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Official URL: <https://doi.org/10.1016/j.algal.2013.11.017>

To cite this version:

Safi, Carl  and Charton, Michael  and Ursu, Alina Violeta and Laroche, Céline and Zebib, Bachar  and Pontalier, Pierre-Yves  and Vaca-Garcia, Carlos  *Release of hydro-soluble microalgal proteins using mechanical and chemical treatments.* (2014) *Algal Research*, 3. 55-60. ISSN 2211-9264

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Release of hydro-soluble microalgal proteins using mechanical and chemical treatments

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ARTICLE INFO

Keywords:

Microalgae
Chemical treatment
Mechanical treatment
Proteins
Amino acid profiles

ABSTRACT

In order to release proteins in the aqueous phase, high-pressure homogenization and alkaline treatments were applied to rupture the cell walls of five intensively grown microalgae. Protein characterisation was carried out by analysing the amino acid profiles of both the crude microalgae and the protein extracts, obtained after both types of treatment. The results showed that the proportion of proteins released from microalgae following both treatments was, in descending order: *Porphyridium cruentum* > *Arthrospira platensis* > *Chlorella vulgaris* > *Nannochloropsis oculata* > *Haematococcus pluvialis*, reflecting the increasingly protective, cell walls. Nonetheless, mechanical treatment released more proteins from all the microalgae compared to chemical treatment. The highest yield was for the fragile cell walled *P. cruentum* with 88% hydro-soluble proteins from total proteins, and the lowest from the rigid cell walled *H. pluvialis* with 41%. The proportion of essential and non-essential amino acids in the extract was assessed and compared to the crude microalgae profile. It was higher after alkaline treatment and much higher after high-pressure homogenization. These results suggest that non-essential amino acids are more concentrated actually inside the cells and that different types of proteins are being released by these two treatments.

1. Introduction

In the 9th century AD the Kanem Empire in Chad discovered the benefits of the cyanobacterium *Arthrospira platensis* and used it as food (called *dihé*) for human consumption [1]. Later on in the 14th century AD, the Aztecs harvested the same species from Lake Texcoco and used it to make a sort of cake called *tecuilatl*. They also used these microorganisms as fodder, fertilisers and remedies. Nowadays, additional species are being industrially and profitably marketed worldwide for the same purposes.

The microalgal industry has grown rapidly over the last decade. Primarily, this is due to the capacity of these micro-organisms to produce lipids suitable for the biodiesel industry, and to grow in a wide variety of geographical and environmental locations, thus precluding competition with arable lands as well as intensive deforestation. Therefore, the major part of microalgal studies has concentrated on enhancing this bioenergy production to the detriment of other high-value biomolecules, but forgetting ancient history and the other advantages of these species.

Today the microalgal bioenergy industry is struggling to find a place in the market due to its uncompetitive cost and its overall unsustainable

production [2–6] sometimes leaving negative footprints on the environment, and public opinion.

Microalgae were originally considered as an important source of protein, a major fraction of their composition; on a dry weight basis the Cyanobacterium *Arthrospira platensis* is composed of 50–70% proteins [7,8], the Chlorophyceae *Chlorella vulgaris* 38–58% [9–11], the Eustigmatophyceae *Nannochloropsis oculata* 22–37% [12], the Chlorophyceae *Haematococcus pluvialis* 45–50% [7], and the Rhodophyta *Porphyridium cruentum* 8–56% protein [13,14]. They have a profile composed of a set of essential and non essential amino acids [10], with relatively similar ratios between species and generally unaffected by growth phase and light conditions [1]. To the best of our knowledge, studies on microalgal proteins have generally either concentrated on finding and proposing the nitrogen to protein conversion factor [10,15–18], in order to avoid incorrect estimations of microalgal total protein content, or focused on determining the best method for protein quantification using colorimetric techniques [19–21]. However, for some species such as the green microalgae *C. vulgaris*, *N. oculata* and *H. pluvialis*, maximising the recovery of proteins requires a unit cell disruption operation to overcome the barrier of their rigid cell wall and release the intracellular biomolecules. Thus, many cell disruption methods were used to break the cell wall of these microalgae, such as

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bead milling, ultrasonication, microwaves, enzymatic treatment and high-pressure homogenization [22–26]. Conversely, fragile cell walled microalgae such as *P. cruentum* and *A. platensis* require milder techniques to enhance recovery.

