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# Characterization and optimization of microwave-assisted extraction of B-phycoerythrin from *Porphyridium purpureum* using response surface methodology and Doehlert design

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ABSTRACT

Microalgae are one of the most promising food source of the future. Nowadays, extracts of high-value active substances of biomass are business aims for the development of food additives in personalized nutrition, in cosmetics and pharmaceuticals. A new-patented vertical farming cultivation technology was used for production of *Porphyridium purpureum*. In this work, microwave assisted extraction was used to extract B-phycoerythrin from *Porphyridium purpureum* biomass. Response surface methodology was implemented for optimization. Numerical optimization established the best point of the experimental domain (biomass/solvent of 16.8 mg/mL, time of 172 s, and temperature of 30 °C) with a desirability value of 0.82. Corresponding experimental responses values of 7.2 mg, 8.5 % and 13,961 PA/μg biomass were obtained for extracted proteins, extraction yield and extracted B-phycoerythrin, respectively. Final freeze-dried product indicated protein content of 55 % using Kjeldahl while targeted mass spectrometry analysis revealed that B-phycoerythrin represented 93 % of the total protein.

## 1. Introduction

The United Nations reports the global increase in population from an estimated 7.7 billion in 2019, to around 8.5 billion in 2030, and further anticipates it to reach 9.7 to 10.9 billion in the years 2050 to 2100 (UN, 2019). On the other hand, we witness a growing restriction of the natural resources including farmland, water resources and nutritional commodities resulting from the traditional agricultural production and practices. These conditions drive the innovation to develop new production methods, which are necessary to accommodate the rising demand of raw materials for food production. One of such next-generation food resources that could be identified as a potential candidate is the utilization of the biomass produced by water organisms. Microalgae among them are recognized as one of the most promising food source of the future, well known for their high content of nutrients like bioactive proteins and carbohydrates (Chanda et al., 2019; Dębowski et al., 2020), omega-3 fatty acids (Gacheva and Gigova, 2014; Koyande et al., 2019) and the presence of pigments, phenolic compounds, vitamins, and minerals (Mysliwa-Kurdziel and Solymosi, 2017; Wang et al., 2015) being further supported by their corresponding potential as health beneficial components. In order to maximize the productivity of microalgae, both the organism and environmental conditions that supports its growth should be optimized with an appropriate scale up cultivation technology (Fabris et al., 2020; Khoo et al., 2019; Koyande et al., 2019). In this context, a new food production method for plants and algae is the so-called vertical farm. The concept advocates the cultivation of plants and microalgae on vertically inclined surfaces beneficial for implementation in urban areas.

The new Institute for Grain Processing Algae Farmhouse technology (IGV - AFT), provides an optimized fitting solution on the horizon of microalgae production as recently documented in a German patent (Huschek and Theisen, 2021).

Nowadays, extracts of high-value active substances of biomass are business aims for the development of food additives in personalized nutrition, in cosmetics and pharmaceuticals (Chanda et al., 2019; Jiang et al., 2017; Mysliwa-Kurdziel and Solymosi, 2017; Wang et al., 2015).

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One of the main examples is the microalgae *Porphyridium purpureum* cultivated to extract high-value substances such as pycobiliproteins (PBPs) and specifically B-phycoerythrin. It was recently reported the market price of 741 USD for 1 mg of highly pure B-phycoerythrin (A545/A280 > 5.0) (Li et al., 2021b).

The pycobiliproteins (PBPs) represent pigmented chromophore-bearing proteins participating in light harvesting in close connection with photosystem II, a schematic illustration of their organization into phycobilisome is given in (Mysliwa-Kurdziel and Solymosi, 2017). Phycobilisomes are composed of two primary structural elements, a core complex and rod-like segments surrounding and bound to this core (Ritter et al., 1999). In most phycobiliproteins, the basic structural unit consists of a heterodimer representing two polypeptides α- and β-chains. The heterodimer is complexed to form an $(\alpha\beta)_3$ trimer, which generally follows a twofold arrangement to an $(\alpha\beta)_6$ hexamer. In addition, a third subunit (γ) often functions as a linker proteins tightly bound to the α- and β-polypeptides, thus resulting into a $(\alpha\beta)_6\gamma$ stoichiometry (Camara-Artigas et al., 2012; Ritter et al., 1999). The core complex of the phycobilisome system consist predominantly of the PBPs allophycocyanin, whereas the major components of the peripheral rods are phycocyanin and phycoerythrin (or phycoerythrocyanin) hexamer assemblies depending on the organism involved (Mysliwa-Kurdziel and Solymosi, 2017) The arrangement and the detailed structure has been elucidated recently, allowing building an accurate atomic model of the *P. purpureum* phycobilisome system (Ma et al., 2020). According to the species involved, different forms of pycobiliproteins exist; e.g. R-forms for Rhodophytes, B-forms for Bangiales (a sub-taxon of red algae) (Pecci and Fujimori, 1970), and C-forms for Cyanophyceae (Cyanobacteria). *Porphyridium cruentum* has been reported exemplarily to contain approximate relative amounts of 5 % allophycocyanin (APC), 11 % R-phycoerythrin (R-PE), 42 % B-phycoerythrin (B-PE), respectively (Camara-Artigas et al., 2012). B-PE can be easily extracted and is also most probably the main component of *Porphyridium purpureum* (Juin et al., 2015). B-PE has a similar hexameric structure $(\alpha\beta)_6\gamma$ as R-PE but differs in its chromophore composition, having only the 2 and 3 phycoerythrobilin (PEB) chromophores in the α and β subunits respectively, whereas γ subunit contains 2 of each PEB and PUB (Fig. 1) (Glazer, 1984; Glazer and Hixson, 1977). The molecular weight of B-PE determined e.g. in *Porphyridium cruentum* was around 240 kDa for the complex $(\alpha\beta)6\gamma$ (Glazer and Hixson, 1977; Ritter et al., 1999). B-PE of *Porphyridium cruentum* was shown to have molecular weights 17.5 kD for the α and β subunits, whereas γ-subunits that of 30.2 kD (Glazer and Hixson, 1977; Ritter et al., 1999)..

