein quantification. NIR spectra were measured on dry seaweed powder using a LabSpec ASD NIR spectrophotometer (Portable Analytical Solutions, Copacabana, NSW, Australia) equipped with a contact probe. Spectra were captured from 350 to 2500 nm at a resolution of 1 nm, after blanking with pure white reference blank supplied by the manufacturer. The spectral region was trimmed to 1000–2500 nm to remove interference from the visible spectrum. Standard normal variate (SNV) normalisation was performed on the cut spectra to

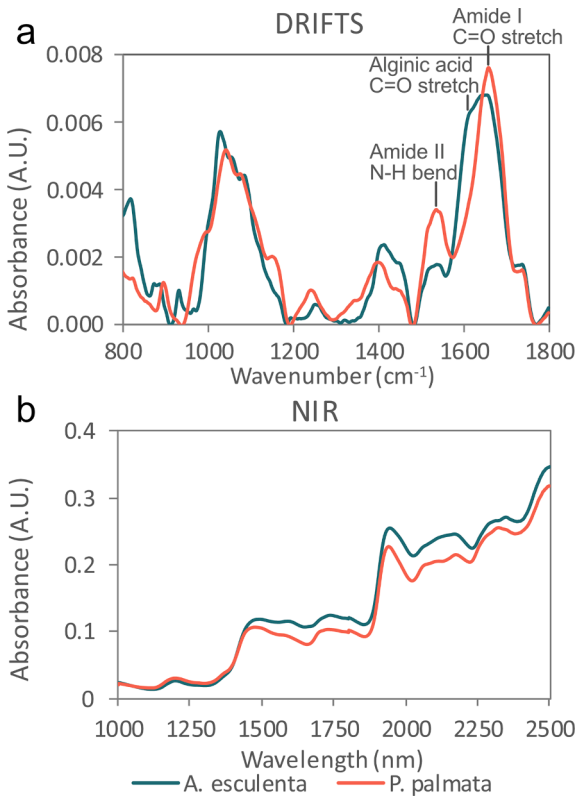


**Fig. 1. Representative spectra of a brown and a red seaweed species.** *A. esculenta* (dark blue line) from the Faroe Islands and *P. palmata* (light red line) from Bodø, Norway. a) Processed DRIFTS spectra. The overlapping bands of from proteins (Amide I) and alginate C=O stretches at approximately 1665 and 1620 $cm^{-1}$, respectively, are indicated. b) SNV-corrected NIR spectra. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

normalise spectral intensities. Measurements were done in triplicate for each sample and the spectra were averaged. Processing was done using Evince software (Prediktera AB, Umeå, Sweden).

The NIR spectrum is not as easily used for univariate prediction as the FTIR spectrum, as the absorbance bands are less characteristic of specific compound classes present in biological materials. As such, the NIR spectra obtained in this study were analysed using only PLSR.

### 2.11. Statistical analyses

#### 2.11.1. ANOVa

A one-way analysis of variance (ANOVA) was performed to determine the statistical significance of discrepancies between protein estimation by amino acid quantification and colorimetric protein assays. A one-way ANOVA was also used to compare the median N ratios of the seaweed species assayed in the study. All ANOVA analyses were performed using Excel (Microsoft, Redmond, WA, USA) with the Analysis ToolPak add-in (Microsoft).

#### 2.11.2. Simple linear and partial least squares regression (PLSR)

Simple linear regression analysis was used to calculate a model for the prediction of protein content using either the integrated area under or the height of the Amide II band from the DRIFTS spectra and the experimentally determined amino acid concentrations. Regression analysis was performed using RStudio software (Rstudio, Boston, MA, USA). Figures were made using the ggplot2 package (https://ggplot2.tidyverse.org) and exported using the Cairo package (https://CRAN.R-project.org/package=Cairo).

Complex biological samples tend to contain a wide variety of compounds with overlapping spectral signatures. For this reason, multivariate statistical methods are often used when quantification of a specific compound or compound class is desired, as they consider multiple variables or spectral bands rather than relying on one specific peak. PLSR is a robust method recommended in cases where the sample contains unknown components and there are many variables (Strachan, Rades, Gordon, & Rantanen, 2007). Spectroscopic quantification by PLSR is typically done by constructing a model using a set of calibration samples with known concentrations of the target analyte. The model was then used to predict a set of external validation samples, and the accuracy of this prediction was used to judge the suitability of the model for the prediction of future samples.

The optimal number of PLSR components was selected by leave-one-out cross-validation (CV). Component numbers from 1 to 10 were cross-validated and the one with the lowest root mean square error of cross-validation (RMSECV) was selected. No more than 10 components were investigated, as an exceedingly high component number in comparison to the number of samples used for calibration increases the likelihood of overfitting by including noise in the model, thereby decreasing the overall general predictive strength. For the DRIFTS data, 7 components were determined to be optimal, while 5 had the lowest RMSECV using NIR data.

For both PLSR and simple linear regression analyses, 10 out of 45 samples were set aside for validation, while the remaining 35 were used to calibrate the prediction model. Validation samples were selected through random number generation (each sample was assigned a random number from 1 to 45, and numbers 36–45 were used for validation). The calculated model was tested against these 10 validation samples to estimate the prediction accuracy. The resulting root mean square error of prediction (RMSEP), as well as the predicted $R^2$ value was used to indicate the predictive capability of the model. PLSR analysis was performed using Rstudio software (Rstudio) with scripts from the PLS package (v. 2.8–0, https://CRAN.R-project.org/package=pls). The mean relative error of the predicted samples was also compared between the different protein prediction methods.

## 3. Results

### 3.1. Total protein and amino acid quantification

Bound and free amino acids in seaweeds were determined by LC-MS/MS, and the distribution of amino acids in five seaweed species is shown in Fig. 2. The quantity of each identified amino acid was summed up to determine total protein contents. The protein contents of the 45 samples analysed had a wide range from 3.19 % to 22.41 %, with a mean standard deviation of 0.22. The majority of samples were within a range of 8.99 % to 13.75 % with a median of 10.20 %. The majority of amino acids were in bound form, which is to say incorporated into peptide chains.

### 3.2. Colorimetric protein assays

The comparability of the BCA and Lowry assays with the total amino acid by the LC-MS/MS analysis with respect to the protein quantification was investigated. Two methods of protein precipitation prior to colorimetric analysis were also compared to the direct analysis of unprecipitated samples. The ratio of the colorimetric estimates to the amino acid measurements was calculated to determine the degree of deviance from the expected result. Fig. 3 shows the calculated ratio of colorimetric protein estimation to total amino acid measurements. Compared to the amino acid contents, the protein contents of the three species of brown seaweed tended to be overestimated by the Lowry assay (1.79, 1.78 and 1.54-fold in *A. esculenta*, *H. elongata* and *S. latissima*, respectively), an effect that was lessened by precipitation with TCA and further improved slightly by combined precipitation with TCA, acetone and 2ME. However, neither precipitation method was able to decrease the

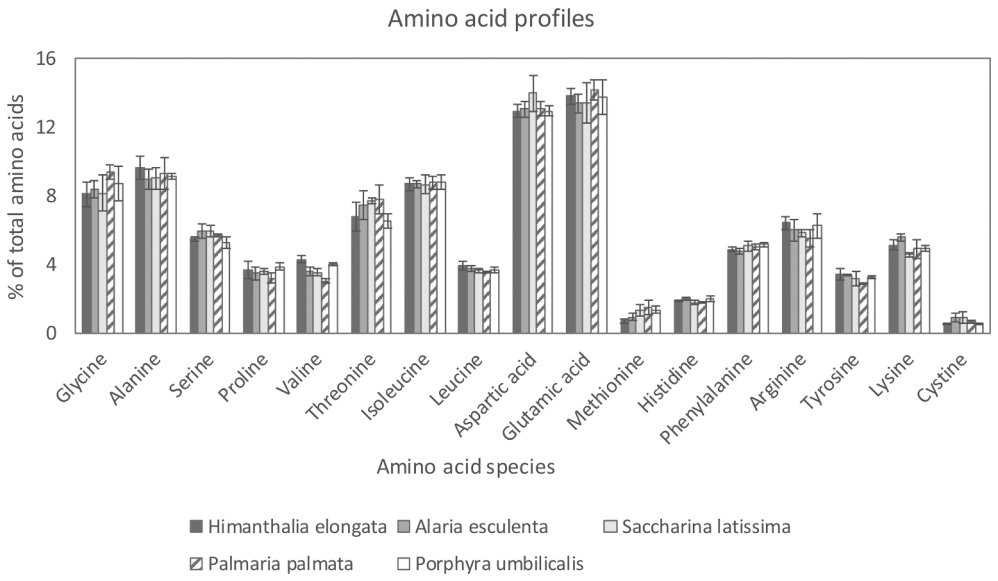


**Fig. 2. Amino acid profiles of five Faroese seaweed species**. Profiles are presented as percentage of total identified amino acids by weight. Error bars indicate standard deviation of triplicate analyses.

