Journal of Food Composition and Analysis 140 (2025) 107289

Contents lists available at ScienceDirect

Journal of Food Composition and Analysis

journal homepage: www.elsevier.com/locate/jfca

Comprehensive amino acids profiling of microalgae by gas chromatography-mass spectrometry analysis after pre-column derivatization

Gabriela Bermudez, Cristina Terenzi, Francesca Medri, Vincenza Andrisano, Serena Montanari *

Department for Life Quality Studies, University of Bologna, Corso D'Augusto 237, Rimini 47921, Italy

ARTICLE INFO

Keywords:
Chlorella and *Spirulina*
GC-MS analysis
Tryptophan
MTBSTFA derivatization
Alkaline hydrolysis
Multivariate analysis

ABSTRACT

Microalgae represent a sustainable protein supply compared to plant and animal sources. However, there is the need to characterize the full microalgae amino acids content, distinguishing among different species. In this work, a new efficient, fast, sensitive, reproducible and convenient gas chromatography-mass spectrometry (GC-MS) method, characterized by protein hydrolysis and derivatization performed in one-single-step, was validated for the determination of all amino acids in microalgal samples, including tryptophan. The overall optimized protein hydrolysis (improved in alkaline conditions due to tryptophan instability at acidic pH), the derivatization step (at neutral pH by using N-(tert-Butyldimethylsilyl)-N-methyltrifluoroacetamide) and the GC-MS method were validated. This methodology resulted selective, accurate (99.201 ± 7.61E-01 %), sensitive, reproducible and with a good recovery (96.20 ± 1.62E+00 %). Four commercial samples of *Spirulina* and three samples of *Chlorella* were analyzed. The resulting data were compared with the daily amino acid requirements identified by World Health Organization. The microalgae were found rich in the essential amino acids, including tryptophan, with a specific pattern associated to the species. Finally, by applying the principal component analysis, the intra/inter species differences of the amino acid profile of microalgae samples address them to specific uses, such as food in substitution of animal proteins or as food supplement.

1. Introduction

Essential amino acids (valine, leucine, isoleucine, methionine, threonine, phenylalanine, lysine, histidine and tryptophan) cannot be synthesized by mammals and must be obtained from the diet (National Research Council, 1989). Hence, the main sources of amino acids for humans come from the diet or from the breakdown of endogenous proteins. Non-essential amino acids, on the other hand, are synthesized by humans starting from carbon skeletons, mainly from glucose (alanine, glycine, proline, serine, aspartic acid, glutamic acid, arginine, cysteine), with the exception of tyrosine, which is synthesized from phenylalanine (Puigserver, 2017).

The quality of dietary proteins can vary considerably, depending on digestibility and the availability of essential amino acids. In fact, due to their higher digestibility in the human gastro-intestinal tract, usually, animal proteins with a richest source of essential amino acids are considered having better nutritional value than plant proteins (Kaur et al., 2022). Likewise, plant proteins are often considered a source of incomplete proteins because usually they contain no more than few essential amino acids. Despite this, there are growing concerns about the high levels of saturated fat and cholesterol found in animal foods, which are linked to the development of cardiovascular disease and diabetes.

Considering the estimates of the average requirement of essential amino acids and assuming an average protein requirement of

Abbreviations: GC-MS, gas chromatography-mass spectrometry; HPLC-UV, high-performance liquid chromatography-UV, WHO, World Health Organization; PCA, principal component analysis; MTBSTFA, N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide ACN, acetonitrile; HPLC-DAD, high-performance liquid chromatography with diode-array detection; BSTFA-TMCS, N,O-bistrimethylsilyltrifluoroacetamide/trimethylchlorosilane; TBDMS, tert-Butyldimethylsilyl; PTFE, polytetrafluoroethylene; EI, electron ionization; LoD, limit of detection; LoQ, limit of quantification; LC-MS, liquid chromatography–mass spectrometer; HPLC, high-performance liquid chromatography.

* Corresponding author.
E-mail address: serena.montanari5@unibo.it (S. Montanari).

https://doi.org/10.1016/j.jfca.2025.107289
Received 22 July 2024; Received in revised form 26 December 2024; Accepted 23 January 2025
Available online 27 January 2025

0.66 g kg^{-1} per day, data reports that the intakes of approximately 0.18 g kg^{-1} per day and 0.48 g kg^{-1} per day respectively of essential and non-essential amino acids should be sufficient to maintain body nitrogen homeostasis in healthy adults (World Health Organization, 2007). In this regard, microalgae are a source of bioactive compounds of great value and interest, such as polyunsaturated fatty acids, vitamins, minerals, antioxidants, pigments and, in particular, they contain a high protein source, often comparable to other protein sources, such as soy and egg. In the food field, microalgae can improve the nutritional content of conventional foods, integrated into products such as snacks, pasta, sweets and drinks, or they can be exploited for the production of food supplements in the form of tablets or capsules (Koyande et al., 2019). Furthermore, since consumer demand is shifting towards alternative and sustainable sources, algal biomass is considered a relevant option, which has numerous advantages such as a high growth rate, low water consumption, no competition with arable land, carbon dioxide consumption and production of many bioactive compounds (Geada et al., 2021; Montanari et al., 2021). Microalgae protein content can reach up to 70 % of dry weight, advantageous for nutraceutical purposes. Therefore, microalgae represent an alternative source to meat, standing out in terms of quality for a balanced amino acids content, comparable to other food proteins of plant origin, even if plant-based proteins often contain low concentrations of essential amino acids (Wang et al., 2021). For this reason, it is necessary to have an efficient, fast, sensitive, reproducible and inexpensive analytical method able to characterize the full amino acid content of the different microalgae species, in order to rank and valorize them for the market depending on their qualities.

In literature, numerous analytical methods for the determination of amino acids in plants, human plasma and animal matrices are reported. Most of them include the use of high-performance liquid chromatography-UV (HPLC-UV), liquid chromatography–mass spectrometer (LC-MS) and GC-MS (Xu et al., 2020). However, all these techniques demonstrate various shortcomings. In fact, it is important to consider the polar nature of amino acids, their inability to either absorbing light or having native fluorescence and their low molecular weight. For these reasons, quantitative analysis by traditional HPLC-UV is impaired by the lack of significant chromophores, even if this issue can be overcome by a pre-derivatization step (Violi et al., 2020). Nevertheless, as reported in literature, HPLC-UV methods often require long chromatographic run (about 2–3 h per sample) (Kaspar et al., 2009; Le Boucher et al., 1997). In addition, these methods sometimes demonstrate a lack of analyte specificity, due to interference of co-eluting compounds, giving rise to non-accurate determination of some amino acids in human plasma, serum, and urine matrices (Dietzen et al., 2008). The analyses of amino acids by GC-MS and LC-MS generate comparable results but, sometimes, LC-MS analyses are not entirely accurate since results may be affected by matrix effect that can lead to ionization suppression or enhancement, resulting in reduced sensitivity (Gałęzowska et al., 2021). Other scientists analyzed only free amino acids present in microalgae, avoiding or reducing the time of the hydrolysis step (Araya et al., 2021; Rodriguez et al., 1997). Another issue found in literature is the impossibility of determining all amino acids, in particular tryptophan, in a single step (Fountoulakis & Lahm, 1998; Mustățea et al., 2019). In fact, the most commonly used protein hydrolysis is carried out in acidic conditions that however causes the complete tryptophan degradation. It is reported that the addition of thioglycolic acid can greatly increase tryptophan stability, but with partial recovery (80–90 %) (Friedman & Finley, 1971; Yokote et al., 1986).

As described in literature, the best way to preserve tryptophan is to carry out alkaline protein hydrolysis, as already reported on various matrices, among which microalgae (La Cour et al., 2019; León-Vaz et al., 2023; Yust et al., 2004). In other papers regarding microalgal matrix, sample preparation is carried out by protein hydrolysis in acid conditions, ignoring tryptophan content (Fountoulakis & Lahm, 1998; Hempel et al., 2012; Kudełka et al., 2021; Rojo et al., 2023). As a compromise, acid hydrolysis and alkaline hydrolysis can be performed in parallel on the same microalgal sample in order to obtain a complete analysis of all amino acids, including tryptophan. However, analysis times, costs and the amount of sample required are very much increased (La Cour et al., 2019; León-Vaz et al., 2023). In literature, a few papers regarding GC-MS are applied to the determination of only free amino acids, but temperature and acid hydrolysis time variations produce data affected by poor sensitivity for some amino acids, including tryptophan (Furlan et al., 2024; Vendruscolo et al., 2018; Y. Zhang et al., 2016).

In light of these premises, this work aimed to validate a new efficient, fast, sensitive, reproducible and convenient GC-MS method, characterized by protein hydrolysis and derivatization, performed in one-single-step, for the determination of all amino acids, including tryptophan, in microalgal samples of *Spirulina* (*Arthrospira Platensis)* and *Chlorella (Chlorella vulgaris)*. Obtained results were compared with the daily amino acid doses suggested by World Health Organization (WHO). Then, an integrated analytical approach with the aid of the principal component analysis (PCA) was applied to profile the commercial microalgal powders of *Spirulina* and *Chlorella* species in terms of their amino acid and protein contents. In particular, the two different species of microalgae were compared for their composition by highlighting the intra and inter-species differences, with the aim of addressing microalgae either to food supplements, functional foods or cosmetics.

2. Materials and methods

2.1. Materials and chemicals

Lyophilized microalgal samples of *Chlorella* were purchased from by Allmicroalgae-Natural Product (Portugal), Archimede Ricerche SRL (Italy), Yanchi Yijian Biochemical (China). Lyophilized microalgal samples of *Spirulina* were purchased from Spirulina IndustryPark (China), Archimede Ricerche SRL (Italy), Spirulina Industry Park, (China), AlghItaly (Italy).

