A validated ultra-performance liquid chromatography with diode array detection coupled to electrospray ionization and triple quadrupole mass spectrometry method to simultaneously quantify taurine, homotaurine, hypotaurine and amino acids in macro- and microalgae

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Abstract

A fast and reliable method for the simultaneous quantification of Taurine, Homotaurine, Hypotaurine and 19 amino acids in algae samples by Ultra-performance liquid chromatography coupled with diode array and tandem mass spectrometry (UHPLC–DAD-MS/MS) was optimized and validated.

Target compounds were chromatographically resolved in less than 15 minutes. (ESI)-MS/MS electrospray ionization and pure analytical standards were used to confirm the identity of all analytes, while quantitation was carried out with diode array detection. Validation parameters of the method were satisfactory: Resolution of peak pairs was always higher than 1.55; all analytical curves showed $R^2 > 0.99$, with working ranges between 0.04 mg/g to 33.1 mg/g and 9.13 mg/g to 107 mg/g and the Lack-of-fit test was not significant. The intra and inter-day precision of the method (expressed as relative standard deviation) were lower than 6 % and recovery values ranged between 95 % and 105 %. The method was demonstrated to be robust to small deliberate variations of seven variables such sample weight, volume of hydrolysis reagent, hydrolysis time and temperature, derivatization time, column temperature and flow rate.

The mean expanded uncertainty for all the target compounds were 0.7 mg/g with a coverage factor of 2.

Method Limits of detection and quantification varied from $0.005 * 10^{-3}$ mg/g to $0.11 * 10^{-3}$ mg/g and $0.01 * 10^{-3}$ mg/g to $0.22 * 10^{-3}$ mg/g respectively, allowing the routine determination of these bioactive compounds in algae extracts. Therefore, the method was successfully applied for the quantitative determination of the 22 target compounds in five seaweed commercial samples.

Relevant compounds were quantified for the first time in the five algae species, namely: *i*) Taurine in *Gracilaria longissima* and *Chlorella* spp., *ii*) **G**amma-aminobutyric acid in *G. longissima* and *L. japonica, iii*) Hydroxyproline in *G. longissima, Ulva lactuca, Porphyra* spp., and *L. japonica* and *iv)* Homotaurine and Hypotaurine in the five species studied.

Highlights

- Simultaneous analysis of Taurine, Homotaurine, Hypotaurine and 19 amino acids
- The method was validated and applied for the analysis of algae extracts
- Quantitation was performed with DAD to support routine analyses
- Homotaurine and Hypotaurine were quantified for the first time in commercial samples

Keywords

Homotaurine, Hypotaurine, Taurine, amino acids, Algae, UPLC-DAD-MS/MS

1. **Introduction**

Amino acids (**AAs**) are the main constituents of proteins and act as precursors for nucleic acids, hormones, vitamins, and other important molecules. Thus, an adequate supply of dietary protein and amino acids is essential to maintain cellular integrity and function, as well as a healthy state at different stages of life [1, 2].

In the latest years, the interest for the algae in the food market has been increasing due to the many positive nutritional properties and health benefits, including their protein fraction. However, there is still a limited knowledge of nutritional composition across algal species, geographical regions, seasons, all of which can substantially affect their dietary value [3].

The protein content of seaweed and microalgae can vary greatly depending on different factors as specie, environmental growth conditions (geographic area, season, temperature, light, available nutrients, etc.) and stage of algal life cycle [4]. As an example, the protein content of brown algae species (*e.g. Laminaria japonica* and *Undaria pinnatifida*) is relatively low, about 7–16 % on dry weight basis [5]. In contrast, red algae (*e.g. Palmaria palmata* and *Porphyra tenera*) contain 21–47 % protein on dry weight basis [6], and freshwater micro-algae, *as Chlorella vulgaris*, can reach concentrations of protein up to 58 % on dry weight basis [7].

High concentrations of Arginine, Asparagine and Glutamic acid are generally found in many seaweed species [6], but algae protein contain also high proportions of all essential amino acids (**EAAs**) [8], and, in some algae species (*e.g.*, *Porphyra* sp.), EAAs concentration compares extremely well with that of soy and egg protein [6, 8].

Additionally, other amino acids (*e.g.* **H**ydroxyproline, **O**rnithine and **C**itrulline), amino acid-like compounds, such as Gamma-aminobutyric acid (GABA), and mycosporine like amino acids have been occasionally found in seaweed species [9]. GABA is a non-protein amino acid, considered a potent bioactive compound, which has been widely studied because of its numerous physiological functions and positive effects on many metabolic disorders. One of the most important is the hypotensive effect that has been demonstrated in animals and in human intervention trials [10]. In the past, Hydroxyproline (Hyp) has been considered to have little nutritional significance, but it is now recognized as a substrate for the synthesis of glycine, pyruvate, and glucose, and an oxidants scavenger which may regulate the redox state of cells [11, 12].

Algae can also be a source of **sulfonic acid derivatives**, like Taurine, Hypotaurine, and Homotaurine (Figure 1), which may play important roles in human and animal health, due to their properties to prevent neurodegenerative diseases.

Taurine (2-aminoethanesulfonic acid) is an amino acid–like compound widely distributed in animals and an essential nutrient in some species. It is involved in the regulation of neuroendocrine functions and nutrition [13], and can show anti-obesity effects in humans [14]. Taurine can effectively prevent glutamate-induced neuronal injury in cultured neurons [15], may play an important role in inflammation associated with oxidative stress [16], and can protect against H_2O_2-induced cell injury in PC12 cell cultures [17].

Homotaurine (3-Amino-propanesulfonic acid), which can be found in the market as “tramiprosate” (Alzhemed™), is a small molecule that is naturally present in different species of marine red algae [18]. This compound (an analog of GABA), has been demonstrated to have a neuroprotective effect and has been evaluated as a possible therapeutic agent for Alzheimer's disease [19]. Both in vitro and in vivo models, tramiprosate provide a relevant neuroprotective effect, by

preventing the formation of Aβ fibrils and the β-sheet conformation and plaque formation in TgCRND8 mice [20]. Moreover, recent studies have demonstrated positive and significant effects of Homotaurine on the reduction of hippocampal volume loss, on the reduction of global cognitive decline in Apo ε4 allele carriers, and on decline in memory function [21, 22].

Hypotaurine (2-aminoethanesulfinic acid), a non-proteinogenic cysteine-oxoform and an intermediate in the biosynthesis of Taurine found in some species of green algae, shows a strong free radical detoxifying action as well as other healthy properties such as antihypertensive and hypocholesterolemic [23]. Fontana et al. [24] pointed out that Hypotaurine is a strong antioxidant in vivo, and a protective agent preventing damage from oxidizing and nitrating agents under physiological conditions, while Araki et al. [25] showed that Hypotaurine may exhibit cytoprotective effect against H_2O_2-induced cell damage by scavenging hydroxyl radicals in placental trophoblast cells.

Analysis of amino acids and sulfonic acid derivatives in algae has been typically carried out by chromatographic methods and hyphenated techniques with pre and post column derivatization, such as ion exchange chromatography [26-29] and high-performance liquid chromatography with UV or fluorescence detection [30].

Typical derivatizing agents include, ortho-phthalaldehyde (OPA), 9-fluorenylmethyl chloroformate (FMOC-Cl), phenyl isothiocyanate (PITC), 1-fluoro-2, 4-dinitrobenzene, 1-fluoro-2, 4-dinitrophenyl-5-L-alanine amide, dansyl and dabsyl chloride. Anyway, many derivatizing agents suffer from some limitation; OPA cannot react with secondary amino acids such as proline, FMOC-Cl is fluorescent by itself, and may give rise to disubstituted derivatization products with Tyrosine and Histidine, and Dansyl and Dabsyl chloride reactions proceed very slowly especially with Proline. On the contrary, formation of PITC derivatives is rapid and complete, with both primary and secondary amino acids [31].

Despite the extensive literature available about total amino acid profile in algae, data are scattered among the multiple possible species, and there is limited information regarding the content of sulfonic acid derivatives and GABA and Hyp. For instance, several authors quantified Taurine and main amino acids in some green, red and brown algae species [27, 32, 33], or quantified only Taurine and Homotaurine by HPLC with fluorescence detection in several non-commercial marine macro algae [30]. Hypotaurine was detected by UPLC-MS/MS, but not quantified, in a metabolomic study including red, brown and green algae [34], as well as by NMR in the green alga *Ulva lactuca* [35]. GABA and Hyp have been previously quantified at low or even trace amounts in several red, green and brown algal species by colorimetric and chromatographic methods, but only in two works both compounds were considered [32, 36, 37, 50].

So, to the best of our knowledge, there is no published analytical procedure allowing the simultaneous quantification of the amino acid profile (including GABA and Hyp) and the main sulfonic acid derivatives (Taurine, Hypotaurine and Homotaurine) in algae samples. Hence, the aim of this work was to develop and validate a fast and reproducible analytical method to simultaneously quantitate the main amino acids plus Taurine, Hypotaurine and Homotaurine in algae samples by UHPLC-DAD-MS/MS.

2. Materials and methods

2.1. Chemicals and reagents

Acetonitrile (ACN) and methanol (MeOH) were HPLC gradient-grade (Merck KGaA (Darmstadt, Germany). Perchloric (60 %) and hydrochloric acid (37 %) were from J.T. Baker (NJ, United States). Sigma-Aldrich Chemie (Sant Quentin Fallavier, France) provided formic acid, ammonium acetate, ammonium formate, phenyl isothiocyanate (PITC), triethylamine (TEA), and pure standards for 19 amino acids, Taurine, Hypotaurine and Homotaurine. Ultrapure water was obtained with a Milli-Q system from Millipore (Bedford, MA, USA).