Different methods for the extraction of high-value products from red algae *Porphyridium purpureum* are reported. Different conventional buffers have been tested to extract B-PE from red algae. Li et al. (2021a) used cold water, 95 % ethanol, and hot water to extract phycoerythrin, lipids, and polysaccharides. Garcia et al. (2021) implemented 0.1 M phosphate buffer pH 5.5 followed by precipitation with ammonium sulfate ((NH4)2SO4) at 60 % saturation, while Bermejo et al. (2013) developed a pilot-scale facility for the extraction and recovery by anion exchange chromatography of B-phycoerythrin from *Porphyridium cruentum*. Microwave-assisted extraction is one of the most simple and effective option recently applied. The primary advantage of microwave-assisted extraction compared to conventional extraction methods is its microwave radiation, which enhances the evaporation of residual water from the material leading to the disruption of plant cell walls in order to promote extraction by internal diffusion (Reddy et al., 2020). It has already been used in *Porphyridium purpureum* for extraction of phycobiliproteins, but in the direction of characterization purposes. Juin et al. (2015) applied microwave to extract phycobiliproteins from *Porphyridium purpureum*. The extraction was performed successively and the maximum extraction yield of PE was obtained after 10 s of microwave assisted extraction at 40 °C. Microwave process has been used to extract

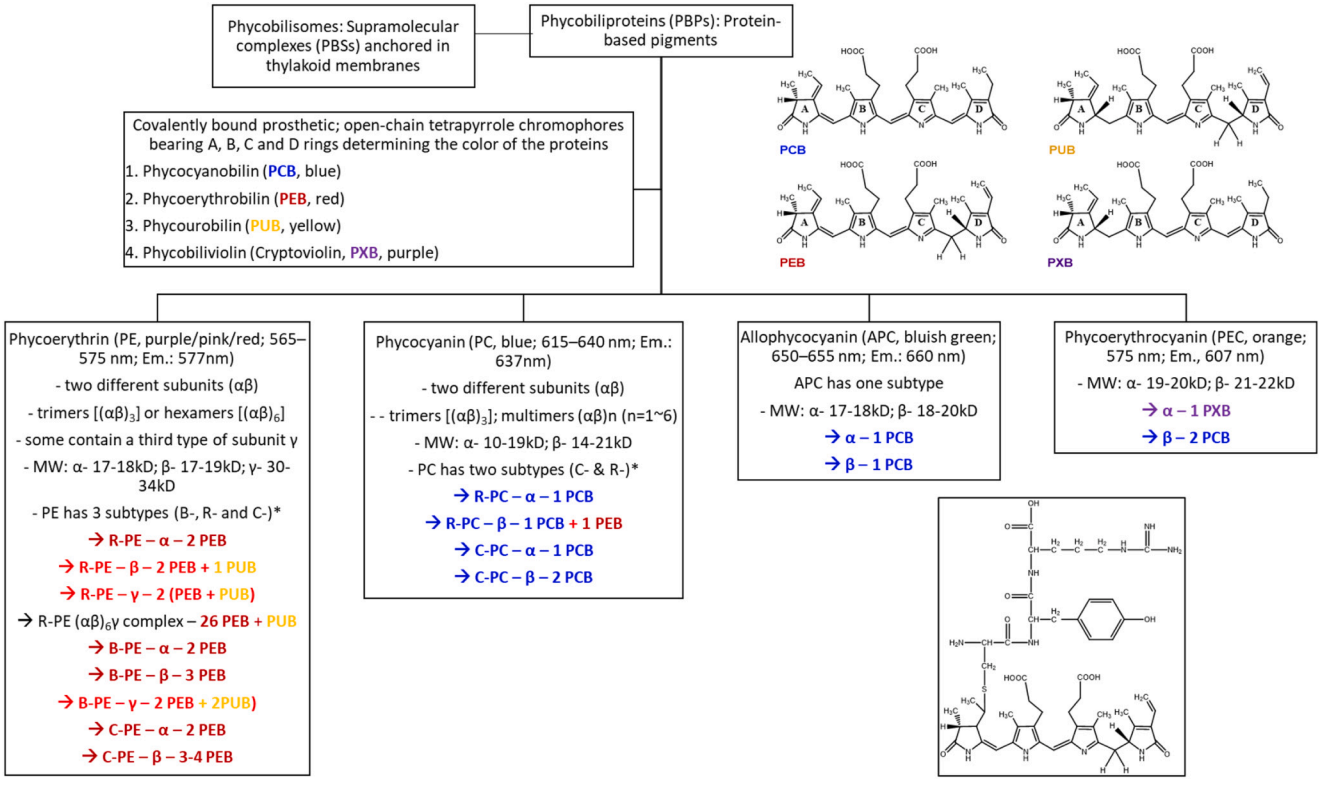


**Fig. 1.** Classification of the pycobiliproteins (PBPs) according to data available (Glazer, 1984; Glazer and Cohenbaz, 1971; Glazer and Hixson, 1977; Huang et al., 2002; Kim et al., 2018; Liu et al., 2012; Mysliwa-Kurdziel and Solymosi, 2017; Sekar and Chandramohan, 2008; Sidler, 1994). *According to the species, different forms exist: R-phycoerythrin (R-PE) for Rhodophytes, B-phycoerythrin and b-phycoerythrin (B-PE and b-PE) for Bangiales, and C-phycoerythrin (C-PE) for Cyanophyceae (Cyanobacteria). The inset shows the thioether link to the peptide isolated after tryptic digestion of the B-PE (Glazer, 1984).

as well as phycoerythrin from *Porphyridium* (Ardiles et al., 2020). In this study three different extraction solvent where tested including sodium phosphate buffer, calcium chloride solution and distilled water. The extraction time, microwave power and extraction solvent were optimized. However, the extraction temperature and the ratio of biomass to solvents were not investigated. Furthermore, recent papers dealing with the extraction of B-PE have been limited to extraction-related investigations. The purification step and the complete characterization (composition and purity determination by mass spectrometry) have not been addressed.. Therefore, it remains interesting to invest more attention in the optimization of the process for production purposes. The aim of this study was then to develop an optimized microwave-assisted extraction and purification process for extraction of B-phycoerythrin from red algae *Porphyridium purpureum*. For this purpose, microwave-assisted extraction followed by HCl precipitation was employed. Design of experiment based on Doehlert matrix was applied in order to optimize the process. The freeze-dried product was characterized performing selected analysis such as protein content, SDS PAGE and targeted mass spectrometry. Solubility assessment of B-phycoerythrin was finally carried out.

