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doi.org/10.1002/cbdv.202200230

RESEARCH ARTICLE

CHEMISTRY & BIODIVERSITY

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Bioactive Metabolites of Microalgae from Canary Islands for Functional Food and Feed Uses

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Three freshwater microalgae (*Spirogyra* sp., *Cosmarium* sp., and *Cosmarium blytii*) collected from several locations in Gran Canaria have been studied to explore their potential as a novel source of bioactive compounds for biotechnological applications. Soluble carbohydrates were quantified after extraction with 3 M HCl at 100 °C, ranging from 35.8 to 43.3 %, and with water at room temperature, ranging from 19 to 22.8 %. Amino acids glutamic acid, proline and aspartic acid were quantified by RP-HPLC. Glutamic acid was the most abundant, ranging from 12.2 to 3.63 mg g^{-1} of dry biomass. *Cosmarium blytii* was the richest sample in amino acids (24.02 mg g^{-1} of dry weight). In addition, *Cosmarium blytii* and *Spyrogira* sp. exhibited higher radical scavenging activity (RSA) against 1,1-diphenyl-2-picrylhydrazyl (DPPH) than that of the synthetic antioxidant butylhydroxytoluene (BHT), commonly used as food additive. These results show a great potential of these microalgae for exploitation in the food, feed and pharmaceutical industries.

Keywords: microalgae, amino acids, carbohydrates, radical scavenging activity, RP-HPLC.

Introduction

The 2019 Revision of World Population Prospects of United Nations estimates that world's population will exceed 9.8 billion and could peak at nearly 11 billion around 2100. This growing population will require 60 % more food by 2050 and therefore, brings additional challenges to eradicate poverty, combat hunger and malnutrition, and strengthen the coverage and quality of health. In addition, high consumption of processed food is associated with higher environmental costs and higher greenhouse gas emissions and has negative impact on health.[1] Changes in human and animal nutrition are essential to achieving several of the Sustainable Development Goals (https://sdgs.un.org/es/goals).

The ability of microalgae in mitigating CO_2 levels together with their production methods at a low cost,[2] make them a novel environmentally friendly source of bioactive compounds with potential use in human and animal healthy diets and in pharmaceutical industries.[2–3] Microalgae can play a very important role in improving nutrition and health in the future, as well as combating hunger and undernutrition due to their high levels of multifunctional compounds.[3] Several health benefits have been attributed to some macro and microalgae components such as phenolic compounds, polysaccharides, proteins, lipids, polyunsaturated fatty acids (PUFAs), and other secondary metabolites. Algal polysaccharides have been described as critically important for industrial and nutritional purposes[4] as their activities include antioxidant, antifungal, antibacterial, antiviral, antitumoral, anti-inflammatory and immunomodulatory properties, among others.[5–6] These polysaccharides derived from microalgae have been used in the food industry as fat substitute for texturizing, thickeners, stabilizers, and emulsifiers[7] and as nutraceuticals and low-sweetness humectants.[8] In addition, balanced diets in amino acids of natural origin and safe sources are strongly recommended as several diseases are linked with their lack or excess.[9] Proteins are one of the main components of microalgae, reaching up to 70 % of dry biomass and containing up

to 50% of essential amino acids, with higher antioxidant capacities compared to common proteins in human diet.[10–11] They also exhibit anti-hypertensive, immune-modulatory, anti-cancer, hepatoprotective, anti-atherosclerotic, anticoagulant, anti-UV radiation, anti-osteoporosis and anti-microbial activities.[12]

As food ingredients, amino acids glutamic and aspartic acids contribute to enhance food flavour of meat, soy sauce, seafood and some processed food.[13–14] Besides, supplementation with glutamic acid could be advantageous due to its beneficial effects in: (i) protecting intestinal function from the oxidative stress, (ii) regulating the intestinal damage cause by mycotoxins, (iii) lowering blood pressure, (iv) reducing cardiovascular disease risk, (v) increasing the efficacy and decreasing the toxicity of anticancer drugs.[15–17] Meanwhile, aspartic acid in human diet is involved in: (i) regulating the secretion of important hormones, (ii) alleviating intestinal damage, (iii) immproving liver energy status, (iv) reducing the oxidative stress in the kidneys.[18–19] Dietary supplementation of proline may also be advantageous under certain physiological and pathological conditions as an important regulator of cell metabolism and physiology, affecting fetal survival, growth, and development.[20–21]

