Modeling and Analysis

Biofpr Biofuels Bioproducts & Biorefining

A successful method for phycocyanin extraction from *Arthrospira platensis* using [Emim] [$EtSO_4$] ionic liquid

Jennifer Sánchez-Laso, Department of Chemical and Environmental Technology, ESCET, Universidad Rey Juan Carlos, Móstoles, Spain
Alejandro Piera, Gemma Vicente, Department of Chemical, Energy and Mechanical Technology, ESCET, Universidad Rey Juan Carlos, Móstoles, Spain
Luis Fernando Bautista, Department of Chemical and Environmental Technology, ESCET, Universidad Rey Juan Carlos, Móstoles, Spain
Rosalía Rodríguez, Juan J. Espada, Department of Chemical, Energy and Mechanical Technology, ESCET, Universidad Rey Juan Carlos, Móstoles, Spain

Received May 31 2021; Revised June 30 2021; Accepted July 14 2021;
View online August 9, 2021 at Wiley Online Library (wileyonlinelibrary.com);
DOI: 10.1002/bbb.2275; *Biofuels, Bioprod. Bioref*. 15:1638–1649 (2021)

Abstract: Research into the extraction of phycobiliprotein from *Arthrospira platensis* is currently ongoing. Most traditional methods of phycobiliprotein extraction include the use of organic solvents, which negatively affect the sustainability of the process. Ionic liquids (ILs) are a promising alternative for phycobiliprotein extraction due to their properties as green solvents. In the present work an imidazolium-based IL and sonication were studied for phycobiliprotein and carbohydrate extraction. A factorial experimental design was used to optimize the amount of extracted phycocyanin. The maximum extraction yield was achieved by using low biomass/solvent ratios combined with high IL/water ratios and sonication powers, and long operation times. The recovery of IL was studied under these conditions using a dialysis-based process to separate the IL from the extracted phycobiliproteins. The results reveal the possibility of using the recovered IL for seven consecutive extraction cycles with an acceptable phycocyanin extraction amount, from 75 mg g^{-1} (fresh IL) to 60 mg g^{-1} (7 cycles of reused IL). The use of [Emim] [$EtSO_4$] + ultrasound is a promising alternative for phycocyanin extraction, enhancing the results obtained with other IL in terms of extracted phycocyanin and the number of times the IL can be reused. © 2021 Society of Chemical Industry and John Wiley & Sons, Ltd

Supporting information may be found in the online version of this article.

Key words: *Arthrospira platensis*; bioproducts; phycobiliprotein; phycocyanin; ionic liquid; extraction

Correspondence to: R Rodríguez, Department of Chemical, Energy and Mechanical Technology. ESCET, Universidad Rey Juan Carlos, 28933 Móstoles, Madrid, Spain. E-mail: rosalia.rodriguez@urjc.es

Introduction

Microalgae and cyanobacteria are microorganisms with advantages for producing a variety of valuable products with applications in different industrial sectors.[1–3] Microalgae can be used to produce biofuels and to generate bioproducts such as pigments (xanthophylls, carotenes, and phycobiliproteins) and polyunsaturated fatty acids (PUFAs).[4–8] Among these products, phycobiliproteins have attracted the interest of researchers as they have remarkable antioxidant and anti-inflammatory properties.[9,10] Phycobiliproteins (phycocyanin, phycoerythrin, and allophycocyanin) are light-harvesting protein pigments present in the chloroplast thylakoid membranes of cyanobacteria and red algae and constitute the essential components of the phycobilisomes.[11,12]

Phycocyanin is the most important phycobiliprotein due to its pharmacological properties, such as its antioxidant and anti-inflammatory effects.[6,13,14] It is a natural blue pigment, mainly present in cyanobacteria, especially in *Arthrospira platensis*, with content up to 13% dry weight.[15] However, its resistant cell wall makes phycocyanin extraction difficult,[11] so it requires a pre-treatment step (freeze and thawing, ultrasound, lysozyme disintegration, etc.) along with the extraction process.[9] These methods are usually combined with each other or with solvents to improve the extraction efficiency of phycocyanin.[11,16] Nevertheless, these methods present some disadvantages concerning environmental sustainability due to the use of organic solvents and the reusability of solvents. To overcome these drawbacks, different non-conventional methods of extraction (supercritical conditions, pulsed electric fields, microwave, etc.) have been reported to enable high added-value bioproducts, such as β-carotene, astaxanthin from microalgae and products for food and medical industry from other biomass.[17–24]

In this scenario, ionic liquids (IL) appear as a promising alternative for phycobiliprotein extraction due to their versatile properties as green solvents, such as low volatility and non-inflammability.[9,25–27] On the other hand, ILs are expensive and present low biodegradability, but they can be reused.[9] The structure of ILs (consisting of protic and anionic parts), which allows their properties to be adjusted, along with the possibility of performing the extraction under mild conditions, also makes these solvents a more attractive option than other non-conventional methods.[5] In this sense, ILs have shown the potential to be applied in many extraction processes involving biomolecules, such as lipids and phycobiliproteins from microalgae, showing promising results.[9,12,25,28–33] Accordingly, these solvents were chosen for use in phycobiliprotein extraction from *A. platensis*.

This work aims to optimize phycobiliprotein extraction conditions from *A. platensis* using 1-ethyl-3-methylimidazolium ethyl sulfate [Emim] [$EtSO_4$] combined with sonication. This IL was chosen based on previous work in which different IL families (pyridinium, phosphonium, and imidazolium) were tested to check their ability to disrupt the microalga cell wall. [Emim] [$EtSO_4$] was the most suitable solvent for this purpose, which agrees with the findings of Orr *et al.*, 2016.[31] This IL presents several advantages, such as its reasonable cost and good electrochemical properties.[34] For this purpose, a 2^4 full factorial design was used, examining the following main variables to maximize phycocyanin extraction: IL/water and biomass/solvent mass ratios, sonication power, and time. The recovery of IL was also studied under the optimal extraction conditions. This issue is crucial in processes involving IL as it is necessary to reduce economic and environmental costs related to raw IL. In this work, a dialysis-based method was used to separate the IL from the extracted phycobiliproteins, which allows the reduction of IL losses and impurities present in IL recovered by other methods.

Materials and methods

Microalgae characterization

Arthrospira platensis was provided as dried powder by AlgaEnergy SA (Madrid, Spain). Table 1 shows its biochemical composition and elemental analysis (Combustion CHNS/O Analyzer, Model Flash 2000, Thermo Fisher Scientific, Waltham, MA, USA). Lipids were extracted and quantified according to an adapted version of Bligh and Dyer's method, using a mixture of chloroform : methanol (2:1 v/v).[35,36] Total carbohydrates were measured with the phenol-sulfuric acid method, using D-glucose as standard and UV detection at 485 nm wavelength.[37] Soluble carbohydrates were determined using the 3,5-dinitrosalicylic acid (DSN) method.[38,39] Proteins

Table 1. Composition of *Arthrospira platensis* (dry weight basis).

