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# **Glycerol-based NaDES as green solvents for ultrasound-assisted extraction of phycocyanin from *Arthrospira platensis*-RSM optimization and ANN modeling**

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## **Abstract:**

Phycocyanin, a natural blue phycobiliprotein extracted from cyanobacteria, recently focused the interest of food industry as promising pigment and antioxidant. Eco-extraction of phycocyanin *via* ultrasound using five glycerol based NaDES was described here. Response surface methodology (RSM) and artificial neural network (ANN) were adapted for the generation of predictive models to simulate and optimize phycocyanin extraction. Input parameters were extraction time (15–60 min), temperature (25–50°C), and water addition (20–60%). Two original glycerol based NaDES, (glycerol: glucose: betaine) (4:1:1) and (glycerol: glucose: proline) (4:1:1), displayed the highest phycocyanin recovery, combined with high free radical scavenging activity. Additionally, a stabilizing effect of NaDES on phycocyanin was highlighted after accelerated ageing. Optimized extraction protocol

developed here allowed then the extraction, pre-formulation and stabilization of phycocyanin in one step, opening the way to a sustainable formulation of this bioactive pigment for food industry.

**Keywords:** Natural Deep Eutectic Solvent, Spirulina, Phycobiliprotein, Response surface methodology, Artificial Neural Network.

## **1. Introduction:**

Due to the exponential increase in global population growth, resources demand such as energy, food and drugs will inadvertently increase. This led the search on underutilized resources such as microalgae, notably *Arthrospira platensis* [1,2]. This water organism is a non-toxic cyanobacterium that have gained a lot of attention for various industries and was commercially grown in order to produce a range of metabolites counting polysaccharides, proteins, lipids, vitamins, pigments, etc [3].

Phycobiliproteins are light harvesting pigments defined as a group of water-soluble proteins containing a number of subunits that enclose a protein backbone and a phycobilin linked *via* a covalent bond that made them highly fluorescent[4]. They are frequently branched into specific categories according to their colour and absorption properties; mainly, phycoerythrins (bright pink colour), allophycocyanins (light blue), and phycocyanins (dark blue) [1,5].

Phycocyanins are the major photosynthetic accessory pigments in the cyanobacterium *Spirulina* [6]. Beside their fluoresce properties, phycocyanins exhibit anti-oxidative, immunity enhancing, anti-viral, anti-tumour, and anti-inflammatory effects [7–13]. Therefore, purified phycocyanin have gained importance over synthesized compound in a wide range of application including; protein markers for electrophoretic techniques, cosmetic application (lipsticks, eyeshadows...), pharmaceutical and nutraceutical, as well as fluorescence probe for

bio-applications [11,14]. They are also used as natural protein dye, due to nutritional and nutraceutical properties that could be exploited for food supplements and healthy foods preparation [15].

Typically, the phycobiliproteins extraction involves cell disruption in order to release the proteins from cell. In fact, phycocyanin extraction from *A. platensis* can be carried out by various conventional techniques like maceration [16]. However, such processes methods do not provide high yields; this is mainly due to this cyanobacteria's resistant cell wall [6,17]. Several studies reported novel methods such as homogenization, sonication, and microwave that efficiently damage the cell wall enhancing extraction process efficacy. Among all, ultrasound assisted extraction (UAE) appears as a promising process according to the resulting yields and the low temperature used, that limit phycocyanin deterioration[14,17,18]. Nowadays, the development of ultrasound assisted extraction is gaining momentum as it shows higher extraction efficiency of bio-compounds. It is a novel clean, and green extraction technology for various molecules including dyes and pigments. It uses acoustic cavitation to produce cavitation bubbles that implodes causing high shear forces, allowing cell wall disruption and deeper solvent penetration into matrices that boost the mass transfer [19,20]. Consequently, UAE process increases the extraction yield, fastened the mass transfer and reduce the plant to solvent ratio [21]. In microalgae biorefinery, UAE is was used to breakdown the cell wall and efficiently extract proteins antioxidants and pigments; notably phycobiliproteins [22].

With the growing concern related to carbon emission, water, and solvent consumption, a high demand for environmental-friendly processes was noticed [23]. Such green technologies seek new substitute to the commonly used organic solvents that are known for their inherent toxicity and high volatility. When it comes to phycobiliprotein, water is the most common solvent used even if phycocyanin stability is limited in water solution. An increase protein

thermal stability was achieved by adding either preservatives, like citric acid, or low sugar concentrations [24]. Recently, protic ionic liquids were screened and demonstrated higher recovery and stabilization of phycocyanin compared to water [3]. Another green alternative that was not fully explored was the use of Natural Deep eutectic solvent (NaDES). NaDES have gained much attention in the scientific community, and the amount of literature has grown exponentially. NaDES were first introduced by Choi et al. [25]. They are defined as organic compounds with a melting point which is considerably lower than that of each component [25,26]. NaDES are obtained by mixing hydrogen bond-donors with a hydrogen-bond acceptor using a suitable molar ratio [26-28], based on natural compounds. They are mostly composed by primary metabolites, such as organic acids, amino acids, and sugars. The NaDES stands out due to the fact that they are solely formed by eco-friendly and often low-cost components; thus, making them attractive for food applications.

Recently, Response surface methodology, has been effectively used in numerous studies for the optimization of different extraction parameters (extraction time, temperature, solid-liquid ratio, solvent composition, particle size...) [29,30]; it is a factorial approach that generate a mathematical model based on the relation between the responses and the variables. However, RSM based models have a linear behaviour, making them useful only for a limited range of input parameters. Whereas, Artificial Neural Network (ANN) is a more fitted tool for non-linear multivariate modelling; it is a powerful optimization approach, used in numerous fields, that employs learning algorithms to define a relation between input and output data using a number of inputs, hidden and an output layer depending on the chosen parameters. Both optimization tools are often used together in synergy [31].

In this study, UAE as eco-friendly process for the extraction of phycocyanin using NaDES has been adopted. The objective of this study was to identify NaDES with the highest phycocyanin extraction yield. An effective respond surface methodology (RSM) and artificial

neural network (ANN) model were developed using the experimental data. Therefore, five NaDES, approved for food applications [32], were investigated and a number of experiments were carried out using Box-Behnken experimental design in order to identify the impact of different extraction parameters (water content, temperature and extraction time) on the phycocyanin yield.

## 2. Material and methods:

### 2.1 Raw material and chemicals

Spirulina (*Arthrospira platensis*) biomass (17.9 % dry matter) was kindly provided by AquaEco culture (Lamballe, France), and stored at -20°C. A voucher specimen was kept in Aqua Eco Culture strain library. Spirulina was cultured in raceway and harvested in August 2020. All extractions were performed between September and December 2020.