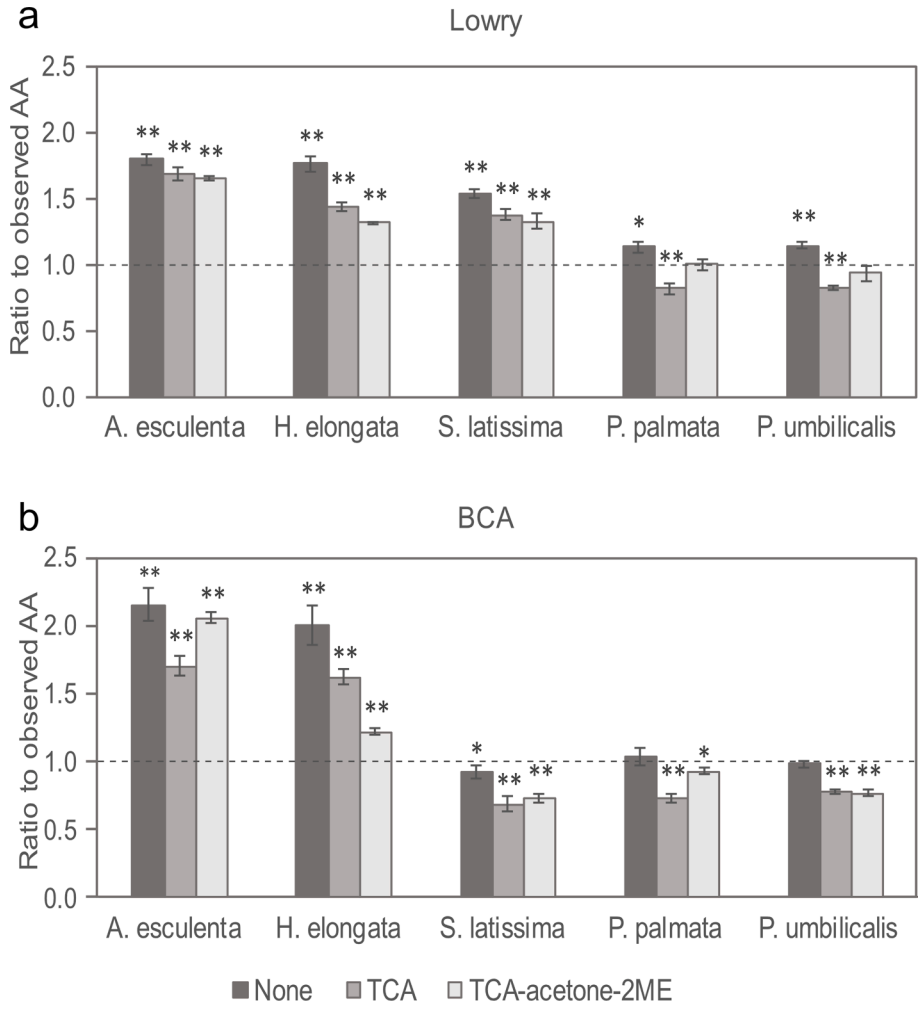


**Fig. 3. Ratios of total protein estimated by colorimetric assays compared to total amino acids.** Protein contents estimated by the a) Lowry and b) BCA assays, using different precipitation methods in five different seaweed species. Y-axis indicates the ratio of estimated protein to AA content, and the dashed line indicates a 1:1 ratio. Error bars indicate standard deviation of triplicate analyses. Asterisks show significant deviation from amino acid contents, * = $p < 0.05$, ** = $p < 0.01$.

overestimation to below 1.3-fold. The two species of red seaweed, in contrast, could be more accurately estimated by the Lowry method, with a relatively lower overestimation in both species (1.12-fold in *P. palmata* and 1.14-fold in *P. umbilicalis*). Precipitation with TCA led to underestimation (1.18-fold in both species), while TCA–acetone–2ME produced no significant difference from the amino acid measurements. The Lowry assay showed high repeatability, with a mean standard deviation of ± 0.44.

The BCA assay resulted in an even greater overestimation in the brown seaweed species (approx. 2-fold in both *A. esculenta* and *H. elongata*), except for *S. latissima*, which was slightly underestimated by this method (1.08-fold). The underestimation was worsened by both precipitation methods (1.31-fold with TCA and 1.27-fold with TCA–acetone–2ME). The red seaweed species were more accurately estimated by the BCA assay than by the Lowry assay, showing no significant difference from the amino acid measurements. However, precipitation had adverse effects on quantification in the red seaweed species, leading to significant underestimation (1.27 and 1.22-fold in *P. palmata* and *P. umbilicalis*, respectively, using TCA and 1.07 and 1.23-fold, respectively, using TCA–acetone–2ME). The BCA assay showed a somewhat higher mean standard deviation than the Lowry assay, at ± 0.53, implying slightly lower repeatability.

### 3.3. Prediction of protein content by nitrogen ratio

The ratio of protein to N content in five seaweed species was determined. The average N-ratio for all 45 samples was 4.14 (±0.43 SD) with a mean relative error of approx. ± 0.0211 %, indicating considerable differences between species. *H. elongata* had the lowest N-ratio (3.46 ± 0.42), while *A. esculenta* had the highest (4.49 ± 0.23), indicating quite a wide span depending on the species in question. All N-ratios are presented in Table 1. One-way ANOVA was performed to compare the N ratios of *A. esculenta*, *S. latissima*, *H. elongata*, *L. digitata* and *P. palmata*, which indicated that there was a statistically significant difference between at least two of the assayed species ($F = 10.83935$, $P = 6.45 \bullet 10^{-6}$). Individual t-tests were performed on each pairwise combination, indicating a significant difference between *A. esculenta* and *S. latissima*, as well as between *A. esculenta* and *L. digitata*. Thus, it appears that a universal N-ratio is not applicable due to certain differences between species.

### 3.4. Seaweed protein estimation by FTIR and NIR spectroscopy

Using univariate linear regression, a protein prediction model was constructed from amino acid data and the intensity of the FTIR Amide II band. Both the integrated area of the band (1485 to 1570 $cm^{-1}$) and the height of its apparent maximum (at 1540 $cm^{-1}$) were investigated as potential univariate predictors of protein content. The peak height and integral performed similarly, with RMSEP of 1.51 and 1.49, respectively. Judging by predictive error, it appears that peak height is more accurate for univariate prediction than peak area. The mean relative error was also calculated, and was approx. ± 0.0261 % and ± 0.0244 % for the peak height and integral, respectively, implying a somewhat higher experimental error for the peak height. Both univariate DRIFTS analyses were also slightly less accurate than the use of N-ratio for protein estimation. The linear regression analysis of the DRIFTS data is summarised in Table 2. The unsuitability of the Amide I band for univariate protein estimation in brown seaweed is demonstrated in Fig. 1 and Fig. 4a, as it so heavily overlaps with an interfering peak from what is likely to be alginate. The example *Palmaria palmata* sample in Fig. 1 contained nearly twice the protein content of the *A. esculenta* sample, but the Amide I peak was of comparable signal intensity. The intensity of the Amide II peak at approximately 1540 $cm^{-1}$, however, appears considerably more representative of the relative protein content.

Protein was also predicted using PLSR of both DRIFTS and NIR spectra. The PLS models constructed from the amino acid data of 35 samples were used to predict the total protein content in 10 validation samples. Leave-one-out CV indicated an RMSEC of 0.92 for DRIFTS calibration using 7 PLS components. The RMSEP for the validation dataset was 1.24, with a protein content range of 4.5–21.8 %. With the same calibration set, the optimal component number for PLSR using NIR data was 5, resulting in an RMSEC of 1.26. The RMSEP for the validation set was 1.19, implying a slightly lower error than the DRIFTS model. The mean relative error calculated for PLSR prediction using DRIFTS and NIR was just below ± 0.01 % for both methods. The key results from the PLSR analyses are summarised in Table 2.

The regression coefficients clearly identified positive correlation to the major protein bands (amide I, II and III, at ca. 1640, 1550 and 1230 cm-1, respectively) (Fig. 4a). On the other hand, negative correlations featured bands originating from -C—H and = C—H vibrations (1330 and 1460 cm-1, respectively) as well as from -C═O (around 1720 cm-1), which can all be associated to (saturated and unsaturated) fatty acids. While the positive correlation to amide bands would suggest that single band intensity evaluations of these bands could be sufficient to protein content estimation, our work shows that this is not that simple. For example, the amide I band that is commonly used for protein content estimation cannot be used in this case. The amide II (which also has a higher correlation to protein content in the PLS model) works better, and indeed can provide a rough estimate quickly, albeit not as accurate as the PLS model (Table 2).

## 4. Discussion

It is well known that commonly used colorimetric assays are prone to over- or underestimation when the protein used as calibration has a considerably different amino acid profile compared to the average protein of the sample (Sapan, Lundblad, & Price, 1999). This is

**Table 1**
Seaweed samples used for prediction modelling (total n = 45).

| Species | Region | Samples (n) | Year of harvest | Wild or cultivated |
|---|---|---|---|---|
| ***A. esculenta*** | Faroes | 5 | 2019, 2020 | cultivated |
| | Bodø | 3 | 2021 | wild |
| | Tromsø | 3 | 2021 | wild |
| | Ireland | 2 | 2020 | cultivated |
| | Greenland | 1 | 2020 | wild |
| ***H. elongata*** | Faroes | 3 | 2018 | wild |
| ***L. digitata*** | Faroes | 1 | 2019 | wild |
| | Bodø | 3 | 2021 | wild |
| | Tromsø | 3 | 2017 | wild |
| ***S. latissima*** | Faroes | 3 | 2020 | cultivated |
| | Bodø | 3 | 2021 | wild |
| | Tromsø | 3 | 2021 | cultivated |
| ***P. palmata*** | Faroes | 1 | 2020 | wild |
| | Bodø | 7 | 2021 | wild |
| | Tromsø | 3 | 2021 | wild |
| ***P. umbilicalis*** | Faroes | 1 | 2020 | wild |

**Table 2**
Linear and PLS regression protein prediction results.

| | RMSEC | RMSEP | Mean rel. error (%) | $R^2$ |
|---|---|---|---|---|
| **DRIFTS** | | | | |
| Amide II Height | 1.4448 | 1.5095 | 0.0261 | 0.8631 |
| Amide II Integral | 1.3792 | 1.4912 | 0.0244 | 0.8752 |
| PLSR 7 comp. | 0.9220 | 1.2376 | 0.0100 | 0.9540 |
| **NIR** | | | | |
| PLSR 5 comp. | 1.2614 | 1.1939 | 0.0095 | 0.9258 |
| **N-ratio** | | | | |
| 4.14 ratio | N/A | 1.2733 | 0.0211 | 0.9176 |