N-(tert-Butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA) 97 % was purchased from VWR International Srl. L-Alanine BioUltra ≥ 99.5 % (NT), Glycine BioUltra, for molecular biology ≥ 99.0 % (NT), L-Valine BioUltra ≥ 99.5 % (NT), L-Leucine BioUltra ≥ 99.5 % (NT), L-Isoleucina BioUltra, ≥ 99.5 % (NT), L-Prolina BioUltra ≥ 99.5 % (NT), L-Metionina BioUltra ≥ 99.5 % (NT), L-Serine BioUltra ≥ 99.5 % (NT), L-Threonine BioXtra ≥ 99.5 % (NT), L-Phenylalanine BioUltra, ≥ 99.0 % (NT), L-Aspartic Acid BioUltra ≥ 99.5 % (NT), L-Glutammic Acid BioUltra ≥ 99.5 % (NT), L-Arginine BioUltra ≥ 99.5 % (NT), L-Lysine BioUltra ≥ 99.5 % (NT), L-Histidine BioUltra ≥ 99.5 % (NT), L-Tyrosine BioUltra, ≥ 99.0 % (NT), L-Tryptophane BioUltra ≥ 99.5 % (NT), L-Cysteine BioUltra ≥ 98.5 % (RT), D,L-Norleucine ≥ 98 %, Sodium Hydroxide puriss. p. a., ACS reagent, reag. Ph. Eur., K ≤ 0.02 %, ≥ 98 %, pellets, water HPLC grade, acetonitrile (ACN) suitable for high-performance liquid chromatography with diode-array detection (HPLC-DAD), N,O-bis(trimethylsilyl) trifluoroacetamide/trimethylchlorosilane (BSTFA-TMCS) were purchased Sigma-Aldrich company (St. Luis, MO, USA).

2.2. Standard solutions

NaOH 4.2 N solution was prepared and stored at room temperature. Standard stock solution in NaOH 4.2 N of amino acids (amino-SD) at the concentration of about 10 mg mL^{-1} was prepared. Internal standard solution was prepared by dissolving D,L-Norleucine (N1) in water to reach a concentration of 4.04 mg mL^{-1}. The obtained solutions have been stored at −20 ºC and kept out from light. The solutions were stable for more than one month after going through many freeze-thaw cycles.

2.3. Sample preparation

Approximately 30 mg of microalgal powder were weighed into

amber glass vials with screw caps and rubber septum and 0.5 mL of N1 were added. The mixture was suspended in 3.5 mL of NaOH 4.2 N, and it was placed under nitrogen atmosphere. Then, the sample was vortexed for 20 seconds and heated at 110°C for 22 hours. The vial was then cooled and vortexed for 20 seconds. The suspension was filtered with a 0.45 μm filter. 1 mL of suspension was taken, and the pH was brought to a range between 6.5 and 7.0 by using diluted HCl (2 N). The aqueous solvent was eliminated using rotavapor. The solid was taken up in acetonitrile and water (3 mL, 2:1) and transferred to a 5 mL Eppendorf tube with a screw cap. The solvent was carefully removed before proceeding with the derivatization step, via nitrogen flow and subsequently with a vacuum pump. Four samples of *Spirulina* (S1-S4) and three samples of *Chlorella* (C1-C3) were analyzed trice. The unknown amino acid concentration of microalgal samples was then calculated by Standard Addition Method (SAM). Calibration lines were obtained by plotting the known analyte concentrations, added to the samples, versus the corresponding chromatographic peaks area/internal standard peak area. The concentration of each single amino acid in microalgae matrix was then calculated by interpolating the ratio chromatographic peaks area/internal standard peak area in the obtained calibration curve.

2.4. Derivatization reaction

In order to prepare the volatile tert-Butyldimethylsilyl (TBDMS) derivatives of amino acids, a pre-derivatization reaction, modified from described procedures (Stenerson, 2007; Pérez-Palacios et al., 2015; Sobolevsky et al., 2003), was performed by adding 2.7 mL of ACN and 300 μL of silylating reagent N-(tert-Butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA) to the amino acid mix, previously dried in a screw cap Eppendorf of 5 mL. The reaction was stirred and heated at 80 ºC a silicon bath for 4 hours. The solution was filtered with a 0.22 μm polytetrafluoroethylene (PTFE) filter and it was analyzed by GC-MS. The optimized reaction was also applied to the samples obtained after the hydrolysis step, by dissolving the dried amino acid extracts in 2.7 mL of ACN and performing the described reaction.

2.5. Gas chromatography-mass spectrometry (GC-MS) method for the amino acid content determination

An Agilent Gas-Chromatograph coupled with a single quadrupole selective mass detector (Agilent 7820 A GC System, Agilent 5977E MSD) in electron ionization (EI) mode (70 eV) under a temperature gradient elution was applied. The gas carrier was helium with a flow rate of 1 mL min^{-1}. An aliquot of 1 μL of the pre-derivatized sample was injected in split mode= 20:1. The MS source temperature was set at 230 °C, the MS quad temperature was adjusted to 150 °C, the AUX-1 temperature was fixed at 280 °C and the Front Inlet temperature at 250 °C. The GC oven temperature program started at 100 °C and the temperature was increased to 210 °C by a linear gradient rate of 8 °C min^{-1} and with hold time of 4 min. Then, the temperature was increased to 219 °C with gradient rate of 2 °C min^{-1} and hold time of 0 min. Therefore, 300 °C were reached with an increasing of 7 °C $min^{-1,}$ and finally temperature was increased to 325 °C rate of 25 °C min^{-1} and hold time of 3 min. The total run time was of 37 min and the analyses were performed in total Ion Scan with a speed scan of 1.562 (N = 2), and MS scanned from: 45–750 Da each 2.1 scans sec^{-1}. Data were acquired with MassHunter GC-MS Acquisition B.07.00, 2013, processed with MassHunter Workstation Software Qualitative Analysis B.06.00, 2012, and compared and identified with NIST Mass Spectral Search Program, 2012. Amino acids identification was performed by comparing the retention times and mass spectra. Calibration curves were then obtained by linear least-squares regression analysis by plotting the ratio of the amino acid/norleucine (internal standard) peak areas versus the analyte concentrations.

2.6. GC-MS method validation

The proposed method consisting in protein hydrolysis with amino acids extraction from microalgae powder, pre-derivatization and GC-MS analysis was validated considering specificity, linearity, sensitivity, precision, accuracy and recovery (European Medicines Agency, 1995).

2.6.1. Specificity

The specificity of the method was determined by using three blank samples of *Spirulina* (S1) microalgae and by comparing the chromatograms obtained after injecting the pre-derivatized non-spiked samples and spiked with standard amino acids respectively. After each sample analysis, two solvent injections were performed to demonstrate the absence of any carry-over effect.

2.6.2. Linearity

Three standard calibration curves were determined by analyzing five different concentrations of amino acid standards.

The amino acid calibration curves were prepared using the commercial standards of L-Alanine, L-Glycine, L-Valine, L-Leucine, L-Isoleucine, L-Proline, L-Methionine, L-Serine, L-Threonine, L-Phenylalanine, L-Aspartic Acid, L-Glutamic Acid, L-Arginine, L-Lysine, L-Histidine, L-Tyrosine, L-Tryptophan and L-Cysteine. The amino acid mix was prepared by weighing approximately 250 mg of each of the amino acids into a 25 mL volumetric flask and making up to volume with NaOH 4.2 N solution. The concentrations obtained from the stock (range 0.156 – 0.856 mg mL^{-1}) are reported in Table S1. As internal standard, N1 was used (stock solution concentration= 4.04 mg mL^{-1}). Five dilutions were used for the amino acid mix and final concentration are reported in Table S1. Each solution was subjected to the pre-derivatization reaction (par. 2.4). The calibration curves were obtained by plotting amino acid derivatives peak area ratio norleucine (internal standard, at the concentration of 0.169 mg mL^{-1}) peak area versus their concentration. As relevant examples, the standard calibration curves of alanine and tryptophan have been included in the SI (Fig. S21-S22).

Further on, linearity was determined by preparing three calibration curves, by analyzing for each curve five samples of blank *Spirulina* (S1) microalgae spiked with amino acid mix (same concentrations reported in Table S1, range 0.156 – 0.856 mg mL^{-1}) and N1 (0.169 mg mL^{-1}). Then, the enriched samples were subjected to the hydrolysis procedure reported in par. 2.3, pre-derivatization (par. 2.4) and subsequently analyzed by GC-MS. Calibration curves of S_std amino acids were obtained by plotting analyte concentrations added to the sample versus the corresponding "derivatized amino acid peak area/derivatized N1 peak area" ratio (R1). Then, the value of the intercept was subtracted from all the R1 values and new calibration curves were constructed. As relevant examples, the spiked calibration curves of alanine and tryptophan have been included in the SI (Fig. S23-S24).

2.6.3. Sensitivity

The limit of detection (LoD= 3 *SE/m) and limit of quantitation (LoQ= 10 *SE/m) values, were obtained by a statistical evaluation, considering the standard signal deviations (Wu et al., 2011).

To compare LoD and LoQ to those reported in literature, the LoD and LoQ were also calculated by apporting sequential dilutions of amino acid standard and by evaluating the signal-to-noise ratios of 3:1 and 10:1 for LoD and LoQ.

2.6.4. Precision

The intra- and inter-day precisions were evaluated by analyzing standard samples at low (range 0.156–0.171 mg mL^{-1}), medium (range 0.469–0.514 mg mL^{-1}) and high (range 0.781–0.856 mg mL^{-1}) amino acids mix concentrations, each containing N1 at a fixed concentration of 0.169 mg mL^{-1}, after the pre-derivatization step (par. 2.4). In addition, the intra- and inter-day precisions were evaluated by analyzing spiked *Spirulina* (**S_std**) microalgae samples at low (range

0.156–0.171 mg mL^{-1}), medium (range 0.469–0.514 mg mL^{-1}) and high (range 0.781–0.856 mg mL^{-1}) amino acid mix concentrations, each containing N1 at a fixed concentration of 0.169 mg mL^{-1}, after the pre-derivatization step (par. 2.4) Spiked *Spirulina* microalgae samples were extracted daily. After the pre-derivatization step (par. 2.4), each final solution was injected twice into the GC-MS. Intra-day analyses were performed three times in a single day (n = 3) at three different concentrations of amino acid mix both as standard solutions and for the ones in spiked S1 microalgae solutions. Inter-day analyses were performed twice a day for three subsequent days (n = 6) on standard solutions and for the spiked S1 microalgae solutions.

2.6.5. Accuracy

Accuracy (n = 3) was determined by calculating the percentage of the deviation between the experimental concentrations of derivatized amino acids of spiked blank S1 microalgae and the nominal ones considering three concentrations: low (range 0.156–0.171 mg mL^{-1}), medium (range 0.469–0.514 mg mL^{-1}) and high (range 0.781–0.856 mg mL^{-1}) each containing N1 at a fixed concentration of 0.169 mg mL^{-1}.