Anhydrous betaine (Acros Organics, 98%), proline (Alfa Aesar, 99%) and  $\alpha$ -D-(+)-glucose (Acros Organics, >99%) and Glycerol (Fisher Scientific, >99%) were purchased from Fisher Scientific SAS (Illkirch, France). Ascorbic acid was purchased from COOPER (Melun, France). Water was purified using a Milli-Q system (Millipore Corporation, Bedford, MA, USA). Ethanol 96% analytical grade were purchased from Carlo Erba (Val de Reuil, France). 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH•) was purchased from Sigma Aldrich (St Louis, MO, USA).

2.2. NaDES preparation  
NaDES were prepared by mixing appropriate ratio of glycerol and HBD, to prepare following NaDES according to the following table:

**Tableau 1:** NaDES composition

	HBA	HBD	HBD	Ratio (mol/mol)
GG	Glycerol	Glucose	-	2:1
GB	Glycerol	Betaine	-	2:2
GP	Glycerol	Proline	-	4:1:1
GGB	Glycerol	Glucose	Betaine	4:1:1
GGP	Glycerol	Glucose	Proline	4:1:1

Water was added when appropriate, ranging from 20 to 60% of NaDES (w/w). The mixture was heated at 70°C and stirred until a colourless liquid was obtained.

## 2.2 Extraction process :

UAE was carried out in an ultrasonic bath RK-100H (Bandelin electronic, Berlin, Germany). The extraction was carried according to different conditions set by the design of experiments by mixing 0.1 g of fresh biomass with 1 g of the NaDES (biomass/solvent ratio of 1/10). Resulting extracts were centrifuged during 20 min at 16 200 g (Rotanta 460R, Hettich) and supernatant were recovered.

## 2.3 Phycocyanin titration :

100 mg of extract were diluted by 1 mL of distilled water and shaken. 250 µL of the resulting dilution were then used for phycocyanin titration.

Phycocyanin absorption spectra was measured using UV–vis microplates spectrophotometer reader (multiskan GO, Thermo fisher scientific SAS). Phycocyanin contents were calculated from the absorbances at 620 and 652 using the following equations [33].

$$C_{CP} = (OD_{620} - 0,474OD_{652}) / 5,34 \quad (\text{Equation 1})$$

n which  $C_{CP}$  (mg/ml) is the phycocyanin concentration and  $OD_{620}$  and  $OD_{652}$  indicates the optical density of the sample at 620 and 652, respectively.

The phycocyanin yield, it was calculated as:

$$Yield = C_{CP} \times V_{NaDES} / M_{DM} \quad (\text{Equation 2})$$

Where the  $V_{NaDES}$  is the NaDES volume,  $M_{DM}$  is the dried matter and the yield of the extracted phycocyanin is expressed in mg phycocyanin per g of dried matter.

## 2.4 DPPH radical scavenging assay :

The DPPH radical scavenging activity of sample extracts was measured after the extracts were diluted with ethanol 50%, 12.5 µL of diluted extract and 87.5 of ethanol 50% were

pipetted into a 96-well-plate. 100  $\mu$ L of DPPH diluted in a 50% ethanol was added to each well. and the plate was shaken for 10 second and the absorbance was measured each minute at 517 nm was then measured using a Microplate reader (Multiskan GO, Thermo Scientific).

Radical scavenging activity is determinate as ascorbic acid equivalent per ml of extract.

### 2.5 RSM experimental design for the phycocyanin optimization

Box Behnken Design (BBD) was adopted for parameters optimization of phycocyanin extraction via UAE. The parameters range were fixed based on carried preliminary experiments; the extraction time (15–60 min); water ratio (20% - 60%) and the temperature (25°C – 50°C). A total of 17 experiments were conducted in this study to optimize phycocyanin extraction parameters. The analysis of variance (ANOVA) was conducted for the validation of the theoretical accounts for the optimization process.

The estimation of optimum conditions was through a second order polynomial equation. The generalized form describe relationship between the responses and the parameters as shown below:

$$Y = \beta_0 + \sum_{i=1}^n \beta_i x_i + \sum_{i=1}^n \beta_{ii} x_i^2 + \sum_{i \leq 1 \leq j} \beta_{ij} x_i x_j + \varepsilon \quad (\text{Equation 3})$$

In the aforementioned equation Y is the respond while  $\beta_0$ ,  $\beta_i$ ,  $\beta_{ii}$ ,  $\beta_{ij}$  are the regression coefficients for intercept, linear, interaction, and quadratic, respectively.  $x_i$  and  $x_j$  represent the independent variables and the number of independent parameters ( $n = 3$ )

The analyses were conducted using Design-Expert 13 (Stat-Ease, Minneapolis, Minnesota, USA). The model adequacy was assessed based on the obtained coefficient of multiple determination ( $R^2$ ), coefficient of variance (CV) and  $p$ -values for the model and lack of fit testing.

### 2.6 Structure of artificial neural network

Artificial Neural Network (ANN) is a mathematical function that automatically looks for the best linear or non-linear relation between input and output data. ANN is consisted of three



functional layers specifically the input, the hidden and the output layer. ANN models with one hidden layer were designed for the five NaDES with three inputs (time, water ratio, and temperature) and one output. Separate ANN was developed to investigate each NaDES response.

Data used for ANN were obtained from the experimental study. The topology of the neural network developed was designated as 3-h-1 representing the three input neurons, a single layer and one output neurons (phycocyanin yield).

The training process was a trial and error run search till a minimum mean square error (MSE) was reached for the validation process of each NaDES. The trained network performance was estimated according to its neural network accuracy to predict outputs that are equivalent to target values. MATLAB 7.11.1 (The Mathworks Inc.) was used in this study in order to train the model to analyze and generate neural network modeling.

#### 2.7 ANN and RSM models comparison:

For the evaluation of RSM and ANN fitting goodness and effective prediction error the correlation coefficients ( $R^2$ ) as well as errors analysis such as the root mean square error (RMSE), standard error of prediction (SEP), relative percent deviation (RPD) were calculated.

The used equations were as followed [34]:

$$R^2 = \frac{\sum_{i=1}^{17} (PCC_{i,p} - PCC_{i,exp})^2}{\sum_{i=1}^{17} (PCC_{i,p} - PCC_{exp})^2} \quad (\text{Equation 4})$$

$$RMSE = \sqrt{\frac{\sum_{i=1}^{17} (PCC_{i,p} - PCC_{i,exp})^2}{17}} \quad (\text{Equation 5})$$

$$SEP = \frac{RMSE}{PCC_{exp}} \quad (\text{Equation 6})$$

$$RPD = 100 / \sum_{i=1}^{17} \frac{|(PCC_{i,p} - PCC_{i,exp})|}{|PCC_{i,exp}|} \quad (\text{Equation 7})$$

where,  $PCC_{i,p}$  the predicted values,  $PCC_{i,exp}$  the equivalent experimental value,  $PCC_{exp}$  is the

experimental data mean value and 17 represent the number of the experimental runs. Overall, small RMSE and SEP values imply high model efficiency and ability.