The main objective of this study is to evaluate the effect of two different cell disruption techniques on aqueous phase protein extractability, in five microalgae with different cell wall characteristics, while simultaneously evaluating and comparing the profile of amino-acids subsequent to these two cell disruption methods.

2. Materials and methods

2.1. Microalgae

The selected microalgae were supplied as frozen paste from Alpha Biotech (Asserac, France): the Cyanobacteria *Arthrospira platensis* (strain PCC 8005), two different Chlorophyceae *Chlorella vulgaris* (strain SAG 211-19), and *Haematococcus pluvialis* (unknown strain), one Rhodophyta *Porphyridium centum* (strain UTEX 161), and the Eustigmatophyceae *Nannochloropsis oculata* (unknown strain).

Each microalga was cultivated on a different culture media; Hemerick media was used for *P. cruentum*, Sueoka media for *C. vulgaris*, Basal media for *H. pluvialis*, Conway media for *N. oculata* and Zarrouk media for *A. platensis*. All were grown in batch mode in an indoor tubular Air-Lift PhotoBioReactor (PBR, 10 L) at 25 °C, inoculated from a prior culture in a flat panel Air-Lift PBR (1 L). Culture homogenization was by sterile air injection at the bottom of the PBR. The pH and temperature were recorded using a pH/temperature probe (Mettler Toledo SG 3253 sensor) monitored by LabVIEW acquisition software. The pH was regulated at 7.5 with CO₂ bubbling. Microalgae were harvested during the exponential growth phase, concentrated by centrifugation, and the biomass which contained 20% dry weight, was then frozen.

2.2. Chemicals

The Lowry kit ((prepared mixture of Lowry reagent plus bovine standard albumin (BSA) standards and 2 N Folin–Ciocalteu reagent)) was purchased from Thermo Fisher Scientific. NaOH and HCl 37% were purchased from Sigma Aldrich and used as received.

2.3. High-pressure cell disruptor

A “TS Haiva series, 2.2-kW” homogenizer from Constant Systems Limited (Northants, UK), was used. For each experiment, a biomass concentration of 2% dry weight (0.5 g of freeze dried cells dispersed in 25 mL distilled water) was passed through the machine twice at a pressure of 2700 bar.

2.4. Alkaline treatment

Mother solutions were prepared with approximately 500 mL of ultrapure water and some drops of 2 N NaOH to adjust to pH 12. A sample of 1 g of freeze-dried biomass was added to 50 mL of mother solution and the mixture heated at 40 °C with stirring for 1 h. Separation of the solid–liquid mixture was conducted by centrifugation at 5000 g for 10 min. Samples of the supernatant were taken for protein analysis by the Lowry colorimetric method and for amino acid analysis.

2.5. Lowry method

The procedure involves reacting proteins with cupric sulphate and tartrate in an alkaline solution, leading to the formation of tetradentate copper protein complexes. The addition of the Folin–Ciocalteu reagent leads to the oxidation of the peptide bonds by forming molybdenum blue with the copper ions. Therefore, a calibration curve was prepared using a BSA concentration range from 0 to 1500 µg mL⁻¹. In order to measure the protein content, 0.2 mL of each standard or samples containing the crude protein extract were taken, and then 1 mL of modified Lowry reagent was added to each sample, which was then vortexed and incubated for exactly 10 min at room temperature. After incubation, 0.1 mL of Folin–Ciocalteu Reagent (1 N) was added and the sample again vortexed and incubated for exactly 30 min at room temperature. The blue colour solution absorbance was then measured at 750 nm with a UV-1800 Shimadzu spectrophotometer, previously zeroed with a blank sample containing all the reagents minus the extract.

2.6. Elemental analysis

The total nitrogen was evaluated by LCC (Laboratoire de Chimie de Coordination, Toulouse-France) using a PerkinElmer 2400 series II elemental analyser. The samples of 2 mg were placed in thin capsules and then heated to 925 °C using pure oxygen as the combustion gas, and pure helium as the carrier gas. The percentage nitrogen was evaluated and converted into protein percentage using the conversion factors obtained for each microalga in another study [10].