## 2. Material and methods

### 2.1. Material

The microalga *Porphyridium purpureum* used for the extraction of B-phycoerythrin was cultivated by the IGV Algae Farmhouse technology (AFT) under the optimized microalgae production system as recently documented in a German patent (Huschek and Theisen, 2021). Hydrochloric acid 37 % used for the precipitation was provided by VWR International GmbH (Darmstadt, Germany). Reducing solution tris(2-carboxyethyl)phosphine hydrochloride (≥98 %) and MS grade acetonitrile (≥99,9 %) used for the LC-MS analysis were obtained from Carl Roth GmbH (Karlsruhe, Germany) while the alkylating reagent iodoacetamide (≥99 %) and proteomics grade trypsin used for the protein digestion were purchased from Sigma Aldrich GmbH (Steinheim, Germany). Other chemicals used in this work were all of analytical grade.

### 2.2. Microwave-assisted extraction

The temperature controlled microwave-assisted extraction of B-phycoerythrin was performed by mixing red algae *Porphyridium purpureum* dried biomass with 5 mL of distilled water (CEM Discover SP-D Microwave, CEM GmbH, Kamp-Lintfort, Germany). The amount of sample used for each experiment is given in the experimental design. The whole sample was transferred into the glass vessel and introduced into the microwave instrument. After a ramping time of 1 min, the extraction was then performed at different time and temperature conditions according to the experimental design defined more detailed in the following part. Subsequently, the mixture was transferred into the 15 mL tube for centrifugation (10,000 ×*g* for 5 min, 4 °C). The supernatant consisting of the B-PE extract was collected and kept at 4 °C for the following analysis.

### 2.3. Optimization of the microwave assisted extraction

The Design of experiment (DoE) applying the response surface methodology (RSM) was used to optimize the conditions of the microwave-assisted extraction of the B-PE from the red algae *Porphyridium purpureum*. The aim was to maximize the extracted B-PE while minimizing the production costs, i.e. minimizing the process inputs and the necessary energy. The selection of the appropriate parameters to be used in the design for efficient data collection and to avoid a number of problems in statistical modeling and optimization constituted the first step. For this purpose, a preliminary study was conducted with initially 7 relevant factors including the type of extraction solvent (distilled water/tap water); the ratio biomass/extraction solvent; salt concentration; energy (microwave); pressure (microwave); extraction temperature and extraction time. From this preliminary study (data not shown), the three main parameters showing the major effect on the extraction process was selected for further optimization: (1) the ratio biomass/solvent, (2) the extraction time, and (3) the extraction temperature. Furthermore, three experimental responses were chosen to assess the performance of the experiments: extracted proteins, extraction yield and extracted B-PE.

The Doehlert matrix was used in the study. It is a practical and economical alternative to other second order experimental matrices; describing a spherical domain for three parameters, which increases the uniformity of the investigated factors (Sagu et al., 2014a; Sagu et al., 2014b; Tchewonpi et al., 2019). Another important consideration here is to study each parameter at a different level and thus requiring few experimental runs for a high efficiency. Table 1 shows the experimental design with the coded values provided by the Doehlert matrix, and the real values of the parameters used for the different extraction experiments. Eq. (1) was used to transform the coded values into the real values.

$$X = X_i + (\Delta X_i \times x_i) \quad (1)$$

With $X$ the real variable; $x_i$ the coded variable given by the Doehlert matrix; $X_i$ the centre of variable and $\Delta X_i$ the increment corresponding to $X_i$.

### 2.4. Purification of B-phycoerythrin

B-phycoerythrin extract obtained at the optimal conditions as determined with the experiment design was purified in one step process by HCl precipitation. For this purpose, 25 µL of 37 % HCl were added to 30 mL of the extract for a final concentration of HCl of 0.03 % (or 10 mM). The whole was shortly mixed then incubated at −20 °C for 20 min. After centrifugation at 10,000 ×*g* for 5 min, 4 °C, the supernatant was removed and discarded. The precipitate was washed by mixing with distilled water to remove residual polysaccharides and to attenuate the effect of HCl during the protein folding. After centrifugation (10,000 ×*g* for 5 min, 4 °C), the supernatant was discarded; the precipitate freeze-dried and then stored in an airtight package for further analysis.

### 2.5. Solubility study

In this work, studies were conducted in order to improve the solubility of the final freeze-dried product. The approach used involved adjustment of the pH and ionic strength of the precipitated B-PE before the freeze-drying step. For this purpose, two buffer solutions (100 mM sodium phosphate and 100 mM ammonium bicarbonate) and three salt solutions (100 mM sodium chloride, 100 mM ammonium sulfate, and 100 mM calcium chloride) were used following different combination. Resulting freeze-dried materials were then gradually applied using distilled water to solubilize them. The mixtures were briefly vortexed and sonicated for 5 min, and the clarity of the solution was visually evaluated.

### 2.6. Analysis

#### 2.6.1. Protein determination

The Lowry method (Lowry et al., 1951) with bovine serum albumin (BSA) as standard was used to determine the protein content.

#### 2.6.2. Sodium dodecylsulfate polyacrylamide gel electrophoresis

The extracted protein composition of each fraction was evaluated by electrophoresis. Similarly, an approximation of the protein content (expressed in percent) of the freeze-dried samples was also assessed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS PAGE). Briefly, 20 µL of each sample was mixed with 20 µL of sample buffer