2.6.6. Recovery

Recovery (n = 3) determination was carried out on S1 sample spiked with three incremental concentrations of amino acids mix (low range= 0.156–0.171 mg mL^{-1}, medium range= 0.469–0.514 mg mL^{-1} and high range= 0.781–0.856 mg mL^{-1}) each containing N1 at a fixed concentration of 0.169 mg mL^{-1}. The recovery values were obtained by the Eq. 1.

Equation 1:

Recovery= [(peak Area amino acid spiked microalgae/peak N1 spiked microalgae) / (peak Area amino acid standard solution/peak area N1 standard solution)] * 100 (1)

2.7. Statistical analysis

Regarding the amino acids content in microalgae, basic hydrolysis and further derivatization were exhausted in triplicate for each sample (Steel et al., 1997). Statistical data regarding the GC-MS method validation and the amino acids content in microalgae were obtained by using Microsoft Office Excel (Microsoft Office LTSC Professional Plus 2021). Data regarding PCA analysis were processed with SIMCA® Multivariate Data Analytics Solution, version 17.0.2.34594, Sartorius Stedim Data Analytics AB software.

3. Results and discussion

Spirulina and *Chlorella* microalgae are naturally rich in lipids, pigments, bioactive compounds. In particular, due to their high content in proteins, they can be exploited as a food source or as a food supplement for human nutrition, farming, as supplements for athletes, in substitution of animal proteins (Davani et al., 2022). In literature, only few methods are reported for the determination of amino acids in microalgae and these techniques demonstrate various shortcomings such as long chromatographic run times (about 2–3 h per sample), lack of analyte specificity due to interference by co-eluting compounds and the the difficulty of determining tryptophan since it is acidic pH sensitive (Andreeva et al., 2021; Araya et al., 2021; Derrien et al., 1998). In this context, the present work was aimed to develop and validate an accurate, fast and reproducible new GC-MS analytical method, useful to determine the full essential amino acid content, including tryptophan, in different commercial samples of microalgae. Under the here proposed method, the use of a single step hydrolysis procedure followed by all amino acids silylation as pre-derivatization sample preparation and GC-MS method allowed a reliable amino acid less time consuming and more accurate, selective and reproducible determination (Fig. 1).

Under the optimized chromatographic GC-MS conditions all 18 amino acids (asparagine and glutamine were determined as aspartic acid and glutamic acid respectively) were determined with a good chromatographic resolution and a short analysis time, as reported in par. 2.5 (Pérez-Palacios et al., 2015; Sobolevsky et al., 2003). In more details, their identification was obtained by comparing the reference standards retention times and their mass spectra matching, by using the NIST 2012 library. Instead, quantitative determination was obtained by using norleucine as internal standard, and by preparing standard calibration curves with standard amino acids. After method validation, spiked microalgae calibration curves were obtained for determining accuracy and the overall method recovery. By applying this validated GC-MS method, it was possible to obtain the complete qualitative-quantitative amino acid profile of seven commercial samples of microalgae, four samples of *Spirulina* (S1, S2, S3, S4) and three samples of *Chlorella* (C1, C2, C3) (of which we previously studied the overall composition in terms of total protein content, lipids, pigments, etc.) (Davani et al., 2022; Oliveira et al., 1999; Rahim et al., 2021; Safi, Zebib, et al., 2014; Spoehr & Milner, 1949). The application of this method was found useful to highlight differences in composition within samples of the same species and between samples belonging to the two species with the aim to select the best strain for specific and different applications.

Concerning sample preparation, since tryptophan is unstable at acidic pH, a protein hydrolysis in basic conditions was optimized (Bellmaine et al., 2020; Kuiken et al., 1947). Then, to volatilize amino acids prior GC-MS analysis, since the silylating reagent is unstable to basic pH, the amino acid solutions were neutralized by using HCl. Then, a pre-derivatization step by using MTBSTFA as silylating agent was optimized to find the best reaction conditions. The resulting solutions containing silylated amino acids were then injected into the GC-MS. The GC-MS analysis was selected since separation of derivatized amino acids by gas chromatography is quicker than by HPLC, still guaranteeing their identification by mass detection (Biermann et al., 1986). Obtained results were compared regarding the essential and non-essential amino acids content, highlighting the different distribution depending on the species and on the sample source. A final comparison of the microalgae contents to the daily amino acid requirements identified by WHO was performed (World Health Organization, 2007).

Finally, by applying a multivariate analysis, the microalgae samples were grouped in clusters, according to the single amino acid results expressed as a percentage of the total microalgal protein content of the specific sample (data reported in Table S10a and S10b) (Davani et al., 2022). The resulting clusters underline intra-inter species differences for specific uses.

3.1. Sample preparation

The amino acid composition analysis is a technique that comprises two steps: the hydrolysis of the substrate and the chromatographic analysis. Fort these reasons, a suitable hydrolysis is a requirement for a comprehensive analysis of all the amino acids contained in microalgae sample. To determine the amino acid content of proteins, acid hydrolysis is generally used and applied to many matrices. (Moore & Stein, 1948; Moore et al., 1958). However, tryptophan is an amino acid difficult to determine because it chemically decomposes during acid hydrolysis, leading to a reduction or complete loss of its quantity. In addition, the stability of tryptophan in solutions can be affected by various environmental factors such as temperature, light exposure, reactive oxygen species. Moreover, tryptophan can react with sugars, vitamins, aldehydes, keto acids and trace metals (Bellmaine et al., 2020). Usually, additional analyses on the specific sample must be performed for tryptophan accurate determination in basic conditions. In fact, tryptophan is stable to alkaline hydrolysis which is usually obtained by using NaOH or KOH at 100–120°C for approximately 24 h, a time similar to that of acid

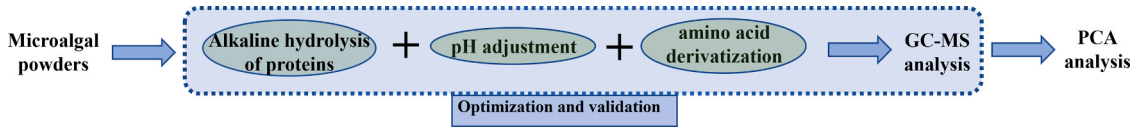

Fig. 1. Scheme of optimization and validation processes.

hydrolysis. In addition, the use of NaOH at high temperature and prolonged exposure time, is advantageous because this treatment guarantees the microalgae complete cell wall disruption (Nunes et al., 2024; Safi et al., 2014).

Taking into account all these issues, in this study, protein basic hydrolysis was applied (Fountoulakis & Lahm, 1998). The potential racemization of the amino acids during the protein hydrolysis step, after the subsequent derivatization with MTSTFA, did not affect the GC-MS analysis results. Hence, a single procedure for the analysis of all 18 amino acids present in the microalgal sample was performed. Asparagine and glutamine, that suffer deamination reactions during hydrolysis and they are converted in aspartic acid and glutamic acid were determined in these forms (Mustățea et al., 2019). Arginine was evaluated as its degradation product, ornithine.

Another issue to solve was that MTBSTFA is sensitive to alkaline pH and to moisture. So, the pH of the hydrolyzed solution was adjusted in order to obtain the best amino acids derivatization conditions. A pH range between 5 and 10 was investigated. Only reactions carried out between pH= 6.5 and pH= 7 preserved all amino acids, including tryptophan. To adjust the pH at this value, many solutions were tested such as phosphate buffer 0.1 M, citrate buffer 0.1 M, HCl and a very large excess (1 mL) of MTBSTFA. In particular, the use of buffers created problems in processing the sample forming a very sticky solid, which compromised water removal. The use of MTBSTFA in large excess gave rise to numerous interferences and increased costs. The best condition in terms of feasibility, cost, stability, repeatability of the reaction, ease of removal of water and interferences decrease, was the addition of a 2 N HCl water solution.

By following this sample preparation procedure (basic hydrolysis and derivatization at pH 6.5–7.0) a high recovery was achieved (recovery range= 93.21 ± 2.67 – 99.77 ± 0.97 %, recovery average= 96.20 ± 1.62 %). Moreover, another advantage regarded the opportunity to inject the obtained microalgae amino acid extract into the GC-MS after the pre-derivatization step, without any further purification. The obtained short run (37 minutes) chromatograms were found free from interferences (Fig. 2).

3.2. Derivatization reaction

In order to transform microalgae amino acids into volatile derivatives, a pre-derivatization reaction was performed. MTBSTFA is a silylating reagent, which produces derivatives more stable to hydrolysis and moisture than those derived from the use of lower molecular weight reagents (Biermann et al., 1986; Stenerson, 2007).

On these premises, after protein hydrolysis, amino acids derivatization using MTBSTFA was achieved by modifying previously described methods (Davani et al., 2023; Pérez-Palacios et al., 2015; Sobolevsky et al., 2003).

During optimization step, we took into consideration that amino acids free carboxyl, amine, and thiol groups can react with TBDMS, therefore producing multiple derivatives. Hence, in order to obtain only one derivative, some modifications in the silylating conditions were introduced. Different reaction temperatures in the range between 70 and 100 °C were combined to different reaction times comprised between 30 and 240 minutes. The best reaction conditions were found to be (0.429 mol L^{-1}) MTBSTFA at 80 °C for 240 minutes.

The identification of the amino acids in the analyzed microalgal samples was performed by comparing sample and reference standard retention times and matching mass spectra with NIST 2012 library. The final selected amino acids pre-derivatization conditions ensured their complete derivatization to just one chromatographic peak. In detail, the obtained amino acids derivatives are reported in Table 1.

Experimental amino acids spectra, compared with those reported in NIST library, are reported in SI par 1, Fig. S1-S19. The complete and stable derivatization reaction allowed the validation of the method and the construction of the calibration curves. Since it was not possible to know the amino acid content in microalgal samples before analyzing them, it was decided to use the MTBSTFA reagent in large excess (300 μL of 0.429 mol L^{-1}MTBSTFA for each microalgae sample).