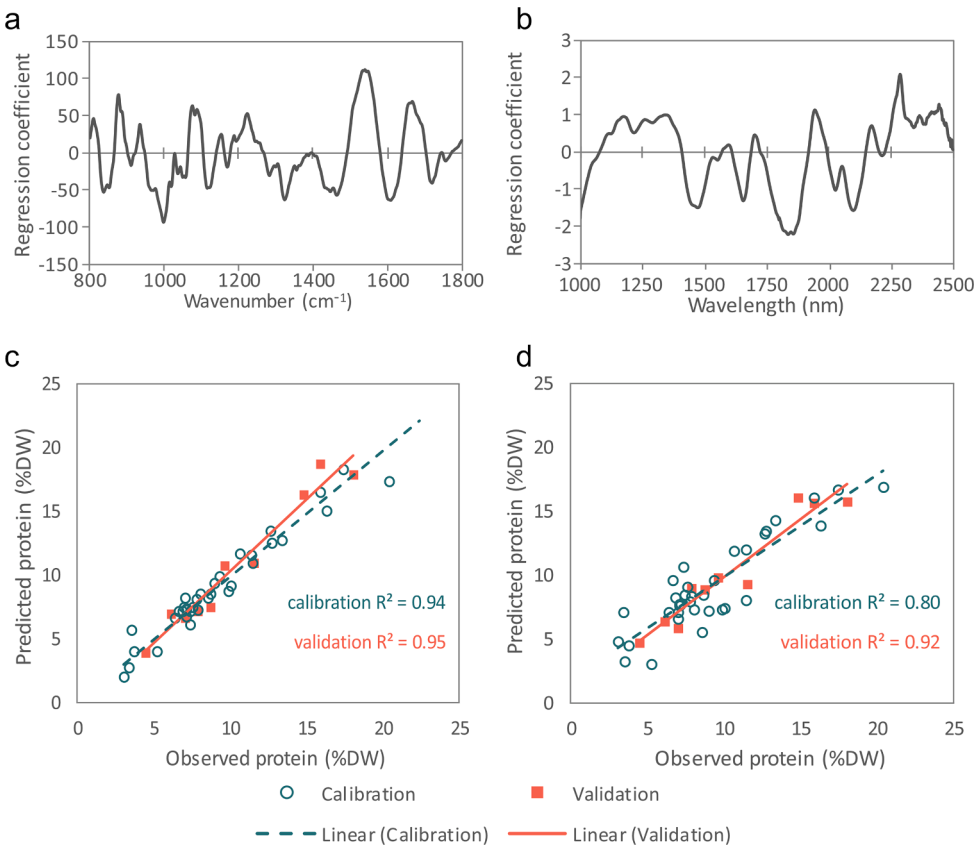


**Fig. 4. PLSR analysis of DRIFTS and NIR spectra for prediction of protein in seaweeds.** Regression coefficient plots of a) DRIFTS and b) NIR spectra, indicating the spectral signatures which impact prediction of protein in seaweed. Modelling and prediction plots for prediction of protein using c) DRIFTS and d) NIR spectra, plotted against protein estimated through amino acid quantification. The circles are calibration samples (n = 35) and the squares are validation samples (n = 10). The dashed line indicates linear correlation of fitted vs observed protein in the calibration samples, while the solid line indicates the linear correlation of predicted vs observed validation samples. The correlation coefficients for the calibration and the validation samples are both indicated in the plots.

undoubtedly the case when comparing BSA to proteins from seaweed samples, as standard proteins, such as BSA, contain considerably higher ratios of aromatic and basic amino acid residues, which tend to be more reactive in colorimetric assays (Barbarino & Lourenço, 2005), than most seaweed (Pangestuti & Kim, 2015). The Bradford assay, which was not included in this study, is particularly sensitive to the amino acid profile of the sample since it relies on dye binding specifically to lysine and arginine residues rather than reacting more generally with peptide bonds, such as in the Lowry and BCA assays (Sapan, Lundblad, & Price, 1999). The typically lower lysine content in seaweed leads to a high degree of underestimation, hence why the Bradford assay was not included. Nevertheless, the considerable, species-dependent error observed in the colorimetric methods used in this study was not due to the relative quantity of basic or aromatic amino acids, as all the species investigated had a very similar distribution of amino acids (Fig. 3). Sapan, Lundblad, and Price (1999) specifically point out tyrosine and tryptophan as contributing to over-estimation in certain samples by reducing copper ions in the Lowry and BCA assays. While tryptophan was not detected in the samples, tyrosine was. The quantity of tyrosine relative to the other amino acids is clearly not the main factor in over-estimation however seeing as the highest tyrosine contents was in the red seaweed (4.01 % in *P. palmata* and 3.81 % in *P. umbilicalis*, relative to total amino acids) which generally were not over-estimated, compared to the brown seaweed (3.44 %, 3.16 % and 3.30 % in *A. esculenta*, *S. latissima* and *H. elongata*, respectively). Thus, interspecies differences in accuracy were unrelated to the amino acid profile and would instead likely depend on the presence of interfering substances. A reliable method for concentrating proteins while removing these interfering substances would thus be useful.

As a precipitation medium, TCA renders proteins insoluble by disruption of both their hydration spheres and intramolecular H-bonds (Koontz, 2014), as well as deactivating proteases and other enzymes that interfere with protein stability and later solubilisation (Mechin, Damerval, & Zivy, 2007). As the acidity caused by the addition of TCA can further complicate solubilisation, pelleted protein extracts are commonly washed with acetone to remove trace amounts of TCA (Koontz, 2014; Mechin, Damerval, & Zivy, 2007). Acetone also removes interfering substances, such as pigments, triglycerides and terpenoids (Mechin, Damerval, & Zivy, 2007), while being considerably easier to remove from the pelleted protein afterwards since it can be evaporated. Adding 2-ME to the precipitation mixture ensures the reduction of intramolecular disulphide bonds, aiding in the unfolding of tertiary structures and further decreasing protein solubility by exposing hydrophobic moieties to the aqueous solvent (Foroumadi & Saeedi, 2014). It therefore seems reasonable that a precipitation medium containing both TCA, 2-ME and acetone would be highly useful for complex samples, such as marine seaweed, combining the beneficial effects of all three. An optimised protocol developed by Méchin et al. (2007) for the precipitation of proteins in higher plants was thus also investigated in the present study, in addition to commonly used TCA precipitation.

From the present study, it appears that the use of both the Lowry and BCA assays for protein determination in seaweed samples tends to be rather unreliable, leading to over- or underestimation, depending heavily on the assayed species (Fig. 3). The use of precipitation techniques to remove interfering substances had varying degrees of success in improving accuracy, again depending on the assayed species. However, we could not discern a clear trend as to which methods work more reliably, as some pre-treatments produce a more accurate reading in some species while having the opposite effect in others. In brown seaweed species, the BCA assay strayed further from the total amino acid measurements than the Lowry assay. Curiously, while the Lowry assay overestimated the protein content in all three brown seaweed species, BCA overestimated only *A. esculenta* and *H. elongata* samples, while having the opposite effect on *S. latissima* (Fig. 3). The two assayed red algae species, however, exhibited similar results using the colorimetric assays. All combinations of assays and pre-treatments lead to similar

patterns of minor (sometimes insignificant) over- or underestimations in both *P. palmata* and *P. umbilicalis*. Due to the availability of only two species of red algae, it was not possible to determine whether this was a general trend among rhodophytes.

Linear regression analysis of N and amino acid contents showed good correlation across all assayed species (Fig. S1), as expected from the existing literature (Angell, Mata, de Nys, & Paul, 2015; Finkel, Follows, Liefer, Brown, Benner, & Irwin, 2016; Lourenço, Barbarino, Lavín, Lanfer Marquez, & Aidar, 2004). Nonetheless, there is a statistically significant difference in the N-ratios of some species, in particular *S. latissima*, *H. elongata* and *L. digitata*, which appeared to have generally lower N-ratios, while *A. esculenta* and *P. palmata* tended to be on the higher end (Table S1). However, this discrepancy is quite minor, and using the apparent N-ratio of 4.14 to estimate protein content in all samples led to an RMSE of 1.27, which is on par with the multivariate spectroscopic models developed in this study. The mean relative error of protein estimation by N-ratio was considerably higher than the PLSR models however, indicating higher experimental error and thus lower reliability. Using the previously suggested N-ratio of 5 (Angell, Mata, de Nys, & Paul, 2015) to predict protein content resulted in a considerable increase in RMSE, up to 2.78, indicating a severe divergence from the amino acid values. Thus, the N-ratio of 4.14 appears to be a better predictor of total amino acid content in North Atlantic seaweed, but these ratios do have a high degree of variability depending on a wide range of environmental factors, in addition to species and geographic location (Forbord, et al., 2020).

The univariate FTIR regression models for the prediction of seaweed protein content showed good prediction capabilities (Fig. 4). Both the approximate integration of the Amide II band and the height of the same peak (determined at a fixed position of 1540 $cm^{-1}$) performed similarly. From these data, using the height (single point intensity) of the Amide II band appears to be more reliable, likely due to overlaps from other nearby spectral bands on the fringes of the Amide II band. PLSR models of amino acid content and FTIR or NIR spectra provided greater predictive strength, as expected from a multivariate model. The mean relative error for the PLSR models especially was considerably lower than all the others, at around ± 0.01 % using both spectroscopic techniques (Table 2). These spectroscopic methods have the distinct benefit of requiring considerably less sample preparation than traditional methods (essentially only drying and milling) and thus provide a protein estimate far faster than any of the other methods, while using little to no chemicals (such as KBr for the DRIFTS analysis) with lower relative error of measurement. This makes spectroscopy a sustainable and economically viable alternative to both colorimetric methods and *N*-conversion factors while being able to perform the measurement and calculate an estimate within seconds with a high degree of accuracy. Multivariate modelling is also far more resilient to errors caused by non-proteinaceous compounds, which interfere with colorimetric assays. However, both spectroscopic methods used in this study were highly sensitive to interference from moisture. Thus, dehydration is always necessary, which increases processing costs and energy usage. A further study should endeavour to develop an in-field method using portable equipment and spectroscopic techniques that are not (or considerably less) sensitive to water, such as attenuated total reflectance (ATR) FTIR spectroscopy, which works well for protein content estimation in, for example, algal samples (Ferro, Gojkovic, Gorzsas, & Funk, 2019). The ability to predict the protein contents of seaweed directly on-site could enable optimisation of harvest timing, ensuring high macronutrient values for the end product.