2.7. Amino acid analysis

The biomass amino acid composition was determined using a well known standard method (Moore and Stein 1948). The samples were hydrolysed with 6 N HCl at 103 °C for 24 h., and the hydrolysed material was then adjusted to pH 2.2 with 6 N NaOH and stabilised with a pH 2.2 citrate buffer solution. The final solution was then filtered over a 0.45 µm PTFE membrane to remove any residual solids remaining in the solution. The analysis was performed using a Biochrom Ltd 32 + (Cambridge, UK) amino acid analyser, equipped with a high pressure PEEK “column + pre-column” (size, 200 × 4.6 mm) packed with Ultropac cation exchange resin containing sodium. The separation of

Table 1

Different protein contents in crude microalgae calculated according to the following equation: Proportion of hydro-soluble protein in total protein for different microalgae.

Microalgae	N _{EA} ^a (%)	NTP ^b (%)	P _{TOTAL}	Chemical treatment		High-pressure homogenization	
				P _{Lowry} ^c (%)	$\frac{P_{Lowry}}{P_{TOTAL}} \times 100$ (%)	P _{Lowry} ^c (%)	$\frac{P_{Lowry}}{P_{TOTAL}} \times 100$ (%)
<i>P. cruentum</i>	9.18 ± 0.61	6.34	58.29 ± 3.78	44.34 ± 0.97	76.07 ± 1.48	51.60 ± 2.45	88.52 ± 1.17
<i>A. platensis</i>	8.76 ± 0.16	6.27	54.92 ± 1.10	37.19 ± 2.67	67.72 ± 1.64	41.75 ± 2.82	76.02 ± 0.75
<i>C. vulgaris</i>	7.98 ± 0.16	6.35	50.67 ± 1.02	21.50 ± 0.34	42.43 ± 0.52	26.18 ± 3.99	51.68 ± 2.03
<i>N. oculata</i>	7.83 ± 0.31	6.28	49.17 ± 2.13	15.52 ± 0.42	31.56 ± 1.06	24.34 ± 0.58	49.50 ± 1.51
<i>H. pluvialis</i>	8.30 ± 0.04	6.25	51.87 ± 0.43	14.23 ± 0.69	27.43 ± 0.49	21.23 ± 3.66	40.93 ± 1.97

P_{TOTAL}: Total protein in microalgae = N_{EA} × NTP.

^a N_{EA}: Total nitrogen % (d.w) obtained by elemental analysis.

^b NTP: Nitrogen-to-protein conversion factors of Safi et al. (2012b) for each microalga.

^c P_{Lowry}: Hydro-soluble protein % (d.w) at pH 12 and 40 °C and by high-pressure homogenization calculated using the Lowry method.

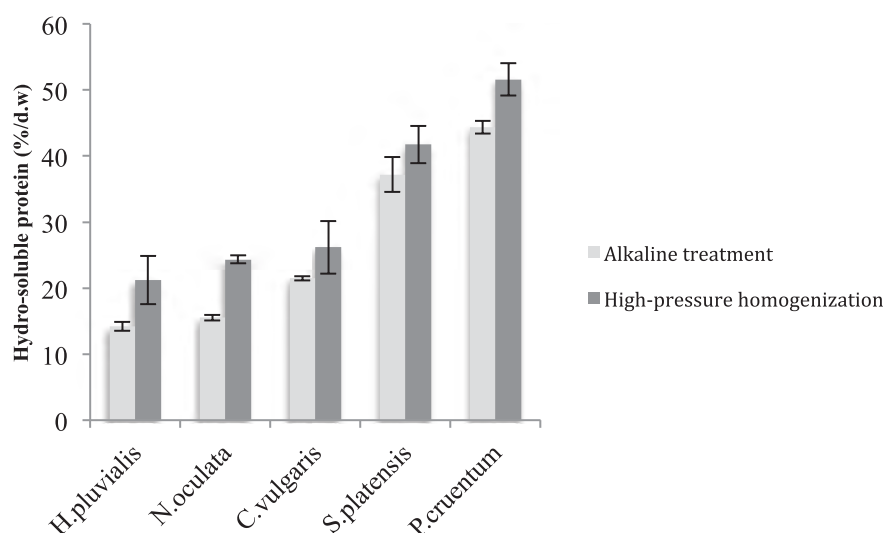


Fig. 1. Protein % d.w. of biomass in water after cell disruption of five microalgae by a mechanical and chemical method. Results are based on 3 replicates for 3 experiments \pm SD ($n = 9$).

amino acids was carried out by elution with loading buffers (flow rate 25 mL.h⁻¹) at different pH's. After reaction with ninhydrin (flow rate 35 mL.h⁻¹), amino acids were detected with a UV detector at a 570 nm wavelength, except for proline, where detection was at 440 nm. Ammonia was added to compensate for the value of some less resistant amino acids, broken down by the strong acid hydrolysis.