3.3. Chromatographic analysis

The chromatographic method was optimized, starting from an

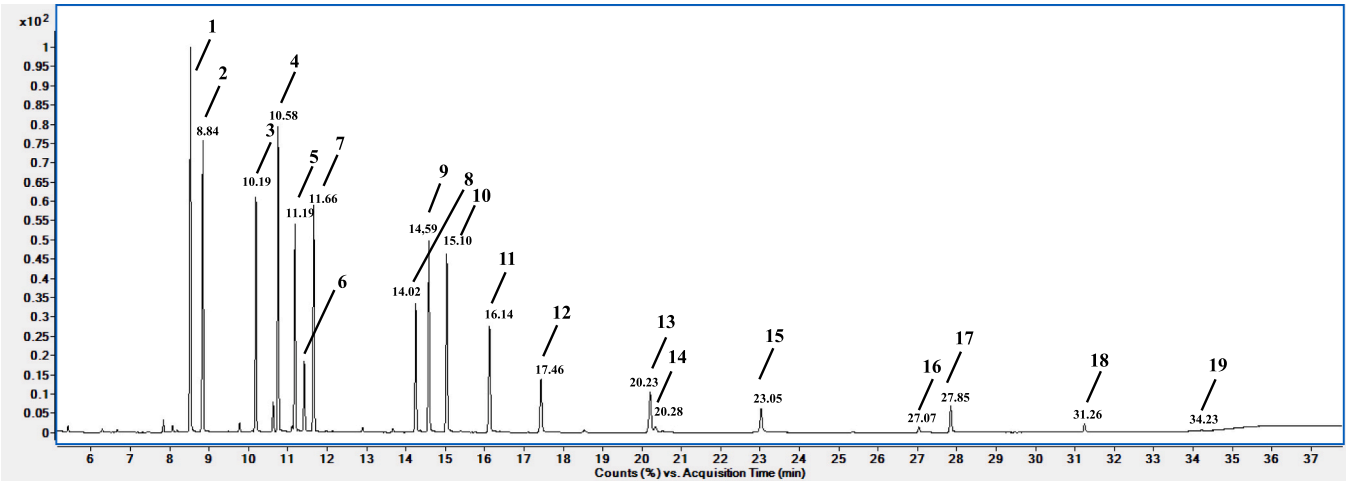

Fig. 2. Chromatogram of amino acids of *Spirulina* microalgae sample S1 obtained with GC-MS analysis. The peaks identify the following amino acids: 1 = Alanine, 2 = Glycine, 3 = Valine, 4 = Leucine, 5 = Isoleucine, 6 = Norleucine, 7 = Proline, 8 = Methionine, 9 = Serine, 10 = Threonine, 11 = Phenylalanine, 12 = L-Aspartic Acid, 13 = Glutamic Acid, 14 = Ornithine (Arginine), 15 = Lysine, 16 = Histidine, 17 = Tyrosine, 18 = Tryptophan, 19 =Cysteine.

Table 1
Amino acids derivatives and their matching scores with NIST 2012 library.

Amino acid	Derivatized amino acid	NIST probability %
Alanine	Alanine, N-(tert-butyldimethylsilyl)-, tert-butyldimethylsilyl ester	88.1
Glycine	Glycine, N-(tert-butyldimethylsilyl)-, tert-butyldimethylsilyl ester	87.8
Valine	Valine, N-(tert-butyldimethylsilyl)-, tert-butyldimethylsilyl ester	82.9
Leucine	Leucine, N-(tert-butyldimethylsilyl)-, tert-butyldimethylsilyl ester	70.3
Isoleucine	Isoleucine, N-(tert-butyldimethylsilyl)-, tert-butyldimethylsilyl ester	70.9
Norleucine	Norleucine, N-(tert-butyldimethylsilyl)-, tert-butyldimethylsilyl ester	64.3
Proline	Proline, 1-(tert-butyldimethylsilyl)-, tert-butyldimethylsilyl ester	98.5
Methionine	Methionine, N-(tert-butyldimethylsilyl)-, tert-butyldimethylsilyl ester	98.7
Serine	Serine, N,O-bis(tert-butyldimethylsilyl)-, tert-butyldimethylsilyl ester	95.8
Threonine	Threonine, N,O-bis(tert-butyldimethylsilyl)-, tert-butyldimethylsilyl ester	93.8
Phenylalanine	Phenylalanine, N-(tert-butyldimethylsilyl)-, tert-butyldimethylsilyl ester	94.8
Aspartic acid	Aspartic acid, N-(tert-butyldimethylsilyl)-, bis(tert-butyldimethylsilyl) ester	98.7
Glutamic acid	Glutamic acid, N-(tert-butyldimethylsilyl)-, bis(tert-butyldimethylsilyl) ester	98.5
Arginine*	Ornithine, N2,N5-bis(tert-butyldimethylsilyl)-, tert-butyldimethylsilyl ester	79.8
Lysine	Lysine, N2,N6-bis(tert-butyldimethylsilyl)-, tert-butyldimethylsilyl ester	97.2
Histidine	Histidine, N1-bis(tert-butyldimethylsilyl)-, tert-butyldimethylsilyl ester	97.5
Tyrosine	Tyrosine, N,O-bis(tert-butyldimethylsilyl)-, tert-butyldimethylsilyl ester	97.9
Tryptophan	Tryptophan, N-(tert-butyldimethylsilyl)-, tert-butyldimethylsilyl ester	97.9
Cysteine	Cysteine, N,N1-bis(tert-butyldimethylsilyl)-, tert-butyldimethylsilyl ester	92.4

already published method (Pérez-Palacios et al., 2015; Sobolevsky et al., 2003; Starke et al., 2001). Under the described selected chromatographic conditions, the GC elution of the 18 derivatized amino acid peaks were found to be comprised in the range 8.53 – 34.23 minutes (Fig. S20), reducing total runtime in respect to those reported by Pérez-Palacios et al. on meat matrix (from 12.86 to 50.18) and by Vendruscolo et al. on microalgal matrix (from 13.60 to 53.11 minutes). Asparagine and glutamine were determined as aspartic acid and glutamic acid respectively, and arginine was quantified as ornithine derivative, its degradation product. The respective chromatographic peaks were baseline resolved and not affected by interferences.

By plotting the five incremental concentrations of the derivatized amino acid standards versus the ratio of "derivatized amino acid peak area/derivatized D,L-norleucine peak area", calibration curves (n = 3) were obtained for each studied amino acid. Linear least-squares regression analysis (Table S2) were obtained with good correlation coefficients (average of R^2= 0.998181 ± 6.38E-04), a similar value to the one reported in literature (average R^2=0.99720 ± 2.32E-03) (Vendruscolo et al., 2018; Villas-Bôas et al., 2003). LoD and LoQ values obtained by a statistical evaluation, considering the standard signal deviations, are reported in the SI (Table S2). Average of LoD and LoQ values, obtained from signal-to-noise ratios of 3:1 and 10:1 respectively, were found to be 0.046010 ± 4.27E-04 μg mL^{-1} and 0.15002 ± 1.41E-03 μg mL^{-1} of the same order of magnitude of those reported in literature (Vendruscolo et al., 2018; Villas-Bôas et al., 2003). However, this is not a parameter object of optimization in this work, since microalgal matrix has a very high amino acid content. Then, the method was validated on microalgae samples in terms of specificity, linearity, sensitivity, precision, accuracy and recovery (par. 3.4) (European Medicines Agency, 1995).

3.4. GC-MS method validation

The specificity of the method was determined by using three blank samples of *Spirulina* microalgae. and comparing the obtained chromatograms after injecting the pre-derivatized non-spiked and the pre-derivatized spiked with standard amino acids samples. After each sample analysis, two solvent injections were performed to demonstrate the absence of any carry-over effect. Results showed a good correlation coefficient (average of R^2 = 0.998801 ± 7.66E-04). LoD and LoQ obtained by a statistical evaluation considering the standard signal deviations are reported in SI (Table S3). LoD and LoQ obtained from signal-to-noise ratios of 3:1 and 10:1 respectively, were of 0.031021 ± 1.39E-04 μg mL^{-1} and 0.100012 ± 4.58E-04 μg mL^{-1} respectively. Therefore, the validated GC-MS method showed a satisfactory and suitable selectivity and very high sensitivity for evaluating the presence of each studied amino acid. Results of the determination of the intra-day and inter-day showed of relative standard deviation (RSD) values of 1.31 ± 1.44E+ 00 % and 1.21 ± 1.23E+ 00 % respectively (Table S4, S6). The average of the RDS for intra-and inter-day of spiked S_std proved to be 2.23 ± 1.11E+ 00 % and 2.44 ± 1.20E+ 00 % respectively (Table S5, S7), better than that reported in literature (intra-day RSD= 6.23 ± 4.17E+00 % and inter-day RSD= 7.37 ± 5.17E+00 %) (Vendruscolo et al., 2018). Accuracy was found to be 99.201 ± 7.61E-01 %, as average of all amino acids (Table S8), thus confirming the closeness between experimental and true value. The amino acids recovery was very satisfactory and comprised between 93.21 ± 2.67E+ 00 % and 99.770 ± 9.67E-01 % (Table S9). Results confirmed the high accuracy of this GC-MS method, including extraction and derivatization steps. In addition, by using this new method, improved data were achieved when compared to those reported by Furlan et al. (accuracy range from 60 % to 120 %) and by Vendruscolo et al. (accuracy range from 68.7 % to 122.7 %).

Resuming, a fast (37 minutes run), selective, accurate (99.201 ± 7.61E-01 %, average of all amino acids), sensitive (LoD and LoQ values of 0.031021 ± 1.39E-04 μg mL^{-1} and 0.100012 ± 4.58E-04 μg mL^{-1} respectively), reproducible and endowing a recovery better than those reported in literature, GC-MS method was validated (Furlan et al., 2024; Vendruscolo et al., 2018).

3.5. Sample analysis

In literature, some publications report the analysis of amino acids in microalgae, but these methods usually regard the use of acid hydrolysis that excludes the quantification of tryptophan (Kudełka et al., 2021). This newly optimized and validated method, involves alkaline hydrolysis, allows the determination of all the amino acids including tryptophan in lyophilized powders of microalgal samples. The estimated amount in terms of percentage of amino acids on dried weight of microalgal powder (S1-S4 and C1-C3) (n = 3), is reported in SI, Table S10a and S10b.