Single stock solutions were prepared for each of the 22 target compounds [Histidine (His), Hypotaurine (Hyptau), Hydroxyproline (Hyp), Taurine (Tau), Homotaurine (HTau), Arginine (Arg), Serine (Ser), Glycine (Gly), Aspartic acid (Asp), Glutamic acid (Glu), Cysteine (Cys), Threonine (Thr), Proline (Pro), Alanine (Ala), Gamma aminobutyric acid (GABA), Lysine (Lys), Tyrosine (Tyr), Methionine (Met), Valine (Val), IsoLeucine (Ile), Leucine (Leu), and Phenylalanine (Phe)] by dissolving the corresponding pure standards in 0.1 M HCl.

Calibration working solutions were prepared by mixing suitable volumes of each stock solution in 0.1 M HCl, to obtain the following calibration levels for each compound: 0.1 mM, 0.5 mM, 1 mM, 1.5 mM and 2.5 mM.

2.2. Algae samples.

Five different species of algae, including macroalgae (red algae *Porphyra and Gracilaria*, green algae *Ulva lactuca*, brown algae *Laminaria japonica*) and green microalga *Chlorella*, were purchased in their dehydrated form in commercial establishments in Girona (Spain). All samples, except microalgae *Chlorella*, were in the form of flakes or sheets, so it was necessary to reduce the sample size before extraction, by using a mixer mill (Retsch GmbH & Co, KG Germany). The powdered samples were stored at ambient temperature under dry and dark conditions.

2.2. Amino acid extraction and derivatization

The samples were processed following the method of Campanella et al **[38]** with some modifications. Briefly, for the quantitation of the total amino acids, 10 mg of seaweed sample were placed in 15 mL falcon tubes and 1 mL of 8 M perchloric acid was added. Hydrolysis was carried out for 24 h at 110 °C. After cooling at room temperature, the samples were filtered through 0.2 µm membrane syringe filters (GMP filter membranes, Merck KGaA, Darmstadt, Germany), and then derivatized.

The derivatization was carried out following the method of Zheng et al. **[39]**, with some modifications. Sample extracts or calibration solutions (40 µL) were pipetted into 10 mL polypropylene tubes and dried under nitrogen at 60 °C. The dried sample was re-suspended with 40 µL of a methanol-water-TEA solution (2:2:1, v/v/v), dried again under nitrogen at 60 °C, added with 40 µL of a methanol-water-TEA-PITC solution (7:1:1:1, v/v/v/v), and vigorously mixed. The derivatization was performed for 20 minutes at ambient temperature, and then the excess reagent was evaporated under nitrogen at 60 ºC.

The derivatized samples were re-dissolved with 24 µL of mobile phase B and 226 µL of mobile phase A, centrifuged at 11,000 × g for 5 min, filtered through a Thomson Single Step Standard Filter Vials (Thomson Instrument Company, CA, USA), and injected into the UHPLC system (4 µL).

2.3. Chromatographic analysis

The chromatographic system consisted of an Acquity UPLC® (Waters, Milford, MA, USA), equipped with a diode array detector (Acquity PDA detector, Waters, Milford, MA, USA), an electrospray (ESI) as a source of ionization and a triple quadrupole mass spectrometer (Acquity TQD, Waters, Milford, MA, USA) operated at unit mass resolution. The system was controlled by MassLynx 4.1 software (Waters, Milford, MA, USA).

Four columns (100 mm x 2.1 mm i.d.), packed with different stationary phases, were tested, namely: *i*) Charged Surface Hybrid particle with C_{18} reversed phase (1.7 µm, CSH-C_{18}), *ii)* Phenyl-Hexyl reversed phase (1.7 µm, CSH-PH), *iii*) Ethylene Bridged Hybrid particle with C_{18} reversed-phase (1.7 µm, BEH-C_{18}), and *iv*) High Strength Silica particle with trifunctional C_{18} alkyl phase bonded (1.8 µm, HSS-T3) (Waters, Milford, MA, USA).

Optimization of the chromatographic performances was carried out by modifying: *i*) the percentage of organic modifiers (methanol or acetonitrile) in the mobile phase, ii) the pH modifiers (ammonium acetate, ammonium formate, formic acid) in the mobile phase, *iii*) the flow rate and the gradient elution program, and *iv*) the column temperature.

Electrospray interface (ESI) was operated in the positive mode; the source temperature was fixed at 135 ºC, the capillary voltage was set at 3.0 kV and the desolvation temperature was set at 350 ºC. The cone gas (nitrogen) flow rate was 350 L/h and cone voltage was set at 30 V. MS experiments were carried out in "Scan" mode to obtain *m/z* values of the molecular ions. MS/MS experiments in "Daughter Ions" mode were also performed, to obtain the fragmentation patterns of molecular ions. The collision energies varied between 10 and 20 eV (Supplementary material, Table A1). The gas used in the collision cell was argon at a flow rate of 0.1 mL/min.

Identity of the peaks in the sample extracts was confirmed by comparing their retention times, UV spectra, MS and MS/MS spectra with the corresponding data obtained from pure standards.

Quantitation of the target compounds was done based on an external calibration curve and taking into account the sample dilution during the extraction and derivatization steps. Calibration curve was made by injecting derivatized amounts of pure standards in the range from 0.1 mM to 2.5 mM, and by plotting the signal obtained from the diode array detector at λ=254 nm versus the corresponding concentrations.

2.4. Method validation

The whole protocol of analysis was validated in terms of selectivity, accuracy (precision, trueness), linearity and working range, robustness / ruggedness, uncertainty and detection and quantification limits according to Harmonized guidelines for single-laboratory validation of methods of analysis (IUPAC Technical Report), ICH, AOAC, EURACHEM and GUM **[40 - 45]**.

Selectivity is the ability to unequivocally assess the target analyte in the presence of other analytes, matrices or other potentially interfering materials that may be expected to be present in the matrix or sample. Peak resolution for each targeted analyte (R_s) was calculated as a function of both the absolute separation distance expressed as retention times

(minutes) of the two peaks, t_{R1} and t_{R2}, and the peak widths at half height, $W1_{1/2}$ and $W2_{1/2}$, of the analyte and nearest peak (Equation 1).

$$R_S = 1.18 \times \left(\frac{t_{R2} - t_{R1}}{W1_{1/2} + W2_{1/2}}\right) \qquad \text{(Eq. 1)}$$

AOAC International has recommended that a suitable Rs value to obtain a usable separation of two peaks is at least 1.5.

The linearity was assessed by checking the following parameters: coefficient of determination (R^2), residual value of replicates, and Lack-of-fit (LoF) test significance. This test, recommended by the IUPAC validation guidelines **[40]**, measure if the regression model fits the data. The extent of deviation of the points from the line caused by random scatter of the points was estimated by the mean sum of squares of random error (MSS_{error}). This was compared to the extent of deviation of the points from the line caused by mismatch of the calibration model (mean sum of squares due to lack of fit MSS_{LOF}; Equation 2).

$$F = \frac{MSS_{LOF}}{MSS_{error}} = \frac{\frac{\sum(\overline{y_i} - \hat{y_i})^2}{n-2}}{\frac{\sum(y_i - \overline{y_i})^2}{n(p-1)}} \qquad \text{(Eq.2)}$$

When the $F_{calculated}$ was lower than $F_{tabulated}$, the model was considered to fit the data.

The linear ranges were assessed by injecting calibration working solutions of pure compounds at different concentrations, ranging from 0.1 mM to 10.0 mM.

The instrument limit of detection (ILOD) and the instrument limit of quantification (ILOQ) were calculated as 3.3σ/b and 10 σ /b, respectively, where "σ" is the Residual Standard Deviation of the Calibration Curve ($S_{x/y}$) and "b" is the slope of regression line from the calibration curves of each compound. The Breush-Pagan test, to establish the presence or absence of heteroscedasticity, was also applied. The method limits of detection and quantification (MLOD and MLOQ, respectively) were estimated from ILOD and ILOQ taking into account the dilution factor and the mass fraction of each sample.

The accuracy of a measurement result describes how close the result is to its true value and includes the effect of both precision and trueness (expressed in the form of bias). Precision, which relates to the repeatability and / or reproducibility condition of the measurement "getting the same measurement each time", was estimated as both intra-day repeatability (RSD_r) and inter-day reproducibility (RSD_R). RSD_r was calculated by analyzing six spiked samples in the same day (n=6), while RSD_R was assessed by analyzing six spiked samples on three different days during the same week (n=18). Precision was expressed by relative standard deviation (RSD %) of the measurements and calculated from Eq. 3

$$RSD\% = \left(\frac{s}{X}\right) x\ 100 \qquad \text{(Eq. 3)}$$

Where "*s*" is standard deviation of replicates and "X" is the arithmetic mean of the measurements.

The repeatability standard deviation varies with concentration, C, that is expressed as a mass fraction. The predicted acceptable value, RSDr, for each concentration is proximate to the value recommended by the FDA Guidelines for the Validation of Chemical Methods for the Food Program, or can be calculated using the Horwitz equation as follows **[40]**:

$$RSDr\ (\%) = 2 * C^{-0.15} \qquad \text{(Eq. 4)}$$

The acceptable values for repeatability are between ½ and 2 times the calculated values.