2.8. Statistical analysis

Three experiments were conducted separately for each microalga. Measurements of three replicates for each sample were repeatable at maximum \pm 5% of the respective mean values.

3. Results

The total protein content of crude microalgae was determined from the value of total nitrogen obtained through elemental analysis, and the conversion factor found for each crude microalga in a separate study (Safi et al. 2012b). In all cases, the total protein content was high and consistent with literature values, ranging from 49 to 58% dry weight (Table 1). The fraction of hydro-soluble proteins released into water after both cell disruption techniques is presented in Fig. 1, after quantification by the Lowry method in cases where the mechanical method recovered more proteins compared to the alkaline treatment. The hydro-

soluble protein fraction of total proteins present in the microalgae was also evaluated, and all these results are shown in Table 1.

The amino acid profile was first determined for the crude microalgae, with Aspartic acid being the highest member for *P. cruentum*, *A. platensis* and *C. vulgaris* and Alanine for *N. oculata* and *H. pluvialis* (Table 2). In addition, this profile was evaluated after alkaline treatment, and here Aspartic acid was the highest for *P. cruentum*, Alanine for *A. platensis*, *C. vulgaris* and *H. pluvialis*, and Proline for *N. oculata* (Table 3). However, with high-pressure homogenization Proline was the highest for *P. cruentum*, *A. platensis* and *N. oculata* and Alanine for *C. vulgaris* and *H. pluvialis* (Table 4). Furthermore, the percentages of essential and non-essential amino acids before and after both cell disruption treatments, were also evaluated and are shown in Table 5. The proportion of non-essential amino acids was higher than that of essential amino acids for all the microalgae after both treatments. Nevertheless, it can be seen (Table 5) that the proportion of non-essential amino acids was much higher with high-pressure homogenization than with the alkaline treatment.

4. Discussion

This study used two different cell wall treatments on five different microalgae followed by quantification of the proteins [27] released in the aqueous phase, and then assessed the amino acid profile of these

Table 2

Results of total amino acids of 5 microalgae expressed in g per 100 g of algal protein representing 3 replicates for 3 experiments \pm SD ($n = 9$).

Freeze-dried microalgae amino acid composition					
Amino acids	<i>P. cruentum</i>	<i>A. platensis</i>	<i>C. vulgaris</i>	<i>N. oculata</i>	<i>H. pluvialis</i>
Aspartic acid	12.41 \pm 0.45	13.10 \pm 0.11	11.20 \pm 0.02	10.13 \pm 0.05	9.76 \pm 0.10
Threonine	6.91 \pm 0.25	6.83 \pm 0.10	6.24 \pm 0.01	6.55 \pm 0.03	5.75 \pm 0.06
Serine	8.98 \pm 0.29	7.59 \pm 0.02	7.97 \pm 0.04	7.23 \pm 0.01	7.31 \pm 0.05
Glutamic acid	9.04 \pm 0.29	11.64 \pm 0.09	9.30 \pm 0.01	11.41 \pm 0.02	10.44 \pm 0.11
Glycine	7.59 \pm 0.28	8.60 \pm 0.06	8.81 \pm 0.01	9.97 \pm 0.01	9.98 \pm 0.09
Alanine	7.39 \pm 3.67	10.99 \pm 0.08	11.17 \pm 0.03	12.11 \pm 0.01	12.44 \pm 0.12
Cysteine	0.37 \pm 0.01	0.20 \pm 0.02	0.20 \pm 0.01	0.22 \pm 0.01	0.25 \pm 0.01
Valine	2.76 \pm 0.10	3.17 \pm 0.02	3.17 \pm 0.01	3.65 \pm 0.02	3.67 \pm 0.04
Methionine	3.08 \pm 0.11	1.91 \pm 0.02	0.66 \pm 0.01	1.66 \pm 0.01	0.71 \pm 0.01
Isoleucine	5.81 \pm 0.24	0.13 \pm 0.01	0.09 \pm 0.01	0.13 \pm 0.01	4.99 \pm 0.04
Leucine	6.46 \pm 0.21	7.79 \pm 0.02	7.68 \pm 0.02	8.99 \pm 0.05	8.92 \pm 0.10
Tyrosine	4.90 \pm 0.18	5.35 \pm 0.05	8.63 \pm 0.01	3.76 \pm 0.02	3.08 \pm 0.04
Phenylalanine	5.54 \pm 0.20	5.34 \pm 0.04	5.96 \pm 0.01	5.59 \pm 0.01	5.42 \pm 0.07
Histidine	1.22 \pm 0.04	1.00 \pm 0.01	1.29 \pm 0.01	1.04 \pm 0.01	0.99 \pm 0.01
Lysine	6.09 \pm 0.21	5.65 \pm 0.62	6.99 \pm 0.07	6.32 \pm 0.01	6.31 \pm 0.08
Arginine	8.62 \pm 0.29	8.52 \pm 0.07	7.57 \pm 0.03	6.58 \pm 0.02	6.73 \pm 0.08
Proline	2.80 \pm 0.17	2.16 \pm 0.05	3.04 \pm 0.07	4.65 \pm 0.07	3.24 \pm 1.15

