



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

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## ARTICLE INFO

### Keywords:

Seaweed  
Spectroscopy  
Protein  
FTIR  
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<sub>280</sub> 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<sub>280</sub> 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<sub>2</sub>CO<sub>3</sub> in 0.1 M NaOH), Lowry reagent B (1 % (W/V) NaK tartrate tetrahydrate) and Lowry reagent C (0.5 % (W/V) CuSO<sub>4</sub> 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<sub>2</sub>) and ground into flakes using a mortar and pestle. The flakes were loaded into a liquid N<sub>2</sub>-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<sub>2</sub>-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  $\text{CuSO}_4$  contents, it was determined to be a suitable initial solubilisation buffer prior to both assays. The samples were heated at  $55\text{ }^\circ\text{C}$  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  $15,000 \times g$  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 resubilised in 1 mL Lowry reagent D. Twenty microliters of the protein extract was transferred to a fresh tube and 980  $\mu\text{L}$  Lowry reagent D was added to reach a volume of 1 mL. After 10 min of incubation at RT, 100  $\mu\text{L}$  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  $\mu\text{L}$  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  $\mu\text{g mL}^{-1}$  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  $\mu\text{g mL}^{-1}$ .

### 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  $\mu\text{L}$  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  $37\text{ }^\circ\text{C}$  for 30 min, as recommended by the manufacturer. Samples were cooled to room temperature for 30 min prior to the transfer of 200  $\mu\text{L}$  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  $\text{cm}^{-1}$  spectral region at a resolution of 4  $\text{cm}^{-1}$ . 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).

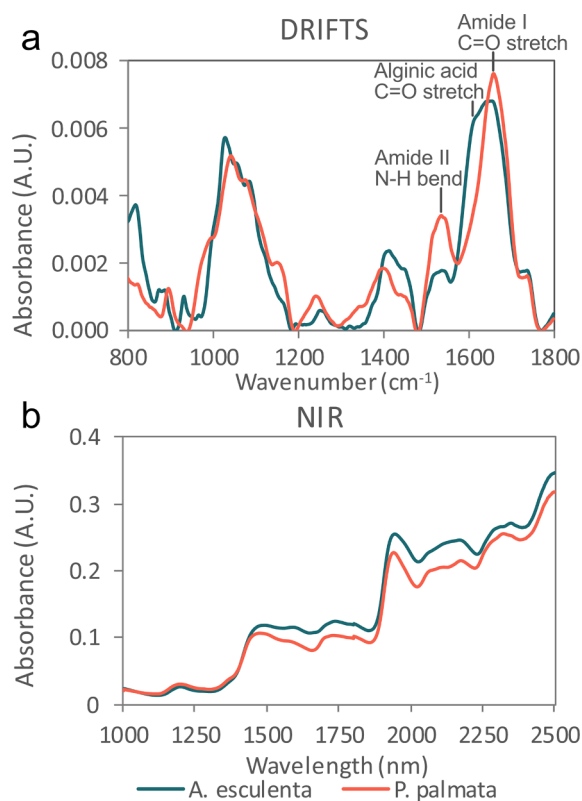
The recorded spectra were exported as .mat files and processed using MCR-ALS GUI, available at the Vibrational Spectroscopy Core Facility, Department of Chemistry, Umeå University (v4c, <https://www.umu.se/en/research/infrastructure/visp/downloads/>) in MATLAB (version R2017b, MathWorks, Natick, MA, USA). All spectra were cut to the 800–1800  $\text{cm}^{-1}$  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  $\text{cm}^{-1}$  (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  $\text{cm}^{-1}$  and 1565  $\text{cm}^{-1}$  (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  $\text{cm}^{-1}$ , 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.