Trueness (or bias) describes the closeness of agreement between the average of an infinite number of replicate measured quantity values and a reference quantity value. As no commercial Certified Reference Material was available, spiked samples were analyzed to evaluate bias. Accuracy of the method was assessed by analyzing a *Chlorella* sample spiked before hydrolysis with known amounts of pure standards at three levels (0.1, 5.0 and 10.0 mM), to cover the working range of the method. Three sample replicates for each spiking level (n=9) were prepared by adding the suitable volume of the standard solution, allowing the samples to settle for 30 min, and then carrying out the hydrolysis, extraction and derivatization procedures as described above.

The robustness or ruggedness of an analytical method is the resistance to change in the results when minor changes are made from the experimental conditions described in the procedure. Robustness was tested by deliberately introducing small changes into the procedure and examining the effect on the results following the work described by Youden et al **[46],** which suggested variations of selected factors at once.

Robustness of the method was determined on the basis of independent assays of a *Chlorella* sample, following a fractional factorial design obtained by taking into account seven factors (sample weight, volume of hydrolysis reagent, hydrolysis time, hydrolysis temperature, derivatization time, column temperature and flow rate), each of them with two levels (nominal value/alternate value), for a total of eight different combinations analyzed in duplicate (n=16) (Supplementary material, Table A2). Once quantified each amino acid, the difference D_i and the standard deviation of the difference SD_i were calculated (Equation 5).

$$SD_i = \sqrt{2x \sum \left(\frac{D_i^2}{7}\right)} \qquad \text{(Eq.5)}$$

D_i = (difference between the mean concentration obtained with the factor at nominal value and the mean concentration obtained with the factor at alternate value)

When SD_i is significantly lower (significance level of 0.05) than the standard deviation of the method carried out under within-laboratory reproducibility conditions (RSDr) can be concluded there is no global effect of the factors on the result, and the method can be considered robust.

The **uncertainty** estimation was carried out using GUMWorkbench 1.3 TrainMiC software package (Metrodata GmbH) [44]. Among the possible sources of uncertainty (Ishikawa diagram, Supplementary material, Fig. 1), those arising from balances and volumetric measuring devices are covered by the precision and recovery studies since all these instruments are controlled under UNE-EN-ISO/IEC 9001. Sample homogeneity and calibration uncertainties are included in the precision uncertainty because various replicates from the same sample were analyzed and standards were injected each day of analysis. The purity of amino acid standards is given by the manufacturer, but the contribution is so small that could be neglected. So, the expanded uncertainty was estimated using the in-house validation data (precision and trueness).

3. Results and discussion

3.1. Method development

Different chromatographic conditions were explored, by varying column stationary phase, mobile phase composition and gradient elution profile, to reach a suitable chromatographic separation of all the target compounds in a short time with a mobile phase compatible with both DAD and MS detection.

Ammonium acetate, ammonium formate and formic acid, which are volatile and may improve amino acids separation as well as peak shape in UPLC chromatography [47, 48] were employed as pH modifiers, while ACN was preferred as organic modifier because provided better peak shapes than methanol. The flow rate was set at 0.4 mL/min and the column temperature was maintained at 30 ºC.

Preliminary trials showed that mobile phases A (7.5 mmol/L ammonium formate, 7.5 mmol/L ammonium acetate and 0.075% formic acid in aqueous solution) and B (1 mmol/L ammonium formate, 1 mmol/L ammonium acetate and 0.075% formic acid in acetonitrile) gave the better chromatographic performances with all the columns.

On the other hand, stationary phases other than BEH needed larger and more complex elution programs to separate some critical peak pairs, while modifications of the mobile phases were limited, to allow the MS detection. Finally, to show the different behavior of the four columns, the same elution program was used. Elution was carried out by varying the proportion of the mobile phases A and B; the program started with an isocratic elution with 11 % B until 1.3 min., then the percentage of B was increased up to 32 % at 15 min. with a linear gradient. Afterwards, the columns were washed with 80 % B for one min. and re-equilibrated to the initial conditions for 2 min.

Figure 2 shows the capacity factor (k) of the 22 target compounds eluted with the four different columns under the same conditions. The BEH-C_{18} showed a stronger retention ability as well as better resolution of critical pairs under the same condition, allowing a satisfactory chromatographic separation of all the compounds including Tau, Htau and Hyptau. CSH-PH showed poor resolution, while CSH-C_{18} could not separate specific pairs of amino acids such as HTau / Arg, Thr / Cys and Ala / GABA. HSS-T3 is ideally suited for the enhanced retention of polar compounds and metabolites by reversed-phase LC, nevertheless His and Hyp nearly coeluted, and critical pairs His / Hyptau, Thr / Cys, Pro/Ala and Leu/Ile were not resolved. Therefore, method validation was carried out only with the BEH-C_{18} column, which gave the best performances.

Figure 3 shows typical chromatographic separations of the 22 target compounds, for a standard solution and an algae extract, with the BEH-C_{18} column. The high efficiency of the UPLC column allowed a complete separation of Hyptau, Tau, Htau and 19 amino acids derivatized with PITC within 13 minutes with a reasonable resolution of all the critical peak pairs.

This result can be considered satisfactory, taking into account that chromatographic separation of PITC amino acid derivatives is extremely challenging. Other authors, operating with conventional HPLC [49], underlined that the mixture of Ser, His, Glu, Thr, and Arg, as well as Tyr and Leu, could not be completely separated, or reported the incomplete separation of a mixture of Asp, Ser, and Hyp, and of Tyr and Leu. Zheng et al. [39] separated PITC derivatives of 15 amino acids in a total run of 28 minutes by using UHPLC-ESI-MS.

In our study the quantification was performed with the DAD, nevertheless experiments with MS/MS in "daughter" mode were performed to support the identification of the peaks, especially in the case of the three sulfonic acid derivatives. Figure 4 shows the MS/MS spectra of Hyptau and Htau PITC derivatives, and their corresponding peaks found in an *Ulva lactuca* extract. In each spectrum can be recognized the molecular ion of the PITC derivative (Hyptau *m/z* = 245 and Htau *m/z* = 275) and the typical fragmentation pattern which includes, in all cases, the molecular mass of each compound Hyptau *m/z* = 110 and Htau *m/z* = 140).

3.2. Validation parameters

The results of method validation and performance parameters are summarized in Table 1.

The **selectivity** expressed as resolution of peak pairs (R_S) for all the target compounds was ranged between 2.07 – 26.9 and 1.55 – 40.5 in standard solution and samples, respectively (Supplementary material Table A3). Satisfactory chromatographic separation (Rs > 1.5) was achieved for all the amino acid pairs.

Calibrations showed R^2 value always higher than 0.994 for all the compounds and fulfilled the homoscedasticity criterion, and the residual standard deviation approach could be applied. Therefore, $F_{calculated}$ values were lower than $F_{tabulated}$ in the *Lack of fit test;* so the calibration model fitted well with the data for all the 22 compounds.

The linear range was initially tested between 0.1 mM to 2.5 mM. Preliminary analysis of samples gave the need to extent the upper limit of the calibration curve to 10 mM for several amino acids. So the working range for all the amino acids was finally established between 0.1 mM to 10.0 mM (0.04 mg/g and 98.3 mg/g) (Supplementary material, Table A4).

The method limits of detection and quantification were comprised between $0.005 * 10^{-3}$ mg/g to $0.11 * 10^{-3}$ mg/g and $0.01 * 10^{-3}$ mg/g to $0.22 * 10^{-3}$ mg/g respectively, for all the target compounds. Gly was detected at the lowest detection and quantification limits. Hyptau was detected at the highest detection and quantification limits.

Precision, expressed as RSD%, ranged from 1.0 % to 4.7 %, (intra-day) and from 1.7 % to 5.8 % (inter-day). The results indicated that there is no remarkable variability in precision at different concentrations measured on the same or in different days.

Method **recoveries** for overall amino acids at different concentrations were found within the range of 95 % and 105 %. These results indicate that the bias due to the effects of operating on the added analyte, which was conducted

independently on different days, were very small. The results largely achieved the accepted value of recovery at certain analyte concentration levels recommended by the IUPAC Technical report [40].

Regarding the **robustness** of the method, all the SD_i values were lower than the relative standard deviation of within lab reproducibility meaning that the method is robust for the 7 factors studied (Table 1). However, there are specific variables that contribute to a larger variation of D_i. For instance, variations in volume of perchloric acid and temperature of hydrolysis have both the major differences for all the amino acids while hydrolysis and derivatization times have the minor effect.

Results for standard and expanded **uncertainty** are summarized in *Supplementary material Table A5*. Expanded uncertainty was calculated for a level of confidence of approximately 95% considering a coverage factor of 2, because when assuming infinite degrees of freedom, t-Student distribution tends to a normal distribution. As shown in Table A5, the values of uncertainty for each amino acid due to within lab reproducibility study, u (RSD_R), ranged between 0.17 mg/g – 3.95 mg/g, where Met and Arg represented the lowest and highest uncertainty of method precision, respectively. Therefore, the uncertainty of method recovery ranged between 0.07 mg/g to 2.75 mg/g. Hence, the precision is the largest contribution to the measurement uncertainty. Since this component is derived from the overall variability in the method, further experiments would be needed to show where improvements could be made. Finally, the expanded uncertainty for all the 22 compounds was ranged between 0.001 mg/g to 2.5 mg/g, and the mean expanded uncertainty was 0.7 mg/g.