Biochemical composition (wt%)		Elemental composition (wt%)	
Total proteins	65.4 ± 1.8	C	47.2 ± 0.2
Phycocyanin	8.84 ± 0.06	H	6.59 ± 0.07
Allophycocyanin	3.25 ± 0.08	N	11.0 ± 0.1
Phycoerythrin	1.69 ± 0.03	S	0.33 ± 0.03
Lipids	11.2 ± 0.8	O	22.1 ± 0.4
Total carbohydrates	19.4 ± 1.3		
Soluble	1.34 ± 0.01		
Ash	5.40 ± 0.15		

| *Biofuels, Bioprod. Bioref.* **15**:1638–1649 (2021); DOI: 10.1002/bbb.2275

were measured by the Lowry method.[40] Ash was determined by calcining the biomass in a CWF 1300 furnace (Carbolite, Hope, UK) using a temperature ramp of 50 °C min^{-1} from room temperature to 750 °C, maintaining the final temperature for 5 h. Then, the ash was weighed and quantified.

Phycobiliprotein and carbohydrate extraction

Ultrasound-assisted extraction was carried out mixing the microalga (0.1, 0.3 or 0.6 g) with 10 mL of [Emim][$EtSO_4$] IL (Sigma-Aldrich, St Louis, MO, USA) and water at different concentrations (0, 15, or 30 wt%). The mixture was stirred for 30 s in a Vortex mixer and then sonicated for different periods (10, 20, or 30 min) at room temperature by using Elmasonic P ultrasound equipment (Elma Schmidbauer GmbH, Singen, Germany) providing a maximum power of 820 W (100% amplitude) at a constant frequency of 37 kHz. Different amplitudes were studied in the experimental design (40%, 60%, or 80%). The mixture was then centrifuged at 10 000 rpm for 10 min in an Eppendorf centrifuge 5910 (Hamburg, Germany) to separate the supernatant (rich in phycobiliproteins and carbohydrates) from the waste biomass.

The extracted amount of each phycobiliprotein was determined by suspending 0.1 g of dry *A. platensis* in phosphate buffer (pH = 7; 0.1 mol L^{-1}) and stirred for 30 s in a Vortex mixer. Then, the suspension was frozen with liquid nitrogen and thawed at 4 °C under stirring. This procedure was repeated five times to ensure that all phycobiliproteins were extracted.

The extracted amounts of phycocyanin (PC), allophycocyanin (APC), and phycoerythrin (PE) (E_{PC}, E_{APC} and E_{PE}) were measured by spectrophotometric absorption (UV–visible–near infrared (NIR) Varian Cary 500, Palo Alto, CA, USA) using adapted equations from previous studies:[4]

$$E_{PC}\left(\frac{mg\ PC}{g\ biomass}\right)=\left(\frac{OD_{615}-0.474\cdot OD_{652}}{5.34}\right)\cdot\frac{V_{sample}(ml)}{m_{Biomass}(g)} \quad (1)$$

$$E_{APC}\left(\frac{mg\ APC}{g\ biomass}\right)=\left(\frac{OD_{652}-0.208\cdot OD_{615}}{5.09}\right)\cdot\frac{V_{sample}(ml)}{m_{Biomass}(g)} \quad (2)$$

$$E_{PE}\left(\frac{mg\ PE}{g\ biomass}\right)=\left(\frac{OD_{562}-2.41\cdot CP-0.849\cdot AP}{9.62}\right)\cdot\frac{V_{sample}(ml)}{m_{Biomass}(g)} \quad (3)$$

The purity of each phycobiliprotein (P_{PC}, P_{APC} and P_{PE}) was calculated as a fraction of the total protein content by using the following equations:[42]

$$P_{PC}=\frac{OD_{615}}{OD_{280}} \quad (4)$$

$$P_{APC}=\frac{OD_{652}}{OD_{280}} \quad (5)$$

$$P_{PE}=\frac{OD_{562}}{OD_{280}} \quad (6)$$

where OD_{615}, OD_{652}, OD_{562}, and OD_{280} are the optical density values (absorbance) at 615, 652, 562 and 280 nm, respectively.

Ionic liquid recovery

Ionic liquid was recovered using a dialysis-based procedure. For this purpose, a membrane with a molecular weight cut-off of 14 kDa (Sigma-Aldrich) was used. Firstly, the membrane was soaked at 40 °C in a distilled water bath for 1 h, keeping a continuous, gentle stirring. The membrane was then submerged into a stirred bath containing an ethanol/water mixture (50% v/v) at 40 °C. Finally, the membrane was immersed overnight in deionized water under continuous stirring to remove ethanol.

The IL recovery process consisted of three dialysis cycles of 4 h according to the following sequence. First, the crude extract, containing IL, water, and phycobiliproteins, was loaded inside the membrane with deionized water (volume ratio crude extract: water 1:4) for 4 h with continuous stirring, allowing the IL to permeate through the membrane. Then, the loaded membrane was placed in fresh deionized water, repeating the operation three times. The water phases containing IL were collected, and water was partially removed. The remaining water was measured by Karl Fischer titration. Finally, the recovered IL was analyzed by attenuated total reflection–Fourier transform infrared

Table 2. Correspondence between coded and actual values of the experimental factors selected for the factorial design.

Coded value	B/S (wt%)	IL/W (wt%)	P (%)	T (min)
−1	10	0	40	10
0	35	15	60	20
1	60	30	80	30

| *Biofuels, Bioprod. Bioref.* **15**:1638–1649 (2021); DOI: 10.1002/bbb.2275

spectroscopy (ATR-FTIR) in an ATI Mattson Infinity Series FTIR spectrometer (Mattson Instruments. Madison, WI, USA) to check its purity.

Statistical analysis

A factorial experimental design was used to study the effect of different variables and their interaction in the extraction of bioactive compounds using [Emim][$EtSO_4$] IL. The experimental design applied to this study was a full 2^4 factorial design. The response surface methodology was applied to optimize the extraction process.

The studied factors were biomass/solvent mass ratio (B/S), ionic liquid/water mass ratio (IL/W), power of the ultrasonic source (P), and ultrasonication time (t). Factors and levels were selected according to previous studies reported elsewhere.[9,11] The chosen levels are summarized in Table 2.

The selected responses for the study were: the extracted amount of phycocyanin (E_{PC}), allophycocyanin (E_{APC}), and phycoerythrin (E_{PE}), as well as their respective purities (P_{PC}, P_{APC} and P_{PE}), along with the extracted amount of carbohydrate (E_{CH}). The factorial design was completed with four central points to evaluate curvature and experimental error. Additional experiments (star points) were included to produce, finally, a face-centered central composite design, resulting in 28 experiments, as shown in Table 3. Experiments were run at random to minimize errors due to possible systematic trends in the variables. The significance level for the difference between the means was 95% (P-value <0.05).

Table 3. Experimental design matrix and results.