## 5. Conclusions

The Lowry and BCA assays are not suitable for protein estimation in seaweed due to apparent interference from substances present in the seaweed, especially brown seaweed. Protein-estimation by use of *N*-conversion factors is limited by differences between species but also by other factors. The Amide II band of DRIFTS spectra can be used to approximate protein content in seaweed by linear regression modelling, but a considerably higher degree of prediction accuracy is possible when using multivariate PLSR modelling of either DRIFTS or NIR spectra. The experimental error of the PLSR analyses was also considerably lower than the univariate analyses and prediction by N-ratio, making these multivariate techniques highly reliable. Spectral protein estimation in seaweed is a rapid and environmentally sustainable alternative to more conventional methods.

## CRediT authorship contribution statement

**Calle Niemi:** Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization. **Agnes Mols Mortensen:** Writing – review & editing. **Ralf Rautenberger:** Writing – review & editing. **Sanna Matsson:** Writing – review & editing. **András Gorzsás:** Methodology, Writing – review & editing. **Francesco G. Gentili:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.134700.

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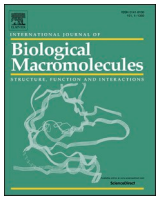

# Quantitative and qualitative saccharide analysis of North Atlantic brown seaweed by gas chromatography/mass spectrometry and infrared spectroscopy

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ABSTRACT

Brown seaweeds contain a variety of saccharides which have potential industrial uses. The most abundant polysaccharide in brown seaweed is typically alginate, consisting of mannuronic (M) and guluronic acid (G). The ratio of these residues fundamentally determines the physicochemical properties of alginate. In the present study, gas chromatography/mass spectrometry (GC/MS) was used to give a detailed breakdown of the monosaccharide species in North Atlantic brown seaweeds. The anthrone method was used for determination of crystalline cellulose. The experimental data was used to calibrate multivariate prediction models for estimation of total carbohydrates, crystalline cellulose, total alginate and alginate M/G ratio directly in dried, brown seaweed using three types of infrared spectroscopy, using relative error (RE) as a measure of predictive accuracy. Diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS) performed well for the estimation of total alginate (RE = 0.12, $R^2$ = 0.82), and attenuated total reflectance (ATR) showed good prediction of M/G ratio (RE = 0.14, $R^2$ = 0.86). Both DRIFTS, ATR and near infrared (NIR) were unable to predict crystalline cellulose and only DRIFTS performed better in determining total carbohydrates. Multivariate spectral analysis is a promising method for easy and rapid characterization of alginate and M/G ratio in seaweed.

## 1. Introduction

Seaweeds represent a rich source of compounds and materials which have a wide array of uses [1]. They contain numerous complex carbohydrates including several types of dietary fibers which have potential health benefits [2], and which have structural properties which make them useful for industrial purposes. Carbohydrates often represent the largest component of seaweed biomass by dry weight (DW), sometimes as much as 70 % in brown seaweed [3]. Understanding and characterizing the polysaccharide contents of seaweed biomass is important for estimating their chemical properties and consequently their potential uses [19].

The unique gelling qualities of many seaweed polysaccharides have led to them being used for thickening or binding in many common food items as well as being used in a variety of research fields and for medical purposes [1,4–6]. Brown seaweed contains a number of unique polysaccharides, including fucoidan, laminarin and alginate. Fucoidan, as the name implies, consists largely of fucose, but with occasional sulfate-modifications. The minor monosaccharides, xylose, galactose, arabinose and rhamnose, can be also found in fucoidan along with fucose as the main sugar in the backbone [7]. Laminarin is a seaweed-specific storage glucan consisting of glucose residues bound together by β-1,3- bonds, with branching β-1,6- bonds [8]. The most abundant polysaccharide, and often the most abundant biomolecule in brown seaweed in general, is alginate [3]. Alginate is a polymer largely specific to brown seaweeds, which is currently used in several industries, particularly for biodegradable food packaging [9]. Alginate and other seaweed phycocolloids are optimal for use as biodegradable films for foods such as fruits, as they are edible, impermeable to oxygen, prevent microbial contamination, and protect the food during transportation [10]. Alginate is also commonly used in the dental industry for taking dental imprints for diagnosis or to be used as molds for prosthetic implants [11]. More recently, the potential for using alginate as a 3D-printing material has also been investigated [12], including the production of 3D-printed

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agar/alginate-supported hydrogels used as scaffolds for bioprinting of live cells for tissue reconstruction [13]. The production of biodegradable plastics incorporating alginate is also being investigated [14].

In addition to the interesting structural uses for alginate, it also has the ability to chelate metal ions which has proven to be useful for wastewater remediation. It has been shown that alginate can be used to remove heavy metal contaminants from wastewater streams, including $Pb^{2+}$, $Cu^{2+}$, $Cd^{2+}$, and that these metals can be recovered from the resulting alginate gel by calcination at elevated temperatures, resulting in metal oxide nanopowders [15]. Furthermore, alginate contains a large number of hydroxylic and carboxylic moieties which can be chemically modified, enabling vast customization of its physicochemical properties [16]. This further indicates the variety of uses for seaweed polymers. Comprising as much as 30–45 % of the total DW of certain brown seaweed species [3], alginate is a very abundant material, suitable for industrial-scale production. Seaweeds are rapid-growing and naturally occurring in a wide range of geographical areas, and its cultivation and harvest on industrial scale is expanding [17].

Structurally speaking, alginate is a linear polymer consisting of the uronic acid residues β-D-mannuronic (M) and α-L-guluronic (G) acid [18]. The ratio and distribution of the M and G residues within the linear chain determines the physicochemical properties of the polymer, so these factors must be considered when using alginate for specific industrial processes [19]. The M and G monomers can be found either in hetero blocks (mixed M and G), M-blocks (stretches of just M-residues) or G-blocks (stretches of G-residues), which heavily influences the rigidity of the resulting gel or film. The C1 and C4 glycosidic bonds within G-blocks have an equatorial conformation which places the carboxylic moieties in a position that facilitates alginate's binding of metal ions (commonly $Na^{+}$, $Ca^{2+}$, $Mg^{2+}$, $Sr^{2+}$, $Ba^{2+}$) in a so-called egg-box structure [9,18]. The glycosidic bonds of M-blocks on the other hand have an axial formation which does not form an ion-binding site. The binding of divalent metal ions in G-blocks enhances the rigidity of the gel, as the electrostatic interactions between the anionic charges of the alginate fiber and the cationic charges of the metal ions allow for intermolecular ionic crosslinking. The M/G ratio is thus one of the main factors determining the properties of alginate gel. This ratio is typically decided through time-consuming methods involving partial hydrolysis and $^{1}H$ liquid-state Nuclear Magnetic Resonance (NMR) spectroscopy or $^{13}C$ NMR, or colorimetric estimations, but it has been shown that the M/G ratio of extracted alginate can also be estimated more rapidly through infrared spectroscopic methods [20,21]. Estimation of M/G ratios directly in brown seaweed has also been performed by calculating ratios between specific absorbance bands in the infrared spectrum [22]. Fourier transform infrared spectroscopy (FTIR) in particular has been used more and more frequently in the last two decades to study alginate, and spectral characterization of seaweed polysaccharides is a growing field of study [23–26].

In the present study, the carbohydrate profiles of four species of brown seaweed from the North Atlantic region are determined, namely *Alaria esculenta*, *Saccharina latissima*, *Laminaria digitata*, and *Himanthalia elongata*, and this experimental data is used to assess the viability of spectral methods for estimating carbohydrates in seaweed. Seaweeds from primarily Norway and the Faroe Islands are characterized, with a few additional samples from Ireland and Greenland. Crystalline cellulose contents are estimated by the Updegraff method and anthrone assay. The total carbohydrate and alginate content as well as detailed monosaccharide composition is estimated by complete sulfuric acid hydrolysis of polysaccharides followed by identification and quantification of monosaccharide species using gas chromatography/mass spectrometry (GC/MS). Moreover, the GC/MS technique was used to quantify M and G contents in the seaweeds, which to the authors' knowledge has not been done previously. The potential for using infrared spectroscopic techniques coupled with multivariate analysis to estimate total carbohydrate, total crystalline cellulose, total alginate contents and the alginate M/G ratio directly in brown seaweed biomass is investigated, using three different spectroscopic techniques and partial least squares regression (PLSR) multivariate analysis. The validity of PLSR predictions is assessed through identification of relevant spectral bands in the regression coefficients of the resulting models. This study therefore serves to show that alginate and M/G ratio can be estimated from brown seaweed biomass without the need for chemical characterization methods.

## 2. Material and method

### 2.1. Sampling and pre-processing of seaweed

The seaweed samples used in the present study have been described in detail previously [27]. Briefly, all samples were dried by either hot-air drying or freeze-drier, and shipped to Umeå, Sweden, for milling and analysis. Samples were milled using a 400 MM Mixer Mill (Retsch GmbH, Haan, Germany) until the whole sample could pass through a 250 μm sieving screen, to ensure small enough particles for efficient extraction. In total, 38 samples of brown seaweed, mostly from the Faroe Islands as well as Tromsø and Bodø in Norway, were analyzed. Biological replicates per species and location are shown in Table 1.