Although several papers can be found about the analysis of total amino acids in algae, few of them include results of validation studies. Considering only published methods which provided data about validation, the overall chromatographic performances of our method are similar or better, especially the higher number of compounds simultaneously quantified and the shorter total run time. For instance, Sanchez-Machado et al. **[50]** reported the separation of 17 amino acids in algae with a total run of 35 min., with RSD% values ranging between 1.3 % and 3.8 % and estimated instrument limits of detection ranged between 6.9 ng/mL and 13 ng/mL. Other authors **[30]** developed an HPLC-FLD method to separate and quantitate only Tau and Htau in a total run of 20 min. In this case the RSD% and recovery values were comprised between 2 % and 6 % and 94 % and 110 %, respectively and instrument limits of detection for Tau and Htau were 30 ng/mL and 15 ng/mL, respectively. Campanella et al **[38]** described an HPLC - UV method to analyze 18 amino acids in algae samples in a total run of 30 minutes with recovery values ranging between 87 % and 102 %. Besides, almost all the non-validated methods found in the literature **[26 – 29, 32, 33]** has been performed by using automated amino acids analyzers with longer run times and less compounds quantified. Finally, none of them include Hyptau, Tau and Htau in a single chromatographic run. (Supplementary material, Table A6).

3.3. Analysis of commercial algae samples

Once validated, the method was used to quantify the levels of 19 amino acids, Tau, Htau and Hyptau in five commercial samples of different algae species. Concentrations of each compound are listed in Table 2.

One of the main results of this study is that some relevant compounds were quantified for the first time in the five algae species, namely: i) Tau in *G. longissima* and Chlorella spp., ii) GABA in *G. longissima* and L. japonica, iii) Hyp in *G. longissima*, *Ulva lactuca*, Porphyra spp., and *L. japonica* and iv) Htau and Hyptau in the five species studied. Comparative results from other authors regarding the amino acid content in algae are summarized in Supplementary material, Tables A7- A10.

The overall concentration of EAAs are in good agreement with other studies, which were carried out with *L. japonica*, *Chlorella* spp. and *Porphyra* spp. **[26 - 28]**. Mc Cusker et al. **[27]** reported a content of 37.54 mg/g of EAAs in *Porphyra* spp., which also agrees with our results (**Table 2**). As a rule, the levels of NEAAs found in the five commercial samples were consistent with previously published data, reporting high levels of glutamic and aspartic acids in *Chlorella* spp., *Porphyra* spp. and *Laminaria japonica* **[26 - 29]**. We detected GABA at significant amounts in *Chlorella* spp. (18.49 mg/g) and *Porphyra* spp. (5.90 mg/g). Concentrations of Hyp were generally lower, and only in the case of *U. lactuca* reached 0.95 mg/g (Table 2). As previously outlined, there is a substantial lack of information about the presence of GABA and Hyp in algae. Anyway, Eun-Sun Hwang et al. **[32]** found 0.31 mg/g of GABA in *Porphyra tenera*, which agrees with our results in *Porphyra* spp. In contrast, Brown et al. **[51]** reported lower contents of GABA and Hyp in *Chlorella* spp. strains than in our study. The concentrations of Tau agreed with previous works highlighting the occurrence of this sulfonic acid derivative mostly in red algae species (**Table 2**) **[27, 28, 32]**.

With the proposed method, Hyptau was detected and quantified in the five algae species at concentrations ranging from 0.55 mg/g (*Chlorella* spp.) to 0.19 mg/g (*L. japonica*), while the amount of Htau varied between 4.26 mg/g (*U. lactuca*) and 0.18 mg/g (*L. japonica*) (**Table 2**). Studies about the content of sulfonic acid derivatives in algae has been overlooked, so the comparison with previous works is limited by the substantial lack of data for many species. For instance, Mehdinia et al. [30] quantified Tau and Htau in several marine macro algae and outlined levels between 0.009 mg/g and 2.5 mg/g for Tau and from 0.0003 mg/g to 0.7 mg/g for Htau. In other previous studies, Hyptau was detected in the green alga *Ulva lactuca*, but authors did not report quantitative data **[23, 34, 35]**.

4. Conclusion

In this study, a validated UPLC-DAD-MS/MS method is proposed to simultaneously quantify 19 amino acids and three sulfonic acid derivatives (Hyptau, Tau and Htau), which have been demonstrated to have interesting bioactive functions, with a short chromatographic run (15 minutes). To the best of our knowledge it is the first time that these amino acids and sulfonic acid derivatives are separated and quantified in a single chromatographic run. Both chromatographic performances and validation parameters were satisfactory in terms of resolution of critical peaks pairs, linearity, working range, LOD, LOQ, accuracy, precision and robustness, indicating that the method is suitable for the routinely assessment of the target compounds in algae sample at trace levels. Moreover, the measurement uncertainty of the entire analytical method is reported. The major contribution to uncertainty arises from precision study and expanded uncertainties of amino acids ranged from 0.001 mg/g to 2.50 mg/g. Our method is based on sample derivatization with PITC and DAD detection; a protocol that can be easily implemented for routine analysis of algae samples. Furthermore, the fast and simultaneous profiling of both amino acids and sulfonic acid derivatives makes the proposed method very useful for high throughput

screening purposes, when the occurrence and concentration of these bioactive molecules should be assessed in a wide number of different algae species. The analyses of five commercial edible algae with the proposed method gave results that were generally in good agreement with other studies reporting the amino acid content of algal samples.

Notwithstanding, with the method developed in the present work we quantified for the first time: i) Tau in *Gracilaria longissima* and *Chlorella* spp.*, ii)* GABA in *Gracilaria longissima* and *Laminaria japónica*, *iii)* Hyp in *Gracilaria longissima*, *Ulva lactuca*, *Porphyra* spp., and *Laminaria japónica,* and *v)* Hyptau and Htau in the five species included in this study.

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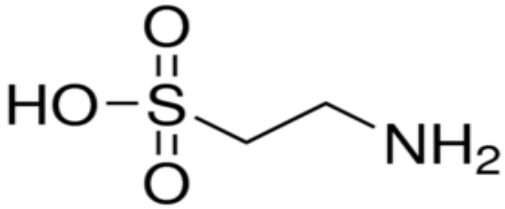
FIGURES & TABLES



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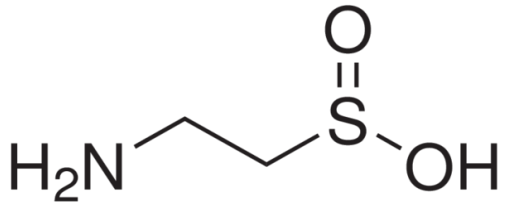
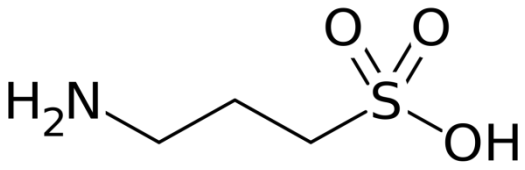


HYPTAU



HTAU

Figure 1. Chemical structure of Taurine, Hypotaurine and Homotaurine.