Run	B/S (wt%)	IL/W (wt%)	P (%)	t (min)	E_{PC} (mg g^{-1})	E_{APC} (mg g^{-1})	E_{PE} (mg g^{-1})	P_{PC}	P_{APC}	P_{PE}	E_{CH} (mg g^{-1})
1	−1	−1	−1	−1	18.4	5.6	1.6	0.2	0.1	0.1	8.8
2	1	−1	−1	−1	19.9	7.5	1.3	0.3	0.2	0.2	13.5
3	−1	1	−1	−1	47.6	8.9	4.0	0.4	0.2	0.2	12.3
4	1	1	−1	−1	46.3	12.6	3.7	0.5	0.2	0.3	17.5
5	−1	−1	1	−1	20.0	4.9	1.0	0.2	0.1	0.1	11.5
6	1	−1	1	−1	22.6	7.1	1.3	0.3	0.2	0.2	16.2
7	−1	1	1	−1	47.6	9.0	4.0	0.4	0.2	0.2	24.0
8	1	1	1	−1	49.1	13.3	3.8	0.5	0.2	0.3	16.5
9	−1	−1	−1	1	24.1	11.9	4.2	0.3	0.2	0.2	15.1
10	1	−1	−1	1	23.3	7.2	1.3	0.3	0.1	0.2	20.4
11	−1	1	−1	1	61.6	10.5	4.9	0.3	0.1	0.2	16.5
12	1	1	−1	1	64.8	19.2	6.1	0.6	0.2	0.3	21.1
13	−1	−1	1	1	28.0	14.1	2.7	0.3	0.1	0.1	14.9
14	1	−1	1	1	22.8	6.9	1.2	0.3	0.1	0.2	19.5
15	−1	1	1	1	67.7	15.3	5.7	0.4	0.2	0.2	14.1
16	1	1	1	1	58.6	20.5	6.0	0.5	0.3	0.3	20.7
17	−1	0	0	0	69.9	19.6	5.5	0.5	0.2	0.3	18.0
18	1	0	0	0	66.2	20.9	4.7	0.6	0.2	0.3	21.4
19	0	−1	0	0	24.1	9.0	1.8	0.3	0.1	0.1	14.8
20	0	1	0	0	67.6	18.5	5.3	0.5	0.2	0.3	15.2
21	0	0	−1	0	71.0	22.9	5.6	0.5	0.2	0.3	25.2
22	0	0	1	0	70.1	21.4	5.3	0.5	0.2	0.3	26.9
23	0	0	0	−1	55.7	16.0	4.3	0.5	0.2	0.2	15.2
24	0	0	0	1	71.7	24.4	6.1	0.5	0.2	0.3	28.6
25	0	0	0	0	68.6	20.9	5.3	0.6	0.2	0.3	16.7
26	0	0	0	0	69.7	22.0	5.4	0.5	0.2	0.3	18.3
27	0	0	0	0	69.1	21.1	5.3	0.6	0.2	0.3	16.3
28	0	0	0	0	67.2	20.9	5.3	0.6	0.2	0.3	16.9

Results and discussion

Optimization of phycobiliprotein extraction conditions

Experimental design

The main objective of the experimental design was to study the recovery of phycobiliproteins from *A. platensis* by maximizing the extracted phycocyanin amount. The standard experimental matrix for the factorial design is shown in Table 3. Columns 2 to 5 represent the 0 and ±1 encoded factor levels in a dimensionless scale, whereas columns 6 to 12 represent the results of all the measured responses.

These experimental values were analyzed statistically, and the main effects and interaction effects of the variables were calculated. The ANOVA results for the extraction model of phycobiliproteins and carbohydrates are summarized in Tables S1–S7 (supplementary material). According to the statistical analysis within the experimental range evaluated, the IL/W ratio, IL/W^2 and ultrasonication time are the most significant factor regarding the amount of extracted phycobiliproteins,

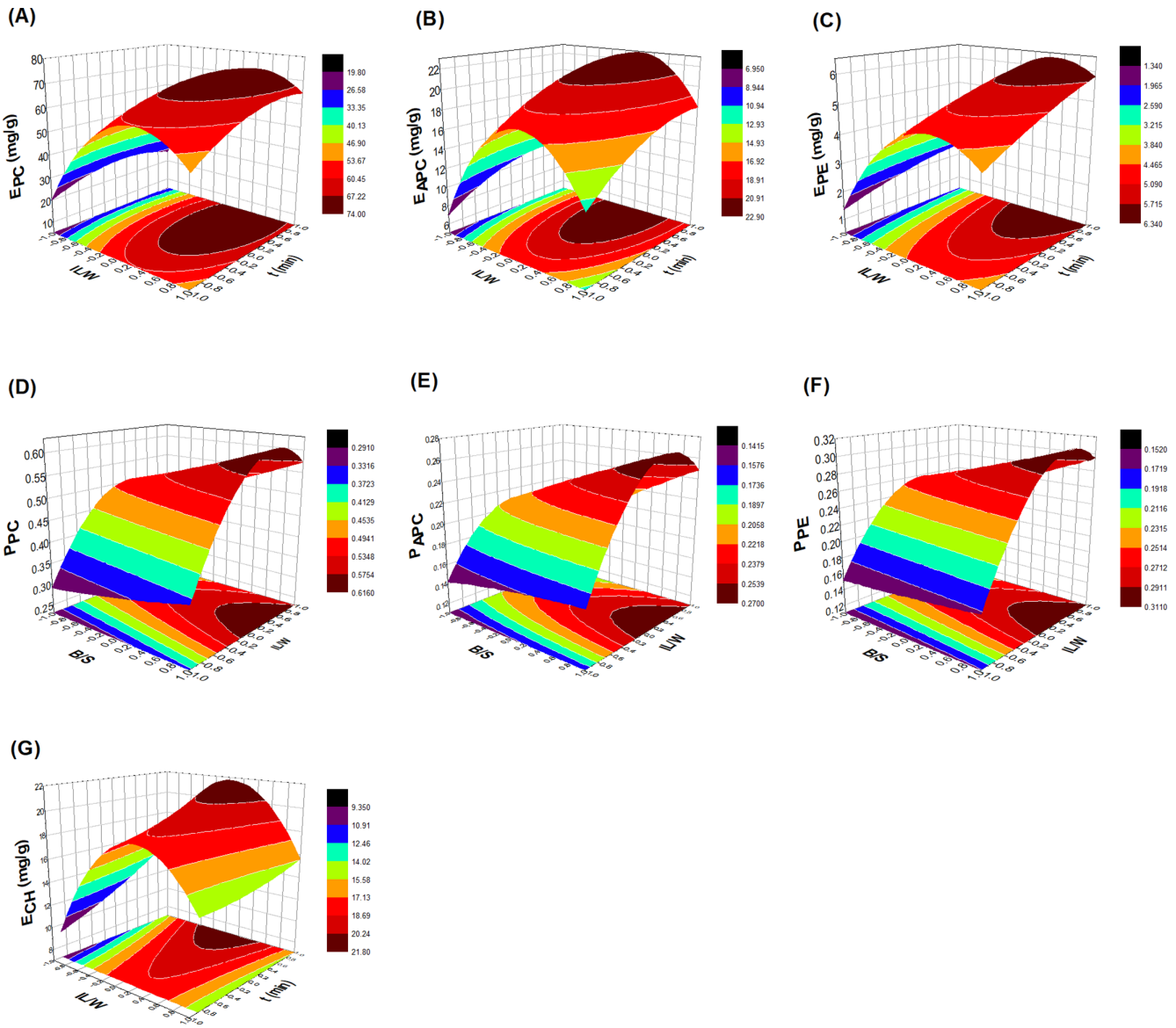

Figure 1. Response surface plot of the extracted amount of phycocyanin (E_{PC}) (A); allophycocyanin (E_{APC}) (B), and phycoerythrin (E_{PE}) (C); purity of phycocyanin (P_{PC}) (D); allophycocyanin (P_{APC}) (E) and phycoerythrin (P_{PE}) (F), and extracted amount of carbohydrates (E_{CH}) (G).

the interaction IL/W – t and the quadratic factor t^2 being only significant for the extracted amount of PC (*P*-value = 0.0021). On the other hand, the interaction between B/S and IL/W ratios is significant for the extracted amount of APC and PE (*P*-value = 0.0025 and 0.028, respectively). Concerning phycobiliprotein purity, the B/S, IL/W, and IL/W^2 factors were the only significant main effects. Regarding the extracted amount of carbohydrate, ultrasonication time is the main significant factor along with the quadratic effect of IL/W ratio.