**Table 1**
Doehlert design with the coded and real values of the parameters as well as the experimental responses.

| N° exp. | Parameters | | | | | | Experimental responses | | |
|---|---|---|---|---|---|---|---|---|---|
| | Coded variables | | | Real variables | | | Extracted proteins | Extraction yield | B-PE content |
| | $x_1$ | $x_2$ | $x_3$ | $X_1$ | $X_2$ | $X_3$ | (mg) | (%) | PA/μg biomass |
| 1 | 0 | 0 | 0 | 22.0 | 155 | 65 | 6.283 ± 0.271 | 5.7 ± 0.3 | 11,477 ± 1411 |
| 2 | 1 | 0 | 0 | 40.0 | 155 | 65 | 5.477 ± 0.065 | 2.5 ± 0.5 | 5941 ± 424 |
| 3 | −1 | 0 | 0 | 4.0 | 155 | 65 | 1.935 ± 0.200 | 9.7 ± 0.1 | 16,700 ± 1515 |
| 4 | 0.5 | 0.866 | 0 | 31.0 | 300 | 65 | 5.100 ± 0.205 | 3.3 ± 0.2 | 7801 ± 365 |
| 5 | −0.5 | −0.866 | 0 | 13.0 | 10 | 65 | 5.650 ± 0.155 | 8.7 ± 0.0 | 11,553 ± 650 |
| 6 | 0.5 | −0.866 | 0 | 31.0 | 10 | 65 | 7.236 ± 0.013 | 4.7 ± 0.4 | 6969 ± 300 |
| 7 | 0.5 | 0.866 | 0 | 13.0 | 300 | 65 | 4.123 ± 0.094 | 6.3 ± 0.1 | 12,963 ± 196 |
| 8 | 0.5 | 0.289 | 0.816 | 31.0 | 203 | 100 | 4.577 ± 0.167 | 3.0 ± 0.5 | 4227 ± 358 |
| 9 | −0.5 | −0.289 | −0.816 | 13.0 | 107 | 30 | 5.385 ± 0.295 | 8.3 ± 0.1 | 13,189 ± 441 |
| 10 | 0.5 | −0.289 | −0.816 | 31.0 | 107 | 30 | 9.219 ± 0.153 | 5.9 ± 0.2 | 12,587 ± 485 |
| 11 | 0 | 0.577 | −0.816 | 22.0 | 252 | 30 | 8.784 ± 0.231 | 8.0 ± 0.5 | 13,512 ± 210 |
| 12 | −0.5 | 0.289 | 0.816 | 13.0 | 203 | 100 | 3.218 ± 0.353 | 3.4 ± 0.5 | 12,886 ± 106 |
| 13 | 0 | −0.577 | 0.816 | 22.0 | 58 | 100 | 3.402 ± 0.075 | 4.2 ± 0.7 | 6608 ± 319 |
| 14 | 0 | 0 | 0 | 22.0 | 155 | 65 | 6.186 ± 0.053 | 5.7 ± 0.5 | 11,287 ± 297 |
| 15 | 0 | 0 | 0 | 22.0 | 155 | 65 | 6.368 ± 0.190 | 5.2 ± 0.3 | 12,207 ± 241 |
| 16 | 0 | 0 | 0 | 22.0 | 155 | 65 | 6.380 ± 0.607 | 5.6 ± 0.1 | 12,333 ± 134 |
| 17 | 0 | 0 | 0 | 22.0 | 155 | 65 | 6.516 ± 0.341 | 5.5 ± 0.3 | 12,371 ± 169 |

$x_1$, $x_2$, $x_3$ and $X_1$, $X_2$ and $X_3$ are the coded and real values of the ratio biomass/solvent, the extraction time and the extraction temperature, respectively. With B-PE the content of B-phycoerythrin as determined by LC-MS/MS and expressed as peak area per μg of biomass.

(Thermo Fischer Scientific, Waltham, MA, USA) in the presence of reducing agent and heated to 95 °C for 5 min. After cooling to room temperature, 15 μL of the mixtures were loaded into the wells of an Invitrogen NuPAGE Bis-Tris 10 % gel (Thermo Fischer Scientific, Waltham, MA, USA) and separation was performed for approximately 60 min at constant current (30 mA). The gels were then stained overnight in brilliant blue R-250 Coomassie solution, and then de-stained for 3 to 4 h with a 10 % acetic acid solution (solution changed regularly). The gels were scanned (Bio-5000 Professional VIS Gel Scanner, SERVA Electrophoresis GmbH, Heidelberg, Germany) and ImageLab software (Bio-Rad Laboratories, Hercules, CA, USA) was used to process and analyze the resulting images.

### 2.6.3. *Liquid chromatography tandem mass spectrometry*

A targeted liquid chromatography tandem mass spectrometry (LC-MS/MS) method was developed in order to identify and characterize the extracted samples. The methodology used and the different steps leading to the method development are similar to those previously described (Huschek et al., 2018; Sagu et al., 2021; Sagu et al., 2020a; Sagu et al., 2020b).

The sample preparation procedure consisted of first reducing 0.4 mL of each sample by mixing with 10 μL of 0.25 M TCEP. After a 20 min incubation period at 50 °C, the samples were then alkylated by incubating for 20 min in the dark in the presence of 10 μL of 0.25 M IAA. Following addition of 135 μL of digestion buffer (100 mM ammonium bicarbonate), 20 μL of a proteomics grade trypsin solution (4 mg/mL) was added and the digestion was carried out at 37 °C overnight under shaking conditions. The reaction was then stopped by adding 15 μL of 40 % formic acid. The clean-up step was performed by solid phase extraction (SPE). Approximately 300 mg of packing material for reversed phase application (sorbent octadecyl C18, MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) was loaded into the columns. Subsequently, after activation (with 6 mL of a 50 % acetonitrile solution containing 0.1 % formic acid) and conditioning the columns (with 6 mL of distilled water), the samples were loaded. 6 mL of distilled water were then introduced into the column for washing and finally the analytes were eluted with 1 mL of acetonitrile containing 0.1 % formic acid. The collected solutions were diluted with distilled water (1:5, *v*/v) prior to analysis.

The Skyline software (MacLean et al., 2010) was used to develop the LC-MS/MS method. For this purpose, the sequences of the 6 phycobiliproteins corresponding to the red algae *Porphyridium purpureum* were downloaded from the online UniProt Knowledge database (UniProt, 2014) in FASTA format (as of 20.01.2022). These proteins included: B-phycoerythrin alpha chain (P11392); B-phycoerythrin beta chain (P11393); R-phycocyanin-1 subunit alpha (P37207); R-phycocyanin-1 beta chain (P37208); Allophycocyanin alpha subunit (W0RYM0) and -Allophycocyanin beta subunit (W0S279). The FASTA files were uploaded to Skyline and an in silico digestion was performed. For peptide settings, trypsin was chosen as the digesting enzyme with a maximum missed cleavages set at 0. The filter was set for peptides containing 4 to 25 amino acid residues, considering the carbamidomethylation of cysteine residues as structural modification. For the transition settings, the y- and b-type ions were selected, with precursor charge +2 and product ion charge +1 (with minimum and maximum *m/z* of 300 and 1500, respectively). Based on these criteria, different methods were initially generated, and after optimization of the collision energy, the final method was achieved and was applied for the analysis of individual samples. Table 2 shows the final optimal parameters including Q1 and Q3 masses, retention times and collision energies.