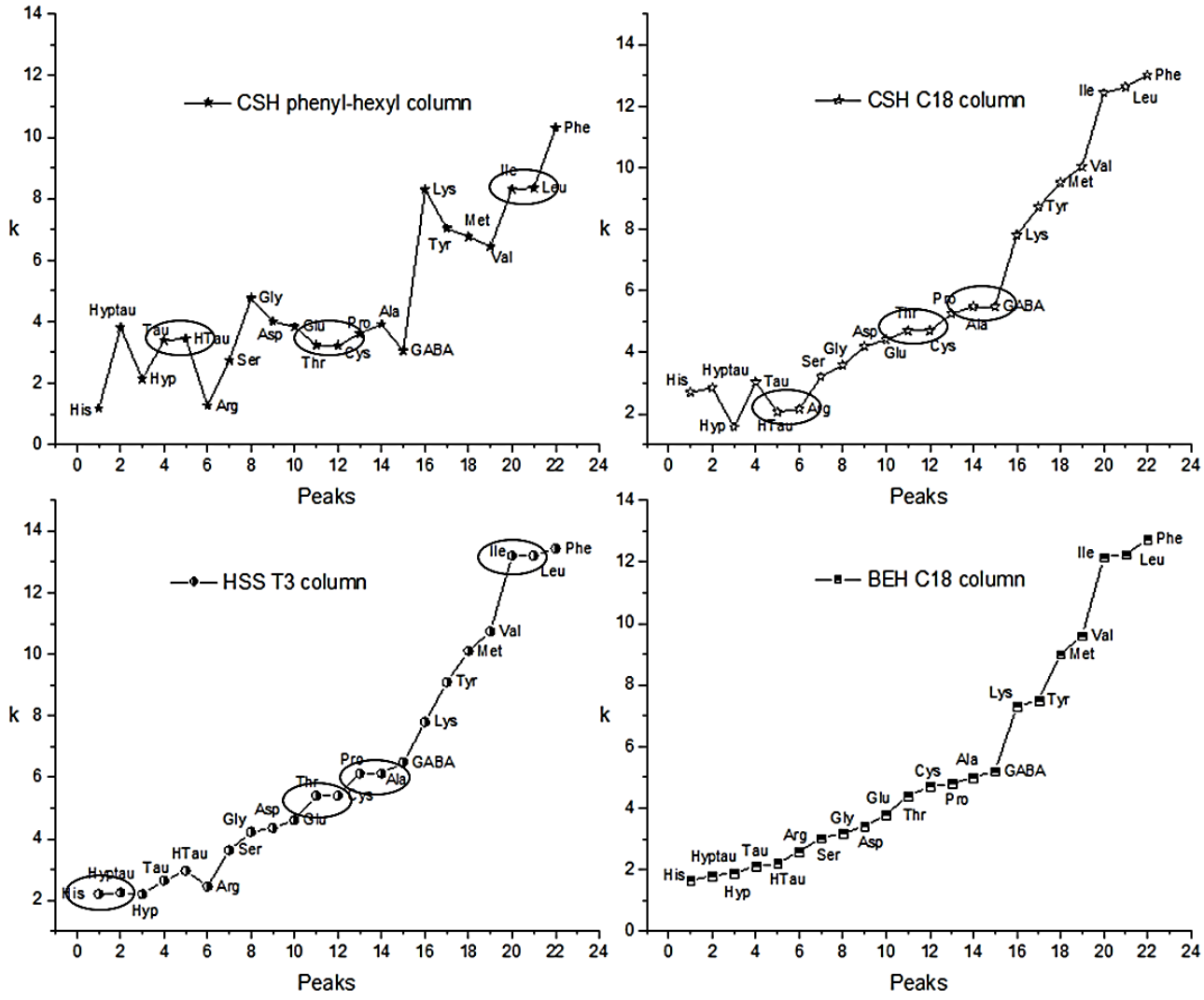


Figure 2. Elution profile of 4 chromatography columns (CSH phenyl - hexyl 1.7 µm, 2.1 mm x 100 mm; CSH C_{18} 1.7 µm, 2.1 mm x 100 mm; HSS T3 1.8µm, 2.1 mm x 100 mm and BEH C_{18} 1.7µm, 2.1 mm x100 mm). (*) Ovals show co-elution or low resolution of critical peak pairs. His (Histidine); Hyptau (Hypotaurine); Hyp (Hydroxy proline); Tau (Taurine); Htau (Homotaurine); Arg (Arginine); Ser (Serine); Gly (Glycine); Asp (Aspartic acid); Glu (Glutamic acid); Cys (Cisteine); Thr (Threonine); Pro (Proline); Ala (Alanine); GABA (Gamma amino butyric acid); Lys (Lysine); Tyr (Tyrosine); Met (Methionine); Val (Valine); Ile (Isoleucine); Leu (Leucine); Phe (Pheylalanine).

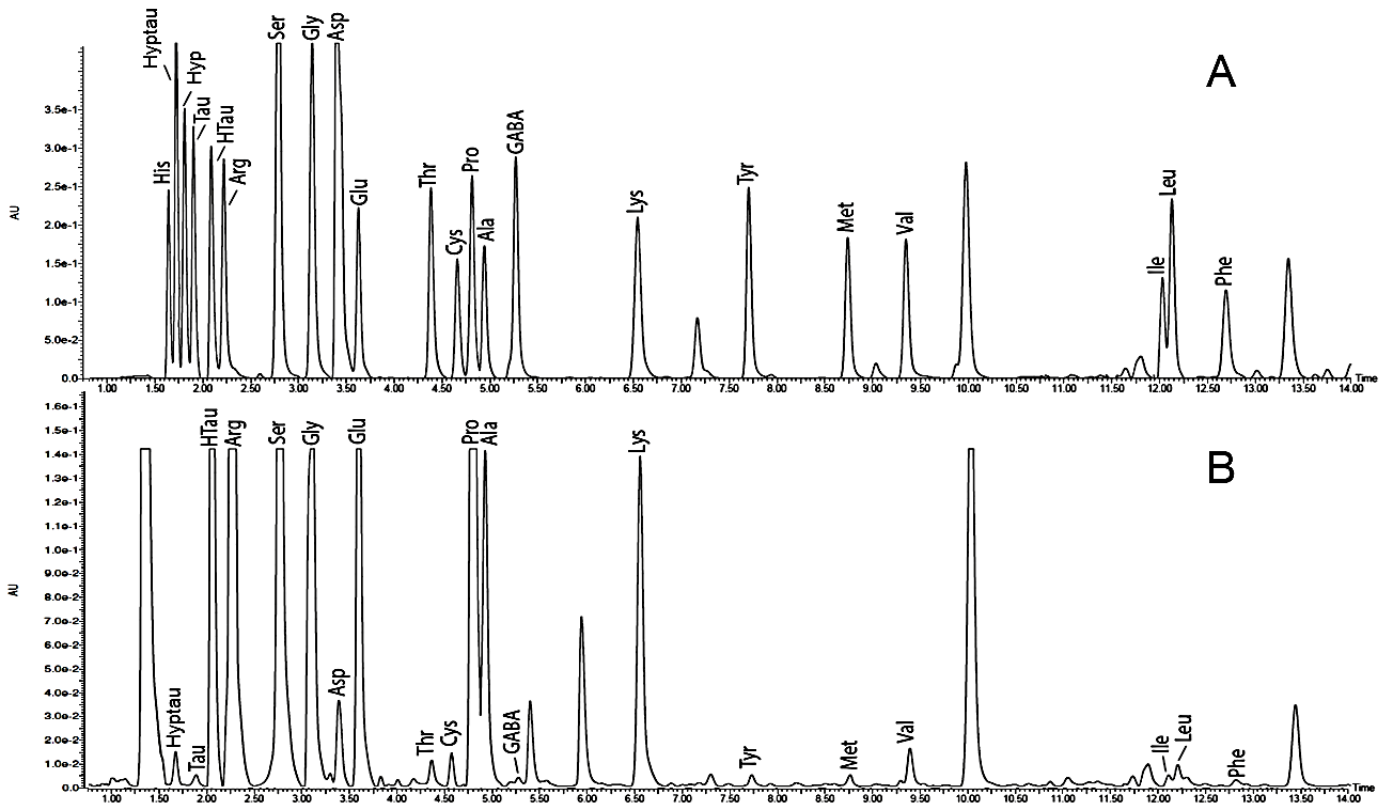
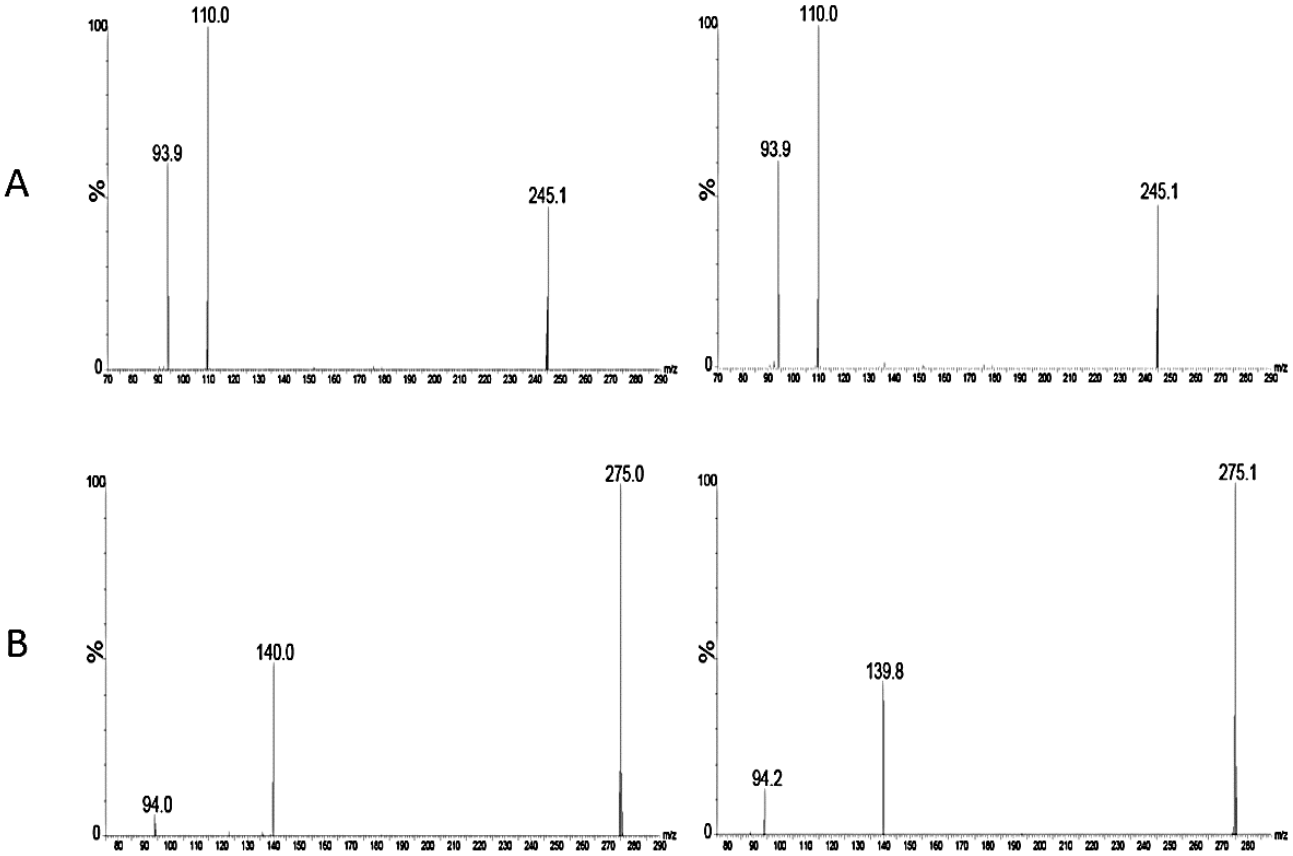


Figure 3. DAD chromatograms of an amino acid standard mixture (A) and a total amino acids profile in *Ulva lactuca* (B). His (Histidine); Hyptau (Hypotaurine); Hyp (Hydroxy proline); Tau (Taurine); Htau (Homotaurine); Arg (Arginine); Ser (Serine); Gly (Glycine); Asp (Aspartic acid); Glu (Glutamic acid); Cys (Cisteine); Thr (Threonine); Pro (Proline); Ala (Alanine); GABA (Gamma amino butyric acid); Lys (Lysine); Tyr (Tyrosine); Met (Methionine); Val (Valine); Ile (Isoleucine); Leu (Leucine); Phe (Pheylalanine).