In Fig. 3, graph (a) reports the amino acid content of analyzed *Spirulina* samples and graph (b) reports the amino acid content of analyzed *Chlorella* samples. It is important to highlight that all the analyzed samples contain, albeit in different quantities, all the amino acids and in particular, all the essential amino acids. The average value of total amino acids in *Spirulina* species is 59.30 ± 3.16E+ 00 % on dry weight, of which 20.89 ± 1.01E+ 00 % of essential amino acids. The average value of total amino acids in *Chlorella* species is 42.16 ± 6.20E+ 00 % on dry weight, of which 17.441 ± 9.59E-01 % of essential amino acids.

In *Spirulina* species the highest content of non-essential amino acids is represented by glutamic acid, aspartic acid, alanine and, in addition by arginine, while the essential amino acid content is characterized by

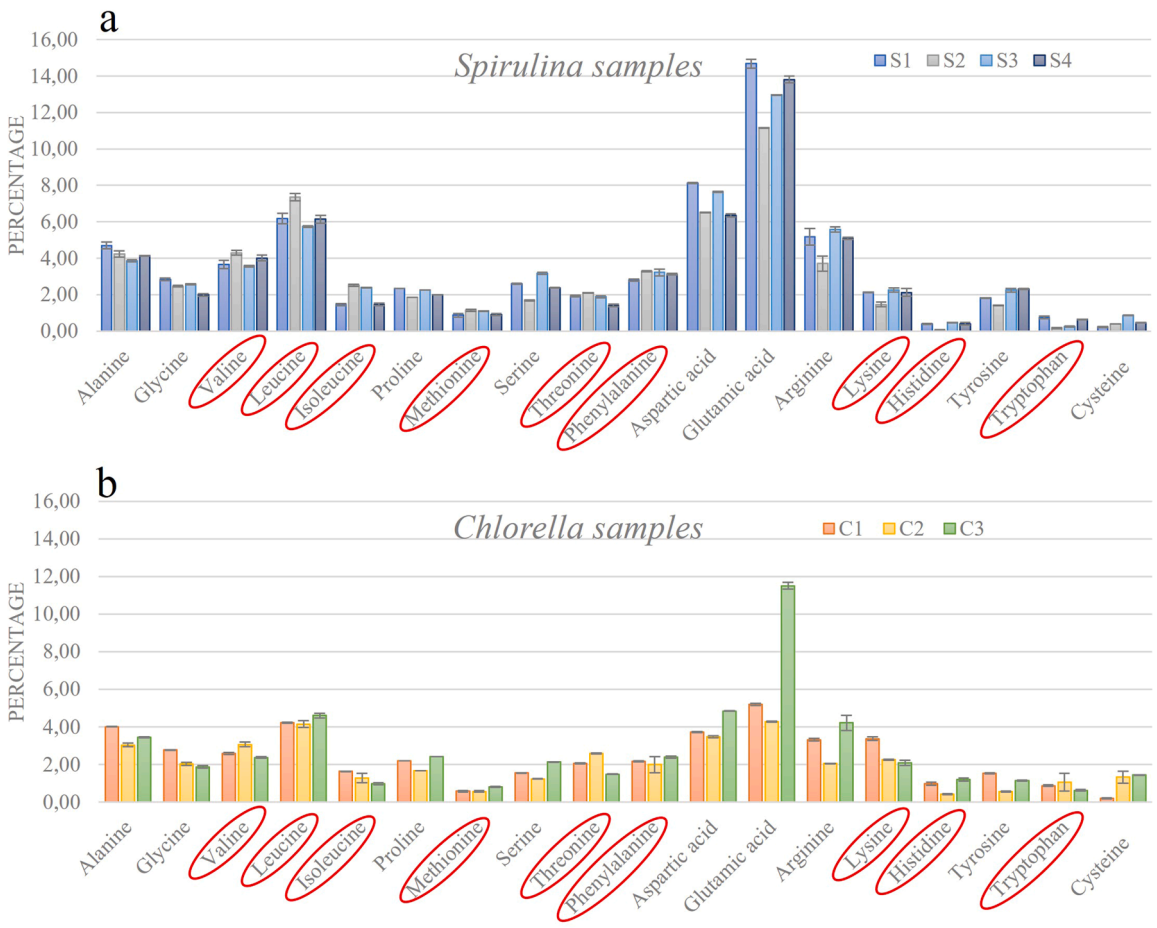

Fig. 3. (a) amino acid content expressed in percentage on dried weight of powder in analyzed *Spirulina* samples, (b) amino acid content expressed in percentage on dried weight of powder in analyzed *Chlorella* samples. Red circle highlights essential amino acids.

leucine, valine and phenylalanine.

Instead, regarding *Chlorella* species, the highest content of non-essential amino acids is represented by glutamic acid, aspartic acid, alanine and arginine, while the essential amino acids include leucine, valine and lysine. In both species of microalgae, glutamic acid is the amino acid present in largest quantity, added to those already present (that derive from the sum of glutamine and glutamic acid), with the highest value equal to 14.680 ± 2.38E-01 % in sample S1 of the *Spirulina* species, while in the Chlorella species reaches 11.502 ± 1.78E-01 % in sample C3.

Table 2: essential amino acids content reported in percentage regarding the protein content. *Grams per day for a person of 70 kg

Table 2
Essential amino acids content reported in percentage on protein content.

Amino acid	% amino acid on protein n = 3							g per day (person 70 kg)*
	S1	**S2**	**S3**	**S4**	**C1**	**C2**	**C3**	
Valine	5.60 ± 3.40E−01	6.90 ± 2.15E−01	4.75 ± 7.07E−02	6.81 ± 2.79E−01	4.97 ± 1.13E−01	5.81 ± 2.21E−01	3.81 ± 5.52E−02	1.82
Leucine	9.46 ± 4.48E−01	11.81 ± 3.20E−01	7.67 ± 5.66E−02	10.44 ± 3.71E−01	8.14 ± 6.94E−02	7.85 ± 3.18E−01	7.41 ± 1.97E−01	2.73
Isoleucine	2.23 ± 7.68E−02	4.02 ± 8.83E−02	3.18 ± 3.59E−02	2.49 ± 1.26E−01	3.14 ± 3.91E−03	2.43 ± 4.72E−01	1.56 ± 7.63E−02	1.40
Methionine	1.34 ± 1.55E−01	1.83 ± 7.51E−02	1.46 ± 1.98E−02	1.55 ± 1.09E−01	1.11 ± 9.50E−02	1.07 ± 7.55E−02	1.30 ± 3.53E−02	0.73
Threonine	2.93 ± 6.49E−02	3.37 ± 2.84E−02	2.50 ± 8.06E−02	2.38 ± 1.24E−01	3.96 ± 7.72E−02	4.88 ± 6.37E−02	2.38 ± 2.82E−02	1.05
Phenylalanine	4.28 ± 8.23E−02	5.28 ± 3.70E−02	4.30 ± 2.47E−01	5.30 ± 6.92E−02	4.16 ± 5.44E−02	3.76 ± 8.01E−01	3.85 ± 1.04E−01	1.75 * *
Lysine	3.27 ± 2.64E−02	2.35 ± 2.05E−01	3.00 ± 1.42E−01	3.60 ± 3.62E−01	6.49 ± 1.89E−01	4.25 ± 4.11E−02	3.35 ± 2.35E−01	2.10
Histidine	0.61 ± 3.41E−02	0.02 ± 9.89E−02	0.64 ± 1.43E−02	0.67 ± 1.23E−01	1.89 ± 1.53E−01	0.79 ± 4.58E−02	1.93 ± 1.27E−01	0.70
Tryptophan	1.14 ± 1.06E−01	0.25 ± 5.65E−02	0.33 ± 5.41E−02	1.08 ± 1.28E−02	1.67 ± 8.69E−02	2.01 ± 8.84E−01	1.00 ± 8.56E−02	0.28

*Grams per day for a person of 70 kg estimated from suggested amount of amino acid by WHO. * * Grams per day of phenylalanine combined with the non-essential amino acid tyrosine for a person of 70 kg estimated from suggested amount of amino acid by WHO.

estimated from suggested amount of amino acid by WHO. * * Grams per day of phenylalanine combined with the non-essential amino acid tyrosine for a person of 70 kg estimated from suggested amount of amino acid by WHO.

Table 2 shows the essential amino acid content for each microalgae sample reported in percentage on protein content, previously determined (Davani et al., 2022). In details, except for histidine content in S1-S4 and tryptophan content in S2, all the analyzed microalgae contained a higher amount of essential amino acids, above the recommended limit for human daily nutrition (World Health Organization, 2007). Leucine was found to be the most abundant amino acid contained in the analyzed microalgal powders (11.810 ± 3.20E-01 % in S2). This result is extremely interesting, because it is usually more abundant in animal sources (Kimball & Jefferson, 2006; Rondanelli et al., 2021; World Health Organization, 2007). Furthermore, it is reported that the intake of food containing leucine stimulates muscle protein synthesis. Valine, that promotes human muscle growth and tissue repair and has a role in the immune response, was found to be in high amount in all analyzed samples and the highest content is in S2 (6.901 ± 2.15E-01 % on protein) (Sharma et al., 2022). Isoleucine, of which the highest content is in S2 too (4.0204 ± 8.83E-02 % on protein), is one of the three branched-chain amino acids (together with leucine and valine) that is involved in enhancing glucose consumption and utilization, by up-regulating intestinal and muscular glucose transporters (Zhang et al., 2017). Usually, branched-chain amino acids are contained in meat and meat products, so this high concentration in microalgae underlines their important nutritional value (Górska-Warsewicz et al., 2018). All analyzed samples demonstrated a high content of methionine, which exhibits antioxidant activity, that was approximately the double of that recommended by the WHO, and, in particular, S2 was found to be the richest (Campbell et al., 2007). Regarding the threonine and phenylalanine contents, that usually are mostly abundant in meat and meat products, each analyzed sample contained almost the double of the value indicated WHO (Górska-Warsewicz et al., 2018). In particular, threonine is particularly important, since it serves as a substrate for protein synthesis in humans. On the other hand, phenylalanine is reported to be needed for the synthesis of proteins, catecholamines, and melanin (Kohlmeier, 2003). In addition, it is reported that phenylalanine is a potent satiety hormone releaser, cholecystokinin, and that its ingestion reduces energy intake (Pohle-Krauza et al., 2008). Lysine, that usually is present in most plant proteins only in low concentrations, showed to be in high concentration in these analyzed microalgal samples (C1 = 6.491 ± 1.89E-01 % on protein) (Eggeling & Sahm, 2011). Histidine, that has important anti-inflammatory, antioxidant, and anti-secretory functions within the body, except for S2, reaches the minimum recommended dose by the WHO in all samples (Peterson et al., 1998). Tryptophan, that is a precursor for important metabolites such as serotonin and nicotinamide, is mostly found in animal products, such as beef, lamb, pork, poultry, and dairy, as well as in nuts and seeds, whole grains, and legumes. It is the least abundant amino acid in the cell, and one of the rarest in the proteome (Barik, 2020; Richard et al., 2009; Zuraikat et al., 2021). Except for S2 and S3, tryptophan was found at a level greater than three times that suggested by WHO. In particular, C1 and C2 (1.6720 ± 8.69E-02 % on protein and 2.011 ± 8.84E-01 % on protein respectively) demonstrated a content approximately six times higher than that of WHO.