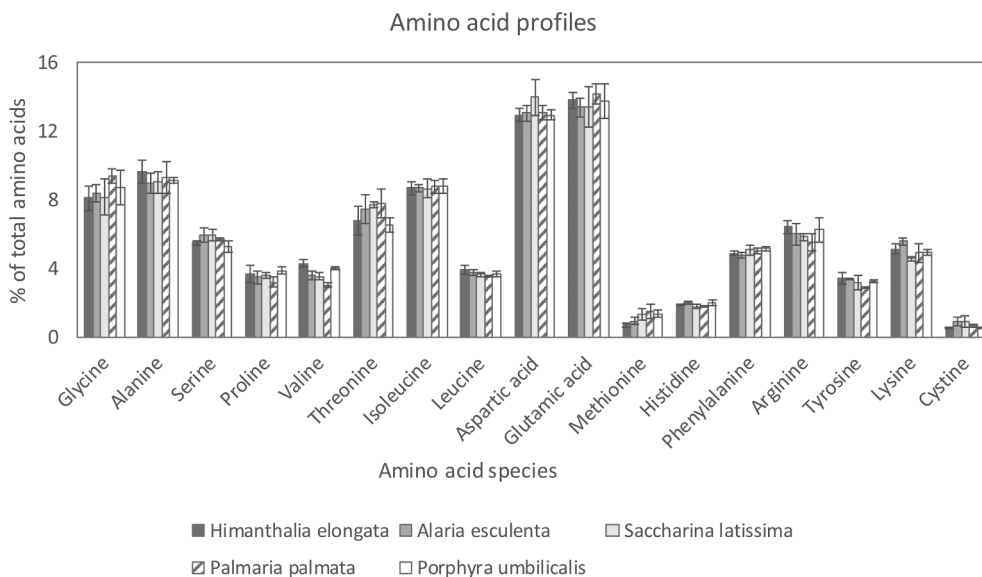
## 3. Results

### 3.1. Total protein and amino acid quantification

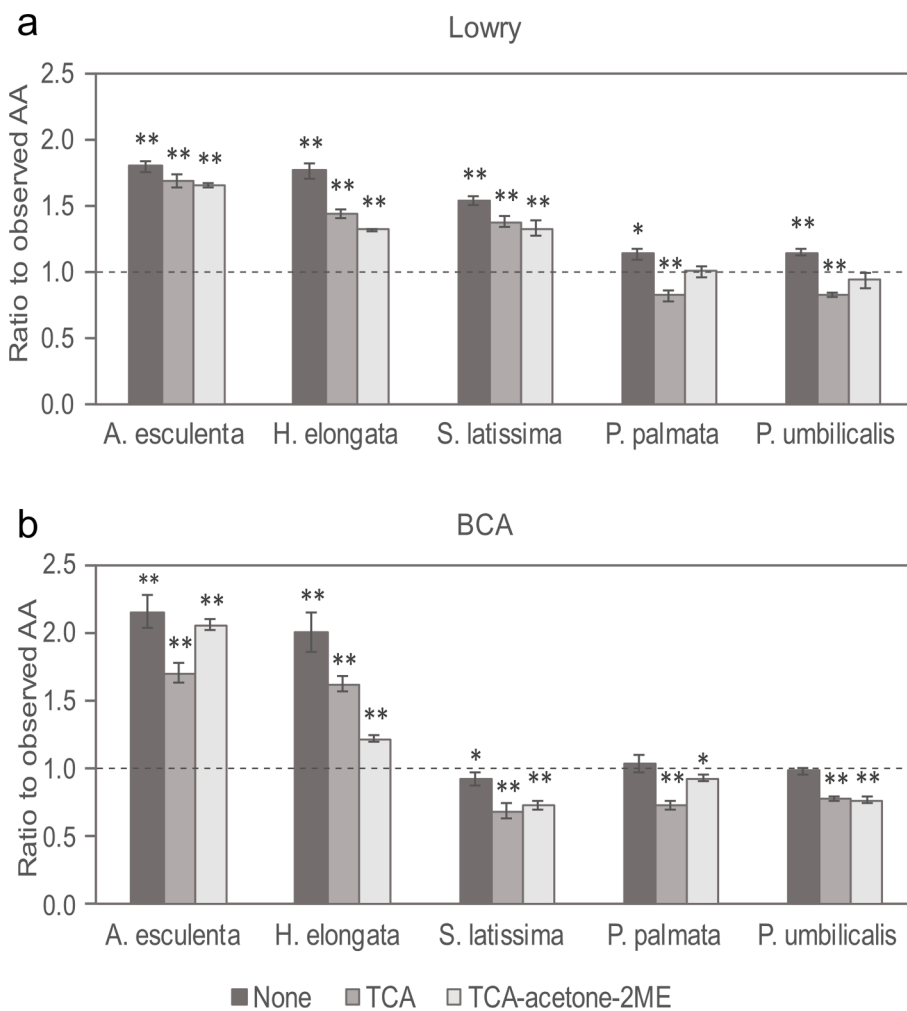
Bound and free amino acids in seaweeds were determined by LC-MS/MS, and the distribution of amino acids in five seaweed species is shown in Fig. 2. The quantity of each identified amino acid was summed up to determine total protein contents. The protein contents of the 45 samples analysed had a wide 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 Faroe 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  $\pm 0.44$ .