### 2.2. Updegraff cellulose and anthrone assay

Amorphous polymers and soluble sugars were removed from samples by suspension of 3 (±0.2) mg algal powder in 1.5 mL Updegraff reagent [28], consisting of acetic acid:nitric acid:water in a 8:1:2 ratio (v/v). Samples were heated at 100 °C for 30 min, and allowed to cool down to room temperature before being centrifuged at approx. 18,700 ×*g* for 10 min at 15 °C. The supernatant was removed, and the pelleted cellulose was washed once with 1.5 mL water and once with 1.5 mL acetone, by centrifuging as previously described. The pellet was dried under vacuum overnight.

Saeman hydrolysis was used to break down the crystalline cellulose into glucose [29], by suspension 72 % sulfuric acid ($H_2SO_4$). Samples were shaken for 30 min, sonicated for 15 min, and shaken for another 15 min. Water was added to dilute the acidic sample, and 20 μL was used for colorimetric quantification using the anthrone assay (Scott and Melvin, 1953). The sample hydrolysate was diluted in deionized water to a total volume of 200 μL, and the same was done with a glucose standard curve of 0, 25, 50 and 100 μg $mL^{-1}$. To both samples and standards, 400 μL 0.2 % anthrone reagent in concentrated sulfuric acid (w/v) was followed by immediate vortexing. Samples were kept under aluminum foil to avoid photodegradation. Samples were heated at 100 °C for 5 min, and cooled down on ice. The absorbance was measured at 620 nm using an Epoch 2 microplate spectrophotometer (BioTek, Winooski, Vermont, U.S.), and the glucose standard curve was used to calculate glucose, and by extension cellulose in the samples.

### 2.3. Trimethylsilyl (TMS)-derivatization and GC/MS analysis of monosugar residues

For the determination of total monosaccharide residues in the seaweed samples, 500 (±30) μg sample was pelleted using a glass capillary (Microcaps, Drummond Scientific Company, Broomall, U.S.), in quadruplicate for each sample and 30 μg inositol was added as internal standard. The monosaccharide standards, consisting of arabinose, rhamnose, fucose, xylose, glucuronic acid, galacturonic acid, mannose, glucose and galactose (Merck KGaA, Darmstadt, Germany) as well the two alginate residues mannuronic (Merck KGaA) and guluronic acid (MCE, Princeton, NJ, USA) were prepared in 10, 20, 50 and 100 μg per each monosaccharide, except for the M and G monosaccharides, for which only 20, 50 and 100 μg were used, since these were expected to be highly abundant and did not need the lowest data point. The water from the standards was evaporated by sparging with $N_2$ gas in a heating block at 60 °C for 15–30 min, until fully dry. For complete polysaccharide

**Table 1**
Carbohydrate contents and sample numbers of analyzed brown seaweeds.

| Species | Region | Sample number | Total carbohydrates | Cellulose | Alginate | M:G ratio |
|---|---|---|---|---|---|---|
| *A. esculenta* | Bodø | 3 | 33.4 ± 3.8 | 3.6 ± 0.1 | 27.3 ± 2.2 | 5.3 ± 0.8 |
| | Tromsø | 3 | 37.3 ± 2.5 | 2.1 ± 0.4 | 29.8 ± 1.8 | 3.9 ± 0.6 |
| | Faroe Islands | 9 | 37.3 ± 8.5 | 2.8 ± 0.6 | 26.4 ± 5.4 | 2.4 ± 0.5 |
| | Ireland | 3 | 59.1 ± 2.2 | 4.6 ± 1.3 | 43.8 ± 5.5 | 3.9 ± 1.2 |
| | Greenland* | 1 | 31.9 ± 4.9 | 4.7 ± 0.2 | 20.9 ± 3.8 | 3.8 ± 0.4 |
| *H. elongata* | Faroe Islands | 3 | 30.6 ± 3.1 | 2.3 ± 0.2 | 21.7 ± 3.0 | 2.5 ± 0.1 |
| *L. digitata* | Bodø | 3 | 51.3 ± 5.8 | 4.5 ± 0.8 | 42.6 ± 5.5 | 3.0 ± 0.1 |
| | Tromsø | 3 | 40.3 ± 6.2 | 4.2 ± 0.3 | 30.1 ± 5.6 | 2.9 ± 0.5 |
| | Faroe Islands* | 1 | 48.0 ± 1.7 | 5.6 ± 1.0 | 34.0 ± 1.2 | 4.7 ± 0.3 |
| *S. latissima* | Bodø | 3 | 39.9 ± 0.7 | 3.6 ± 0.7 | 32.4 ± 0.1 | 4.9 ± 0.6 |
| | Tromsø | 3 | 60.3 ± 7.9 | 4.7 ± 0.9 | 45.1 ± 7.0 | 7.0 ± 1.7 |
| | Faroe Islands | 3 | 19.1 ± 3.0 | 2.9 ± 2.3 | 12.2 ± 1.6 | 2.5 ± 0.5 |

All units are in %DW, except the M/G ratio. The SD of technical and biological replicates is reported, except for the single-replicate samples labeled with *, for which only the SD of technical replicates are reported.

hydrolysis, 72 % sulfuric acid was added to all samples and standards, followed by sonication for 30 min. The hydrolysates were incubated at room temperature for 2 h. The acidic hydrolysate was diluted with ultrapure water, and the slurry was boiled at 100 °C for 150 min. After cooling down, the hydrolysates were centrifuged at approx. 18,700 ×*g* for 5 min, and the supernatant was collected for further processing.

The acidic hydrolysates were neutralized by addition of calcium carbonate ($CaCO_3$). The samples were centrifuged at 18,700 ×*g* for 10 min and the supernatant was collected. To further clear up the samples, centrifugation was repeated and the supernatant was collected to 6 mL glass tube, which was then dried by sparging with $N_2$ gas in a heating block at 60 °C, and in a vacuum chamber with phosphorus pentoxide desiccant overnight, to ensure minimal water content for the following methanolysis. 600 μL 2 M HCl/MeOH was added as methanolysis reagent, flushed briefly with $N_2$ gas, the cap was screwed on and the samples were incubated at 85 °C for 24 h.

The solvent was evaporated by sparging with $N_2$ at 40 °C. The dry sugars were washed twice with 300 μL methanol, evaporating the methanol between washes as previously described. Silylation of the methanolysed monosaccharide residues [30] was performed by addition of 200 μL silylating reagent (85,431; Merck KGaA), followed by heating at 80 °C for 20 min. The tubes were allowed to cool, and most of the solvent was evaporated under a stream of $N_2$. The pellet was dissolved in 1 mL hexane, centrifuged at 18,700 ×*g* for 5 min and filtered through glass wool. The filtrate was concentrated down to approx. 100–200 μL of which 0.5 μL was used for quantification by GC/MS (7890A/5975C; Agilent Technologies, Santa Clara, U.S.) [30]. The separation of silylated monosaccharides were performed on a J&W DB-5MS column (30 m length, 0.25 mm diameter, 0.25 μm film thickness; Agilent Technologies, Santa Clara, U.S.) with the oven program: 80 °C followed by a temperature increase of 20 °C/min to 140 °C, holding for 2 min, then 2 °C/min to 200 °C, holding for 5 min, then 30 °C/min to 250 °C for 5 min. The total run time was 47 min.

Raw data MS files from GC/MS analysis were converted to NetCDF format in Agilent Chemstation Data Analysis (Version E.02.00.493) and exported to RDA (version 2016.09; Swedish Metabolomics Centre (SMC), Umeå, Sweden). Data pretreatment procedures, such as baseline correction and chromatogram alignment, peak deconvolution and peak integration followed by peak identification was performed in RDA. Certain peaks associated with M and G residues overlapped with other monosaccharides within the standard mixture, which was confirmed by running these standards separately. Most notably, the highest-intensity M peak between 1116.4 and 1127.7 s overlapped with two minor unspecific peaks from other monosaccharide standards. This overlap could not be fully eliminated, but it was reduced by selectively integrating 216.5–217.5 *m/z*, as the 217 *m/z* ion fragment was the major fragment in M while being less pronounced in these two minor peaks (Supplementary Fig. 1a–b). The brown seaweed samples barely showed indication of these peaks after deconvolution, indicating little influence in quantification of M residues. Further, the most intense galactose peak, the alpha-pyranosyl (α-p) peak between 1262.5 and 1276.5 s, overlapped heavily with G and M, making it unsuitable for quantification in brown seaweed samples (Supplementary Fig. 1c). The beta-pyranosyl (β-p) peak between 1338.7 and 1353.9 s also overlapped with a signal from M residues, but this overlap was eliminated by selectively integrating mass fragments between 203.5 and 204.5, as the 204 *m/z* ion fragment was unique to β-p galactose (Supplementary Fig. 1d). This peak was thus used for galactose quantification.

### 2.4. Infrared spectroscopies

#### 2.4.1. Diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS)

DRIFTS was evaluated as a potential method for polysaccharide analysis in dried macroalgae. Measurements were carried out using a previously established protocol [27,31]. DRIFTS measurements were performed using an IFS 66 v/S vacuum spectrometer (Bruker Optik GmbH, Ettlingen, Germany) on dried seaweed samples mixed with KBr in an approximate 1:10 sample:KBr ratio by volume. Spectra were recorded over the region of 4000–400 $cm^{-1}$ at a resolution of 4 $cm^{-1}$, co-adding 128 scans per sample with pure KBr subtracted as background using the manufacturer's software (OPUS, version 5, Bruker Optik GmbH). Spectra were processed using the MCR-ALS GUI, available at the Vibrational Spectroscopy Core Facility, Department of Chemistry, Umeå University (v4c, https://www.umu.se/en/research/infrastructure/visp/downloads/) in MATLAB (version R2017b, MathWorks, Natick, MA, USA). The 800–1800 $cm^{-1}$ fingerprint region contains spectral bands that are strongly correlated to basic biochemical components found in algal biomass, including carbohydrates, and further analysis was limited to this region. All spectra were baseline corrected using asymmetric least squares (AsLS) (lambda = 20,000, $p = 0.001$), and subsequently normalized over the total area of the cut spectral range. Processed DRIFTS spectra were used for PLSR modeling to predict total carbohydrates, cellulose, total alginate and alginate M/G ratio in seaweed.