The analysis was performed on an HPLC system (Agilent Technologies GmbH & Co. KG, Waldbronn, Germany) consisting of a DEAEQ20522 vial sampler, a DEAEP005250 binary pump and a DEAED 10080 column oven. The HPLC was equipped with a DEACEX10164 UV detector and a 6470 Triple Quad LC/MS detector (Agilent Technologies GmbH & Co. KG, Waldbronn, Germany). Column temperature of 30 °C and flow rate of 0.5 mL/min were applied for total time of 32 min including an additional post run time of 4 min. The analysis was performed on a Kinetex C8 column (150 × 4.6 mm, 2.6 μm, 100 Å; Phenomenex, Torrance, CA, USA) and with gradient mode (0.1 % formic acid in water and acetonitrile used as eluents A and B, respectively). The elution program was set as follows: from 0 to 1 min, 0 % solvent B; at 2 min, 5 % solvent B; at 18 min, 50 % solvent B; at 19 min, 95 % solvent B; from 19 to 22 min, 95 % solvent B; at 23 min, 0 % solvent B and from 23 to 28 min, 0 % solvent B. The detection was performed in multiple reaction monitoring (MRM) mode while using an electrospray ionization (ESI) source operating in positive polarity. The temperature of the desolvation gas in the ionization source was 275 °C, the nebulizer pressure 35 psi, the gas flow rate 11 L/min, the fragmentor 120 for a Dwell time of 20 ms. Nitrogen was used as the collision gas. The peak areas were used to express the relative abundance of the targeted compounds and the results were expressed as peak area/mg sample (PA/mg).

**Table 2**
Selected biomarkers and their corresponding characteristics according to the optimized multiple reaction monitoring method.

| Protein | Biomarkers | Fragment | Q1-Mass | Q3-Mass | CE [eV] | Retention time [min] |
|---|---|---|---|---|---|---|
| P11392 | LAGNHEAVVK | G [y8] | 519,2905++ | 853,4526+ | 17.1 | 7.1 |
| | | H [y6] | | 682,3883+ | 17.1 | |
| | | V [b8] | | 792,3999+ | 17.1 | |
| | | V [b9] | | 891,4683+ | 17.1 | |
| P11393 | DGEIILR | I [y4] | 408,2347++ | 514,3711+ | 13.7 | 10.6 |
| | | L [y2] | | 288,2030+ | 13.7 | |
| | | G [b2] | | 173,0557+ | 13.7 | |
| | | E [b3] | | 302,0983+ | 13.7 | |
| P37207 | FLSNTELQAVNGR | E [y8] | 724,8782++ | 886,4741+ | 23.5 | 10.9 |
| | | L [y7] | | 757,4315+ | 23.5 | |
| | | Q [y6] | | 644,3474+ | 23.5 | |
| | | A [y5] | | 516,2889+ | 23.5 | |
| P37208 | DMEIVLR | E [y5] | 438,2364++ | 629,3981+ | 14.6 | 11.9 |
| | | I [y4] | | 500,3555+ | 14.6 | |
| | | L [y2] | | 288,2030+ | 14.6 | |
| | | M [b2] | | 247,0747+ | 14.6 | |
| W0RYM0 | YLSPGELDR | S [y7] | 525,2667++ | 773,3788+ | 17.3 | 10.2 |
| | | P [y6] | | 686,3468+ | 17.3 | |
| | | G [y5] | | 589,2940+ | 17.3 | |
| | | S [b3] | | 364,1867+ | 17.3 | |
| W0S279 | EVTGSLVGSDAGK | G [y10] | 610,3119++ | 890,4578+ | 19.9 | 9.2 |
| | | V [y7] | | 633,3202+ | 19.9 | |
| | | G [y6] | | 534,2518+ | 19.9 | |
| | | A [y3] | | 275,1714+ | 19.9 | |

Q1 = precursor mass; Q3 = transition mass; CE = collision energy.

### *2.7. Statistical analysis*

All experiments were performed in triplicate. The results of the analysis were reported as mean values ± standard deviation. Statistical analysis of the data was performed using GraphPad Prism 8 software (GraphPad Software, Inc., San Diego, CA, USA), and the results were considered statistically significant for *P*-values $< 0.05$.

## 3. Results and discussion

### *3.1. Preliminary assessments – selection of the parameters for optimization*

The use of the microwave for the extraction of high value compounds from different biological materials is now a well-established procedure. This method has a considerable advantage in reducing the extraction time substantially (within a few seconds or minutes) compared to conventional extraction methods which may require much longer extraction times. In order to efficiently optimize the microwave-assisted extraction of B-PE from biomass, the first step consisted in a preliminary study to select the most important parameters, where included among other investigated parameters, the type of extraction solvent (Distilled water/tap water), ratio biomass/extraction solvent, salt concentration, energy/power (microwave), pressure (microwave), extraction temperature and time were considered.

The microwave energy allows cell disruption and solvent penetration into the sample layers, leading to an increase in extraction yields. Two energy levels were tested, 800 and 1600 W. The obtained extracts were analyzed for protein and B-phycoerythrin content, and the results are presented (supplementary data). Similar protein levels of 0.567 ± 0.008 and 0.579 ± 0.013 mg/mL were obtained for energy levels of 800 and 1600 W, respectively. Furthermore, although the analysis of variance showed a significant difference between the B-phycoerythrin beta chain concentrations, the other five protein components analyzed indicated that there were no significant differences.

The type of extraction solvent was also considered. The diffusion of the solvent into the sample matrix and the release of the compounds of interest into the solvent are important mechanisms in microwave assisted extraction. The microwave absorption in the matrix being closely related to the dielectric constant of the solvent used (the higher the dielectric constant, the stronger the absorption and vice versa). In this work, the extraction was performed with distilled water and tap water. No significant difference was observed between the protein and B-PE levels extracted with the two types of solvents. Using distilled water, addition of non-reactive neutral salt at final concentrations of 1.2; 6; and 12 mg; corresponding to 1.25, 5 and 10 % *w*/w salt to biomass was performed to modify the ionic strength of the solvent. The results reported once again showed similar levels of protein and B-PE content for the different samples analyzed.

At the end of the preliminary study, the ratio biomass/extraction solvent, extraction temperature and extraction time presented the most significant effects on the extraction process (data not shown). These three parameters were therefore selected for further optimization. The other parameters were kept constant throughout the work as follows: extraction solvent - distilled water; microwave energy - 800 W and no salt addition.

### *3.2. Optimization of the B-PE microwave-assisted extraction*

The Doehlert design was used to optimize the microwave-assisted extraction process of B-PE. Three parameters were selected for this study namely the ratio biomass/extraction solvent, extraction temperature and time. Seventeen experiments were generated and investigated. It was clearly observed a notable difference in appearance of the extracts, demonstrating that the implementation of varied operating conditions (change in the ratio biomass/extraction solvent, extraction temperature and time) significantly impacted the quality of the extracts obtained. The observed color spectrum ranging from light yellow to dark red. Different analyses were performed on the extracts obtained and the results are presented (supplementary data). It can be observed, among other interesting results, that the extracted volumes ranged from 1.7 mL (experiment 8) to 4.4 mL (experiment 3), starting with the same volume of initial solvent. Similarly, if the highest extraction yield (9.7 ± 0.1 %) as well as the highest extracted B-PE content (16,700 ± 1515 PA/μg biomass) were achieved with experiment 3, the same experiment showed the lowest extracted protein content (1.935 ± 0.200 mg compared to 9.219 ± 0.153 mg obtained in experiment 10). Looking at the extract composition, the proportion of B-PE remained relatively homogeneous, ranging from 87.3 ± 0.6 % for experiment 11 to 91.1 ± 0.7 % for experiment 7.