The experimental results were fitted to a non-linear multiple regression analysis, assuming a second-order polynomial model obtaining the mathematical models. All the factors are expressed in coded units and the equations are valid within the range [−1, +1]. Equations 7–13 show these models including only the significant factors (Eqns 7–13).

$$E_{PC} = 69.220 + 17.089 \cdot IL/W + 5.312 \cdot t - 23.792 \cdot IL/W^2 + 2.802 \cdot IL/W \cdot t - 5.882 \cdot t^2 \left(R^2 = 0.9901\right). \quad (7)$$

$$E_{APC} = 21.546 + 2.979 \cdot IL/W + 2.485 \cdot t + 1.858 \cdot B/S \cdot IL/W - 8.014 \cdot IL/W^2 \left(R^2 = 0.9506\right). \quad (8)$$

$$E_{PE} = 5.339 + 1.503 \cdot IL/W + 0.735 \cdot t + 0.332 \cdot B/S \cdot IL/W - 1.802 \cdot IL/W^2 \left(R^2 = 0.9555\right). \quad (9)$$

$$P_{PC} = 0.539 + 0.051 \cdot B/S + 0.091 \cdot IL/W - 0.126 \cdot IL/W^2 \left(R^2 = 0.9516\right). \quad (10)$$

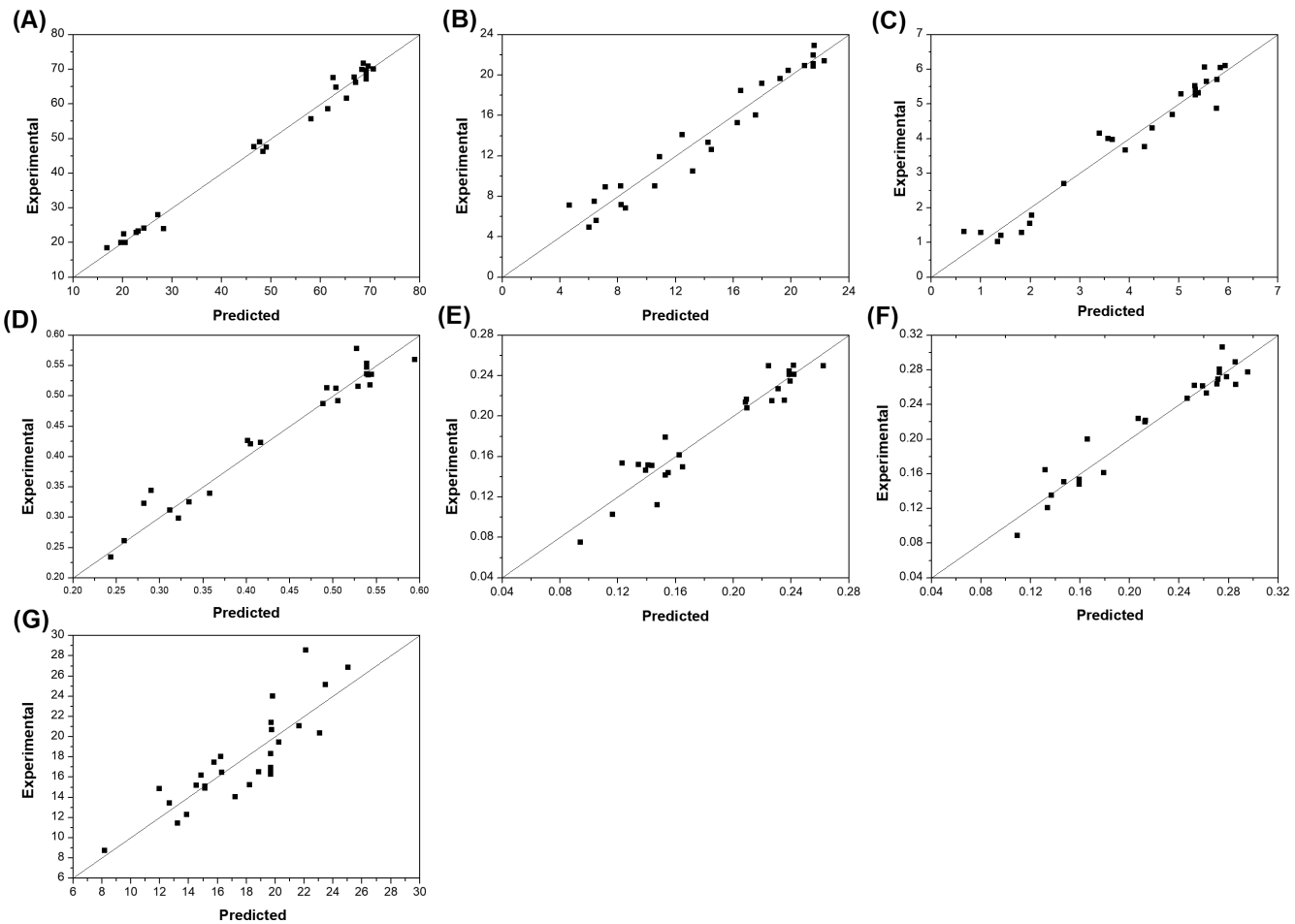

Figure 2. Experimental versus predicted values of the extracted amount of phycocyanin (E_{PC}) (A); allophycocyanin (E_{APC}) (B) and phycoerythrin (E_{PE}) (C); purity of phycocyanin (P_{PC}) (D); allophycocyanin (P_{APC}) (E) and phycoerythrin (P_{PE}) (F), and extracted amount of carbohydrates (E_{CH}) (G).

| *Biofuels, Bioprod. Bioref.* **15**:1638–1649 (2021); DOI: 10.1002/bbb.2275

$$P_{APC} = 0.239 + 0.027 \cdot B/S + 0.027 \cdot IL/W + 0.016 \cdot B/S \cdot IL/W - 0.057 \cdot IL/W^2 \left(R^2 = 0.9167\right). \quad (11)$$

$$P_{PE} = 0.2724 + 0.0215 \cdot B/S + 0.050 \cdot IL/W - 0.063 \cdot IL/W^2 \left(R^2 = 0.9238\right). \quad (12)$$

$$E_{CH} = 19.686 + 1.954 \cdot t - 6.428 \cdot IL/W^2 \left(R^2 = 0.7261\right). \quad (13)$$

In all cases, except for the extracted amount of carbohydrates, the R^2 values associated with the models fitting were satisfactory ($R^2 > 0.9$). Regarding the amount of extracted carbohydrates, the lower R^2 value (0.73) can be probably due to experimental measurement uncertainties associated with the very low content of soluble carbohydrates (1.34 wt%) in the microalgae biomass (Table 1).