### **3. Results and discussion:**

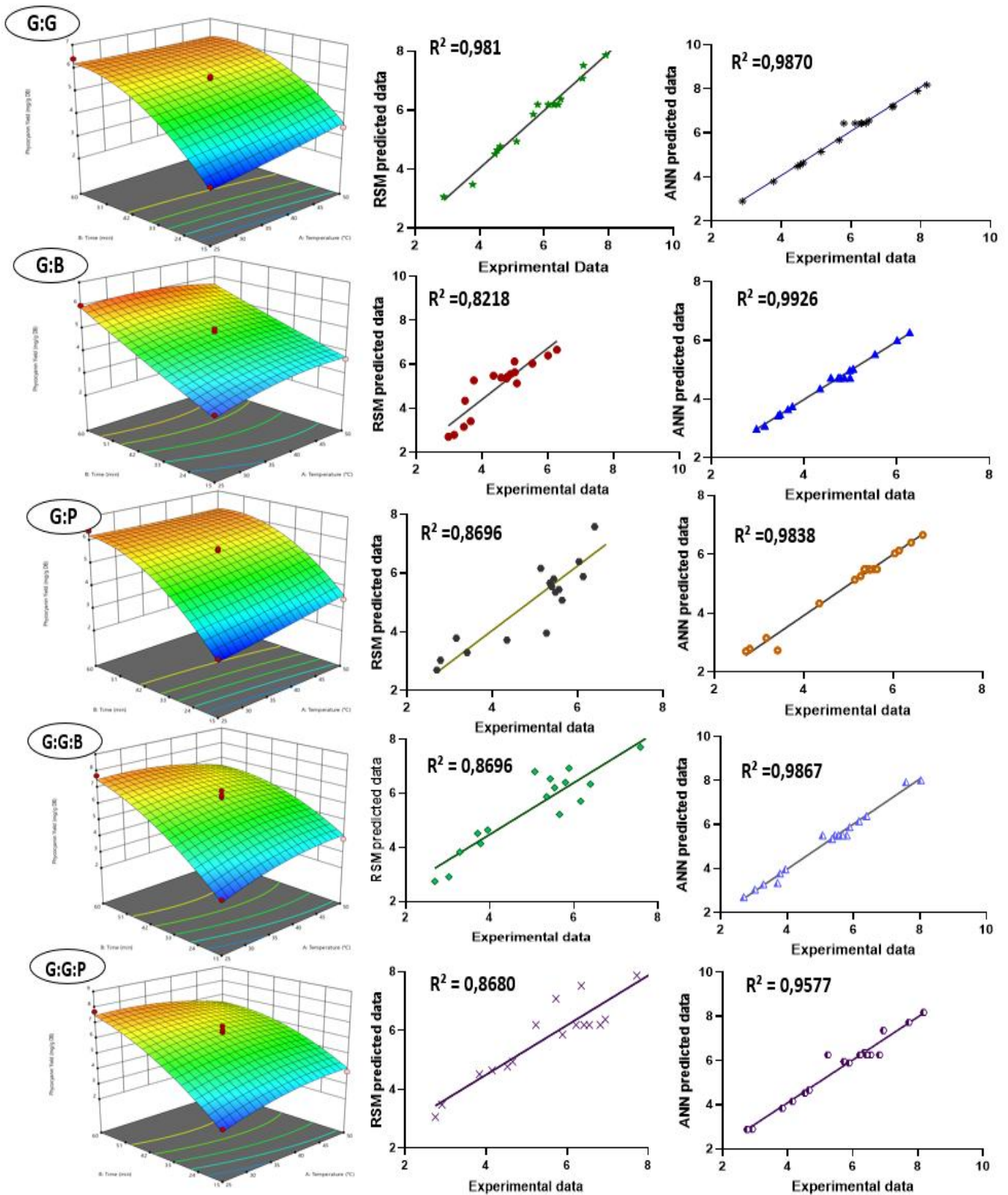
In order to select the most appropriate NaDES for phycocyanin extraction, a preliminary screening was carried out on 60 NaDES from our library, all food-compatible. A wide range of composition was explored as sugars, amino acids, polyols, organic acids, and terpenes combination. Among all, glycerol-based NaDES were identified as the best solvents for phycocyanin extraction. Five NaDES were then selected: glycerol/glucose (2:1) (G:G), glycerol/betaine (2:1) (G:B), glycerol/proline (2:1) (G:P), glycerol/glucose/betaine (4:1:1) (G:G:B) and glycerol/glucose/proline (4:1:1) (G:G:P). Those NaDES were then subjected to RSM to optimize phycocyanin recovery from *A. platensis*.

#### 3.1 RSM model fitting and statistical analysis:

Response surface methodology (RSM) has been established as a mathematical and statistical methods for designing and generating experimental models, while allowing the evaluation of the relative significance of each chosen variable. It also used to define the optimum condition for the set predicted response [35].

Results obtained by performing the UAE experiments on spirulina biomass according to the Box-Behnken are shown in Table 2 and the results were summarized in Figure 1.

Phycocyanin yield (mg/g of dry matter) was used as discriminant factor.



**Figure 1:** Respond surface methodology illustration the effect of time and temperature on the phycocyanin yield of each NaDES and Comparison of experimental with predicted values of RSM and ANN models.

**Table 2:** Three-variable experimental design applied for phycocyanin UAE with experimental and RSM/ANN predicted results