573

574 **Figure 4**. Daughter ion spectra for Hyptau (A) and Htau (B) from a standard solution (left), and from an *Ulva lactuca*
575 sample (right) respectively.

Table 1. Validation results for the analysis of total amino acids with the proposed method. His (Histidine); Hyptau (Hypotaurine); Hyp (Hydroxy proline); Tau (Taurine); Htau (Homotaurine); Arg (Arginine); Ser (Serine); Gly (Glycine); Asp (Aspartic acid); Glu (Glutamic acid); Cys (Cisteine); Thr (Threonine); Pro (Proline); Ala (Alanine); GABA (Gamma amino butyric acid); Lys (Lysine); Tyr (Tyrosine); Met (Methionine); Val (Valine); Ile (Isoleucine); Leu (Leucine); Phe (Pheylalanine).

(#) y = signal intensity; x = compound concentration

(*) LoF = lack of fit test (Ftab: 2.69)

(**) S_{Di} = standard deviation of differences

Amino acid	RT (min)	Regression equation#	LoF*	R^2	Precision (RSD %)		Youden robustness test	MLOD	MLOQ	% Recovery (n=9)
					Intraday (n=6)	Interday (n=6, in 3 days)	SDi (%) **	(µg/g)	(µg/g)	(mean ± SD)
His	1.67	$y = 2.1 * 10^2 x - 33.2$	0.25	0.996	1.2	3.6	1.1	0.043	0.085	99.3 ± 3.2
Hyptau	1.78	$y = 7.5 * 10^2 x - 11.8$	0.32	0.998	1.0	4.7	0.2	0.115	0.220	99.4± 2.5
Hyp	1.88	$y= 5.7 * 10^2 x + 17.7$	0.24	0.999	1.2	2.4	0.1	0.042	0.084	98.6 ± 2.1
Tau	1.96	$y = 2.7 * 10^2 x + 28.1$	0.45	0.999	1.9	3.2	0.1	0.036	0.073	103.1 ± 3.4
HTau	2.12	$y = 5.0 * 10^2 x + 73.5$	0.48	0.999	1.0	1.9	0.1	0.066	0.131	97.2 ± 1.6
Arg	2.27	$y = 6.6 * 10^2 x + 19.8$	0.12	0.999	2.7	4.0	3.5	0.040	0.078	96.7 ± 1.4
Ser	2.85	$y = 1.4 * 10^2 x + 60.3$	0.54	0.999	2.8	3.0	2.3	0.022	0.043	97.9 ± 1.9
Gly	3.31	$y = 1.7 * 10^2 x + 22.1$	0.24	0.999	1.2	1.7	1.3	0.005	0.010	99.2 ± 5.5
Asp	3.52	$y = 8.7 * 10^2 x + 12.3$	0.36	0.998	2.8	3.3	1.9	0.036	0.071	98.3 ± 6.1
Glu	3.74	$y = 7.1 * 10^2 x - 24.74$	0.24	0.999	2.1	5.4	5.2	0.026	0.052	102.2 ± 2.4
Cys	3.88	$y = 4.7 * 10^2 x - 11.29$	0.28	0.996	2.5	3.2	1.4	0.063	0.119	95.4 ± 1.9
Thr	4.46	$y = 7.9 * 10^2 x + 16.35$	0.29	0.998	1.5	3.2	0.9	0.039	0.078	97.6 ± 1.6
Pro	4.92	$y = 9.4 * 10^2 x - 31.41$	0.27	0.999	1.3	2.2	1.9	0.022	0.044	96.6 ± 2.6
Ala	5.05	$y = 7.0 * 10^2 x + 60.39$	0.27	0.997	2.2	4.0	2.1	0.033	0.064	99.1 ± 7.7
GABA	5.37	$y = 4.5 * 10^2 x + 44.53$	0.24	0.999	3.3	4.5	0.1	0.042	0.084	98.2 ± 2.3
Lys	7.23	$y = 5.4 * 10^2 x - 16.33$	0.44	0.998	4.7	5.8	4.6	0.042	0.084	96.1 ± 3.1
Tyr	7.88	$y = 1.0 * 10^2 x + 40.09$	0.41	0.998	2.1	2.5	0.5	0.048	0.094	104.8 ± 0.9
Met	8.91	$y = 8.4 * 10^2 x + 10.46$	0.47	0.999	3.4	3.6	0.4	0.026	0.053	97.4 ± 5.4
Val	9.49	$y = 9.0 * 10^2 x + 53.91$	0.21	0.999	1.7	3.3	2.3	0.021	0.041	103.2 ± 2.8
Ile	12.23	$y = 1.8 * 10^2 x + 12.65$	0.23	0.997	2.4	3.7	3.5	0.092	0.179	103.4 ± 6.3
Leu	12.33	$y = 1.6 * 10^2 x + 11.29$	0.28	0.998	2.6	4.8	0.3	0.070	0.131	99.7 ± 3.8
Phe	12.78	$y = 8.0 * 10^2 x + 63.01$	0.44	0.999	3.1	5.1	0.6	0.090	0.173	99.6 ± 3.1

585 **Table 2**. Concentration (mg/g dry weight) of amino acids and sulfonic acid derivatives in the five algae
586 samples included in the study (values are means of *n*=3 independent determinations ± standard deviation).
587 (º) ΣEAA = Sum of essencial amino acids; (#) ΣNEAA= Sum of non-essential amino acids; ($) ΣSAD = Sum
588 of Tau, Hyptau and Htau. His (Histidine); Hyptau (Hypotaurine); Hyp (Hydroxy proline); Tau (Taurine); Htau
589 (Homotaurine); Arg (Arginine); Ser (Serine); Gly (Glycine); Asp (Aspartic acid); Glu (Glutamic acid); Cys
590 (Cisteine); Thr (Threonine); Pro (Proline); Ala (Alanine); GABA (Gamma amino butyric acid); Lys (Lysine);
591 Tyr (Tyrosine); Met (Methionine); Val (Valine); Ile (Isoleucine); Leu (Leucine); Phe (Pheylalanine).
592

	Gracilaria longissima (red)	***Ulva lactuca*** (green)	***Chlorella spp.*** (green)	***Porphyra spp.*** (red)	***Laminaria japonica*** (brown)
Ile	4.11±0.04	4.47±0.31	3.82±0.18	5.72±0.22	3.87±0.04
Leu	8.59±0.07	8.42±0.58	10.10±0.74	12.08±0.40	7.90±0.40
Lys	11.63±0.19	17.24±0.39	21.21±0.17	10.54±0.21	3.95±0.07
Met	0.17±0.01	1.17±0.07	0.77±0.01	1.88±0.04	6.68±0.92
Phe	7.81±0.12	6.51±0.17	6.92±0.07	8.79±0.34	6.80±0.04
Thr	4.44±0.16	10.77±0.10	30.51±1.23	3.33±0.09	2.78±0.02
Val	11.34±0.07	18.76±1.18	10.26±0.92	11.76±0.21	4.13±0.06
Arg	3.12±0.16	3.17±0.27	2.15±0.07	4.41±0.08	3.34±0.07
His	1.02±0.07	7.26±1.28	22.96±1.29	1.24±0.04	8.90±0.46
[º]ΣEAA	52.23±4.23	77.72±6.03	108.72±10.43	59.73±4.25	48.35±2.22
Ala	2.78±0.19	15.05±0.84	96.80±1.44	19.62±1.04	4.42±0.18
Tyr	2.38±0.10	1.62±0.06	12.84±0.83	12.20±0.37	3.52±0.19
Asp	86.46±1.61	29.15±1.36	98.18±3.42	39.02±1.22	14.01±0.28
Cys	1.49±0.07	1.40±0.08	4.01±0.13	2.93±0.18	0.71±0.01
Glu	18.15 ±0.27	33.08±0.85	89.14±1.73	26.63±0.20	3.72±0.19
Gly	13.31±0.71	14.83±0.25	15.97±0.87	18.78±1.03	5.83±0.37
Pro	9.38±0.91	78.02±1.25	35.28±1.23	18.61±1.27	2.23±0.15
Ser	12.83±0.15	12.27±0.18	13.98±0.75	19.72±1.27	10.21±0.26
GABA	2.56±0.11	0.86±0.04	18.49±1.48	5.90±0.10	0.45±0.03
Hyp	0.08±0.01	0.95±0.01	0.24±0.01	0.09±0.01	0.04±0.01
[#]ΣNEAA	149.42±25.87	187.23±23.85	384.93±39.94	163.49±11.66	45.14±4.50
Hyptau	0.24±0.01	0.35±.016	0.55±0.02	0.21± 0.01	0.19±0.01
Tau	13.03±0.71	0.17±0.02	0.66 ±0.04	6.30±0.12	0.05±0.01
HTau	0.19±0.01	4.26±0.01	0.76± 0.06	0.51±0.03	0.18±0.03
[$]ΣSAD	13.46±7.40	4.78±2.31	1.97±0.11	7.02±3.43	0.42±0.08

593

A validated ultra-performance liquid chromatography with diode array detection coupled to electrospray ionization and triple quadrupole mass spectrometry method to simultaneously quantify taurine, homotaurine, hypotaurine and amino acids in macro- and microalgae

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Table A1. Precursor/products ions and parameters for Daughter-MS/MS experiments.