Figure 1 shows the response surfaces for the predicted values of the selected variables (Fig. 1(A)–(C): phycobiliprotein extracted amount; Fig. 1(D)–(F): phycobiliprotein purity and Fig. 1(G): carbohydrates extracted amount) as a function of two of the factors with the other two in their central value (coded as 0). As observed, there is a maximum in all the phycobiliprotein extracted amount (Fig. 1(A)–(C)) centered around intermediate values of IL/W. By analyzing these responses with the time, it can be inferred that all of them increase with the time though it tends to saturation. By analyzing phycobiliprotein purity (Fig. 1(D)–(F)), it can be observed how an increase in the IL/W factor increases the purity values significantly. With regard to carbohydrate amount, the IL/W factor has a great effect at low values, but it is detrimental at high values. The time only shows an influence at lower IL/W values.

Figure 2 depicts the comparison between experimental versus predicted values for all the responses: phycobiliproteins extracted amount (Fig. 2(A)–(C)); phycobiliproteins purity (Fig. 2(D)–(F)) and carbohydrates extracted amount (Fig. 2(G)). For all the responses, values calculated with the

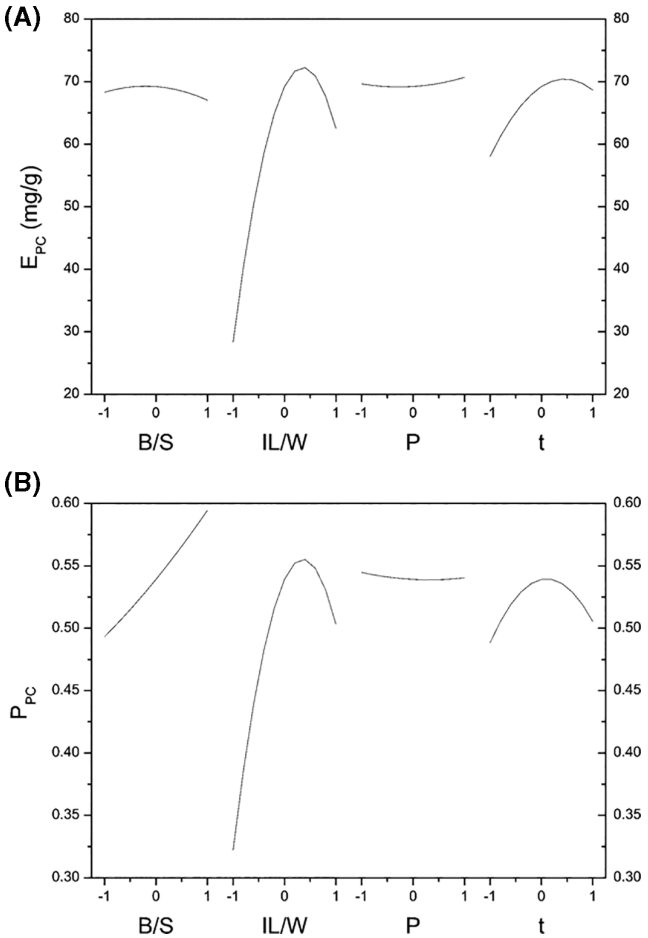

Figure 3. Plot of main effects of the extracted amount (E_{PC} (A) and purity (P_{PC}) (B) of phycocyanin.

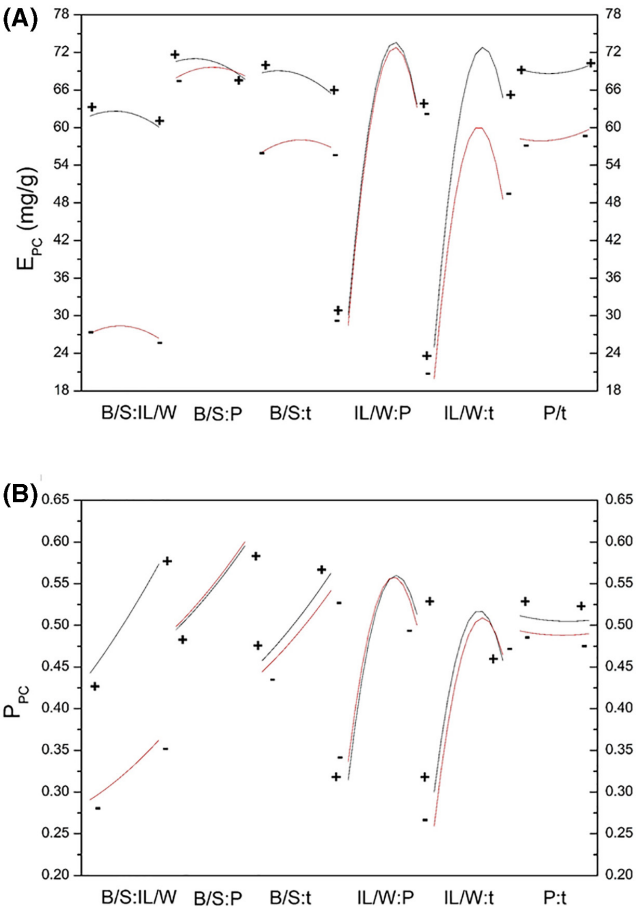

Figure 4. Interaction plots the extracted amount (E_{PC}) (A) and purity (P_{PC}) (B) of phycocyanin.

Table 4. Optimum coded values of the factors maximizing each response.

Factor	Responses						
	E_{PC}	E_{APC}	E_{PE}	P_{PC}	P_{APC}	P_{PE}	E_{CH}
B/D	−0.68	0.12	−0.27	1.00	1.00	1.00	0.33
IL/W	0.39	0.25	0.46	0.45	0.44	0.53	−0.04
Power	1.00	1.00	−0.88	1.00	1.00	1.00	−1.00
Time	0.58	0.99	1.00	0.05	0.22	−0.13	1.00
Maximum	75.9 mg g^{-1}	24.2 mg g^{-1}	6.5 mg g^{-1}	0.62	0.28	0.32	28.1

predictive non-linear models are very close to those obtained experimentally, indicating the accuracy of the models, except for the carbohydrate extracted amount as explained above.

Influence of operating conditions on the phycocyanin extraction

Both the PC amount and purity (E_{PC} and P_{PC}) were analyzed. Figure 3 shows the main effects on the amount of phycocyanin extracted (Fig. 3(A)) and purity (Fig. 3(B)). As shown in Fig. 3(A), the most significant factor for the amount of phycocyanin extracted is the IL/W ratio which is in agreement with the analysis of variance. The effect of IL/W on the amount of PC is positive. The sharp increase observed with the amount of IL is noticeable, achieving a maximum (E_{PC} ~ 72 mg g^{-1}) at the center value of this factor, which corresponds to an IL/W of 15%. However, the quadratic effect of the IL/W ratio has a significant negative influence on the extracted amount of PC (Table S1, Eq. 7). The increase in this operating variable therefore does not produce a constant rise in the extracted amount of PC as shown in Fig. 3. The decrease observed at higher values of IL/W ratio is related to the increase of pH (>8) measured in the experiment (pH = 7.2 at the center point). This pH leads to protein denaturation as we found in the laboratory, and it is reported in the literature.[9]

It was also observed that long ultrasound time values increase the extraction of PC, the quadratic effect of sonication time being significantly negative (Table S1, Eqn 7). Thus, there is a maximum value for this response (E_{PC} ~ 70 mg/g) close to the center value of the sonication time (20 min). The influence of this variable is no longer significant at higher ultrasound times.