### 3.2.1. Mathematical models

Among the analysis performed, three of them were selected and used as experimental responses including the extracted proteins, the extraction yield and the B-PE content. Second-order polynomial mathematical models with interaction between parameters were developed (Eqs. (2), (3), and (4)), and a summary of the statistical analysis is given in Table 3. The analysis of variance (ANOVA) exhibited coefficients of correlation (R-squared) of 0.939, 0.975 and 0.989 as well as determination (adjusted R-squared) of 0.904, 0.922 and 0.974 for the extracted protein, extraction yield and B-PE content, respectively. These results demonstrate that all proposed models provided a good representation of >90 % of the experimental observations. This allowed the validation and use of the models for the simulation of the response surfaces showing the effect of the variables on the respective experimental responses as well as for the subsequent numerical optimization.

Equation of the fitted model:

$$\begin{aligned} Extracted\ proteins\ (mg) = {} & 0.983 + 0.590 \bullet X_1 + 0.002 \bullet X_2 - 0.010 \\ & \bullet X_3 - 0.008 \bullet X_1^2 - 0.0001 \bullet X_1 \bullet X_2 - 0.001 \bullet X_1 \\ & \bullet X_3 - 0.0005 \bullet X_2^2 - 0.0001 \bullet X_2 \bullet X_3 - 0.0004 \\ & \bullet X_3^2 \end{aligned} \quad (2)$$

$$\begin{aligned} Extraction\ yield\ (\%) = {} & 14.2 - 0.37 \bullet X_1 + 0.01 \bullet X_2 - 0.03 \bullet X_3 + 0.01 \\ & \bullet X_1^2 + 0.01 \bullet X_1 \bullet X_2 + 0.001 \bullet X_1 \bullet X_3 + 0.0001 \\ & \bullet X_2^2 - 0.0002 \bullet X_2 \bullet X_3 - 0.0002 \bullet X_3^2 \end{aligned} \quad (3)$$

$$\begin{aligned} Extracted\ BPE\ \left(PA{\cdot}\mu g\ biomass^{-1}\right) = {} & 6903.8 + 224.9 \bullet X_1 + 34.4 \\ & \bullet X_2 + 153.5 \bullet X_3 - 1.9 \bullet X_1^2 - 0.1 \bullet X_1 \\ & \bullet X_2 - 6.2 \bullet X_1 \bullet X_3 - 0.09 \bullet X_2^2 + 0.03 \\ & \bullet X_2 \bullet X_3 - 0.7 \bullet X_3^2 \end{aligned} \quad (4)$$

With $X_1$, $X_2$ and $X_3$ the ratio biomass/solvent, the extraction time and the extraction temperature, respectively.

### 3.2.2. Effect of parameters on the experimental responses: estimated response surfaces

The models were found to adequately represent interaction between the experimental response values and the three variables. Therefore, the surface plots were generated, keeping the extraction temperature constant at its low level (−0.816). Response surface plot showing the effect of the ratio biomass/solvent and the extraction temperature on the extracted protein, extraction yield and extracted B-phycoerythrin is presented (supplementary data). Fig. 2a shows the effect of biomass/solvent ratio and extraction time on extracted proteins. It is observed that the extracted proteins increased exponentially with the biomass. For example, for an extraction temperature set at 30 °C and for extraction time of 30 s, the amount of extracted protein increased from about 2.5 to 9.3 mg as biomass ranged from 4 to 30 mg/mL. Above 30 mg/mL, the increase in extracted protein was no longer proportional to the quantity of biomass supplied and a slight drop in protein content was even observed around 40 mg/mL. In contrast, the extraction time exhibited no significant effect on protein extraction. The ratio biomass/solvent (*P*-value = 0.0025), extraction temperature (P-value = 0.0005) and the quadratic term of the ratio biomass/solvent (P-value = 0.0059) contributed with a significant effect on the extracted protein level. These contributions were found to be positive for the biomass and negative for the extraction temperature. It is well known that proteins are heat sensitive macromolecules and are easily degraded when exposed to high temperatures. Similarly, an increase in the extraction temperature could have led to the gelation of polysaccharides and even proteins contained in the biomass, thus negatively affecting the extraction efficiency of the proteins in the solvent. It can also be noted from these data that no interaction between the parameters proved to be significant. This suggests that there is no real synergistic action and that each parameter operates independently.

The extraction yield was mainly affected by the ratio biomass/solvent (*P*-value = 0.0001) and the extraction temperature (P-value = 0.0006) while with the extracted B-phycoerythrin, in addition to the biomass (P-value = 0.0000) and temperature (P-value = 0.0000), the interaction between biomass and temperature (P-value = 0.0002) as well as the quadratic term of time (0.0006) were also significant. Interestingly, the negative sign of all coefficients indicates that these parameters negatively affected extraction yields and extracted B-PE. These negative effects are also noticeable on the response surface plot. For an extraction temperature of 30 °C and an extraction time of 30 s, a gradual reduction of the yield from 11.8 to only 3.4 % was reported as the biomass increased from 4 to 40 mg/mL (Fig. 2b). In addition, at the same extraction conditions, a slight increase followed by a decrease in extracted B-PE content was documented (Fig. 2c).