Therefore, under the here described GC-MS method, all amino acids, including tryptophan, were quantified with a single analysis. This allowed the comprehensive amino acid characterization of the microalgae samples. Thus, it was demonstrated that all the samples contained all essential and non-essential amino acids in very high quantities and, in particular, that the microalgal samples had a content similar or better to the daily amino acid requirements identified by WHO (0.18 g kg^{-1} per day and 0.48 g kg^{-1} per day respectively of essential amino acids) (World Health Organization, 2007). This study highlights how microalgae can be considered a substitute food for proteins of animal origin and a food supplements of plant origin.

3.6. Principal component analysis

The use of principal component analysis is essential to emphasize not only the amino acids differences of the two microalgae strains, but also within the same species. This analysis can underline specific claims for each class, addressed to define final users, tracing a profile of the intrinsic microalgae characteristics for peculiar uses.

The PCA plot presented in Fig. 4 shows the amino acids composition of the two commercial microalgae strains, *Spirulina* and *Chlorella*. In particular, this PCA analysis correlates *Chlorella* (C1-C4) and *Spirulina* (S1-S5) samples with the analytical results regarding nineteen variables: percentage of the eighteen single amino acids on protein amount and the percentage of protein amount on dry microalgae mass.

Obtained data revealed clearly detectable inter-species differences and intra-species correlations. First, all *Chlorella* samples (C1-C3) are located in the opposites side of the graph compared to *Spirulina* (S1-S4) species so microalgae can be clustered in terms of their amino acid content (Fig. 4a). As reported in Fig. 4b, loadings highlighted that the amino acid content is distributed differently between the two microalgal species. In particular, *Chlorella* is richer in the essential amino acids such as threonine, lysine, tryptophan and histidine, while *Spirulina* stands out for its content of the essential amino acids, namely isoleucine, valine, leucine, phenylalanine and methionine. In particular, as indicated by the purple spot, *Spirulina* is also characterized by a higher total protein content and, in addition, it contains a higher content of other non-essential amino acids compared to *Chlorella*.

As regards intra-species differences, both *Chlorella* and *Spirulina* demonstrated a different content of essential amino acids depending on the analyzed sample. Specifically, C1 stands out for its higher content of lysine and histidine, C2 has a higher content of tryptophan and threonine, while C3 has slightly lower contents of essential amino acids compared to the other two samples. So, C1 can be exploited for its anti-inflammatory and antioxidant properties and C2 can be used to regulate appetite, sleep, mood and to contribute to maintain the proper protein balance in the body. In addition, since lysine, that is contained in high amount in all *Chlorella* samples, has a pleasant bitter-sweet taste it can be considered to mask bitter aftertaste of potassium chloride when it used as a salt replacer in dietary supplements (Gascon, 2007). To sum up, *Chlorella* can be taken to counteract aging processes and to contribute to a correct lifestyle (sleep-wake rhythm, mood control) especially addressed to aged individuals and vegetarians.

Likewise, *Spirulina* also demonstrated differences. In particular, the best sample is S2, which stands out for the highest content of isoleucine, valine, leucine, phenylalanine and methionine. S4 also has a similar valine and phenylalanine content to S2 and also a high leucine content. Thus, *Spirulina* samples can be used as food supplement for athletes and sporty people to improve the glucose metabolism, enhance energy, increase endurance, aid in muscle tissue recovery and repair, increase muscle growth and lean body mass, favour the release of the satiety hormone cholecystokinin (and limiting calorie consumption) and for the antioxidant activity.

As regards the comparison between the protein contents, obtained from our previous work, and the amino acid contents of analyzed samples, it is necessary to take into account the origin of the data from two totally different analytical methods (in particular the analytical method for the quantification of proteins depends on the concentration of aromatic amino acids) and the standard deviations of each data (Davani et al., 2022). Despite these differences, the percentage of total amino acids on total protein content in the analyzed microalgae (data shown in Table S10a and S10b) demonstrate a good correlation. In particular, *Spirulina* showed the highest percentage (from 81.57 % up to 98.97 %), while *Chlorella*, although with lower percentages (from 67.50 % to 82.30 %), presented acceptable values.

Therefore, this new GC-MS analytical method, combined with basic

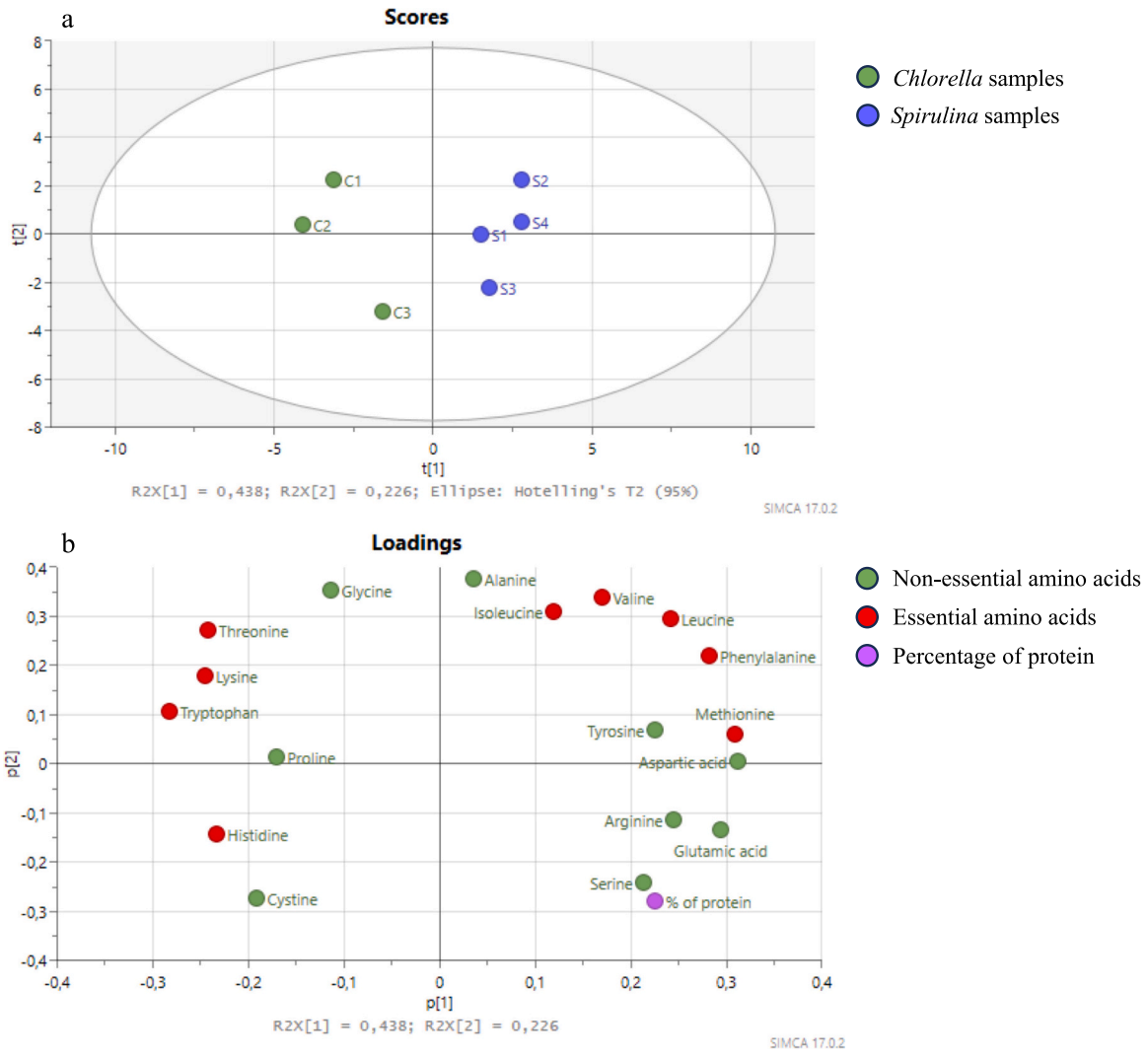

Fig. 4. PCA score plot showing amino acids composition of four *Spirulina* samples (S1-S4) and three *Chlorella* samples (C1-C4). (a) scores of PCA analysis, *Spirulina* samples are identified by blue spots and *Chlorella* samples are identified by green spots; (b) loadings of PCA analysis related to scores. Essential amino acids are highlighted in red, non-essential amino acids are reported in green, the percentages of proteins are reported in purple (Davani et al., 2022).

microalgae protein hydrolysis and pre-derivatization step, can be useful for the comprehensive amino acids profiling of the two species of microalgae. Furthermore, the multivariate analysis, emphasizes the amino acids different contents in the two microalgae strains for a more accurate use.

4. Conclusions

Microalgae represent an interesting sustainable protein source compared to current plant and animal sources, and they can be exploited as dietary supplements. Seen the necessity to characterize the full amino acid content, especially the essential ones and considering the lack in literature, a new selective, accurate, sensitive, reproducible and endowing a good recovery GC-MS method was validated. This method was found to be suitable for the comprehensive determination of all essential and non-essential amino acids in microalgal samples including tryptophan which is usually lost. An optimized basic protein hydrolysis, pH adjustment and pre-derivatization step by using MTBSTFA was found to be an accurate sample preparation that guarantees the complete recovery of all amino acids. All the analyzed microalgae samples contained all essential and non-essential amino acids in high quantities, although the distribution of amino acids was significantly different depending on the species and depending on the sample. Data demonstrated that the microalgal samples had a content similar or better than the daily amino acid uptake suggested by WHO. The use of a multivariate analysis of data allowed to correlate the amino acid pattern of analyzed samples. Results showed that microalgae can be exploited as a source of food or as a food supplement, both directly as microalgal powder (for example in baked preparations or in capsules/tablets), and as a protein extract and that they can be used by athletes, young people, vegetarians, vegans and aged individuals to promote a wellness status. The new GC-MS method, together with the PCA, may be useful for the pharmaceutical, cosmetic, food supplements and food industries, because it allows the comprehensive characterization of the amino acid profile of microalgae and therefore gives the possibility to direct them towards different market fields and different uses according to their peculiarities. Another interesting perspective is the use of this method to check how microalgae growing conditions, in terms of light, medium composition and heat can affect their ammino acids composition.