The main effects of PC purity are plotted in Fig. 3(B). The purity of PC is enhanced by using IL, increasing from 0.33 without IL to 0.55 at the center point of this operating condition (IL/W = 15 wt%). At a higher IL/W ratio, the PC purity decreases as a result of the negative influence of the quadratic effect of IL/W. The PC purity is enhanced from 0.493 to 0.594 with the increase of B/S ratios from 10 to 60 wt%. Lower B/S makes the solvent less selective towards PC yielding lower purity values.

Table 5. Composition of the extraction fractions (dry weight basis).

Component (wt%)		Extract phase	Waste biomass
Total proteins		52.2 ± 2	13.2 ± 1
Phycobiliproteins	Phycocyanin	7.2 ± 0.2	1.6 ± 0.2
	Alophycocyanin	1.63 ± 0.09	1.6 ± 0,3
	Phycoerythrin	1.00 ± 0.05	0.7 ± 0.1
Lipids		2.4*	8.8 ± 1
Total carbohydrates		3.7*	15.7 ± 4
Ash		2.6*	2.8 ± 0.2

*Calculated by mass balance.

Figure 4 depicts the binary interactions between the extracted amount of PC and purity (E_{PC} and P_{PC}). Each pair of interaction ± curves is obtained *using the coded model where the first factor acts as a variable and the second factor is fixed at +1 and −1, respectively*. Parallel curves indicate that binary interactions between these two factors are not significant. In this case, therefore, the only significant interaction effect can be observed between IL/W and time for E_{PC} (Fig. 4(A)), whereas there are no significant interactions for P_{PC} (Fig. 4(B)). This is corroborated by the ANOVA table.

Optimal conditions

The optimization of the process conditions was focused to maximize E_{PC} maintaining reasonable purity values, obtaining the optimal values shown in Table 4. Using the mathematical models (equations 7–13 above shown), the theoretical values for all responses were calculated for these optimum values (Table 4). Under these operating conditions, the predicted values for the rest of the responses were high enough to justify this choice: E_{APC} and E_{PE} are expected to reach 93% and 94% of their maximum values, respectively, whereas the PC purity is 0.62.

The extraction procedure was carried out in triplicate with the above optimal conditions, yielding an experimental value for E_{PC} of 76.6 ± 0.4 mg g^{-1}, which was in good agreement

| *Biofuels, Bioprod. Bioref.* **15**:1638–1649 (2021); DOI: 10.1002/bbb.2275

with the predicted value of the E_{PC} (75.9 mg g^{-1}, see Table 4). Furthermore, the extracted phase and the waste biomass fraction obtained under these conditions were characterized. Table 5 summarizes the results expressed on a dry weight basis. As expected, phycobiliproteins were mostly in the extract (72%), indicating the suitability of the proposed method for extracting this kind of proteins, specially in the case of phycocyanin for which a extraction yield value of 82% was achieved. This parameter is rarely studied in the literature, as most authors usually report phycocyanin concentration.[9,11,25] Finally, as expected, lipids and carbohydrates remained mainly in the residual biomass (79% and 81%, respectively). This residual biomass could be further used for producing bioenergy (biogas, bioethanol, etc.) and/or bioproducts (pigments, valuable lipids, nanocellulose, etc.) in a biorefinery scheme within a circular economy framework.

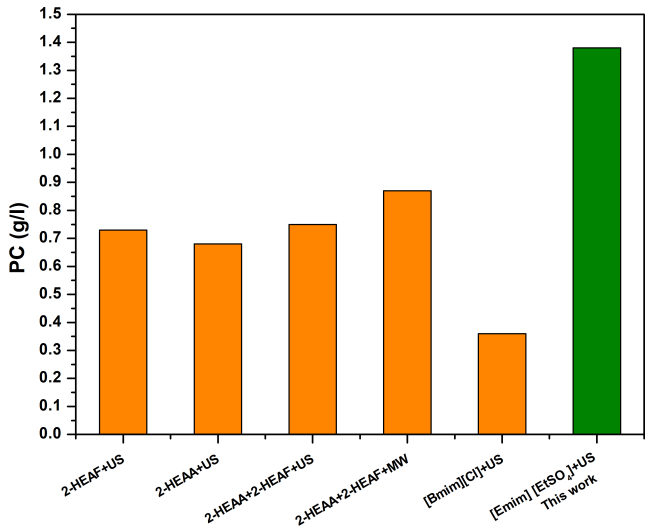

Figure 5. Comparison between PC values obtained by Pinto *et al*.[9,25] and the present work (US, ultrasound; MW, microwave).

Figure 5 compares the PC value achieved in this work and those reported in the literature, which were obtained with other ILs (2-hydroxyethylammonium acetate(2-HEAA), 2-hydroxyethylammonium formate (2-HEAF), its mixture (2-HEAA+2-HEAF), and 1-butyl-3-methyl imidazolium chloride ([Bmim][Cl])).[9,25] It can be seen that the use of [Emim][$EtSO_4$] + ultrasound enhances PC extraction remarkably.

Ionic liquid reuse

The IL used in the extraction process was recovered using a dialysis-based procedure, and reused in subsequent extractions. Figure 6 shows the cycles of reuse of [Emim][$EtSO_4$] in the extraction process. To study the integrity of IL, it was analyzed by ATR-FTIR obtaining the results depicted in Fig. 7. As can be seen, the IL can be reused successfully in seven different extraction cycles, showing the same characteristic IR spectrum of the fresh IL (except the peak centered around 3500 cm^{-1} corresponding to water remaining in the recover IL). After the last cycle, the IL did not show

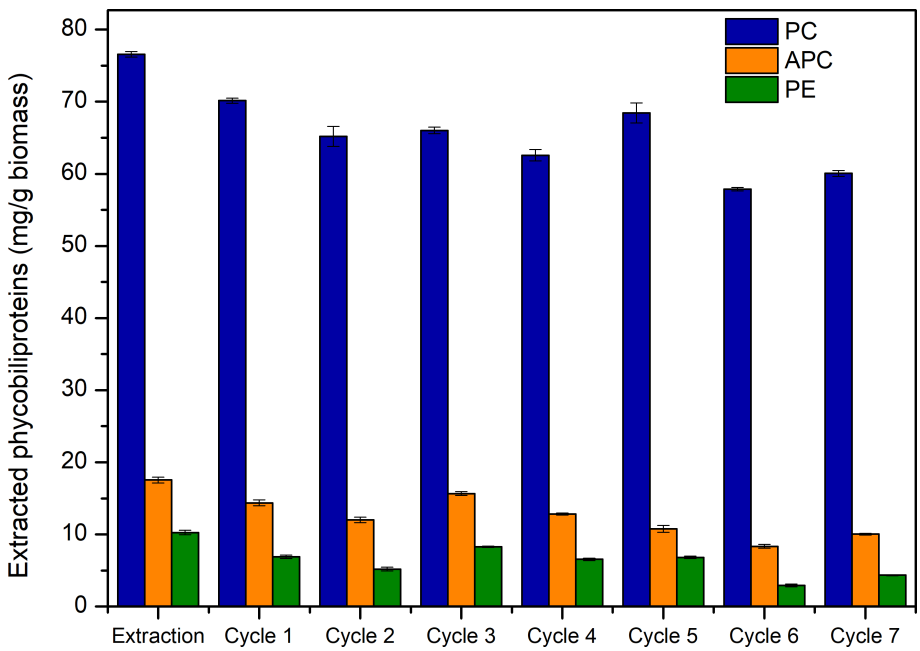

Figure 6. Cycles of reuse of ionic liquid during the extraction of phycobiliproteins.