Amino acid	Retention time	MW	PITC-Amino acid derivative $[M+H]^+$ (m/z)	Main Fragments (m/z)	Cone voltage eV	Collision energy eV
His	1.67	155	291	156, 109	30	20
Hyptau	1.78	109	245	110, 94	30	10
Hyp	1.88	131	268	132, 56	30	20
Tau	1.96	125	261	126, 94	30	20
Htau	2.12	139	275	140, 94	30	20
Arg	2.27	174	310	175	30	20
Ser	2.85	105	241	106, 88	30	20
Gly	3.31	75	211	76	30	20
Asp	3.52	132	268	133, 115, 89	30	20
Glu	3.74	147	283	148, 129, 102, 83	30	20
Thr	3.88	118	254	119, 102, 85	30	20
Cys	4.46	121	257	122, 74	30	20
Pro	4.92	115	251	116, 84	30	10
Ala	5.05	89	225	90	30	10
GABA	5.37	103	239	136, 128, 104, 86	30	20
Lys	7.23	147	283	148, 101	30	10
Tyr	7.88	181	317	182, 165, 146, 90	30	20
Met	8.91	149	285	150, 132, 104, 77	30	20
Val	9.49	117	253	118, 72	30	10
Ileu	12.23	131	267	132, 86, 75	30	10
Leu	12.33	131	267	132, 86	30	20
Phe	12.78	165	301	166, 120, 82	30	10

Minor Changes using the Youden Method								
Sample Number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
A/a	A	A	A	A	a	a	a	a
B/b	B	B	b	b	B	B	b	b
C/c	C	c	C	c	C	c	C	c
D/d	D	D	d	d	d	d	D	D
E/e	E	e	E	e	e	E	e	E
F/f	F	f	f	F	F	f	f	F
G/g	G	g	g	G	g	G	G	g
Results	**H**	**I**	**J**	**K**	**L**	**M**	**N**	**O**

Minor Changes using the Youden Method								
Sample Number	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
A/a	11 mg	11 mg	11 mg	11 mg	9 mg	9 mg	9 mg	9 mg
B/b	1.1 ml	1.1 ml	0.9 ml	0.9 ml	1.1 ml	1.1 ml	0.9 ml	0.9 ml
C/c	26 h	22 h	26 h	22 h	26 h	22 h	26 h	22 h
D/d	120ºC	120ºC	100 ºC	100 ºC	100 ºC	100 ºC	120ºC	120ºC
E/e	25 min	15 min	25 min	15 min	15 min	25 min	15 min	25 min
F/f	35 ºC	25 ºC	25 ºC	35 ºC	35 ºC	25 ºC	25 ºC	35 ºC
G/g	0.45 ml/min	0.25 ml/min	0.25 ml/min	0.45 ml/min	0.25 ml/min	0.45 ml/min	0.45 ml/min	0.25 ml/min
Results	**H**	**I**	**J**	**K**	**L**	**M**	**N**	**O**

		Variables				
		sample processing			chromatography	
1	2	3	4	5	6	7
sample weight	**vol. HClO4**	**hydrolysis time**	**hydrolysis temperature**	**derivatization time**	**column temperature**	**flow rate**
A= 11 mg	B=1.1 ml	C= 26 h	D= 120ºC	E= 25 mins	F =35 ºC	G =0.45 ml/min
a = 9mg	b = 0.9 ml	c= 22 h	d= 100ºC	e= 15 mins	f= 25 ºC	g =0.35 ml/min

Calculation for Robustness for factor A/a:

Differences:

$$D_{A/a} = \left(\frac{H+I+J+K}{4}\right) - \left(\frac{L+M+N+O}{4}\right)$$

Standard deviation of differences:

$$SD_i = \sqrt{2x\sum\left(\frac{D_i^2}{7}\right)}$$

Table A2. Fractional factorial design. Youden robustness experiment.

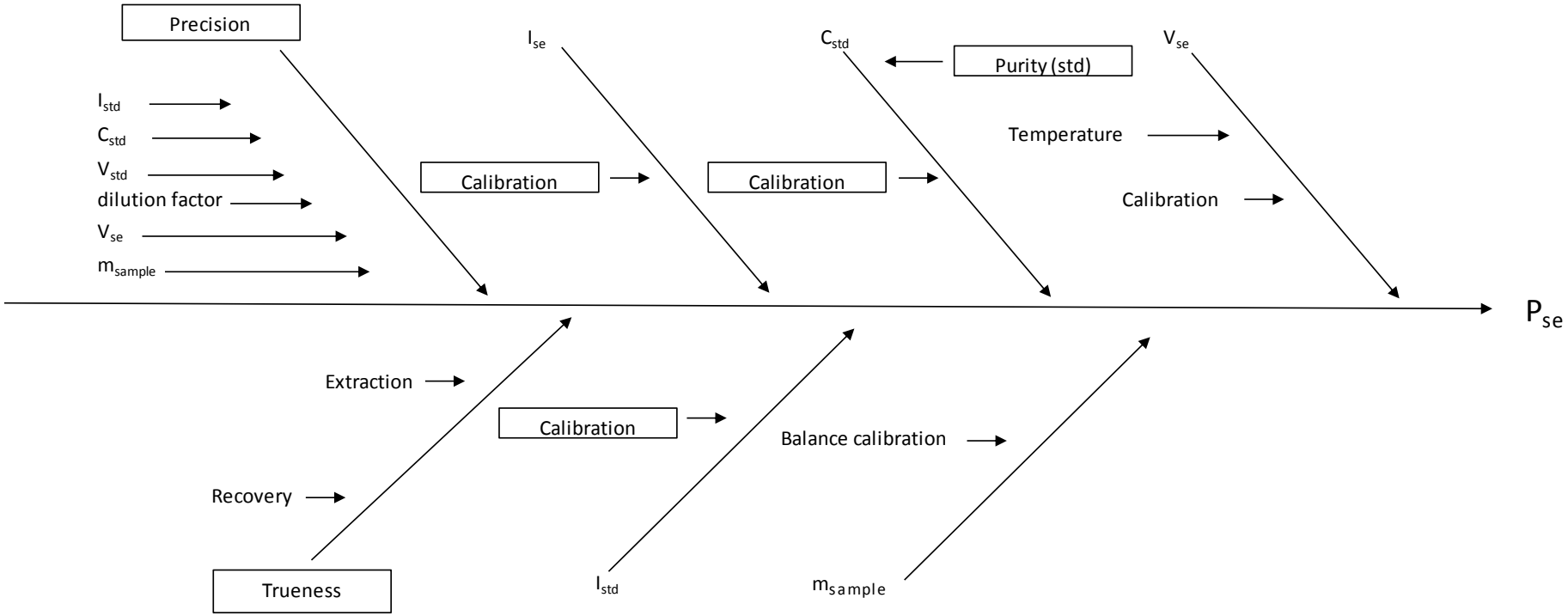

Fig. 1. Ishikawa diagram of the procedure of amino acids determination in algae samples by UPLC-DAD-MS/MS method.

* I_{se} = Peak intensity of the sample extract

I_{std} = Peak intensity of the amino acids standard

m_{sample} = Mass of the sample

m_{std} = Mass concentration of the amino acid standard

V_{se} = Final volume of the extract

V_{std} = Volume of the amino acid standard

P_{se} = Mass fraction of amino acid in the sample

Table A3. Results of selectivity test on standard stock solution and samples.

Amino acid	Peak Resolution, RS			
Peak pairs	Reference std	*Chlorella*	*Porphyra*	*Laminaria*
His / Hyptau	2.16	2.12	2.36	2.60
Hyptau / Hyp	2.15	4.05	3.25	3.54
Hyp / Tau	2.36	2.36	2.07	4.86
Tau / Htau	3.78	2.53	4.25	4.02
Htau / Arg	2.21	2.87	3.30	2.53
Arg / Ser	4.03	6.84	6.73	7.08
Ser / Gly	3.10	4.83	5.43	5.64
Gly / Asp	2.25	1.97	2.48	3.39
Asp / Glu	2.88	1.73	1.85	2.07
Glu / Thr	2.07	1.65	1.72	1.18
Thr / Cys	4.56	3.87	3.89	3.15
Cys / Pro	4.18	2.66	3.54	3.88
Pro / Ala	2.56	1.97	1.92	3.07
Ala / GABA	4.20	2.70	2.36	2.60
GABA / Lys	10.45	10.9	8.04	9.10
Lys / Tyr	2.79	2.88	2.26	2.93
Tyr / Met	4.96	6.40	4.43	4.05
Met / Val	2.85	3.89	3.11	2.21
Val / Ileu	23.0	19.1	18.8	14.7
Leu / le	2.36	1.57	1.77	1.52
Ile / Phe	2.95	3.70	4.52	4.18

Table A4. Working ranges for all amino acids.

Amino acid	Working range (mg/g)
His	0.19 - 30.1
Hyptau	0.14- 6.81
Hyp	0.04 - 1.64
Tau	0.04 - 15.4
Htau	0.17 - 8.69
Arg	1.09 - 10.8
Ser	3.28 - 33.1
Gly	2.34 - 23.4
Asp	9.13 - 107
Glu	1.84 - 98.3
Thr	1.48 - 44.7
Cys	0.30 - 7.56
Pro	1.44 - 94.4
Ala	1.11 - 101
GABA	0.26 - 12.8
Lys	1.84 - 28.4
Tyr	1.13 - 22.6
Met	0.09 - 9.31
Val	1.46 - 34.6
Ileu	0.82 - 8.19
Leu	4.09 - 16.4
Phe	1.03 - 10.3

Table A5. Uncertainty results.