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  $\pm 0.53$ , 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 ( $\pm 0.43$  SD) with a mean relative error of approx.  $\pm 0.0211$  %, indicating considerable differences between species. *H. elongata* had the lowest N-ratio ( $3.46 \pm 0.42$ ), while *A. esculenta* had the highest ( $4.49 \pm 0.23$ ), 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 ( $F = 10.83935$ ,  $P = 6.45 \cdot 10^{-6}$ ). 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  $1570$   $\text{cm}^{-1}$ ) and the

height of its apparent maximum (at  $1540$   $\text{cm}^{-1}$ ) 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.  $\pm 0.0261$  % and  $\pm 0.0244$  % 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  $1540$   $\text{cm}^{-1}$ , 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  $\pm 0.01$  % 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  $\text{cm}^{-1}$ , respectively) (Fig. 4a). On the other hand, negative correlations featured bands originating from C–H and =C–H vibrations (1330 and 1460  $\text{cm}^{-1}$ , respectively) as well as from C=O (around 1720  $\text{cm}^{-1}$ ), 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

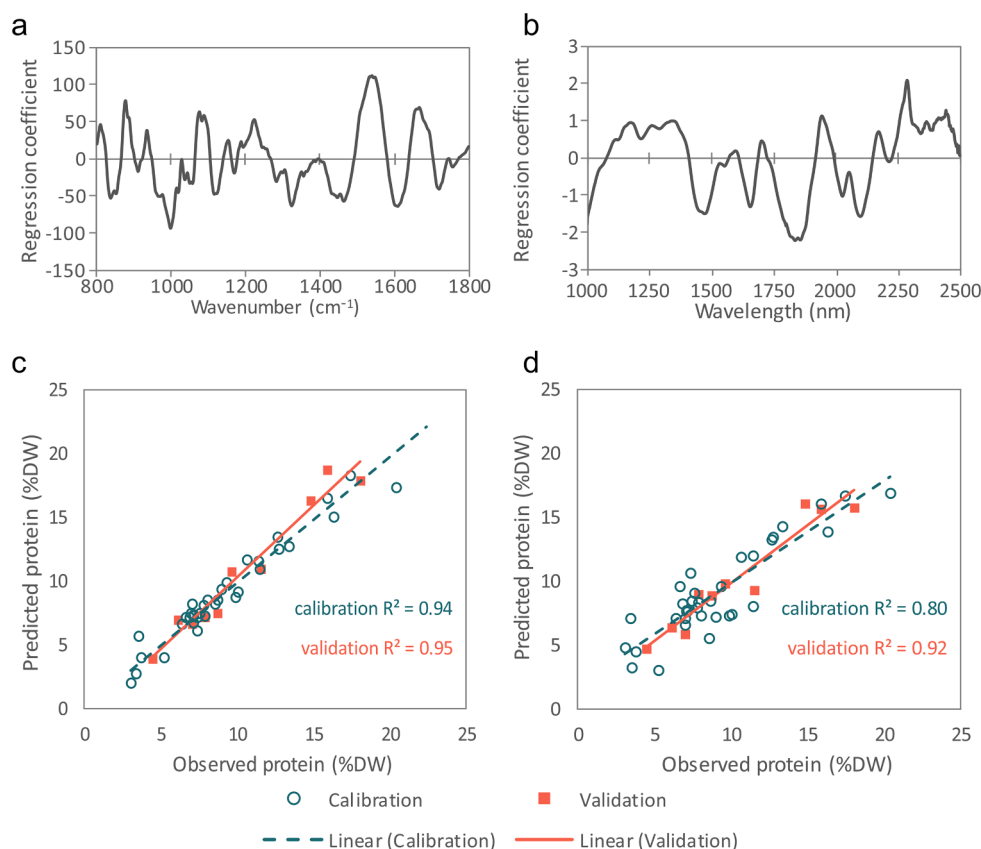
It is well known that commonly used colorimetric assays are prone to over- or underestimation when the protein used as calibration has a considerably different amino acid profile compared to the average protein of the sample (Sapan, Lundblad, & Price, 1999). This is

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

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

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

	RMSEC	RMSEP	Mean rel. error (%)	R <sup>2</sup>
<b>DRIFTS</b>				
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
<b>NIR</b>				
PLSR 5 comp.	1.2614	1.1939	0.0095	0.9258
<b>N-ratio</b>				
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  $1540\text{ cm}^{-1}$ ) 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  $\pm 0.01\%$  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 seaweeds 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 other 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.

## Acknowledgements

The authors would like to thank Paul Geladi and Josefina Nyström at the Department of Forest Biomaterials, Swedish University of Agricultural Sciences, for advice on interpretation of NIR spectra. Furthermore, the authors thank Ann Ruddy, Redrose Developments Ltd, for providing additional seaweed samples. The authors would like to thank the Northern Periphery and Arctic programme of the European Union SW-GROW project for financial support.

## 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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