#### 2.4.2. Attenuated total reflectance Fourier transform spectroscopy (ATR)

ATR FTIR spectra were recorded in the same range and with the same resolution as DRIFTS spectra, using a Vertex 80v FT-IR vacuum spectrometer (Bruker, GmbH). As ATR spectral intensities vary as a function of wavenumbers, baseline correction by AsLS may be suboptimal. Thus, ATR spectra were baseline-corrected in OPUS (version 7, Bruker Optik GmbH) using the built-in 64-point rubberband option, excluding $CO_2$ bands. After baseline correction, the spectra were cut to the 800–1800 $cm^{-1}$ range, total area normalized and used for predictive modeling in the same way as the DRIFTS spectra.

#### 2.4.3. Near-infrared spectroscopy (NIR)

NIR analysis was performed as described previously [27]. In brief, NIR spectra of dried seaweed was captured between 350 and 2500 nm at a resolution of 1 nm using a LabSpec ASD NIR spectrophotometer (Portable Analytical Solutions, Copacabana, NSW, Australia) equipped with a contact probe. Background was removed by blanking with Spectralon white Teflon reference blank supplied by the manufacturer. The visible spectrum was removed, and spectral analysis was limited to 1000–2500 nm, followed by normalization using standard normal variate (SNV) correction. Spectra were averaged over three measurements per sample. Processing was done using Evince software (Prediktera AB, Umeå, Sweden).

### 2.5. Partial least squares regression (PLSR)

Multivariate prediction modeling with spectral data was performed using PLSR, using a method described in Niemi, Mortensen, Rautenberger, Matsson, Gorzsas and Gentili [27]. Briefly, 30 out of 38 samples were selected by random number generation to be used as calibration samples. PLSR prediction models were created from these calibration samples using RStudio Desktop software (RStudio, Boston, Massachusetts, U.S.) with scripts from the PLS package (v. 2.8–0, https://CRAN.R-project.org/package=pls). The optimal component number for each predicted compound and spectroscopic method was determined by leave-one-out cross-validation, and the component with the lowest RMSE of cross-validation was chosen. The models were used to predict the total carbohydrates, total cellulose, total alginate, and alginate M/G ratios of the remaining 8 samples. The accuracy of prediction for all four predicted variables was evaluated in terms of the root mean square error of prediction (RMSEP), the relative error (RE) and the correlation coefficient $R^2$ when comparing known values to predicted values.

## 3. Results and discussion

### 3.1. Crystalline cellulose

Crystalline cellulose contents were determined by the Anthrone assay after removal of amorphous and soluble sugars. In the four species investigated, small quantities of crystalline cellulose were detected, between 2.1 and 4.7 %DW (Table 1). Crystalline cellulose was therefore a minor component of the total carbohydrate profile, compared to alginate as shown by GC/MS. Cellulose contents in all seaweed samples were low compared to higher plant sources of cellulose, including terrestrial energy crops like *Miscanthus* and pine trees but also aquatic weeds like cattail, where cellulose is a primary component of the cell wall and can comprise close to or >40 % of the DW [32]. This seaweed cellulose would thus be of limited industrial use as it can be harvested in larger quantities from other, readily available crops.

### 3.2. Total carbohydrates and monosaccharide profile

The sum of monosaccharide residues determined by TMS of seaweed hydrolysates and GC/MS analysis was used to estimate the total carbohydrate content. The assayed brown seaweed samples ranged in total carbohydrates from approx. 19.1–60.3 % by DW, showing major differences between species but also regional differences (Table 1). *A. esculenta* from most regions included in this study contained between 31.9 and 37.3 %, but the Irish *A. esculenta* had notably higher contents at 59.1 %. Similarly high carbohydrate contents were measured in the *S. latissima* samples from Tromsø, at 60.3 %. The Bodø *S. latissima* samples contained 39.9 % carbohydrates, considerably lower than the Tromsø ones, despite also originating on the Norwegian coast. The Faroese *S. latissima* seaweed had the lowest carbohydrate contents by far at 19.1 %, exhibiting a wide variation within this species potentially depending on growth region. The *L. digitata* samples from both Bodø, Tromsø and the Faroe Islands contained high quantities at 51.3, 40.3 and 48.0 %, respectively. Faroese *H. elongata* had a carbohydrate content of 30.6 %.

Regarding the monosaccharide profiles, all monosugar hydrolysates largely consisted of the two alginate uronic acid residues, comprising approx. 65–86 % of the total monosaccharides by weight (Fig. 1). The third most abundant monosaccharide was glucose, in the range of 10.5–22.4 %DW. Glucose-based polysaccharides besides cellulose, like laminarin and starch, were not specifically measured, but based on existing literature it can be inferred that the majority of non-cellulose glucose residues identified in the samples stem from laminarin, as this is the primary carbon-storage molecule in brown seaweed as opposed to starch [33]. Besides glucose, all samples contained some amount of fucose, approx. 1.4–5.4 %DW in *A. esculenta*, *L. digitata* and *S. latissima*, with *H. elongata* standing out with 13.8 %DW. This fucose was likely stemming from fucoidan. While not exceedingly high in quantity in these samples, fucoidan and laminarin have both been suggested to have potential health benefits and pharmaceutical uses [7,8], and so these polysaccharides also have potential use as high-value extractives in these seaweeds.

Mannose and galactose were also present in minor quantities, approx. 1.2–3.0 and 0.7–2.2 %DW, respectively. Arabinose, rhamnose, xylose, galacturonic acid and glucuronic acid were largely <1 %DW with the exception of the Faroese *S. latissima* which contained 2.1 % galacturonic acid and 2.5 % glucuronic acid.

### 3.3. Alginate contents and M/G ratios

Presuming that the vast majority of M and G residues are present in their polymer form, the total alginate content was estimated from GC/MS measurements of these two monosaccharides. These monosaccharides are not typically measured using GC/MS, but the M and G standards used for calibration and identification of GC/MS data showed strong correlation between signal intensity and concentration, with $R^2$ values at 0.9729 and 0.9769, respectively, indicating the suitability of this method of detection (Supplementary Fig. 2). The validity of the M and G standards was further confirmed by comparing to an alginate standard (A7003; Merck KGaA) processed and analyzed in triplicate using the same procedure as the seaweed samples. The peaks of the M and G standards were confirmed to share positions with the alginate standard (Supplementary Fig. 1).

Alginate was thus estimated to a range of 12.2–45.1 %DW for the samples in this study, typically proportional to the aforementioned carbohydrate contents (Table 1). The Tromsø *S. latissima*, Irish *A. esculenta* and Bodø *L. digitata* samples had the highest alginate content, 45.1, 43.8 and 42.6 %, respectively, while the lowest recorded contents were 12.25 % in *S. latissima* form the Faroe Islands. The vast majority of carbohydrates, and in a few cases over 40 % of the total DW of the seaweed, thus consisted of alginate, presenting a viable resource for industrial purposes.

The measured M/G ratios of most samples were relatively high, in the range of 2.4–7.0 (with individual outliers at 1.79 and 8.25). M/G ratios for alginate are typically reported in the range of 0.5–2.5 [21,23], however higher ratios above 6 have also been reported [25]. These high M/G ratios imply that resultant gel structures would be of low rigidity and high elasticity [19], favoring their use in situations where a high degree of structural rigidity is not necessary, such as biofilms and soft gels. There was a considerable difference in M/G ratio in different regions of origin, with *A. esculenta* from Bodø having an M/G ratio of 5.3, while the other *A. esculenta* samples ranged between 2.4 and 3.9 (Table 1). Also of note is that the three seaweeds with the highest alginate contents had very different M/G ratios, at 7.0, 3.9 and 3.0, respectively. All three of these seaweeds are comparably good sources of alginate, but of likely very differing properties due to stark differences in M/G ratio [19].

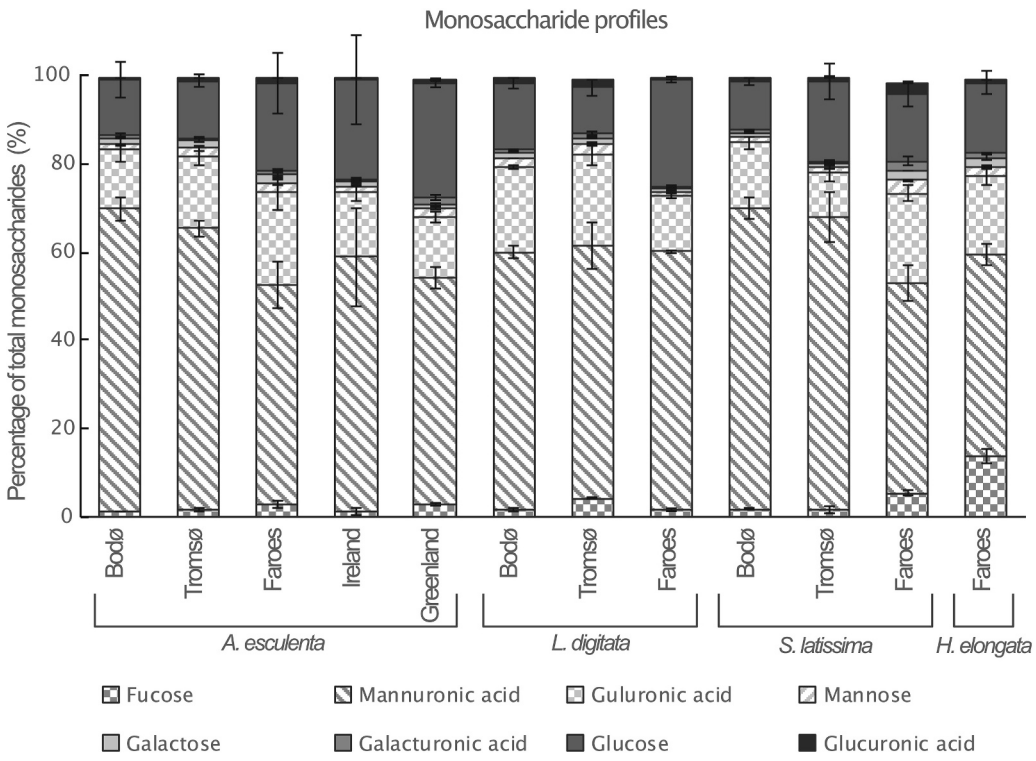


**Fig. 1.** Distribution of major monosaccharide species in brown seaweed. Monosaccharide profiles were measured by GC/MS, and are presented in terms of percentage of total monosaccharides. Error bars indicate standard deviation.