**Table 3**
Statistical analysis summary of mathematical models.

| Statistical parameters | Mathematical models | | |
|---|---|---|---|
| | Extracted protein | Extraction yield | B-PE content |
| R-squared | 93.8524 | 97.5111 | 98.8633 |
| Adjusted R-squared | 90.377 | 92.1682 | 97.4018 |
| Standard error | 0.808885 | 0.80263 | 534.835 |
| Mean absolute error | 0.372347 | 0.412353 | 303.618 |
| Durbin-Watson statistic | 2.54829 | 3.08775 | 1.77322 |
| P-value | 0.0457 | 0.0018 | 0.1479 |

### 3.2.3. Numerical optimization

A numerical optimization of the microwave-assisted extraction process of B-PE from *Porphyridium purpureum* was applied in this work. For this purpose, mathematical models developed were applied with STATGRAPHICS Centurion software (The Plains, VA, USA). The procedure was conducted in order to determine the combination of experimental factors that simultaneously optimize the three experimental responses analyzed and by maximizing a desirability function. The objectives of the individual responses were defined to maximize the extracted proteins, the extraction yield and the extracted B-PE. The low and high levels set for each of the parameters indicating the region over which the optimization was performed were 4–40 mg/mL, 10–300 s and 30–100 °C for biomass/solvent ratio, extraction time and extraction temperature, respectively. The best point of the experimental domain was found with an optimum desirability value of 0.82. The combination of the factors at which that optimum was achieved is: Ratio Biomass/solvent 16.8 mg/mL, Extraction time of 172 s, and extraction temperature of 30 °C. The corresponding optimal experimental responses were: Extracted proteins 7.2 mg, extraction Yield 8.5 % and extracted B-PE 13961 PA/μg biomass. Investigations were furthermore carried out at optimal conditions in order to validate experimentally the numerically obtained data. The experimental response values obtained were 7.6 ± 0.4 mg, 8.9 ± 0.2 % and 14,112 ± 726 PA/μg biomass for extracted protein, extraction yield and B-PE content, respectively. Comparing these values with those of the literature, the performances obtained in this work showed improved yields. Bermejo et al. (2013) et al. using different buffer systems obtained an extraction yield of total biliproteins between 2.34 and 3.64; the maximum percentage recovery being obtained with distilled water (3.64 %). Ardiles et al. (2020) using conventional microwave extraction achieved a protein content in *P. cruentum* of 42.9 % *w*/w and a maximum phycoerythrin extraction yield of 33.85 mg/g. After 10 s of microwave extraction at 40 °C, Juin et al. (2015) obtained a PE extraction yield of 73.7 μg/mg biomass.

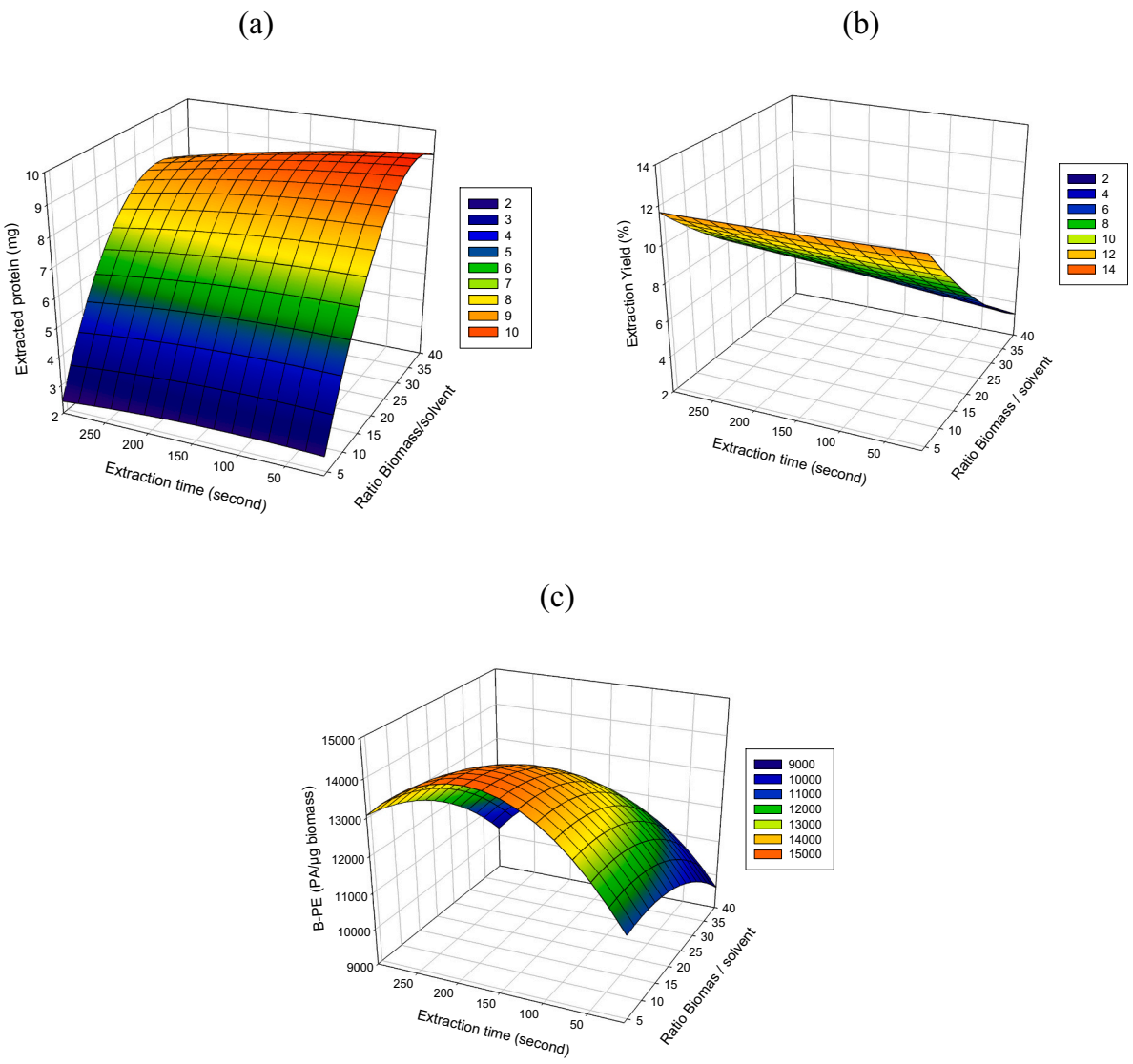


**Fig. 2.** Response surface plot showing the effect of the ratio biomass/solvent and the extraction time on (a) the extracted protein, (b) extraction yield and (c) extracted B-phycoerythrin. The extraction temperature was monitored at the central point of the experimental domain.