Food products incorporating microalgae improve their nutritional and textural properties.[22–25] In fact, evidences of health benefits and enhanced nutritional properties of different food formulations incorporating microalgae have been reported.[22] Gouveia et al.[23] observed an improved resistance to oxidation of food emulsions enriched with microalgal biomass of *Chlorella vulgaris* and *Haematococcus pluvialis* (up to 2.00% w/w). Recently, microalgae have also been introduced as protein resource to produce meat analogs, which are considered healthier than red meat.[24] Žugčić et al.[25] reported that beef patties prepared with microalgal proteins (with 1% *Chlorella* or with 1% *Spirulina* of 60 and 70% of purity, respectively) showed increased concentrations of all amino acids, especially aspartic and glutamic acids, which were the predominant amino acids, concluding that microalgae proteins could be useful candidates for new meat products in the food and feed industries.

On the other hand, microalgae are more nutritious than the traditional sources of animal and aquatic feed.[26] The increasing limitations in wild fish harvest and the benefits of microalgae components described above as well as the economically advantageous production are increasing the demand for microalgae-based feed in aquaculture[27] Besides, inclusion of microalgae in feed could improve animal growth and meat quality. Broiler chickens fed with the same dose (1 g/kg diet) of microalgae *Chlorella vulgaris*, *Spirulina platensis*, and *Amphora coffeaformis* for 36 days, showed a positive effect on performance and improved antioxidant status and meat quality of breast muscle.[28] Kirchgessner et al.[29] found that proline supplementation (0.35, 0.7, 1.05, 1.4, and 2.1%) of young pigs improved their daily growth rate and feed efficiency and reduced their concentrations of urea in plasma.

Microalgae components levels are strongly dependent on several factors such as culture media composition and available nitrogen[30] light photoperiod and light intensity applied during culture[31] and microalgae growth phase of harvesting, among others.[32] Therefore, manipulation of microalgae culture medium and/or conditions can increase the production of bioactive metabolites.[33] Sui et al.[31] studied the impact of two light:dark illumination cycles (12:12 and 24:0 h) on *Dunaliella salina* protein composition, concluding that continuous illumination led to higher suspended protein content, with enhanced proportion of all individual essential amino acids (up to 44% of the total protein content), while cells cultured under 12:12 h light:dark cycle showed 30% of essential amino acids. Permanent illumination stimulated the production of several essential amino acids affecting the free and total amino acid profiles of cells.

The Canary Islands are mountainous with a subtropical volcanic origin, supporting high levels of solar radiation all year round. These environmental conditions generate highly diverse habitats and ecosystems, forcing microorganisms to adapt and accumulate metabolites that might be interesting from a biotechnological approach. The three microalgae strains analysed in the present study were collected from different locations and environments and studied to investigate their potential in developing food products with high nutritional and therapeutic values. Microalgae *Spirogyra* sp., *Cosmarium* sp. and *Cosmarium blytii* were provided by the Spanish Bank of Algae. They were selected because of the wide variety of reported activities for these strains collected from other regions.[34–35] Besides, they showed high values for growth and biomass productivities in previous studies performed by the Spanish Bank of Algae in order to select new species with possibilities from the biotechnological point of view. For this purpose, microalgae extracts were screened for their antioxidant activities by inhibition of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and compared with those of

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synthetic pure compounds with known antioxidant activities and widely used as food additives (butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT)).[36] The total carbohydrates content was determined by the anthrone method, and amino acids proline, glutamic and aspartic acids were identified and quantified using RP-HPLC. The present study contributes to consider this microalgae collection as a potential source of bioactive components for future developments in the food, feed, cosmetic and pharmaceutical industries.