Table 3Results of total amino acids of 5 microalgae expressed in g per 100 g of algal protein representing 3 replicates for 3 experiments \pm SD ($n = 9$).

Protein extract amino acid composition after chemical treatment					
Amino acids	<i>P. cruentum</i>	<i>A. platensis</i>	<i>C. vulgaris</i>	<i>N. oculata</i>	<i>H. pluvialis</i>
Aspartic acid	11.65 \pm 0.02	11.27 \pm 0.02	7.52 \pm 0.34	5.02 \pm 0.04	7.30 \pm 0.09
Threonine	5.88 \pm 0.01	6.05 \pm 0.05	4.60 \pm 0.23	3.57 \pm 0.05	4.92 \pm 0.02
Serine	8.15 \pm 0.03	7.92 \pm 0.03	7.42 \pm 0.33	4.38 \pm 0.09	6.32 \pm 0.02
Glutamic acid	9.85 \pm 0.01	12.71 \pm 0.02	12.85 \pm 0.63	25.42 \pm 0.30	15.13 \pm 0.03
Glycine	8.35 \pm 0.01	9.19 \pm 0.02	10.77 \pm 0.52	9.89 \pm 0.14	12.28 \pm 0.02
Alanine	11.38 \pm 0.02	11.95 \pm 0.02	18.58 \pm 0.92	15.77 \pm 0.20	21.34 \pm 0.04
Cysteine	0.30 \pm 0.01	0.22 \pm 0.01	0.27 \pm 0.02	0.42 \pm 0.01	0.45 \pm 0.01
Valine	3.43 \pm 0.01	3.62 \pm 0.01	4.04 \pm 0.20	2.66 \pm 0.04	3.13 \pm 0.01
Methionine	2.58 \pm 0.01	1.86 \pm 0.01	1.42 \pm 0.06	1.38 \pm 0.03	1.16 \pm 0.01
Isoleucine	5.81 \pm 0.02	0.14 \pm 0.01	2.60 \pm 0.07	1.21 \pm 0.01	3.42 \pm 0.02
Leucine	7.94 \pm 0.01	8.75 \pm 0.02	7.90 \pm 0.40	4.62 \pm 0.06	5.58 \pm 0.03
Tyrosine	4.00 \pm 0.01	4.74 \pm 0.01	2.83 \pm 0.13	2.42 \pm 0.09	2.13 \pm 0.02
Phenylalanine	4.50 \pm 0.01	4.65 \pm 0.01	3.03 \pm 0.16	1.84 \pm 0.03	2.94 \pm 0.01
Histidine	0.86 \pm 0.03	0.81 \pm 0.01	0.59 \pm 0.03	0.32 \pm 0.01	0.48 \pm 0.01
Lysine	6.08 \pm 0.01	6.88 \pm 0.01	6.81 \pm 0.33	2.64 \pm 0.10	3.88 \pm 0.01
Arginine	7.21 \pm 0.03	7.46 \pm 0.01	6.46 \pm 2.96	2.20 \pm 0.11	7.68 \pm 0.01
Proline	2.03 \pm 0.02	2.03 \pm 0.09	2.33 \pm 0.14	16.21 \pm 0.44	1.80 \pm 0.04