Run order	temperature (°C)	time (min)	water (%)	Experimental value					Predicted by RSM					Predicted by ANN				
				G:G	G:B	G:P	G:G:B	G:G:P	G:G	G:B	G:P	G:G:B	G:G:P	G:G	G:B	G:P	G:G:B	G:G:P
1	25	37.5	60	4.632	3.484	4.345	3.713	4.527	4.772	3.647	4.512	4.003	4.705	4.632	3.486	4.334	3.341	4.527
2	37.5	37.5	40	6.434	4.872	5.566	5.438	6.547	6.193	4.794	5.479	5.502	6.247	6.434	4.726	5.497	5.516	6.254
3	37.5	37.5	40	6.295	4.996	5.640	5.070	6.818	6.193	4.794	5.479	5.502	6.247	6.434	4.726	5.497	5.516	6.254
4	50	60	40	7.217	5.531	6.035	6.391	6.353	7.521	6.642	7.155	8.060	8.807	7.217	5.530	6.035	6.392	6.353
5	25	15	40	3.783	3.155	2.787	3.027	2.915	3.479	2.532	2.163	2.083	1.606	3.783	3.094	2.787	3.047	2.895
6	37.5	60	60	7.186	5.062	5.140	6.163	5.725	7.084	5.719	5.919	7.166	7.509	7.186	5.030	5.140	6.164	5.958
7	37.5	15	20	4.543	3.446	3.159	3.783	4.151	4.645	2.789	2.379	2.780	2.367	4.543	3.443	3.159	3.774	4.151
8	25	60	40	7.911	6.008	6.403	7.581	7.721	7.873	6.409	6.697	8.101	8.622	7.911	6.008	6.403	7.929	7.721
9	50	15	40	4.473	3.655	3.411	3.287	3.837	4.511	2.765	2.621	2.043	1.791	4.476	3.645	2.736	3.286	3.837
10	37.5	15	60	2.891	2.977	2.702	2.694	2.752	3.055	2.216	1.918	1.536	1.019	2.893	2.982	2.702	2.702	2.880
11	50	37.5	60	5.143	3.752	5.271	3.950	4.659	4.942	3.693	5.108	3.815	4.429	5.143	3.754	5.264	3.961	4.659
12	37.5	37.5	40	5.798	4.779	5.442	5.798	6.419	6.193	4.794	5.479	5.502	6.247	6.434	4.726	5.497	5.516	6.254
13	25	37.5	20	5.663	4.349	5.481	5.353	5.888	5.864	4.407	5.644	5.487	6.117	5.663	4.358	5.481	5.352	5.888
14	50	37.5	20	6.516	4.988	6.132	5.884	6.942	6.375	4.826	5.965	5.594	6.764	6.558	4.988	6.132	5.886	7.365
15	37.5	37.5	40	6.124	4.581	5.395	5.543	6.221	6.193	4.794	5.479	5.502	6.247	6.434	4.726	5.497	5.516	6.254
16	37.5	37.5	40	6.314	4.740	5.353	5.659	5.233	6.193	4.794	5.479	5.502	6.247	6.434	4.726	5.497	5.516	6.254
17	37.5	60	20	8.182	6.279	6.663	8.027	8.174	8.019	7.040	7.446	9.185	9.907	8.182	6.279	6.662	8.026	8.174
<b>R<sup>2</sup></b>									0.981	0.857	0.895	0.865	0.755	0.984	0.992	0.981	0.986	0.956
<b>RMSE</b>									0.779	2.174	2.222	3.258	5.169	0.732	0.351	0.717	0.660	1.313
<b>SEP(%)</b>									13.368	48.211	44.482	63.405	92.619	12.556	7.783	14.357	12.841	23.533
<b>RPD(%)</b>									3.104	9.595	10.056	14.069	21.036	1.233	0.965	1.741	2.019	2.994

Comparing binary NaDES performances, (G:G) appeared as the best combination with a phycocyanin rate almost 20% higher than with (G:B) and (G:P). Ternary NaDES, containing both glucose and amino-acid HBD combined with glycerol, exhibited extraction performances similar to (G:G) alone.

An increase of extraction time lead to increasing yield for temperature below 50°C as shown in Figure 1 whereas, longer extraction time (60 min) coupled with high temperature (50°C) led to lower phycocyanin rates compared to results obtained at 25°C for all NaDES. This may imply a possible degradation of phycocyanin during a long UAE extraction time coupled with high temperature.

The results also depict the effect of water ratio on phycocyanin. The most described solvent for phycobiliproteins extraction is water [36], thus it was hypothesized that the increase of water addition in NaDES will increased the phycocyanin recovery. As we can noticed Table 1, phycocyanin yield increased with a decrease of water ratio. In fact, beyond 40%, for all NaDES, a notable decrease in phycocyanin yield was observed. This might be linked to the hydrogen-bond network damaging with high amount of water. If addition of water is well described as efficient tailoring method of NaDES properties, there is still debate on the amount beyond which the NaDES is destabilized [25]. In our case, this could explain the results obtained with the 60% water addition.

The quadratic model was chosen as the optimal model for phycocyanin extraction for all NaDES with a linear term that is significant ( $p < 0.0001$ ) as depicted in Table3 of the regression analysis.

**Table3:** Optimal conditions for each NaDES

NaDES	Optimale conditions			
	T (°C)	Time (min)	Water ratio (%)	PC (mg/g DB)
G:G	34.55	60	29.48	8.12
G:B	37.04	59.78	26.28	6.30
G:P	27.33	57.29	21.33	6.73
G:G:B	28.56	59.78	22.74	8.03
G:G:P	36.90	59.4	20.21	8.18

The generated polynomial equation illustrates the interaction between the set parameters for each NaDES as shown below:

$$\begin{aligned} \text{G:G-Yield(mg/g DM)} = & -2.239 + (0.208 * A) + (0.124 * B) + (0.053 * C) - \\ & (0.001 * A * B) - (3.411 * 10^{-4} * A * C) - (3.640 * 10^{-4} * B * C) - (0,002 * A^2) + \\ & (1.332 * 10^{-4} * B^2) - (0.001 * C^2) \end{aligned} \quad (\text{Equation 8})$$

$$\begin{aligned} \text{G:B- Yield(mg/g DM)} = & 2.756 + (0.177 * A) + (0.096 * B) + (0.086 * C) - \\ & (8.682 * 10^{-4} * A * B) - (3.721 * 10^{-4} * A * C) - (4.156 * 10^{-4} * B * C) - (0,002 * A^2) + \\ & (8.977 * 10^{-5} * B^2) - (9.959 * 10^{-4} * C^2) \end{aligned} \quad (\text{Equation 9})$$

$$\begin{aligned} \text{G:P-Yield(mg/g DM)} = & -1.355 + (0.023 * A) + (0.251 * B) + (0.029 * C) - \\ & (8.820 * 10^{-4} * A * B) + (2.752 * 10^{-4} * A * C) - (5.922 * 10^{-4} * B * C) + (2.282 * \\ & 10^{-4} * A^2) - (0.002 * B^2) - (5.189 * 10^{-4} * C^2) \end{aligned} \quad (\text{Equation 10})$$

$$\begin{aligned} \text{G:G:B-Yield(mg/g DM)} = & -4.176 + (0.268 * A) + (0.150 * B) + (0.054 * C) - (0.001 * \\ & A * B) - (2.946 * 10^{-4} * A * C) - (4.307 * 10^{-4} * B * C) - (0.003 * A^2) + (1.18 * 10^{-5} * \\ & B^2) - (8.522 * 10^{-4} * C^2) \end{aligned} \quad (\text{Equation 11})$$

$$\begin{aligned} \text{G:G:P-Yield(mg/g DM)} = & -6.947 + (0.298 * A) + (0.279 * B) + (0.084 * C) - (0.002 * \\ & A * B) - (9.225 * 10^{-4} * A * C) - (5.835 * 10^{-4} * B * C) - (0.002 * A^2) - (0.001 * B^2) - \\ & (9.365 * 10^{-4} * C^2) \end{aligned} \quad (\text{Equation 12})$$

In those equations A represents temperature (°C), B extraction time (min) and C water ratio (%). The aforementioned equations illustrated that the phycocyanin yield increased with the

increased extraction time. The optimum conditions were somewhat similar between the five NaDES in term of time (57.29 to 60 min) (Table 3).