Results and Discussion

The previous treatments for effective mechanical extraction of metabolites from cells and their storage[37–39] have an important impact in the quantification of the cell's components. Cell wall disruption is a necessary preliminary step to quantify the total content of each metabolite and to prevent wrong measurements. It depends on the species being investigated (including cell wall type) and their physiological state.

Visca et al.[37] compared two previous drying methods of cells for hydrolysing and extracting carbohydrates from *Scenedesmus* sp. and *Chlorella* sp. biomass: (i) cells were dried at 105 °C for 12 h; (ii) cells were freeze-dried. Under the first drying conditions, *Scenedesmus* sp. and *Chlorella* sp. showed 30.5 and 17.7 % carbohydrates, respectively. The authors found that *Scenedesmus* sp. reached much higher content of carbohydrates (58.7 and 51.8 %, depending on the extracting solvent) when freeze drying pretreatment was used and lipids were previously removed. However, the carbohydrate content of *Chlorella* sp. was not affected because a simple freeze-drying step was not enough to break the robust cell wall, which requires methods combining freeze-drying with sonication or ball-milling.[40] Kröger et al.[38] observed that freeze-drying produced cell wall damage of microalgae *Scenedesmus rubescens*, improving the extraction yields. Besides, several drying methods have also been studied by de Farias Neves et al.,[39] who confirmed that freeze-drying is the most suitable microalgae drying method without bioactive compounds loss.

In this study, cells were freeze-dried and the cell wall rupture was confirmed by making observations with microscope to ensure accurate quantification of metabolites.[41]

RSA of Microalgae Extracts

DPPH free radical scavenging assay was used for screening the antioxidant activity of samples (microalgae extracts and pure compounds). Methanol was selected as extracting solvent because previous studies performed in our laboratory showed the highest levels of natural antioxidants such as phenolic compounds associated to this solvent in the extraction of algal material.[42] According to the currently food additive regulations, synthetic antioxidants BHA and BHT are lawful for using individually or in combination as preservatives in many food at a maximum level of 0.02 %, or 200 ppm.[43] Therefore, this concentration was used to test their antioxidant activity. In the last decades, there has been a great interest to replace these synthetic antioxidants with additives of natural origin to prolong the shelf life of foodstuffs because BHA and BHT are classified as cancer promoters.[44] In the present study, microalgae *Cosmarium blytii* and *Spyrogira* sp. extracts exerted higher RSA than BHT (21.2, 21.1 and 14.4 % inhibition rate, respectively) but lower than BHA (33.4 %) at the permitted level (0.02 %). Results are summarized in *Figure 1*.

The antiradical activity observed in the extracts of microalgae is very important due to the deleterious role of free radicals in food and in biological systems. Antioxidants present in the extracts react with free radicals by donating an electron or hydrogen radical to reduce them to a stable molecule or a less reactive radical, helping to mitigate the oxidative stress.[3]

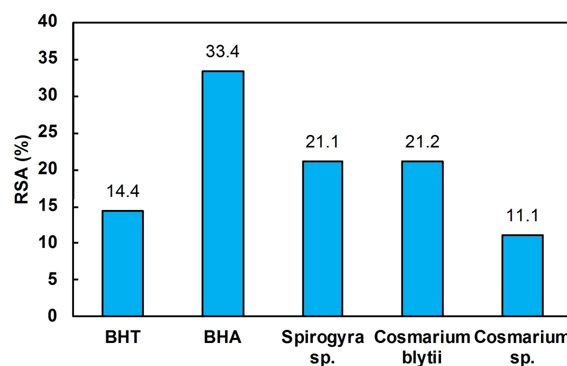

Figure 1. Radical scavenging activities (RSA) of microalgal samples and synthetic antioxidants BHT and BHA calculated as follows: RSA = 100 × (1 − absorbance in the presence of sample/absorbance in the absence of sample) and expressed as percentage inhibition.

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Carbohydrates Quantification

Acid hydrolysis conditions have an important effect in the quantification of carbohydrates, alongside the pretreatment and storage of microalgae cells. Carbohydrates were quantified using the anthrone assay.[45] They were heated with concentrated sulfuric acid producing furfural derivatives, which reacted with the anthrone reagent to yield a blue-green complex. The concentrations of carbohydrates were calculated from a calibration curve prepared with glucose as described below, and the results are shown in *Table 1*.