### *3.4. PLSR prediction of carbohydrate contents by infrared spectroscopies*

PLSR modeling of NIR, DRIFTS and ATR spectra was used to predict carbohydrate contents in brown seaweed. For total carbohydrates, all three methods resulted in an RE of approx. 0.11, with NIR and ATR having an $R^2$ just below 0.8 and DRIFTS at just over 0.8 (Table 2). While having very slightly higher correlation between predicted and observed values, the DRIFTS method had the highest RMSEP at 5.72 with the other two both being approx. 5.2. None of the methods were successful in predicting crystalline cellulose contents, having relatively high predictive error and an $R^2$ between 0.2 and 0.3. This is likely due to the spectral signatures of cellulose being almost completely drowned out by overlapping absorbance bands of the far higher quantities of alginate, and the fact that cellulose lacks a unique spectral signature as it is not the only glucose-based polymer in the samples. The presence of other polysaccharides consisting of glucose thus makes it far more impractical to isolate crystalline cellulose from a complex spectrum. The potential difficulties of quantifying polysaccharides with overlapping spectral bands are well documented and it is known that multivariate methods can overcome these issues [34], but in this case the amount of crystalline cellulose is likely too low compared to the much more abundant alginate.

**Table 2**
Prediction results from PLSR modeling of carbohydrate contents using different infrared spectroscopies.

| Predicted quantity | Spectra | Comp. number[a] | RMSEP | RE | $R^2$ |
|---|---|---|---|---|---|
| Carbohydrates | NIR | 5 | 5.20 | 0.11 | 0.79 |
| | DRIFTS | 5 | 5.72 | 0.11 | 0.81 |
| | ATR | 5 | 5.21 | 0.11 | 0.79 |
| Cellulose | NIR | 3 | 1.16 | 0.29 | 0.24 |
| | DRIFTS | 2 | 1.14 | 0.26 | 0.21 |
| | ATR | 3 | 1.16 | 0.26 | 0.26 |
| Alginate | NIR | 3 | 6.75 | 0.19 | 0.52 |
| | DRIFTS | 6 | 4.89 | 0.12 | 0.82 |
| | ATR | 6 | 5.16 | 0.14 | 0.70 |
| M:G ratio | NIR | 5 | 0.70 | 0.19 | 0.68 |
| | DRIFTS | 5 | 0.81 | 0.16 | 0.74 |
| | ATR | 5 | 0.65 | 0.14 | 0.86 |

[a] Comp. number indicates the number of PLSR components used in the model.

Prediction of alginate was most accurate when using DRIFTS, with RMSEP = 4.89, RE = 0.12 and $R^2$ = 0.82, while the other two methods had $R^2 < 0.8$ and higher predictive error. The higher accuracy of DRIFTS prediction of alginate and potentially total carbohydrates when compared to ATR can likely be explained by the fact that the ATR spectrum loses intensity at higher wavenumbers due to decreased sample penetration depth. DRIFTS spectra have more linear correlation between absorbance intensity and quantity of the measured analyte across the spectrum, so it is typically more capable of quantitative analysis. It is worth noting that for prediction of alginate, the NIR model incorporated far fewer components than the FTIR methods, meaning that the risk of overfitting is considerably lower but the model will also be far more simplistic and less comparable to the other models [35]. Using cross-validation, 3 components was calculated to be optimal for NIR while 6 components were calculated for the other two. For the sake of comparison, 6 components was also attempted for NIR modeling of alginate, but this resulted in far higher predictive error (RMSEP = 8.99, RE = 0.27, $R^2$ of prediction = 0.15), likely due to excessive overfitting. The best-performing model is thus reported in Table 2.

For prediction of M/G ratios, ATR proved most accurate with an RMSEP = 0.65, RE = 0.14 and $R^2$ = 0.86. The predicted M/G ratios correlated poorly to the expected ones when using the other two methods, both scoring $R^2 < 0.8$, and the prediction error was considerably higher than with ATR. This could be a potential advantage of the non-linear absorbance intensity of ATR, as it emphasizes bands in the lower wavenumbers of the fingerprint region, including those originating from glycosidic linkages in polysaccharides [34]. This region has more bands which are specific to poly-M or poly-G segments, so decreasing the proportional contribution from higher wavenumber regions could be the reason why ATR fared better in prediction of M/G ratios. The spectra shown in Fig. 2 indicate a clearer correlation between

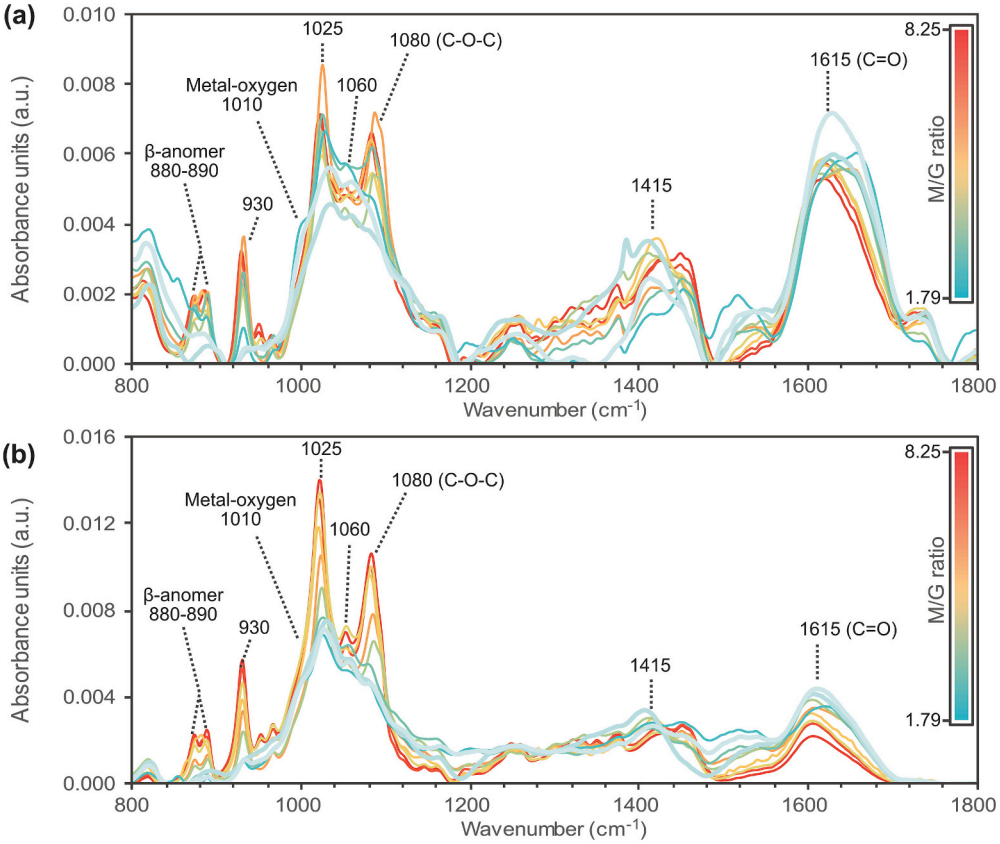


**Fig. 2.** The effect of differing M/G ratios on spectral FTIR absorbance in brown seaweed. (a) DRIFTS spectra and (b) ATR spectra. Potential absorbance bands which are relevant to determining M/G ratio or that are known to be associated with alginate are labeled. The heatmap indicates the M/G ratio of the sample, with red being the highest and blue being the lowest.

M/G ratios and the intensities of certain signature peaks in the lower wavenumbers than in DRIFTS.

The validity of PLSR analysis of spectral data can be verified by comparing the regression coefficients to the presence of bands which are expected to be relevant to predicting the analyte of interest [35]. PLSR coefficients should in principle show strong correlation to peaks in the spectrum which stem from the compound that is being predicted. If the coefficients contain numerous correlations to irrelevant spectral regions, this could be an indicator that the model has been overfitted. For NIR spectra, these coefficients are hard to interpret as they do not contain clearly resolved bands, but FTIR spectra on the other hand contain relatively specific absorbance signatures for specific molecular bonds and functional groups.