### *3.3. Solubility determination*

Solubility is a critical parameter, as it is well known that protein unfolding generally leads to the formation of biologically insoluble protein aggregates; resulting in a loss of functional characteristics. It is a thermodynamic characteristic of protein concentration in a saturated solution under a given set of conditions: The solubility is generally dependent on extrinsic conditions such as pH, ionic strength, temperature as well as intrinsic properties, predominantly the composition of amino acids present on the surface of the protein (Kramer et al., 2012; Schein, 1993). The solubility of freeze-dried B-phycoerythrin was tested in both water and 100 mM ammonium bicarbonate buffer. The ionic strength of the precipitate obtained after purification under acidic conditions was also adjusted prior to the freeze-drying step using different compounds including 100 mM phosphate and ammonium bicarbonate buffers as well as 100 mM of sodium chloride, ammonium sulfate and calcium chloride. Freeze-dried b-phycoerythrin was not soluble in distilled water. In contrast, the product was effectively redissolved in 100 mM ammonium bicarbonate buffer up to a concentration of 3 mg/mL. Attempts to change the ionic strength prior to freeze-drying in order to improve the solubility in water were not successful. A recommendation for the use of the product is therefore made to first dissolve it in ammonium bicarbonate buffer, before subsequently diluting in distilled water. Further studies to improve the solubility of lyophilized B-phycoerythrin will be undertaken.

### *3.4. Composition of the final freeze-dried B-phycoerythrin*

The final product after freeze drying was characterized. For this purpose, different analyses were carried out, including the determination of the protein content, the purity and the composition in terms of phycobiliproteins. The Kjeldahl method was used here to measure the crude protein content. For this purpose, the measurement of the total nitrogen content was performed and then used to estimate the crude protein content by applying a conversion factor of 6.25. The final freeze-dried product showed a protein content of 54.9 g/100 g. In addition, the water content of the freeze-dried protein was tested and found to be in the range of 4–7 %. The mass of phycoerythrobilin (chromophores) was further determined. In fact, according to Glazer and Hixson (1977), B-PE carries six phycoerythrobilin groups with each having 35,000 Da. The mass of phycoerythrobilin (Formula: C33H38N4O6) being 586.7 units, the protein/phycoerythrobilin ratio was then found to be equal to 9.94, which allowed the mass of phycoerythrobilin to be established as 5.5 g/100 g in the freeze dried product. The sum of the three components

(protein, water content and phycoerythrobilin) accounted for a total of about 65–70 g/100 g. This suggested the presence in the freeze-dried product of compounds other than protein, probably polysaccharides. However, this result is above the protein content of the currently marketed B-PE, which is usually in the range of 20–40 %. It confirms the efficiency of the one-step purification process by HCl precipitation implemented in the present work.

Solution of 2 mg/mL of lyophilized B-PE was prepared and analyzed by SDS PAGE at denaturing conditions. The gel was analyzed using ImageLab software. A main band with molecular weight of 18.4 kDa can be observed. This corresponds to the molecular weight of the B-phycoerythrin beta chain (18,554.18 Da, average mass) as reported in the Uniprot online database (data assessed for the last time on 15.07.2022). Furthermore, an attempt for absolute quantification of the protein content in the freeze-dried sample was undertaken based on electrophoresis. For this purpose, myoglobin was used as reference protein. The choice of myoglobin was basically made on the basis of its molecular weight. As myoglobin has a mass of 16,946.40 Da (average mass) which is similar to that of B-PE, the two proteins were found in the same region of the gel at the end of the separation process (supplementary data). Thus, different amounts of myoglobin were loaded into the gel in order to perform the calibration. Similarly, three levels of the freeze-dried sample (5, 10 and 15 μg) were used. Following the absolute quantification, protein contents in the range of 49.8–51.6 % were obtained. It should be mentioned that these protein levels are similar to that obtained by the Kjeldahl method (55 %).

Analyses were also undertaken to determine the different proportions of phycobiliproteins in the freeze-dried sample. For this purpose, a targeted mass spectrometry based method was developed. One biomarker peptide was determined for each of the six phycobiliproteins. The selection of the biomarker peptides was made based mainly on specificity (unique peptide) as well as on the strength of the intensity and the proper coverage of the different transitions selected. The peptides were then used as quantifiers to evaluate the different proportions. Selected biomarkers were: LAGNHEAVVK, DGEIILR, FLSNTELQAVNGR, GEFLSNTQIDALSK, YLSPGELDR, and EVTGSLVGSDAGK for B-phycoerythrin alpha subunit, B-phycoerythrin beta subunit, R-phycocyanin-1 alpha subunit, R-phycocyanin-1 beta subunit, allophycocyanin alpha subunit, and allophycocyanin beta subunit, respectively. In addition, two to three other peptides per protein were also included in the final method and used as qualifiers to further support the identification. Proper validation of the developed method was successfully carried out. Among other parameters analyzed were linearity, selectivity, repeatability, reproducibility, recovery and limits of detection and quantification (data not shown). Table 4 presents results of the composition in term of the phycobiliproteins of the final freeze-dried product. It is shown that >93.5 % of the proteins were constituted by the B-phycoerythrin beta subunit. Allophycocyanin constituted about 6 % while the R-phycocyanin fraction represented <0.5 %. These results demonstrate once again the efficiency of the procedure set up to extract (microwave-assisted) efficiently and purify (HCl-precipitation) selectively B-phycoerythrin from *Porphyridium purpureum* (Red alga) powder.

## 4. Conclusion

In this work, microwave-assisted extraction of B-phycoerythrin from the red alga *Porphyridium purpureum* cultivated in new-patented IGV Algae Farmhouse technology (AFT) was investigated. The ratio biomass/solvent, the extraction temperature as well as the extraction time were found to significantly affect the extraction process and were thus optimized. Furthermore, B-phycoerythrin was purified under acidic conditions and then lyophilized. The freeze-dried product was subsequently characterized (protein content, SDS PAGE and mass spectrometry analysis). Solubility assessments were finally performed. Scaling up studies of the process developed in this work for commercial applications is part of the on-going work.

**Table 4**
Composition in term of the phycobiliproteins of the final freeze-dried product and according to the targeted mass spectrometry analysis.

| Protein | Biomarker | Peak area | Proportion (%) |
|---|---|---|---|
| B-phycoerythrin alpha subunit | LAGNHEAVVK | 492 ± 13 | 0.4 |
| B-phycoerythrin beta subunit | DGEIILR | 120,672 ± 927 | 93.2 |
| R-phycocyanin-1 alpha subunit | FLSNTELQAVNGR | 60 ± 11 | 0.0 |
| R-phycocyanin-1 beta subunit | GEFLSNTQIDALSK | 501 ± 28 | 0.4 |
| Allophycocyanin alpha subunit | YLSPGELDR | 6005 ± 294 | 4.6 |
| Allophycocyanin beta subunit | EVTGSLVGSDAGK | 1714 ± 89 | 1.3 |

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biteb.2022.101212.

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