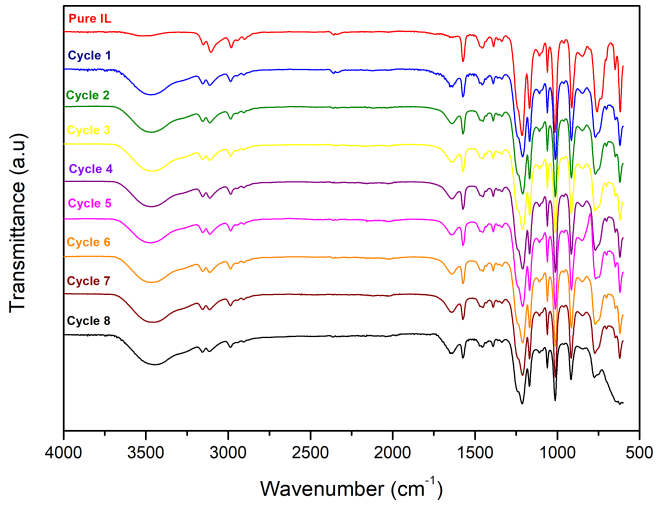

Figure 7. ATR-FTIR analyses of reused IL.

the peak centered at 680 cm^{-1}. The amount of phycocyanin extracted in the seventh cycle was 22% lower than after the first use so the problem of the high price of the IL can be attenuated because of the repeated use of the IL.

Using other ILs, Pinto *et al.*[9] reported a value below 0.8 $g\,L^{-1}$ (fresh 2-HEAA +2-HEAF + ultrasound) falling to 0.05 $g\,L^{-1}$ (after 3 cycles). Furthermore, Pinto *et al.*[25] reported values from 0.87 $g\,L^{-1}$ (fresh 2-HEAA +2-HEAF+microwave) to 0.18 $g\,L^{-1}$ (after three cycles). Our results are higher, with values above 1 $g\,L^{-1}$ of phycocyanin after seven cycles of IL reuse. By comparing the performance of [Emim] [$EtSO_4$] with other solvents, it shows comparable values of extracted phycocyanin (~80 $mg\,g^{-1}$)[11] but the IL-based method presents the possibility of reusing the solvent for subsequent extraction cycles. These results are of relevance in this field because the use of [Emim] [$EtSO_4$] allows the reduction of solvent consumption, enhancing the sustainability of the process. Accordingly, this process could be an interesting option for integration into a biorefinery to obtain phycocyanin from *Arthrospira platensis*.

Conclusions

A low biomass/solvent ratio with a high IL/water ratio, high power amplitude of the ultrasound equipment, and a long period of time are required to maximize the amount and purity of the extracted phycobiliproteins in a combined imidazolium-based ionic liquid ([Emim] [$EtSO_4$]) and sonication procedure for phycobiliprotein extraction. These conditions lead to an extract from *A. platensis* that is rich in phycocyanin (higher than 80%). A dialysis-based process was used to separate the IL from the extracted phycobiliproteins and reuse it in consecutive cycles. The results reveal that it is possible to use the IL for seven cycles with a loss of the extracted phycocyanin of 22 wt%. The results are similar or even better than other extraction methods reported with other ILs in terms of extracted phycocyanin and the number of IL reuses.

Acknowledgements

The authors acknowledge the support from ALGATEC-CM (S2018/BAA-4532) project, financed by Comunidad de Madrid and co-financed by the European Social Fund and the European Regional Development Fund and RENUWAL network (320RT0005) financed by the CYTED Program.

References

1. da Silva B, Botelho J, Greque M and Vieira JA, Microalgae as a new source of bioactive compounds in food supplements. *Curr Opin Food Sci* **7**:73–77 (2016).
2. Sathasivam R, Radhakrishnan R, Hashem A and Abd_Allah EF, Microalgae metabolites: a rich source for food and medicine. *Saudi J Biol Sci* **26**:709–722 (2017). https://doi.org/10.1016/j.sjbs.2017.11.003.
3. Han J, Thomsen L, Pan K and Thomsen C, Two-step process: enhanced strategy for wastewater treatment using microalgae. *Bioresour Technol* **268**:608–615 (2018).
4. Chu W-L, Biotechnological applications of microalgae. *IeJSME* **6**:24–37 (2012).
5. Markou G and Nerantzis E, Microalgae for high-value compounds and biofuels production: a review with focus on cultivation under stress conditions. *Biotechnol Adv* **31**:1532–1542 (2013).
6. Tang DYY, Khoo KS, Chew KW, Tao Y, Ho SH and Show PL, Potential utilization of bioproducts from microalgae for the quality enhancement of natural products. *Bioresour Technol* **304**:122997 (2020).
7. Rodrigues DB, Menezes CR, Mercadante AZ, Jacob-Lopes E and Zepka LQ, Bioactive pigments from microalgae Phormidium autumnale. *Food Res Int* **77**:273–279 (2015).
8. de Jesus MF, Santos Costa RM and Bernardo AMM, Health applications of bioactive compounds from marine microalgae. *Life Sci* **93**:479–486 (2013).
9. Pinto RD, Carolino F, Saraiva R and Ponte MV, Ultrasound-assisted extraction of phycobiliproteins from *Spirulina (Arthrospira) platensis* using protic ionic liquids as solvent. *Algal Res* **31**:454–462 (2018).
10. Manirafasha E, Ndikubwimana T, Zeng X, Lu Y and Jing K, Phycobiliprotein: potential microalgae derived pharmaceutical and biological reagent. *Biochem Eng J* **109**:282–296 (2016).
11. İlter I, Akyıl S, Demirel Z, Koç M, Conk-Dalay M and Kaymak-Ertekin F, Optimization of phycocyanin extraction from *Spirulina platensis* using different techniques. *J Food Compos Anal* **70**:78–88 (2018).
12. Martins M, Vieira FA, Correia I, Ferreira RAS, Abreu H, Coutinho JAP *et al.*, Recovery of phycobiliproteins from the red macroalga: Gracilaria sp. using ionic liquid aqueous solutions. *Green Chem* **18**:4287–4296 (2016).
13. Soheili M and Khosravi-Darani K, The potential health benefits of algae and micro algae in medicine: a review on *Spirulina platensis*. *Curr Nutr Food Sci* **7**:279–285 (2011).
14. Prabakaran G, Sampathkumar P, Kavisri M and Moovendhan M, Extraction and characterization of phycocyanin from *Spirulina platensis* and evaluation of its anticancer, antidiabetic and antiinflammatory effect. *Int J Biol Macromol* **153**:256–263 (2020).