proteins for each treatment. The characteristics of the microalgal cell walls play an important role in the release of these biomolecules. Nonetheless, regardless of cell wall characteristics we have shown that at the 95% confidence level using three replicates for each microalga, all the latter have statistically equivalent protein values (Table 1). It should however be noted that the total nitrogen estimation includes other nitrogenous compounds, such as intracellular inorganic materials [18] pigments, nucleic acids, glucosamine and amines that could account for about 10% of the overall nitrogen content in microalgae [7,28]. After conducting both cell wall treatments, the highest content of hydro-soluble proteins in the extract was from *P. cruentum* which has a pseudo-cell wall composed of exopolysaccharide mucilages [29–31] making it very fragile and offering very little resistance to any treatment. Conversely, the lowest microalgae protein content in this study was obtained from *H. pluvialis*, known for its cell wall composed of cellulose and sporopollenin, which is remarkably resistant to chemical and mechanical treatment [10,25]. Moreover, if we observe the decrease in protein recovery, we can see that this mirrors the increasing rigidity of the cell walls (Table 1) in all the microalgae. Nonetheless, compared to alkaline treatment, mechanical treatment gave more aqueous phase protein recovery for all the microalgae, with the lowest increase reserved for the fragile cell walled microalgae; 11% and 14% calculated for *A. platensis* and *P. cruentum* respectively. Indeed, both of these offer very little resistance to cell disruption treatment, and this small increase

in protein recovery suggests more effective disruption of protein aggregates by high-pressure homogenization, leading to better solubilisation of hydro-soluble proteins in the aqueous phase. Similarly, a higher increase in protein recovery for the rigid cell walled microalgae was also detected, with 18%, 33% and 36% for *C. vulgaris*, *H. pluvialis* and *N. oculata* respectively. Here, the mechanical treatment applied in this study, is more effective at breaking the cell walls and protein aggregates, allowing more protein to be solubilised.

Furthermore, the alkaline treatment does have an effect on protein recovery, because the chemical action acts in synergy with the mechanical characteristics of the cell wall (Safi et al. 2012b). Similarly, as mentioned earlier, the sporopollenin contained in the most rigid cell wall (*H. pluvialis*) is known to be extremely resistant to chemical agents [32]. But for cellulose-rich cell walls, such as in *C. vulgaris* and *N. oculata*, the sodium hydroxide is able to penetrate the cellulose microcrystalline structure to form alcoholates in a process similar to mercerisation, and can also dissolve the hemicelluloses attached to the cellulose. Partial permeation of this kind of cell wall can therefore occur by alkaline action, favouring solubilisation of cell wall proteins but making it difficult to recover cytoplasmic and chloroplastic proteins. Finally, *A. platensis* is a gram-negative cyanobacteria with a thin cell wall rich in amino sugars cross-linked with oligopeptide chains. Under alkaline conditions, the former are labile by deamidation of the *N*-acetylglucosamine while the latter are soluble. Therefore the cell wall becomes highly permeable

Table 4Results of total amino acids of 5 microalgae expressed in g per 100 g of algal protein representing 3 replicates for 3 experiments \pm SD ($n = 9$).

Amino acid composition in the protein extract after high-pressure homogenization					
Amino acids	<i>P. cruentum</i>	<i>A. platensis</i>	<i>C. vulgaris</i>	<i>N. oculata</i>	<i>H. pluvialis</i>
Aspartic acid	12.15 \pm 0.14	8.67 \pm 0.02	7.47 \pm 0.01	7.27 \pm 0.34	8.26 \pm 0.10
Threonine	3.39 \pm 0.03	4.66 \pm 0.25	5.13 \pm 0.02	4.45 \pm 0.26	4.36 \pm 0.09
Serine	5.57 \pm 0.07	5.00 \pm 0.03	5.46 \pm 0.01	4.43 \pm 0.05	5.59 \pm 0.12
Glutamic acid	9.76 \pm 0.51	13.85 \pm 0.05	10.23 \pm 0.06	11.51 \pm 0.12	11.41 \pm 0.11
Glycine	6.72 \pm 0.09	7.67 \pm 0.01	10.07 \pm 0.05	9.15 \pm 0.13	10.62 \pm 0.09
Alanine	11.69 \pm 0.02	10.10 \pm 0.04	16.93 \pm 0.11	10.28 \pm 0.04	17.05 \pm 0.14
Cysteine	1.21 \pm 0.05	0.42 \pm 0.01	0.39 \pm 0.01	0.74 \pm 0.20	0.87 \pm 0.03
Valine	7.06 \pm 0.20	5.63 \pm 0.02	6.19 \pm 0.05	5.36 \pm 0.02	5.33 \pm 0.10
Methionine	4.34 \pm 0.10	1.09 \pm 0.01	1.05 \pm 0.02	1.43 \pm 0.11	0.92 \pm 0.41
Isoleucine	3.91 \pm 0.06	4.14 \pm 0.04	3.19 \pm 0.03	3.05 \pm 0.26	2.52 \pm 0.33
Leucine	5.59 \pm 0.06	7.13 \pm 0.01	7.03 \pm 0.04	7.14 \pm 0.07	5.10 \pm 0.17
Tyrosine	1.74 \pm 0.07	2.27 \pm 0.03	2.31 \pm 0.01	1.09 \pm 0.10	1.35 \pm 0.02
Phenylalanine	2.01 \pm 0.07	2.90 \pm 0.04	2.74 \pm 0.03	3.07 \pm 0.08	2.31 \pm 0.02
Histidine	0.92 \pm 0.02	1.15 \pm 0.01	0.98 \pm 0.02	1.48 \pm 0.03	0.96 \pm 0.01
Lysine	3.13 \pm 0.04	3.12 \pm 0.04	4.95 \pm 0.04	3.37 \pm 0.18	3.61 \pm 0.04
Arginine	4.26 \pm 0.02	4.06 \pm 0.03	3.89 \pm 0.03	3.20 \pm 0.27	5.92 \pm 0.05
Proline	16.52 \pm 0.80	18.11 \pm 0.17	11.98 \pm 0.15	22.93 \pm 0.47	13.79 \pm 0.19