The extracts prepared by heating with 3 M HCl at 100 °C for 5 h yielded the highest amounts of carbohydrates, confirming that the yield of hydrolysis of microalgae biomass is highly dependent on temperature, time and acid concentration. In these conditions, the glycosidic bonds that maintain monosaccharides joined as polysaccharides are broken and the concentration of carbohydrates increases. These results are consistent with previous finding,[46] where drastic hydrolysis conditions (3 M H_2SO_4, 4 h, 100 °C) were required to quantify total monosaccharide content of flaxseed polysaccharides, and milder conditions (0.2 M H_2SO_4, 28 h, 80 °C) gave much lower rate of polysaccharides degradation.

Cosmarium sp. yielded the highest content of carbohydrates followed by *Cosmarium blytii* and *Spirogyra* sp. (43.2, 41.8 and 35.9 %, respectively). Our results also align well with those reported by Mutripah et al.,[47] who hydrolysed the samples in an autoclave at 121 °C for only 15 min with lower concentration of sulfuric acid (0.2 M), reaching up to 46.9 % carbohydrates.

In this work, microalgae samples extracted with water for 1 h at room temperature showed between 19 and 22 % carbohydrates. Our results agree partially with those reported by Kostas et al.,[48] who used an optimized hydrolysis protocol (11 M concentrated sulfuric acid at 37 °C for 60 min) for extracting carbohydrates from seaweed *Laminaria digitata* and *Chondrus crispus* biomass previously dried in a fan oven at 80 °C and milled (21 and 40 % carbohydrates, respectively). However, Militão et al.[49] found lower amounts of carbohydrates than those found in this study, in microalgae *Pseudopediastrum boryanum* and *Scenedesmus obliquus* cultivated at three different temperatures, oven dried at 65 °C, and not subjected to acid hydrolysis. *Pseudopediastrum boryanum* cultured at 30 °C gave the highest content of total carbohydrates followed by *Scenedesmus obliquus* cultivated at 20 °C (6.17 and 3.33 $mg\,g^{-1}$ of biomass, respectively).

Algal polysaccharides have shown numerous health benefits cited above to be used as functional food or nutraceuticals.[5–6,8] The high content of polysaccharides quantified in this study in samples of novel microalgae confirms their potential possibilities to be considered as novel sources of bioactive components in food, feed and biomedical applications.

Free Amino Acids Analysis

Amino acids require derivatization prior to HPLC analysis to introduce chromophores in their chemical structures in order to improve their sensitivity to UV absorption and fluorescence detection. Therefore, the reagent phenyl isothiocyanate (PITC) was used to prepare amino acids derivatives in this work. A simple, rapid, accurate and economical RP-HPLC method was developed to determine the content of glutamic and aspartic acids, and proline in freshwater microalgae extracts. Good linearity was observed in the range of concentration 20 to 800 $\mu g\,mL^{-1}$ with regression coefficients not lower than 0.9964. Recoveries were found in the range between 101 ± 9 and 108 ± 2%, and relative standard deviations (RSD) between 0.73 and 2.31 %. The amounts determined for each amino acid are given in *Table 2*.

Cosmarium blytii showed the highest content of each amino acid, being glutamic acid the most abundant (12 $mg\,g^{-1}$ of dry weight). As compared to

Table 1. Total carbohydrate content expressed as mg of glucose equivalent per g of dry biomass of three freshwater microalgae.

Algae sample	Kaiser method (%)* 3 M HCl at 100 °C for 5 h	Jansen method (%)* Water for 1 h at room temperature
Spirogyra sp.	359±1 (35.9)	227.97±0.02 (22.8)
Cosmarium blytii	417.72±0.07 (41.8)	210.96±0.03 (21.1)
Cosmarium sp.	432.9±0.1 (43.3)	190.9±0.1 (19)

Results are expressed as mean ± standard deviation of three measurements. * In parentheses, results mean gram of glucose equivalent per 100 g of dry biomass.

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Table 2. Free amino acids contents, expressed as mg g^{-1} of dry weight, of three freshwater microalgae extracted with aqueous methanol.