Studies have shown that phycocyanin yield increase with an increasing temperature until it reaches an optimum level [2,16]. The results of the study conducted by Hadiyanto and Sutrisnorhadi [16] showed that optimum yield was obtained at the temperature of 45°C with an extraction time of 42 min; whereas, another study on phycocyanin stated that temperature beyond 40°C lead to phycocyanin instability and denaturation after temperature reaches 55 °C[2]. In this work, the optimal temperature ranged from 27.33°C for (G:P) to 37.04°C for (G:B) with and extraction time of 60 minutes. Those low temperatures were in favor of a low phycocyanin degradation during extraction process. The optimal range for water ratio in this study, was between 20.21% (G:G:B) and 29.48% for (G:G) (Table 3), corresponding to regular amount found in the literature, for tailoring NaDES viscosity. The value found exhibit a trend for optimal conditions that could be exploited for glycerol based NaDES aimed at phycocyanin ultrasound assisted extraction.

In fact, an extraction time of 60 min was highlighted as a common factor for all NaDES, as well as water ratio between 20 and 30%, and temperature of 30-35°C was shown to be optimal. For all NaDES, ANOVA highlighted general trends for some parameters (Table 4). Both time and water ratio had low  $p$ -values ( $p < 0.05$ ) and are significant model terms for phycocyanin extraction.

Both adequacy and fitness of the model were tested by analysis of variance ANOVA. The results demonstrated that the models adapted in this study were significant, meaning that the adapted equation sufficiently represent the relation between the independent parameters and the response (Table S1). The model F-value varied from 16.14 to 67.31 assigned to a  $p$ -value of 0.0007 for (G:G:P) and <0.0001 for the other four NaDES.

**Table 4:** ANOVA for the RSM model.

Source	Sum of squares					df	Mean of squares					F-value					P-value				
	G:G	G:B	G:P	G:G:B	G:G:P		G:G	G:B	G:P	G:G:B	G:G:P	G:G	G:B	G:P	G:G:B	G:G:P	G:G	G:B	G:P	G:G:B	G:G:P
<b>Model</b>	32.631	14.930	24.850	36.930	37.890	9	3.630	1.660	2.760	4.100	4.210	40.550	53.050	67.310	40.240	16.140	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.0007
<b>A-T°C</b>	0.231	0.108	0.420	0.003	0.069	1	0.231	0.108	0.420	0.003	0.069	2.590	3.460	10.240	0.033	0.263	0.1518	0.1052	0.0151	0.8621	0.6241
<b>B-Time</b>	27.403	11.630	18.550	29.540	25.630	1	27.400	11.630	18.550	29.540	25.630	306.450	372.050	452.180	289.670	98.230	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
<b>C-ratio</b>	3.188	1.790	1.980	5.330	7.020	1	3.190	1.790	1.980	5.330	7.020	35.650	57.320	48.190	52.230	26.900	0.0006	0.0001	0.0002	0.0002	0.0013
<b>AB</b>	0.479	0.239	0.246	0.525	1.310	1	0.479	0.239	0.246	0.525	1.310	5.350	7.630	6.000	5.150	5.030	0.0539	0.0280	0.0441	0.0575	0.0599
<b>AC</b>	0.029	0.035	0.019	0.022	0.213	1	0.029	0.035	0.019	0.022	0.213	0.325	1.110	0.462	0.213	0.816	0.5863	0.3277	0.5187	0.6586	0.3965
<b>BC</b>	0.107	0.140	0.284	0.150	0.276	1	0.107	0.140	0.284	0.150	0.276	1.200	4.470	6.920	1.470	1.060	0.3096	0.0722	0.0339	0.2642	0.3380
<b>A<sup>2</sup></b>	0.329	0.268	0.005	0.800	0.573	1	0.329	0.268	0.005	0.800	0.573	3.680	8.550	0.131	7.840	2.200	0.0965	0.0222	0.7286	0.0265	0.1820
<b>B<sup>2</sup></b>	0.019	0.009	3.080	0.000	1.900	1	0.019	0.009	3.080	0.000	1.900	0.214	0.278	75.170	0.002	7.290	0.6576	0.6142	< 0.0001	0.9703	0.0306
<b>C<sup>2</sup></b>	0.761	0.668	0.181	0.489	0.591	1	0.761	0.668	0.181	0.489	0.591	8.510	21.370	4.420	4.800	2.260	0.0225	0.0024	0.0736	0.0646	0.1760
<b>Residual</b>	0.626	0.219	0.287	0.714	1.830	7	0.089	0.031	0.041	0.102	0.261										
<b>Lack of Fit</b>	0.382	0.124	0.230	0.409	0.351	3	0.128	0.041	0.077	0.136	0.117	2.090	1.730	5.310	1.790	0.318	0.2437	0.2984	0.0703	0.2889	0.8134
<b>Pure Error</b>	0.244	0.095	0.058	0.305	1.470	4	0.061	0.024	0.014	0.076	0.369										
<b>Cor Total</b>	33.257	15.150	25.140	37.640	39.720	16															



Therefore, the significance of the models was specified by low  $p$ -values ( $p < 0.05$ ) [37]. The lack of fit value was not significant for all models this implies that the models are good fits.

The Lack of Fit F-value of 2.09, 1.730, 5.310, 1.790, and 0.318 for (G:G), (G:B), (G:P), (G:G:B), and (G:G:P), respectively, was not significant relative to the pure error.