Author statement

I hereby certify that all authors have seen and approved the final version of the manuscript being submitted. The article is the authors' original work, has not received prior publication and is not under consideration for publication elsewhere.

CRediT authorship contribution statement

Medri Francesca: Data curation. **Montanari Serena:** Writing – review & editing, Supervision, Software, Methodology, Investigation, Conceptualization. **Andrisano Vincenza:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition. **Terenzi Cristina:** Methodology. **Bermudez Gabriela:** Writing – original draft, Validation, Software.

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.

Acknowledgements

The authors are grateful for financial support from the University of Bologna, CIRI-MAM, Eni Spa and Uni.Rimini Spa.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jfca.2025.107289.

Data availability

Data will be made available on request.

References

Andreeva, A., Budenkova, E., Babich, O., Sukhikh, S., Ulrikh, E., Ivanova, S., Prosekov, A., Dolganyuk, V., 2021. Production, purification, and study of the amino acid composition of microalgae proteins. Molecules 26 (9), 2767. https://doi.org/10.3390/molecules26092767.

Araya, M., García, S., Rengel, J., Pizarro, S., Álvarez, G., 2021. Determination of free and protein amino acid content in microalgae by HPLC-DAD with pre-column derivatization and pressure hydrolysis. Mar. Chem. 234, 103999. https://doi.org/10.1016/j.marchem.2021.103999.

Barik, S., 2020. The uniqueness of tryptophan in biology: properties, metabolism, interactions and localization in proteins. Int. J. Mol. Sci. 21 (22), 8776. https://doi.org/10.3390/ijms21228776.

Bellmaine, S., Schnellbaecher, A., Zimmer, A., 2020. Reactivity and degradation products of tryptophan in solution and proteins. Free Radic. Biol. Med. 160, 696–718. https://doi.org/10.1016/j.freeradbiomed.2020.09.002.

Biermann, C.J., Kinoshita, C.M., Marlett, J.A., Steele, R.D., 1986. Analysis of amino acids as tert.-butyldimethylsilyl derivatives by gas chromatography. J. Chromatogr. A 357, 330–334. https://doi.org/10.1016/S0021-9673(01)95837-6.

Campbell, K.C.M., Meech, R.P., Klemens, J.J., Gerberi, M.T., Dyrstad, S.S.W., Larsen, D. L., Mitchell, D.L., El-Azizi, M., Verhulst, S.J., Hughes, L.F., 2007. Prevention of noise- and drug-induced hearing loss with d-methionine. Hear. Res. 226 (1–2), 92–103. https://doi.org/10.1016/j.heares.2006.11.012.

Davani, L., Tassinari, E., Chiaberge, S., Siviero, A., Serbolisca, L., Tumiatti, V., Terenzi, C., De Simone, A., Andrisano, V., Montanari, S., 2023. Safety issues in nutraceutical exploitation of chlorella vulgaris, arthrospira platensis and scenedesmus sp. microalgae. J. Food Compos. Anal. 123, 105568. https://doi.org/10.1016/j.jfca.2023.105568.

Davani, L., Terenzi, C., Tumiatti, V., De Simone, A., Andrisano, V., Montanari, S., 2022. Integrated analytical approaches for the characterization of spirulina and chlorella microalgae. J. Pharm. Biomed. Anal. 219, 114943. https://doi.org/10.1016/j.jpba.2022.114943.

Derrien, A., Coiffard, L.J.M., Coiffard, C., De Roeck-Holtzhauer, Y., 1998. Free amino acid analysis of five microalgae. J. Appl. Phycol. 10 (2), 131–134. https://doi.org/10.1023/A:1008003016458.

Dietzen, D.J., Weindel, A.L., Carayannopoulos, M.O., Landt, M., Normansell, E.T., Reimschisel, T.E., Smith, C.H., 2008. Rapid comprehensive amino acid analysis by liquid chromatography/tandem mass spectrometry: comparison to cation exchange with post-column ninhydrin detection. Rapid Commun. Mass Spectrom. 22 (22), 3481–3488. https://doi.org/10.1002/rcm.3754.

Eggeling, L., Sahm, H., 2011. Amino acid production. Comprehensive Biotechnology. Elsevier, pp. 563–571. https://doi.org/10.1016/B978-0-444-64046-8.00186-5.

European Medicines Agency. (1995). ICH guideline Q2(R1) validation of analytical procedures: text and methodology. Retrieved from ⟨https://www.ema.europa.eu/en/documents/scientific-guideline/ich-guideline-q2r1-validation-analytical-procedures-text-methodology-step-5-first-version_en.pdf⟩. Accessed July 12, 2024.

Fountoulakis, M., Lahm, H.-W., 1998. Hydrolysis and amino acid composition analysis of proteins. J. Chromatogr. A 826 (2), 109–134. https://doi.org/10.1016/S0021-9673(98)00721-3.

Friedman, Mendel, Finley, J.W., 1971. Methods of tryptophan analysis. J. Agric. Food Chem. 19 (4), 626–631. https://doi.org/10.1021/jf60176a010.

Furlan, J.M., Da Silva Cunha, J.P., Ribas, V.B., Da Silva, K.F., Dos Santos, B.N., Centenaro, G.S., Wagner, R., Vendruscolo, R.G., 2024. Microalgae amino acids: method validation using gas chromatography coupled to mass spectrometry. J. Appl. Phycol. 36 (3), 1153–1167. https://doi.org/10.1007/s10811-023-03168-9.

Gałęzowska, G., Ratajczyk, J., Wolska, L., 2021. Determination of amino acids in human biological fluids by high-performance liquid chromatography: critical review. Amino Acids 53 (7), 993–1009. https://doi.org/10.1007/s00726-021-03002-x.

Gascon, M., 2007. Masking agents for use in foods. Modifying Flavour in Food. Elsevier, pp. 232–242. https://doi.org/10.1533/9781845693367.232.

Geada, P., Moreira, C., Silva, M., Nunes, R., Madureira, L., Rocha, C.M.R., Pereira, R.N., Vicente, A.A., Teixeira, J.A., 2021. Algal proteins: production strategies and nutritional and functional properties. Bioresour. Technol. 332, 125125. https://doi.org/10.1016/j.biortech.2021.125125.

Górska-Warsewicz, H., Laskowski, W., Kulykovets, O., Kudlińska-Chylak, A., Czeczotko, M., Rejman, K., 2018. Food products as sources of protein and amino acids—the case of Poland. Nutrients 10 (12), 1977. https://doi.org/10.3390/nu10121977.

Hempel, N., Petrick, I., Behrendt, F., 2012. Biomass productivity and productivity of fatty acids and amino acids of microalgae strains as key characteristics of suitability for biodiesel production. J. Appl. Phycol. 24 (6), 1407–1418. https://doi.org/10.1007/s10811-012-9795-3.

Kaspar, H., Dettmer, K., Gronwald, W., Oefner, P.J., 2009. Advances in amino acid analysis. Anal. Bioanal. Chem. 393 (2), 445–452. https://doi.org/10.1007/s00216-008-2421-1.

Kaur, L., Mao, B., Beniwal, A.S., Abhilasha, Kaur, R., Chian, F.M., Singh, J., 2022. Alternative proteins vs animal proteins: the influence of structure and processing on their gastro-small intestinal digestion. Trends Food Sci. Technol. 122, 275–286. https://doi.org/10.1016/j.tifs.2022.02.021.

Kimball, S.R., Jefferson, L.S., 2006. Signaling pathways and molecular mechanisms through which branched-chain amino acids mediate translational control of protein synthesis. J. Nutr. 136 (1), 227S–231S. https://doi.org/10.1093/jn/136.1.227S.

Kohlmeier, M., 2003. Phenylalanine. Nutrient Metabolism. Elsevier, pp. 314–321. https://doi.org/10.1016/B978-012417762-8.50051-X.

Koyande, A.K., Chew, K.W., Rambabu, K., Tao, Y., Chu, D.-T., Show, P.-L., 2019. Microalgae: a potential alternative to health supplementation for humans. Food Sci. Hum. Wellness 8 (1), 16–24. https://doi.org/10.1016/j.fshw.2019.03.001.

Kudełka, W., Kowalska, M., Popis, M., 2021. Quality of soybean products in terms of essential amino acids composition. Molecules 26 (16), 5071. https://doi.org/10.3390/molecules26165071.

Kuiken, K.A., Lyman, C.M., Hale, F., 1947. Factors which influence the stability of tryptophan during the hydrolysis of proteins in alkaline solution. J. Biol. Chem. 171 (2), 551–560. https://doi.org/10.1016/S0021-9258(17)41065-9.

La Cour, R., Jørgensen, H., Schjoerring, J.K., 2019. Improvement of tryptophan analysis by liquid chromatography-single quadrupole mass spectrometry through the evaluation of multiple parameters. Front. Chem. 7, 797. https://doi.org/10.3389/fchem.2019.00797.

Le Boucher, J., Charret, C., Coudray-Lucas, C., Giboudeau, J., Cynober, L., 1997. Amino acid determination in biological fluids by automated ion-exchange chromatography: performance of Hitachi L-8500A. Clin. Chem. 43 (8), 1421–1428. https://doi.org/10.1093/clinchem/43.8.1421.

León-Vaz, A., Giráldez, I., Moreno-Garrido, I., Varela, J., Vigara, J., León, R., Cañavate, J. P., 2023. Amino acids profile of 56 species of microalgae reveals that free amino acids allow to distinguish between phylogenetic groups. Algal Res. 74, 103181. https://doi.org/10.1016/j.algal.2023.103181.