Regression coefficients for DRIFTS and ATR spectra indicated several relevant bands for determining alginate (Fig. 3). With both techniques, the band at 1615 $cm^{-1}$ appeared highly correlated with alginate contents, as is to be expected due to the strong C═O vibrations of alginate in this spectral region [25]. The negative correlation to regions immediately above and below correspond to the Amide I and II bands of protein [36]. The coefficients also showed considerable contribution from areas in the 1100–1400 $cm^{-1}$ range, which is a more amorphous region with very few assigned absorbance bands and thus harder to attribute to specific vibrational signatures. The ATR model also heavily emphasized the 1025 $cm^{-1}$ peak, known to correlate to M [20], likely due to the aforementioned relative decrease in high-wavenumber intensities leading to emphasis of lower wavenumber bands.

Using FTIR spectra, PLSR coefficients indicate that two peaks at 880 and 890 $cm^{-1}$ have particularly strong positive correlation to the M/G ratio (Fig. 3B). Peaks in this region are known to be indicative of β-anomeric bonds in saccharides [34], and has been suggested to distinguish M from G in alginate as well [23] since M-residues are β-D-pyranoses while G residues are α-L-pyranoses. In Fig. 2, these bands do appear to increase in intensity and definition along with the increasing M/G ratio, and this appears to be reflected in previous studies of purified alginates as well [20,21].

Three absorbance bands in the 900–1100 $cm^{-1}$ region (930, 1025 and 1080 $cm^{-1}$) stand out as being highly pronounced in samples with high M/G ratios while almost being absent at the lowest recorded ratios. Firstly, the 930 $cm^{-1}$ band increases notably in intensity and sharpness as M-content increases, although the ATR regression coefficient shows considerably lower dependence on this band than DRIFTS. To the authors' knowledge, this band has no previously described association to M content, but it has been shown to be a significant band in alginate and alginate-containing seaweed before [22]. The peak at 1025 $cm^{-1}$ is related to C-O-C stretching in pyran rings [34] and has previously been shown to decrease in relation to a band at approx. 1010 $cm^{-1}$ associated with metal-oxygen interactions, as the M/G ratio decreases [20]. While the 1010 $cm^{-1}$ band is not clearly resolved in these spectra due to the complex composition of the seaweed biomass and the low G-contents of the studied seaweeds, it does appear to be present as a shoulder on the 1025 $cm^{-1}$ peak, shifted closer to 1000 $cm^{-1}$ (Fig. 2). Consistent with existing literature, this shoulder peak appears more pronounced in low M/G samples since there are more binding sites for metal ions in samples with a higher proportion of G-residues. When this shoulder peak increases, the 1025 $cm^{-1}$ band decreases, as described previously by Sakugawa et al. [20].

The sharp peak at 1080 $cm^{-1}$ is also attributed to C-O-C stretching in pyran rings [34], and has been described previously as being associated with both M and G residues, only changing slightly with different M/G ratios in purified alginate [20]. In the present study however, the intensity of this band appears highly dependent on the M/G ratio, as the band is very consistently of higher intensity at higher M/G ratios, while

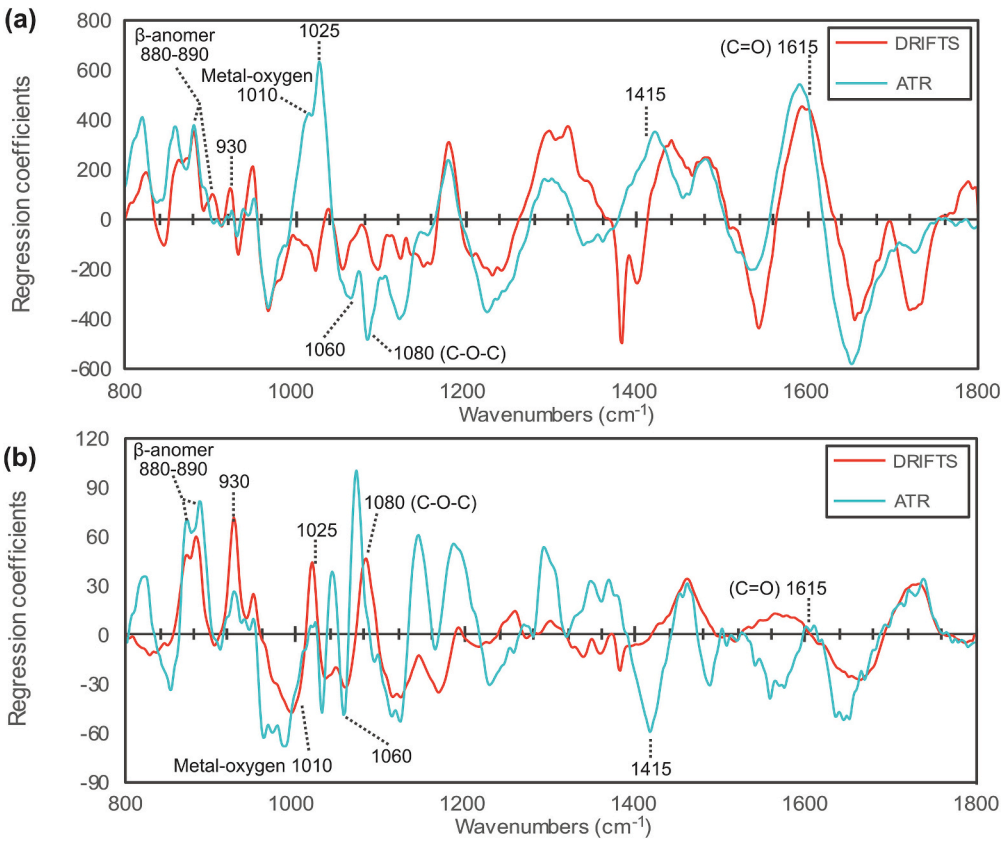


**Fig. 3.** Regression coefficients from PLSR prediction modeling. (a) Total alginate and (b) M/G ratios. Positive values imply positive correlation and negative values imply negative correlation to alginate contents or M/G ratio.

appearing almost absent in both DRIFTS and ATR spectra at the lowest recorded ratios (Fig. 2).

The regression coefficient for ATR indicates a strong negative correlation between M/G contents and a band at approx. 1415 $cm^{-1}$ (Fig. 3B), while DRIFTS does not. There is a band in this area which has been observed in other FTIR analyses of alginate [22,23,25], and it is known that metal-carboxylate compounds have a low-intensity absorbance peak at these wavenumbers [37]. The ATR spectra show a slight increase in this band along with a significant shift towards lower wavenumbers at lower M/G ratios too, while the band is less defined in DRIFTS.

The peak ratios of individual absorbance bands in the FTIR-ATR spectrum have been previously used to achieve approximate estimations of M/G ratios in alginate [20,22]. Due to the complex nature of biological material like seaweed biomass however, the use of multivariate methods can improve the accuracy of prediction as they take into account a far wider array of absorbance signatures [38]. Importantly, the position of alginate's FTIR absorbance peaks are subject to shifts depending on which metal ion the polymer forms salts with [20]. The M/G ratio has been determined previously with good accuracy by calculating the ratio of band intensities at either 1030 and 1080 $cm^{-1}$ or 1010 and 1025 $cm^{-1}$, depending on whether the alginate is in salt form with $Ca^{2+}$ or $Mg^{2+}$, respectively [20]. This was done in purified alginate where the salt type was known however, which works very well, but in its natural form in the seaweed cell wall alginate tends to bind a variety of metals. This likely makes the peaks broader and less defined, in addition to potential overlap with bands from other compounds in the seaweed. Peaks might also be shifted in a more complex chemical environment compared to spectra of pure compounds, as can be seen in the present study where several peaks appear shifted compared to their expected positions. Thus, the PLSR method described here could provide a more accurate estimation of M/G ratio than peak ratio analysis by also taking peak position into account. Beratto-Ramos et al. [39] showed that multivariate curve resolution-alternating least squares (MCR-ALS) could be used to isolate pure spectra of alginate and other polysaccharides from brown seaweed samples, and subsequently for determination of M/G ratio. PLSR has been previously shown by Jensen et al. [21] to be useful for M/G ratio determination in purified alginate, attaining very high accuracy of prediction (RMSEP = 0.07, $R^2 = 0.98$), while also being more reliable for prediction of M/G contents in alginate of different salt types. The present study further demonstrates the use of IR spectral analysis coupled with PLSR to determine M/G ratio directly in seaweed biomass, showing that its usefulness is not limited to extracted alginate.

## 4. Conclusions

The present study provides a detailed account of saccharide contents in North Atlantic brown seaweed of several different species by GC/MS analysis, including the identification and quantification of mannuronic acid and guluronic acid contents by this method. Further, it is shown that there is potential in using infrared spectroscopy coupled with PLSR analysis for the quantification of both total carbohydrates and alginate in brown seaweed biomass, but also for estimating the ratio of M and G residues in the alginate present in this biomass. This multivariate technique could have uses in the seaweed industry, as the M/G ratio heavily influences the properties of the extracted alginate and therefore its potential uses. With a larger data set of a wider range of species, a more complete, general model for prediction of M/G ratios in brown seaweed could be generated, and the technique could be used to predict alginate composition on a routine, large scale. Alternatively, to reduce interference from other compounds, species-specific models could be made which would likely offer higher accuracy but be useful for a narrower range of samples. ATR spectroscopy holds particular promise for future research as it is much less sensitive to moisture than DRIFTS is, and the

use of portable ATR spectrophotometers for direct M/G ratio estimation in wet seaweed could thus be highly useful for determining which seaweed could be used for further industrial processing. Spectral prediction methods also benefit from environmental sustainability due to requiring minimal or no chemical use, as well as being quick and simple to perform.

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## CRediT authorship contribution statement

**Calle Niemi**: Conceptualization, Methodology, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Visualization.

**Junko Takahashi**: Methodology, data processing, Writing - Review & Editing.

**Andràs Gorzás**: Methodology, data processing, Writing - Review & Editing.

**Francesco Gentili**: Conceptualization, Methodology, Resources, Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

## Declaration of competing interest

The authors declare that they have no conflict of interest.

## Data availability

Data will be made available on request.

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