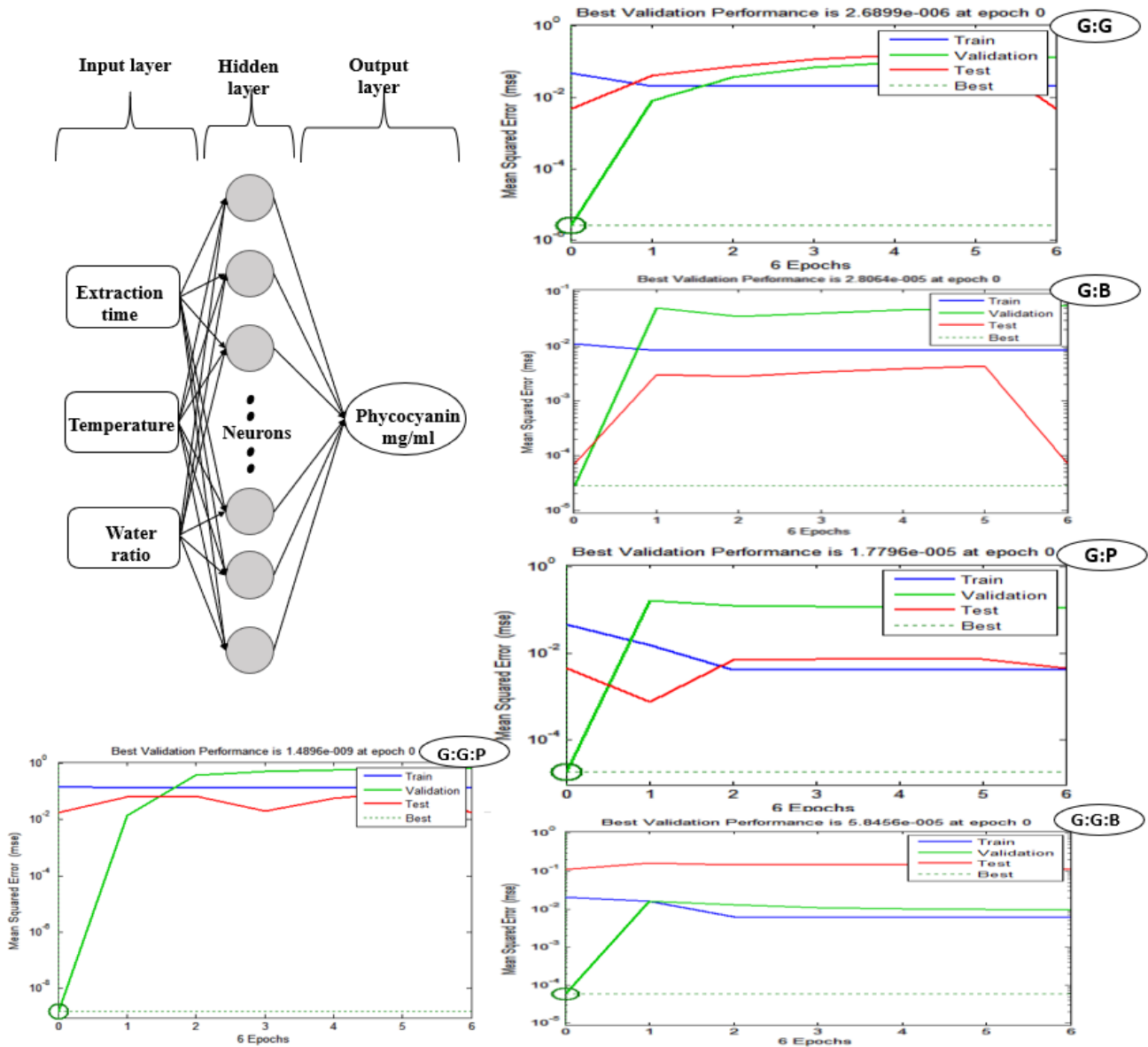
Considering the RSM statistical fit, the value of  $R^2$  varied, for the five NaDES between 0,954 and 0,989 for (G:G:P) and (G:P), respectively (See Table S1). As for the predicted  $R^2$ , it was in reasonable agreement with the adjusted  $R^2$  for all the studied NaDES, which indicated that the predicted data were compatible with the set of experimental data. Coefficients of variation that varies from 3.92 to 9.15 further authenticated the reliability of the obtained results (Qadir et al., 2019).

### 3.2 ANN model analysis:

Nowadays, ANN has proven itself as a powerful tool for simulation and optimization in the extraction field [32–34]. In this study an ANN based model was used via a trial and error process to describe UAE of phycocyanin from spirulina. For that purpose, a three-layer ANN with one hidden layer was used (Figure 2). Various researchers have reported that ANN performs better than RSM when it comes to predictive capabilities [38,39]. Hence, in this study a non-linear relationship between the three input (time, water ratio, and temperature) variables and response (phycocyanin yield) was developed via ANN-based model. The model was generated using the experimental data. To generate the model, data were divided into training data (70%), validation (15%), and testing data (15%).

The model training was performed using Levenberg–Marquardt algorithm for 17 experiments set. The training goal was set to  $10^{-5}$ . To batch train, the data goes through the learning algorithm concurrently in one epoch before updating the weights. Thus, in 5 epochs, the goal is to minimize the mean square error. Figure 2 displays the validation performance after 5 epochs of each NaDES. MSE was  $2.69 \cdot 10^{-6}$ ,  $2.81 \cdot 10^{-5}$ ,  $1.80 \cdot 10^{-5}$ ,  $5.84 \cdot 10^{-5}$ ,  $1.49 \cdot 10^{-9}$  with an

overall  $R^2$  value of 0.992, 0.991, 0.992, 0.994, and 0.958 for (G:G), (G:B), (G:P), (G:G:B), and (G:G:P), respectively, meaning that ANN model is a very suitable model. Figure 2 depicts a plot between the values of the experiment and of ANN predicted data showing the effectiveness of the model.



**Figure 2:** ANN model and NaDES ANN validation.

Additionally, the values predicted by ANN and RSM model were plotted against the matching experimental values to display the abilities of each model as shown in Figure 1. According to the results, fitting  $R^2$  shows better fit for ANN compared to RSM for all the used NaDES. The weights can be defined as coefficients among the artificial neurons that similar to the real

synapse strengths that occurs between the axons and dendrites. Meaning that the weight influences the transmitted signal ratio into the neuron's structure. The ANN weights of this study are listed in Table S2, and training performances are summarized Figure S1.

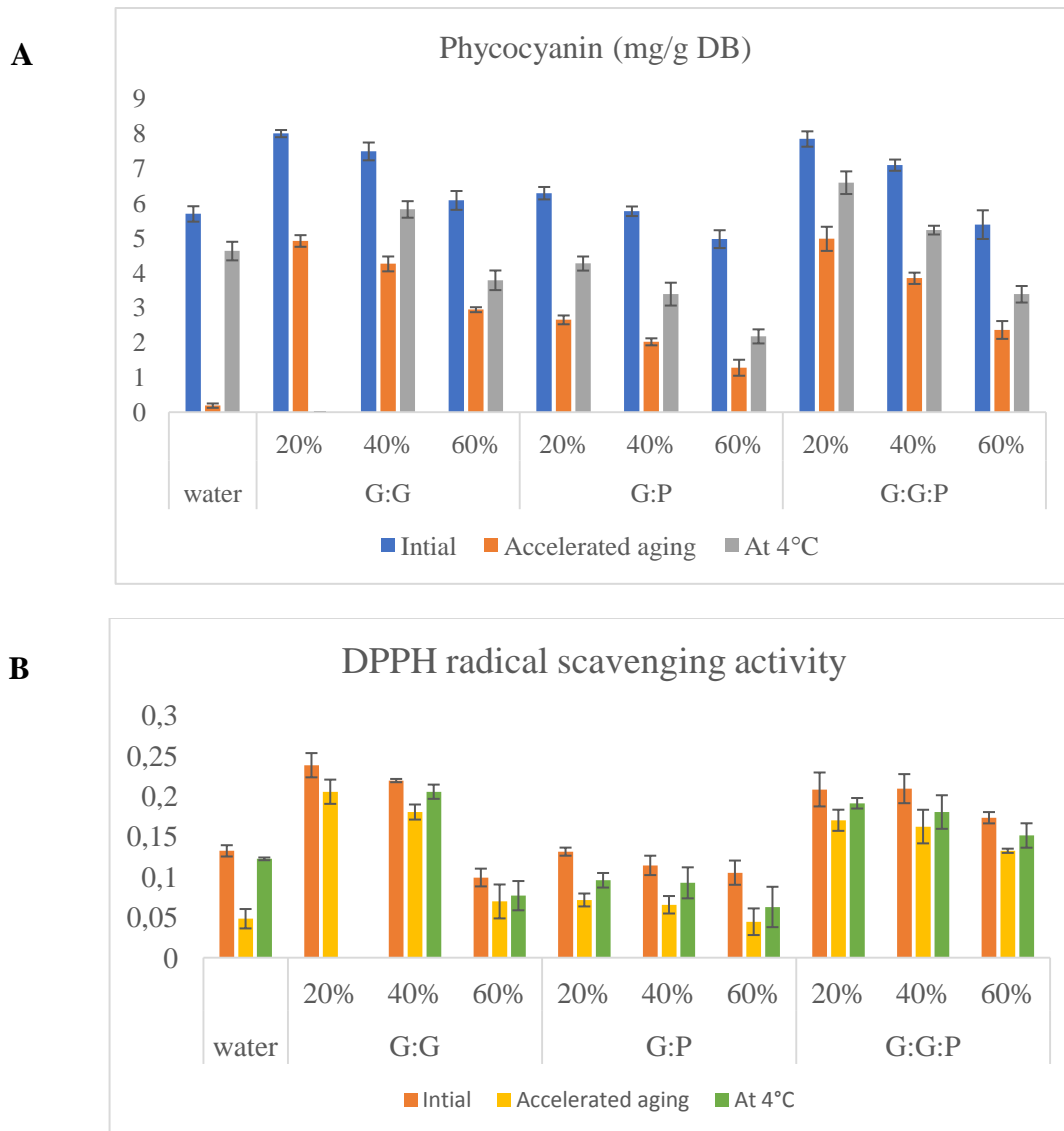
### 3.3 ANN and RSM comparison:

The comparison between the predictive effectiveness of ANN and RSM model for each NaDES is presented in this section based on the data sets developed by Box-Benshen - Behnken (17 runs). The model performance comparison was based on the correlation coefficient ( $R^2$ ), the root means square error (RMSE), relative percent deviation (RPD%), and standard error of prediction (SEP%), displayed in Table 2. In this case the coefficient of correlation  $R^2$  for each NaDES were higher for ANN models in comparison to the corresponding RSM values ranging for ANN between 0.95 to 0.99 for (G:G:P) and (G:B), respectively, and for RSM between 0.75 for (G:G:P) and 0.98 for (G:G). The RMSE, SEP, and RPD for ANN were way lower than those for RSM for each NaDES thus indicating that the RSM model exhibit higher deviation compared to ANN. The result also implies that while both models performed highly, the experimental data fitting was more accurate via ANN model. The difference between the accuracy prediction of RSM and ANN is due to RSM limitation to a second order polynomial while ANN has the ability to estimate the nonlinearity of the model [34].

According to RSM and ANN prediction models, optimal parameters for the extraction of phycocyanin were water ratio 20% to 30%, 60 minutes of extraction at 27 to 37°C. These conditions were thus applied to each NaDES to confirm the predictions. The phycocyanin concentration was found to be  $7.98 \pm 0.103$ ,  $5.86 \pm 0.145$ ,  $6.27 \pm 0.178$ ,  $7.53 \pm 0.161$ , and  $7.82 \pm 0.220$ , for (G:G), (G:B), (G:P), (G:G:P), and (G:G:B), respectively, Those results were somewhat similar to the results predicted by the models and depicted in Table 2, highlighting the adequacy of our models.

### 3.4 DPPH radical scavenging activity and stability:

DPPH radical scavenging assay data was performed in order to compare between different NaDES and water, as phycocyanin exhibit strong antioxidant properties. DPPH was essential in order to investigate if the antioxidant properties were preserved in NaDES media after UAE. For this study, extracts obtained at 25°C and 60 min of extraction were selected. DPPH assay and phycocyanin yield were measured before and after 30 days in two conditions: stored at 4°C and accelerated aging conditions (at 40°C, 75% humidity), which is equivalent to 6 months at room temperature. The results of this study were illustrated in Figure 3.



**Figure 3:** Phycocyanin yield (A) and DPPH radical scavenging activity (B) value at different conditions of NaDES and water extract.

For the antioxidant activity, it was noticed that the NaDES, (G:G), (G:P), and (G:G:P), enhanced the free radical scavenging activity of PC compared to water at both 20% and 40% water ratio. In fact, the antioxidant activity was at it highest at 20% water, and the values were  $0.132 \pm 0.007$ ,  $0.238 \pm 0.015$ ,  $0.131 \pm 0.005$ ,  $0.218 \pm$  for water, (G:G), (G:P) and (G:G:P), respectively. This could be linked to the higher concentration of PC and/or to the potentiation of the antioxidant activity by the NaDES. It was interesting to noticed that the glucose-based NaDES exhibited the best antioxidant activity, compared to the (G:P) system, highlighting a crucial role of this sugar.

This antioxidant potentiation was already described for other metabolites [40] but was observed for the first time here for phycocyanin.

Similar results were found in a study conducted by Pavic et al [41], that focuses on the assessment of total phenolic content and antioxidant activity of *Ruta graveolens* L. extracts obtained by choline chloride based NaDES. In this work it was stated that the extract obtained with 20% water had the highest total phenolic content and antioxidant activity. The stability study showed that the extracts conserved at 4°C had higher preservation value of phycocyanin and antioxidant activity, except for the (G:G) at 20% water ratio where a crystallisation of the extract was observed. It was also observed that all the extract with glucose concentrations showed higher preservation of phycocyanin. In fact, the water extract had lost all colour after 30 days of accelerated aging and had the lowest value of phycocyanin yield ( $0.048 \pm 0.003$  mg/g of DB).

High glucose concentration increase the thermal stability of phycocyanin since the latter is sensitive to light, pH, and temperature which leads to a visible blue colour loss that was observed after 30 days of accelerated aging conditions compared to the one conserved at 4°C for water, (G:G:P), at 60%, and (G:P). These results were in agreement with previous work that highlighted the preservative effect of sugars addition on phycocyanin during food processing [15,43].

### 3.5 Industrial and environmental process feasibility study:

The application of NaDES for UAE in the industrial food processing is in the initial phases since it is still a “young technology” and many features has yet to be discussed and factored into the economic and environmental impact of both NaDES and UAE. This part will focus on the feasibility study for an industrialized valorization of phycocyanin as a grade natural blue colorant from spirulina, using these two technologies.

Although, the physical properties of those NaDES may restrict their utilization in food

processing such as viscosity that will lead to an increase in the energy needed for stirring and pumping, the same property can be altered by water addition. In fact, for sugar based NaDES, with optimal water, the viscosity allows stable molecular interaction [44].

Another restriction of NaDES utilization lies on the fact that they have almost zero vapor pressure, which is environmentally attractive since this implies less air pollution, but it also rises a problem when it comes to bio-compound recovery. However, by using NaDES approved for food utilization, such as those proposed in this study [32], it is possible to use the extract directly for the final formulation thus reducing the solvents removing and purification steps.