Montanari, S., Davani, L., Tumiatti, V., Dimilta, M., Gaddi, A.V., De Simone, A., Andrisano, V., 2021. Development of an UHPLC-diode arrays detector (DAD) method for the analysis of polydatin in human plasma. J. Pharm. Biomed. Anal. 198, 113985. https://doi.org/10.1016/j.jpba.2021.113985.

Moore, S., Stein, W.H., 1948. Photometric ninhydrin method for use in the chromatography of amino acids. J. Biol. Chem. 176 (1), 367–388. https://doi.org/10.1016/S0021-9258(18)51034-6.

Moore, Stanford, Spackman, D.H., Stein, W.H., 1958. Chromatography of amino acids on sulfonated polystyrene resins. An improved system. Anal. Chem. 30 (7), 1185–1190. https://doi.org/10.1021/ac60139a005.

Mustăţea, G., Ungureanu, E., Iorga, E., 2019. Protein acidic hydrolysis for amino acids analysis in food—progress over time: a short review. J. Hyg. Eng. Des. 26 (49), 81–87.

National Research Council, 1989. Recommended dietary allowances, 10th ed. National Academies Press. https://doi.org/10.17226/1349.

Nunes, A.L.F., Lima, V.S., Miranda Júnior, J.R., Resende, M.E.T., Silva, C.A.S.D., Martins, M.A., Coimbra, J.S.D.R., 2024. Cell disruption of microalgae: advances and perspectives. Ciência Rural 54 (5). https://doi.org/10.1590/0103-8478cr20220330.

Oliveira, M.A.C.L.D., Monteiro, M.P.C., Robbs, P.G., Leite, S.G.F., 1999. Growth and chemical composition of spirulina maxima and spirulina platensis biomass at different temperatures. Aquac. Int. 7 (4), 261–275. https://doi.org/10.1023/A:1009233230706.

Pérez-Palacios, T., Barroso, M.A., Ruiz, J., Antequera, T., 2015. A rapid and accurate extraction procedure for analysing free amino acids in meat samples by GC-MS. Int. J. Anal. Chem. 2015, 1–8. https://doi.org/10.1155/2015/209214.

Peterson, J.W., Boldogh, I., Popov, V.L., Saini, S.S., Chopra, A.K., 1998. Anti-inflammatory and antisecretory potential of histidine in Salmonella-challenged mouse small intestine. Lab. Investig. ; a J. Tech. Methods Pathol. 78 (5), 523–534.

Pohle-Krauza, R.J., Navia, J.L., Madore, E.Y.M., Nyrop, J.E., Pelkman, C.L. Effects of L-phenylalanine on energy intake in overweight and obese women: interactions with dietary restraint status. Appetite, 51(1), 111–119. https://doi.org/10.1016/j.appet.2008.01.002.

Puigserver, P., 2017. Signaling transduction and metabolomics. Hematology, 7th ed. Elsevier, pp. 68–78. https://doi.org/10.1016/b978-0-323-35762-3.00007-x.

Rahim, A., Çakir, C., Ozturk, M., Şahin, B., Soulaimani, A., Sibaoueih, M., Nasser, B., Eddoha, R., Essamadi, A., El Amiri, B., 2021. Chemical characterization and nutritional value of Spirulina platensis cultivated in natural conditions of Chichaoua region (Morocco). South Afr. J. Bot. 141, 235–242. https://doi.org/10.1016/j.sajb.2021.05.006.

Richard, D.M., Dawes, M.A., Mathias, C.W., Acheson, A., Hill-Kapturczak, N., Dougherty, D.M., 2009. L -tryptophan: basic metabolic functions, behavioral research and therapeutic indications. Int. J. Tryptophan Res. 2. https://doi.org/10.4137/ijtr.s2129.

Rodriguez, J.R.B., Reina, G.G., Rodriguez, J.J.S., 1997. Determination of free amino acids in microalgae by high-performance liquid chromatography using pre-column fluorescence derivatization. Biomed. Chromatogr. 11 (6), 335–336. https://doi.org/10.1002/(SICI)1099-0801(199711)11:6< 335::AID-BMC684> 3.0.CO;2-T.

Rojo, E.M., Filipigh, A.A., Bolado, S., 2023. Assisted-enzymatic hydrolysis vs chemical hydrolysis for fractional valorization of microalgae biomass. Process Saf. Environ. Prot. 174, 276–285. https://doi.org/10.1016/j.psep.2023.03.067.

Rondanelli, M., Nichetti, M., Peroni, G., Faliva, M.A., Naso, M., Gasparri, C., Perna, S., Oberto, L., Di Paolo, E., Riva, A., Petrangolini, G., Guerreschi, G., Tartara, A., 2021. Where to find leucine in food and how to feed elderly with sarcopenia in order to counteract loss of muscle mass: practical advice. Front. Nutr. 7. https://doi.org/10.3389/fnut.2020.622391.

Safi, C., Ursu, A.V., Laroche, C., Zebib, B., Merah, O., Pontalier, P.-Y., Vaca-Garcia, C., 2014. Aqueous extraction of proteins from microalgae: effect of different cell disruption methods. Algal Res. 3, 61–65. https://doi.org/10.1016/j.algal.2013.12.004.

Safi, C., Zebib, B., Merah, O., Pontalier, P.-Y., Vaca-Garcia, C., 2014. Morphology, composition, production, processing and applications of Chlorella vulgaris: a review. Renew. Sustain. Energy Rev. 35, 265–278. https://doi.org/10.1016/j.rser.2014.04.007.

Sharma, S., Zhang, X., Azhar, G., Wei, J., 2022. Effect of L-valine treatment on sirtuin (SIRT1 and SIRT2) isoforms. Innovation in Aging, 6(Supplement_1), 732. https://doi.org/10.1093/geroni/igac059.2667.

Sobolevsky, T.G., Revelsky, A.I., Miller, B., Oriedo, V., Chernetsova, E.S., Revelsky, I.A., 2003. Comparison of silylation and esterification/acylation procedures in GC-MS analysis of amino acids. J. Sep. Sci. 26 (17), 1474–1478. https://doi.org/10.1002/jssc.200301492.

Spoehr, H.A., Milner, H.W., 1949. The chemical composition of chlorella; effect of environmental conditions. Plant Physiol. 24 (1), 120–149. https://doi.org/10.1104/pp.24.1.120.

Starke, I., Kleinpeter, E., Kamm, B., 2001. Separation, identification, and quantification of amino acids in L-lysine fermentation potato juices by gas chromatography–mass spectrometry. Fresenius' Journal of Analytical Chemistry, 371(3), 380–384. https://doi.org/10.1007/s002160100941.

Steel, R.G.D., Torrie, J.H., Dickey, D.A., 1997. Principles and procedures of statistics: a biometrical approach, 3rd ed. McGraw-Hill.

Stenerson, K. (2007). The derivatization and analysis of amino acids by GC-MS. Sigma-Aldrich. Retrieved from ⟨https://www.sigmaaldrich.com/IT/it/technical-documents/technical-article/analytical-chemistry/gas-chromatography/the-derivatization⟩. Accessed July 12, 2024.

Vendruscolo, R.G., Facchi, M.M.X., Maroneze, M.M., Fagundes, M.B., Cichoski, A.J., Zepka, L.Q., Barin, J.S., Jacob-Lopes, E., Wagner, R., 2018. Polar and non-polar intracellular compounds from microalgae: methods of simultaneous extraction, gas chromatography determination and comparative analysis. Food Res. Int. 109, 204–212. https://doi.org/10.1016/j.foodres.2018.04.017.

Villas-Bôas, S.G., Delicado, D.G., Åkesson, M., Nielsen, J., 2003. Simultaneous analysis of amino and nonamino organic acids as methyl chloroformate derivatives using gas chromatography–mass spectrometry. Anal. Biochem. 322 (1), 134–138. https://doi.org/10.1016/j.ab.2003.07.018.

Violi, J.P., Bishop, D.P., Padula, M.P., Steele, J.R., Rodgers, K.J., 2020. Considerations for amino acid analysis by liquid chromatography-tandem mass spectrometry: a tutorial review. TrAC Trends Anal. Chem. 131, 116018. https://doi.org/10.1016/j.trac.2020.116018.

Wang, Y., Tibbetts, S., McGinn, P., 2021. Microalgae as sources of high-quality protein for human food and protein supplements. Foods 10 (12), 3002. https://doi.org/10.3390/foods10123002.

World Health Organization, Food and Agriculture Organization of the United Nations, & United Nations University. (2007). Protein and amino acid requirements in human nutrition: report of a joint FAO/WHO/UNU expert consultation (No. 935). World Health Organization. Retrieved from ⟨https://iris.who.int/handle/10665/43411⟩. Accessed July 12, 2024.

Xu, W., Zhong, C., Zou, C., Wang, B., Zhang, N., 2020. Analytical methods for amino acid determination in organisms. Amino Acids 52 (8), 1071–1088. https://doi.org/10.1007/s00726-020-02884-7.

Yokote, Y., Arai, K.M., Akahane, K., 1986. Recovery of tryptophan from 25-minute acid hydrolysates of protein. Anal. Biochem. 152 (2), 245–249. https://doi.org/10.1016/0003-2697(86)90405-7.

Yust, M.M., Pedroche, J., Girón-Calle, J., Vioque, J., Millán, F., Alaiz, M., 2004. Determination of tryptophan by high-performance liquid chromatography of alkaline hydrolysates with spectrophotometric detection. Food Chem. 85 (2), 317–320. https://doi.org/10.1016/j.foodchem.2003.07.026.

Zhang, S., Zeng, X., Ren, M., Mao, X., Qiao, S., 2017. Novel metabolic and physiological functions of branched chain amino acids: a review. J. Anim. Sci. Biotechnol. 8 (1), 10. https://doi.org/10.1186/s40104-016-0139-z.

Zhang, Y., Liu, Y., Cao, X., Gao, P., Liu, X., Wang, X., Zhang, J., Zhou, J., Xue, S., Xu, G., Tian, J., 2016. Free amino acids and small molecular acids profiling of marine microalga Isochrysis zhangjiangensis under nitrogen deficiency. Algal Res. 13, 207–217. https://doi.org/10.1016/j.algal.2015.12.001.

Zuraikat, F.M., Wood, R.A., Barragán, R., St-Onge, M.-P., 2021. Sleep and diet: mounting evidence of a cyclical relationship. Annu. Rev. Nutr. 41 (1), 309–332. https://doi.org/10.1146/annurev-nutr-120420-021719.