Amino acid	*Spyrogyra* sp.	*Cosmarium blytii*	*Cosmarium* sp.
Glutamic acid	1.10±0.03	12±1	2.9±0.1
Aspartic acid	2.47±0.03	8.4±0.6	0.74±0.02
Proline	0.63±0.06	3.62±0.05	0.8±0.1
Total	4.20	24.02	4.44

Results are expressed as mean ± standard deviation of three measurements.

Cosmarium sp., *Spyrogyra* sp. yielded lower amount of proline and glutamic acid but higher level of aspartic acid.

Numerous previous studies focused on the determination of total and free amino acid composition of seaweeds and microalgae showed that aspartic and glutamic acids were the most abundant (up to 26% of the free amino acid fraction).[11,50] Kolmakova and Kolmakov[51] reviewed the results available in the literature on the experimental determinations of the total content of essential and nonessential amino acids of green and diatom microalgae and cyanobacteria, concluding that the percentage composition of both types of amino acids is stable for all these microorganisms under consideration. Derrien et al.[52] studied the amino acid profile of five microalgae (*Tetraselmis suecica*, *Skeletonema costatum*, *Chaetoceros calcitrans*, *Thalassiosira* sp. and *Isochrysis galbana*) showing that arginine, tyrosine, aspartic and glutamic acids were responsible for more than 60% of the total amino acid concentration in all five species. These authors reported higher amounts of aspartic acid in microalgae *Tetraselmis suecica*, *Skeletonema costatum* and *Chaetoceros costatum* (20, 11.8 and 11.4 mg g^{-1} of dry weight, respectively) than those found here, and lower amounts in microalgae *Isochrysis galbana* and *Thalassiosira* sp. (0.3 and 1.5 mg g^{-1} of dry weight, respectively). However, *Cosmarium blytii* in this study showed lower content of glutamic acid than those reported by Derrien et al.[52] for *Thalassiosira* sp., but higher content than those in the other four microalgae. Dewi et al.[53] tested six different methods for extracting glutamic acid from *Spirulina* sp., reaching contents (in mg g^{-1} of biomass) between 83.7 in fresh biomass dried at temperature below 60°C for 10 h, and 23.5 in dried biomass macerated with 50% ethanol (1:20, w/v) at room temperature for 4 days. However, five different samples of three microalgae species, *Spirulina platensis*, *Dunaliella salina*, and *Tetraselmis suecica*, yielded lower contents of glutamic and aspartic acids than *Cosmarium blytii* here and lower content of aspartic acid than those found in this study in *Cosmarium* sp. and *Syporiga* sp.[54] Tibbets et al.[55] studied the amino acid profile of *Nannochloropsis granulate* subjected to supercritical CO_2 extraction at 70 and 90°C. These authors found lower amounts of aspartic acid (from 0.052 to 0.093 mg g^{-1} of dry weight) and glutamic acid (from 0.812 to 1.093 mg g^{-1} of dry weight) but higher content of proline (from 11.97 to 12.25 mg g^{-1} of dry weight) than those found here.

The present investigation shows the potential of *Cosmarium blytii*, *Cosmarium* sp. and *Syporiga* sp. for application in the food and feed industry.

Conclusions

The high contents of carbohydrates and the presence of glutamic and aspartic acids, and proline in the extracts derived from green microalgae *Cosmarium blytii*, *Cosmarium* sp. and *Syporiga* sp. confirm their potential possibilities as food and feed sources in future human and animal diets. In addition, *Cosmarium blytii* and *Syporiga* sp. exhibited higher RSA than BHT, and their inclusion in feed and human food could improve their antioxidant activity and nutritional values. Our results provide sufficient evidence for further research about the viability of these microalgae in the healthy food, feed and pharmaceutical industries. This could become a revulsive for the growth of the local economy by developing new commercial and industrial activities aimed at feed production for aquaculture companies in the Canary Islands.