Table 5

Proportion of amino acids before and after treatment for five microalgae.

Microalgae	No treatment		Chemical treatment		High-pressure homogenization	
	Essential AA	Non-essential AA	Essential AA	Non-essential AA	Essential AA	Non-essential AA
<i>P. cruentum</i>	50.29	49.71	48.48	51.28	30.36	69.64
<i>S. platensis</i>	44.94	55.06	43.53	56.22	29.83	70.17
<i>C. vulgaris</i>	44.07	55.93	39.02	60.43	31.26	68.74
<i>N. oculata</i>	43.30	56.70	33.48	65.90	29.36	70.63
<i>H. pluvialis</i>	46.52	53.48	32.37	67.14	25.13	74.87

allowing alkaline extraction of proteins by penetration of the cytoplasmic and chloroplastic space, enhancing protein recovery.

The proteins' amino acid profile was also evaluated by analysing the crude microalgae (Table 2), the alkaline treatment protein extracts (Table 3) and the high-pressure homogenization extracts (Table 4). The proportion of essential and non-essential amino acids was also evaluated (Table 5), and showed that the percentage of non-essential amino acids derived from both treatments was higher than essential amino acids. This suggests that non-essential amino acids are more concentrated inside the cell wall barrier, and also that it is not the same proteins being released in the aqueous phase when comparing both treatments. However, compared to the alkaline treatment, high-pressure homogenization increased the percentage of the non-essential amino acids for the fragile cell walled species from 20% to 26% for *A. platensis* and *P. cruentum* respectively. Similarly, for the rigid cell walled green species, they increased by 7%, 10% and 12% for *N. oculata*, *H. pluvialis* and *C. vulgaris* respectively. Moreover, for the latter species it is noteworthy that after alkaline treatment, the proportions of essential to non-essential amino acids was statistically the same compared with those for the crude fragile microalgae, and this was not the case after mechanical treatment of the same species. However, from the literature, few studies have distinguished between cell wall and intracellular amino acids of microalgae. It has been reported for instance, that after isolating and purifying the cell wall of *C. vulgaris* from the cytoplasmic medium, this contained peptides rather than proteins, although the amino acid profile was limited to their detection without quantifying the proportions [33].

In conclusion, it has been noticed that after both treatments, essential and non-essential amino acids were present but in different ratios, suggesting that the quality and quantity of proteins in the extract depends on the effectiveness of the cell disruption method, and also on the structural morphology of each microalgal cell wall. Therefore, mechanical treatment is more effective than chemical treatment due to its capacity to disrupt the cell walls and protein aggregates. And the logical next step will be to conduct high performance liquid chromatography in order to identify the type of proteins released after cell disruption.

At present, the FAO and WHO recommend microalgal proteins for human consumption because they contain all the necessary amino acids, however, the reported presence of toxins in microalgae [34], reopens the debate on this biomass as a supplementary food product. Notwithstanding, microalgal technology is still in its infancy and has a promising future in tomorrow's food industry, although additional clarification is required to include microalgae in the daily food intake.

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