Amino acid	Pse $_{(mg/g)}$ #	u(RSD_R) [%] *	u(rec) [%] **	u (Pse) [%] **	Coverage factor	Coverage
His	22.9	2.95	1.68	0.78	2	95% (t-table 95,45%)
Hyptau	0.55	1.43	0.72	0.01	2	95% (t-table 95,45%)
Hyp	0.24	0.52	0.22	0.001	2	95% (t-table 95,45%)
Tau	0.66	3.21	2.75	0.03	2	95% (t-table 95,45%)
Htau	0.76	3.24	0.51	0.02	2	95% (t-table 95,45%)
Arg	50.2	3.95	2.39	2.30	2	95% (t-table 95,45%)
Ser	54.0	1.22	0.90	0.83	2	95% (t-table 95,45%)
Gly	36.0	1.47	0.85	0.59	2	95% (t-table 95,45%)
Asp	98.2	1.48	0.18	1.40	2	95% (t-table 95,45%)
Glu	89.1	2.94	1.19	2.50	2	95% (t-table 95,45%)
Thr	30.5	2.28	0.29	0.69	2	95% (t-table 95,45%)
Cys	35.0	1.26	0.95	0.54	2	95% (t-table 95,45%)
Pro	35.3	2.50	0.78	0.92	2	95% (t-table 95,45%)
Ala	96.8	1.27	0.83	1.50	2	95% (t-table 95,45%)
GABA	18.5	3.27	1.01	0.63	2	95% (t-table 95,45%)
Lys	1.97	0.30	0.07	0.01	2	95% (t-table 95,45%)
Tyr	12.8	0.58	0.32	0.09	2	95% (t-table 95,45%)
Met	0.77	0.17	1.13	0.01	2	95% (t-table 95,45%)
Val	10.3	3.54	1.02	0.38	2	95% (t-table 95,45%)
Ile	32.1	3.75	0.22	1.20	2	95% (t-table 95,45%)
Leu	33.2	3.75	0.68	0.38	2	95% (t-table 95,45%)
Phe	9.14	1.50	0.82	0.15	2	95% (t-table 95,45%)

$Pse_{(mg/g)}$ = amino acid concentration (mg/g)

* $u(RSD_R)$ = standard uncertainty for within lab reproducibility

** $u(rec)$ = standard uncertainty for recovery

*** $u(Pse)$ = Expanded uncertainty

Table A6. Comparative chromatographic performances of total amino acids in algae.

	Validated methods									Non-validated methods						
HPLC parameters	Our results			[49]		[30]			[38]	[26]	[27]	[28]	[29]	[32]	[33]	[50]
Detection mode	DAD-MS/MS			DAD		FLD			DAD	Automated amino acid analyzer						DAD
Total Run time (min)	18			35		20			30	102						
No amino acids	22			17		2			18	18	11	21	17	13	20	21
	MLOD*	RSD	Recovery	ILOD**	RSD	ILOD**	RSD	Recovery	Recovery							
amino acid	ng/g	%	%	ng/mL	%	ng/mL	%	%	range (%)							
His	43	1.2	99	12	2.8											
Hyptau	115	1.0	99													
Hyp	42	1.2	99													
Tau	36	1.9	103			30	3.9	101								
HTau	66	1.0	97			15	4.0	98								
Arg	40	2.7	97	10	2.5											
Ser	22	2.8	98	8.1	1.7											
Gly	5.0	1.2	99	13	2.9											
Asp	36	2.8	98	8.9	2.0											
Glu	26	2.1	102	7.5	2.0											
Cys	63	2.5	95						(87 - 102)							
Thr	39	1.5	98	6.9	2.2											
Pro	22	1.3	97	8.3	2.9											
Ala	33	2.2	99	1.4	2.7											
GABA	42	3.3	98													
Lys	42	4.7	96	8.5	1.7											
Tyr	48	2.1	105	7.6	3.1											
Met	26	3.4	97	8.9	3.9											
Val	21	1.7	103	6.9	2.8											
Ile	92	2.4	103	7.8	1.3											
Leu	70	2.6	100	7.9	2.3											
Phe	90	3.1	100	13	2.5											

* Method limit of detection

** Instrument limit of detection

Table A7. Comparative results in *Porphyra sp.* Values expressed as mg/g d.w.

*data has been converted from g/16 g N units to mg/g d.w. taking into account that 16 g N (nitrogen) correspond to approximately 100 g protein (*Food composition Data*, FAO, 2003) and considering the protein content (in g / 100 g d.w.) in the algal sample.

Porphyra spp. (red)	*Our results*	*Dawczynski et al. [28] **	*Mišurcová et al. [26] **	*Sanchez-machado et al. [49]*	*McCusker et al. [27]*
Ile	5.72	8.37	9.10	4.60	3.51
Leu	12.1	14.9	15.2	7.10	6.16
Lys	10.5	13.2	10.3	7.70	5.18
Met	1.88	4.86	8.34	1.60	1.66
Phe	8.79	8.91	11.3	16.6	4.25
Thr	3.33	14.3	13.1	9.70	3.69
Val	11.7	14.0	14.6	7.20	4.79
Arg	4.41	15.9	19.5	7.60	6.93
His	1.24	7.02	5.24	8.30	1.27
ΣEAA	59.7	102	107	70.4	37.4
Ala	19.6	16.7	18.1	14.5	
Tyr	12.2	9.18	7.67	4.40	
Asp	39.0	22.9	27.2	11.5	
Cys	2.93	3.24	7.56		
Glu	26.6	27.5	28.9	12.7	
Gly	18.8	13.7	14.9	9.40	
Pro	18.6	9.45	9.72	8.40	
Ser	19.7	10.8	12.4	6.70	
GABA	5.90				
Hyp	0.09				
ΣNEAA	163	114	127	67.6	
Total	223	215	233	138	
Hyptau	0.21				
Tau	6.30	11.6			1.22
HTau	0.51				
$ΣSAD	7.02				

Table A8. Comparative results in *Laminaria japonica.* Values expressed as mg/g d.w.

*data has been converted from g/16 g N units to mg/g d.w. taking into account that 16 g N (nitrogen) correspond to approximately 100 g protein (*Food composition Data*, FAO, 2003) and considering the protein content (in g / 100 g d.w.) in the algal sample.

Laminaria japonica (brown)	***Our results***	***Dawczynski et al. [28] ****	***Mišurcová et al. [26] ****	***McCusker et al. [27]***
Ile	3.87	1.70	1.58	4.34
Leu	7.90	3.09	2.78	8.39
Lys	3.95	2.46	2.02	9.97
Met	6.68	0.57	1.25	2.17
Phe	6.80	2.02	1.76	5.48
Thr	2.78	2.21	2.22	7.01
Val	4.13	2.39	2.37	6.68
Arg	3.34	2.08	2.09	5.42
His	8.90	1.39	0.77	2.21
ΣEAA	48.3	17.9	16.8	51.7
Ala	4.42	3.59	3.83	
Tyr	3.52	1.07	0.9	
Asp	14.0	7.88	5.32	
Cys	0.71	0.76	1.34	
Glu	3.72	14.9	9.69	
Gly	5.83	2.52	2.38	
Pro	2.23	1.95	3.18	
Ser	10.2	2.08	1.85	
GABA	0.45			
Hyp	0.04			
ΣNEAA	45.1	34.8	28.5	
Total	93.5			
Hyptau	0.19			
Tau	0.06	0.19		0.02
HTau	0.18			
$ΣSAD	0.42			

Table A9. Comparative results in *Ulva lactuca* Values expressed as mg/g d.w.

Ulva lactuca (green)	***Our results***	***McCusker et al. [27]***
Ile	4.47	9.26
Leu	8.42	16.8
Lys	17.2	11.6
Met	1.17	4.47
Phe	6.51	11.7
Thr	10.8	14.3
Val	18.8	16.3
Arg	3.17	16.0
His	7.26	4.52
%EAA	77.8	105
Ala	15.1	
Tyr	1.62	
Asp	29.1	
Cys	1.40	
Glu	33.1	
Gly	14.8	
Pro	78.0	
Ser	12.3	
GABA	0.86	
Hyp	0.95	
%NEAA	187	
Total	265	
Hyptau	0.35	
Tau	0.17	0.01
HTau	4.26	
$ΣSAD	4.78	

Table A10. Comparative results in *Chlorella sp.* Values expressed as mg/g d.w.

Chlorella sp. (brown)	***Our results***	***Kent et al. [29]***	***Brown et al. [50]***
Ile	3.82	44.0	42.0
Leu	10.1	92.0	74.0
Lys	21.2	88.9	61.0
Met	0.77	22.3	23.0
Phe	6.92	54.7	58.0
Thr	30.5	47.4	53.0
Val	10.3	61.0	63.0
Arg	2.15	71.5	69.0
His	23.0	24.3	19.0
%EAA	109	506	462
Ala	96.8	47.4	85.0
Tyr	12.8	41.6	42.0
Asp	98.2	93.6	
Cys	4.01	4.35	8.70
Glu	89.1	128	
Gly	16.0	53.8	60.0
Pro	35.3	47.8	
Ser	14.0	40.4	49.0
GABA	18.5		8.10
Hyp	0.24		1.70
%NEAA	385	457	255
Total	494	963	717
Hyptau	0.55		
Tau	0.66		
HTau	0.76		
ΣSAD	1.97		