Experimental Section

Chemicals

Methanol and acetonitrile (HPLC grade), hydrochloric acid (37%), sodium carbonate and sodium bicarbonate (analytical grade) were purchased from Scharlab

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(Barcelona, Spain). PITC (reagent grade), DPPH, BHA, BHT, by Sigma–Aldrich (St. Louis, MO, USA). Amino acids standards of analytical grade (aspartic acid, glutamic acid and proline) were provided by Merck (Darmstadt, Germany). Ultrapure water was obtained from a Milli-Q system from Millipore (Bedford, MA, USA).

Algal Material

Microalgae *Cosmarium blytii* BEA0204B, *Cosmarium* sp. BEA0208B and *Spyrogyra* sp. BEA0666B were isolated and supplied by the Spanish Bank of Algae (located in Taliarte, SE coast of Gran Canaria), where the identification by microscopy techniques and molecular methods was carried out according to their stablished procedures. This microalgae Collection is recognized by the World Intellectual Property Organization (WIPO) as one of the twelve accredited international authorities for identifying and deposit of microorganisms (algae) through the Budapest Treat. The samples were collected from several locations in Gran Canaria (Canary Islands) in March 2016: *Cosmarium blytii* (road GC 323 to El Hornillo); *Cosmarium* sp. (thirst in Lugarejos Dam) and *Spyrogyra* sp. (Barranco de Azuaje) and were grown to 2 L Erlenmeyer flasks under controlled conditions (temperature: 23 ± 2 °C; light irradiance: <100 micromols photons $m^{-2}s^{-1}$ and photoperiod 16:8 h light:dark in Waris-H media. All screened microalgae were collected in the exponential phase of growth. Then, they were centrifuged (15 min, 8000 rpm), freeze dried and reserved in darkness until analysis.

RSA of Microalgae Extracts

RSA was determined by measuring the loss of color of radical DPPH after reaction with the extracts derived from microalgae.[56] Freeze-dried microalgae (10 mg) were mixed with methanol (1 mL) using a vortex (Vortex Ika Genius 3) for 20 min. The mixture was heated at 40 °C for 10 min and sonicated for 10 min (this step was performed twice). Then, the extracts were centrifuged for 10 min at 9000 rpm in a microcentrifuge (Thermo Scientific, Heraeus fresco 17) and filtered. The supernatant was evaporated to dryness in a rotary vacuum evaporator, the residue was resolved in methanol (100 μL) and 25 μL of this solution were mixed with DPPH solution (975 μL 0.1 mM). After 20 min incubation, absorbance was measured at 515 nm using a Shimadzu 1800 UV/VIS spectrophotometer. The inhibition percentage was calculated by application of the equation: RSA = 100 (1 – absorbance in the presence of sample/absorbance in the absence of sample). The concentration 0.2 $g\,L^{-1}$ was used to test the antioxidant activity of pure compounds BHA and BHT following the same procedure described above. All assays were carried out in triplicate and the results are expressed as average values.

Carbohydrates Quantification

Freeze-dried algal material (30 mg) was extracted: (i) with 12 mL of 3 M HCl for 5 h (100 °C) for the determination of total carbohydrates according to Kaiser and Benner;[57] (ii) with water at room temperature during 1 h according to Jansen[58] with modifications. After centrifugation of each extract at 3500 rpm for 10 min, the supernatant was collected and stored at 4 °C.

The amounts of carbohydrates in the extracts were determined using the colorimetric method described by Brooks et al.[45] with modifications. Anthrone reagent was prepared fresh daily by dissolving anthrone (200 mg) in concentrated sulfuric acid (100 mL). This reagent (2 mL) was mixed with 1 mL of each sample (microalgae extracts and standard solutions), vortexed for 30 seconds and heated for 10 min in a boiling water bath. The test tubes were cooled in an ice bath for 10 min and the absorbance was recorded at 625 nm on a Shimadzu UV-1800 spectrophotometer. A standard calibration curve was prepared with solutions of glucose in the range of concentrations from 100 to 180 $\mu g\,mL^{-1}$ Three replicates were used for the determination of carbohydrates concentrations from the regression line equation ($y=0.0103x+0.7353$; $R^2=0.9954$). The results are expressed as mg of glucose equivalent g^{-1} of dry algal biomass and as percentage of glucose per 100 g of dry biomass.