15. Rizwan M, Mujtaba G, Memon SA, Lee K and Rashid N, Exploring the potential of microalgae for new biotechnology applications and beyond: a review. *Renew Sustain Energy Rev* **92**:394–404 (2018).
16. Tavanandi HA, Mittal R, Chandrasekhar J and Raghavarao KSMS, Simple and efficient method for extraction of C-Phycocyanin from dry biomass of *Arthospira platensis*. *Algal Res* **31**:239–251 (2018).
17. Carullo D, Abera BD, Casazza AA, Donsì F, Perego P, Ferrari G *et al*., Effect of pulsed electric fields and high pressure homogenization on the aqueous extraction of intracellular compounds from the microalgae *Chlorella vulgaris*. *Algal Res* **31**:60–69 (2018).
18. Casazza AA, Pettinato M and Perego P, Polyphenols from apple skins: a study on microwave-assisted extraction optimization and exhausted solid characterization. *Sep Purif Technol* **240**:116640 (2020).
19. Lopresto CG, Petrillo F, Casazza AA, Aliakbarian B, Perego P and Calabrò V, A non-conventional method to extract D-limonene from waste lemon peels and comparison with traditional Soxhlet extraction. *Sep Purif Technol* **137**:13–20 (2014).
20. Lopresto CG, Meluso A, di Sanzo G, Chakraborty S and Calabrò V, Process-intensified waste valorization and environmentally friendly d-limonene extraction. *Euro-Mediterranean J Environ Integr* **4**:1–12 (2019).
21. Rammuni MN, Ariyadasa TU, Nimarshana PHV and Attalage RA, Comparative assessment on the extraction of carotenoids from microalgal sources: astaxanthin from H. pluvialis and β-carotene from *D. salina*. *Food Chem* **277**:128–134 (2019).
22. Kapoore RV, Butler TO, Pandhal J and Vaidyanathan S, Microwave-assisted extraction for microalgae: from biofuels to biorefinery. *Biology* **7**:1–25 (2018).
23. da Silva MF, Casazza AA, Ferrari PF, Aliakbarian B, Converti A, Bezerra RP *et al*., Recovery of phenolic compounds of food concern from *Arthrospira platensis* by green extraction techniques. *Algal Res* **25**:391–401 (2017).
24. Sarkar S, Manna MS, Bhowmick TK and Gayen K, Extraction of chlorophylls and carotenoids from dry and wet biomass of isolated chlorella Thermophila: optimization of process parameters and modelling by artificial neural network. *Process Biochem* **96**:58–72 (2020).
25. Pinto RD, Sousa A, Viana TA, Pessoa AK, Saraiva R and Ponte MV, Application of protic ionic liquids in the microwave-assisted extraction of phycobiliproteins from *Arthrospira platensis* with antioxidant activity. *Sep Purif Technol* **252**:117448 (2020).
26. Chang YK, Show PL, Lan JCW, Tsai JC and Huang CR, Isolation of C-phycocyanin from *Spirulina platensis* microalga using ionic liquid based aqueous two-phase system. *Bioresour Technol* **270**:320–327 (2018).
27. Freire MG, Cláudio AFM, Araújo JMM, Coutinho JAP, Marrucho IM, Canongia Lopes JN *et al*., Aqueous biphasic systems: a boost brought about by using ionic liquids. *Chem Soc Rev* **41**:4966–4995 (2012).
28. Egorova KS and Ananikov VP, Fundamental importance of ionic interactions in the liquid phase: a review of recent studies of ionic liquids in biomedical and pharmaceutical applications. *J Mol Liq* **272**:271–300 (2018).
29. He A, Dong B, Feng X and Yao S, Extraction of bioactive ginseng saponins using aqueous two-phase systems of ionic liquids and salts. *Sep Purif Technol* **196**:270–280 (2018).
30. Orr VCA and Rehmann L, Ionic liquids for the fractionation of microalgae biomass. *Curr Opin Green Sustain Chem* **2**:22–27 (2016).
31. Orr VCA, Plechkova NV, Seddon KR and Rehmann L, Disruption and wet extraction of the microalgae *Chlorella vulgaris* using room-temperature ionic liquids. *ACS Sustain Chem Eng* **4**:591–600 (2016).
32. Lee SY, Show PL, Ling TC and Chang JS, Single-step disruption and protein recovery from *Chlorella vulgaris* using ultrasonication and ionic liquid buffer aqueous solutions as extractive solvents. *Biochem Eng J* **124**:26–35 (2017).
33. Pinto RD, Fernandes P, Saraiva R and Valderez M, Evaluation of protic ionic liquids as potential solvents for the heating extraction of phycobiliproteins from *Spirulina (Arthrospira) platensis*. *Algal Res* **38**:101391 (2019).
34. Li F, Liu Y and Lin W, Phase equilibrium and protein partitioning in aqueous two-phase systems containing imidazolium ionic liquids and surfactant at low voltage levels. *J Mol Liq* **256**:372–379 (2018).
35. Rezende R, Mendonça D, Kunigami CN, Gomes DA and Lapa CML, Comparison between several methods of total lipid extraction from *Chlorella vulgaris* biomass. *Ultrason Sonochem* **22**:95–99 (2015).
36. Bligh EG and Dyer WJ, A rapid method of total lipid extraction and purification. *Can J Biochem Physiol* **37**:911–917 (1959).
37. Dubois M, Gilles KA, Hamilton JK, Rebers PA and Smith F, Colorimetric method for determination of sugars and related substances. *Anal Chem* **28**:350–356 (1956).
38. Miller GL, Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal Chem* **31**:426–428 (1959).
39. Miller GL, Slater R, Birzgalis R and Blum R, Application of different colorimetric tests to cellodextrins. *Anal Biochem* **2**:521–528 (1961).
40. Lowry OH, Rosebrough NJ, Lewis Farr A and Randall RJ, Protein measurement with the Folin phenol reagent. *Anal Biochem* **217**:220–230 (1951).
41. Bennett A and Bogobad L, Complementary chromatic adaptation in a filamentous blue-green alga. *J Cell Biol* **58**:419–435 (1973).
42. Abalde J, Betancourt L, Torres E, Cid A and Barwell C, Purification and characterization of phycocyanin from the marine cyanobacterium Synechococcus sp. IO9201. *Plant Sci* **136**:109–120 (1998).

Jennifer Sánchez Laso

Jennifer Sánchez Laso is a PhD student at Rey Juan Carlos University, Spain. Her research is focused on the integral use of microalgae, combining the extraction of phycocyanin using ionic liquids, biocrude production by hydrothermal liquefaction (HTL), and biogas production by anaerobic digestion of the resulting waste biomass.

Alejandro Piera

Alejandro Piera Ruiz is a PhD student at the Rey Juan Carlos University. His research interest lies in the area of biofuels. He is currently involved in the study of microalga cultivation and the extraction of valuable compounds using experimental and simulation approaches.

Gemma Vicente

Dr Gemma Vicente is associate professor of chemical engineering at the Rey Juan Carlos University, Spain. Her main area of research is the integral use of oleaginous microorganisms for the production of biofuels and by-products.

Rosalía Rodríguez

Dr Rosalía Rodríguez is associate professor of chemical engineering at the Rey Juan Carlos University, Spain. Her research is focused on the production of sustainable biofuels and bioactive products from oleaginous microorganisms evaluating the environmental feasibility of these processes.

Luis Fernando Bautista

Dr L. Fernando Bautista is associate professor of chemical engineering at the Rey Juan Carlos University, Spain. His research interest is the development of chemical and biotechnological processes for microalgae biorefineries and for biodegradation of toxic pollutants from the environment.

Juan J. Espada

Dr Juan J. Espada is associate professor of chemical engineering at the Rey Juan Carlos University, Spain. His research areas are related to bio-based products and biofuel production from microalgae, wastewater treatment through bioelectrochemical processes as well as environmental and techno-economic analysis of biorefinery-based processes.

| *Biofuels, Bioprod. Bioref.* **15**:1638–1649 (2021); DOI: 10.1002/bbb.2275