Amino Acids Determination

Free amino acids were extracted according to Sommaruga and Tartarotti[59] with modifications. Freeze-dried microalgae (400 mg) were mixed with 8 mL of aqueous methanol (20 %) in a test tube and incubated in a water bath at 45 °C for 2 h. Then, the test tubes were sonicated in an ultrasonic bath (J.P. Selecta) for 10 min and centrifuged at 3500 rpm for 15 min at room temperature. The supernatant was collected and evaporated in a rotary vacuum evaporator (Eppendorf,

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Concentrator plus) at 30 °C (6800 rpm). The residues were resolved in 200 μL of ultra-pure water.

Phenyl isothiocyanate (PITC) derivatization according to Vemuri[60] was performed as follows: the samples (200 μL) were mixed with 100 μL of a solution of PITC in acetonitrile (4% v/v) and 100 μL of solution of sodium bicarbonate (5% w/v) using a vortex for 1 min. Then, the mixture was heated at 40 °C in a water bath for 10 min. Later, 100 μL of 1% w/v sodium carbonate solution were added and the resulting mixture was heated again at 40 °C for 5 min. The samples were allowed to reach room temperature and finally, 400 μL of aqueous methanol (30%) were added, and the solution was filtered through a 45 μm nylon syringe to be injected into the HPLC system.

The content of glutamic and aspartic acids, and proline in freshwater microalgae cells was determined by RP-HPLC according to Shi et al.[61] with modifications. Therefore, it was used a Jasco LC-4000 HPLC equipment connected to a ChromNav software for data acquisition and provided with a PU-4180 quaternary pump, a vacuum degasser, an AS-4150 autosampler, a MD-4015 photodiode array detector and a LC-NetII interface. A reverse phase Luna-C18 (250 × 4.6 mm, 5 μm) column was used with a guard column (10 × 4.6 mm, 5 μm) from Phenomenex and a gradient system involving two mobile phases (eluent A was ultra-pure water with 0.1 M sodium acetate buffer and eluent B was methanol) at 30 °C. The samples were injected in triplicate with a flow rate of 1.0 mL min^{-1}. The injection volume was 20 μL and the elution conditions were as follows: 0–4 min, isocratic 100% A; 4–8 min, linear gradient from 0% to 2% B; 8–17 min, linear gradient from 2% to 30% B; 17–27 min, linear gradient from 30% to 50% B; 27–37 min, linear gradient from 50% to 100% B and, finally, washing and reconditioning of the column. The detection was carried out at 254 nm. All the analyses were performed in triplicate and the results were expressed as average value ± standard deviation. Each standard was individually tested to determine its retention times (RT). The calibration curves were prepared with standard solutions of proline, and aspartic and glutamic acids at five different concentrations (from 20 to 800 μg mL^{-1}). The linearity was assessed by linear regression analysis, which was calculated by the least square method. Reproducibility was expressed as RSD of six replicate samples of each standard and the accuracy was expressed as the recovery of the standards.

Acknowledgements

This study received financial support from the ATOPFe project (CTM2017-83476-P) given by the Ministerio de Economía y Competitividad from Spain, and from the Universidad de Las Palmas de Gran Canaria (PIFULPGC-2019). The participation of Paula Santiago was funded through a PhD scholarship from the Universidad de Las Palmas de Gran Canaria (PIFULPGC-2019) to join the Ph.D. Program in Oceanography and Global Change (DOYCAG), promoted by the Institute of Oceanography and Global Change (IOCAG). The authors would like to thank the Plataforma Oceánica de Canarias – Consorcio público – ICTS (plocan.eu) for allowing us to use the chromatographic equipment.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contribution Statement

Paula Santiago-Díaz has performed the acquisition and analysis of data and participated in writing the article; Milagros Rico and Argimiro Rivero have made conception and design, analysis of data and written the manuscript; Magdalena Santana-Casiano has contributed with project administration and funding acquisition, provision of resources; review and editing. All the authors have critically revised and approved the submitted manuscript.

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Received March 14, 2022
Accepted August 15, 2022

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