To further valorize the process for phycocyanin extraction, the six principals of green extraction were used as a reference for an environment-friendly, safe and economic method to obtain the targeted extract [45].

- Principle 1: Innovation in plant resources- Spirulina considered as renewable biomass
- Principle 2: Use of alternative solvents-NaDES
- Principle 3: Reduce energy consumption- UAE bath can use up to 40% less energy than conventional processes [46]
- Principle 4: Reduce waste- After phycocyanin extraction the spirulina can be further valorized for lipid extraction [47]
- Principle 5: Controlled process- optimized extraction conditions
- Principle 6: non-denatured and biodegradable extract with no contamination- Safe and preformulated extract with high stability in the chosen NaDES.

It should be noted that the NaDES price is in the same range off conventional solvents; according to [48] the average price estimation of NaDES is around 2\$/kg, making this solvent economically attractive. Additionally, different company has developed reactor with a range from 30 to 1000L coupled with pumping system to fill, stir, and empty the system [49]. Thus,

it is beneficial and possible to efficiently move the extraction process from a laboratory scale to industry level.

Essentially, ultrasound is nowadays considered as a common operation unit in the food processing due to their efficient extraction, degassing, or/and cutting capacity. Thus, for a decent use of UAE in food processing it is imperial to install a Hazard Analysis and Critical Control Points (HACCP) program with defined critical control point (CCP). The later is a food safety approach based on seven principles to detect and prevent hazardous occurrences during food processing and suppling. As for CCPs they are crucial to prevent, eliminate, or reduce any food safety risks to suitable level so that possible hazards can be controlled. Those hazards can either be biological, chemical or physical hazards. During UAE with NaDES, the critical factors are expected to be the type of biomass, the quantity to be processed, the composition, and the extraction time and temperature; ultrasound bath can also contaminate the final product by causing chemical mitigation. Thus, the identification and control of those factors is an important part of HACCP process.

Hazard and operability (HAZOP) system focuses on the potential operation deviations that could occur during the processing and may lead to operation problems. For UAE, the operators are exposed to numerous risks such as exposure to ultrasonic waves, immersion of a body part in an ultrasound treated liquid, electrical shock since ultrasound is an electrical device, and ear damage. Therefore, operator needs to take precautions while dealing with the device per example sound proof helmets.

Based on all these aspects, this study advocates for the use of ultrasound for the extraction of food grade phycocyanin in a preformulated extract that allows the the preservative of this blue pigment while also reducing the extraction eco-footprint in comparison with conventional processes.

**Conclusion:**



In this study, ultrasound-assisted extraction of blue-green natural dye from spirulina was studied. The effects of extraction time, temperature and water ratio were investigated via two modeling tools RSM and ANN. The finding of this study allows the prediction of phycocyanin yield under three different experimental conditions (extraction time, water ratio%, temperature) via ultrasound assisted extraction for 5 different glycerol-based NaDES. Optimal extraction conditions were predicted using RSM and ANN, that were common to the five NaDES tested: temperature 27-30°C, water ratio between 20 and 30 % and extraction duration 60 min. The crucial role of water ration was highlighted and surprisingly the increase of water content damaged the transfer of phycocyanin. Overall, the box-Behnken design was effectively employed to generate the optimized list of conditions for the maximum PCC. The ANN model exhibited efficient modelling strength. And for both tools simulated and experimental data values were in good agreement.

NaDES extracts also exhibited enhance antioxidant power and phycocyanin stabilization ability compared to water extract. Among all, Glycerol:Glucose (2:1, mol/mol) was highlighted as the most performant for both extraction and stabilization of phycocyanin. The preservation of this phycocyanin for the food application is also very crucial during food process, and glycerol based NaDES represent a great opportunity to develop a stable preformulated phycocyanin extract ready to be used for the food industry.

Food grade NaDES combined with UAE could also decrease the eco-footprint of microalgae food processing, which is still a main concern to recover.

### **CRedit authorship contribution statement**

**Soukaina Hilali:** Methodology, Formal analysis, Investigation Writing - original draft preparation. **Laura Wils:** Experiment preparation, Investigation. **Alya Chevalley:** Resource.

**Barbara Clément-Larosière** : resource, methodology. **Leslie Boudesocque-Delaye** : conceptualization, supervision, funding acquisition ; writing – original draft.

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## Figures captions

Figure 1: Respond surface methodology illustration of the effect of time and temperature on the phycocyanin yield of each NaDES and Comparison of experimental with predicted values of RSM and ANN models.

Figure 4: ANN model and NaDES ANN validation.

Figure 3: Phycocyanin yield (A) and DPPH radical scavenging activity (B) of (G:G); (G:P) and (G:G:P) extract at 25°C and 60 min, for different water ratio.

## Tables

**Tableau 2:** NaDES composition

	HBA	HBD	HBD	Ratio (mol/mol)
GG	Glycerol	Glucose	-	2:1
GB	Glycerol	Betaine	-	2:2
GP	Glycerol	Proline	-	4:1:1
GGB	Glycerol	Glucose	Betaine	4:1:1
GGP	Glycerol	Glucose	Proline	4:1:1

**Table 2:** Three-variable experimental design applied for phycocyanin UAE with experimental and RSM/ANN predicted results

Run order	temperature (°C)	time (min)	water (%)	Experimental value					Predicted by RSM					Predicted by ANN				
				G:G	G:B	G:P	G:G:B	G:G:P	G:G	G:B	G:P	G:G:B	G:G:P	G:G	G:B	G:P	G:G:B	G:G:P
1	25	37.5	60	4.632	3.484	4.345	3.713	4.527	4.772	3.647	4.512	4.003	4.705	4.632	3.486	4.334	3.341	4.527
2	37.5	37.5	40	6.434	4.872	5.566	5.438	6.547	6.193	4.794	5.479	5.502	6.247	6.434	4.726	5.497	5.516	6.254
3	37.5	37.5	40	6.295	4.996	5.640	5.070	6.818	6.193	4.794	5.479	5.502	6.247	6.434	4.726	5.497	5.516	6.254
4	50	60	40	7.217	5.531	6.035	6.391	6.353	7.521	6.642	7.155	8.060	8.807	7.217	5.530	6.035	6.392	6.353
5	25	15	40	3.783	3.155	2.787	3.027	2.915	3.479	2.532	2.163	2.083	1.606	3.783	3.094	2.787	3.047	2.895
6	37.5	60	60	7.186	5.062	5.140	6.163	5.725	7.084	5.719	5.919	7.166	7.509	7.186	5.030	5.140	6.164	5.958
7	37.5	15	20	4.543	3.446	3.159	3.783	4.151	4.645	2.789	2.379	2.780	2.367	4.543	3.443	3.159	3.774	4.151
8	25	60	40	7.911	6.008	6.403	7.581	7.721	7.873	6.409	6.697	8.101	8.622	7.911	6.008	6.403	7.929	7.721
9	50	15	40	4.473	3.655	3.411	3.287	3.837	4.511	2.765	2.621	2.043	1.791	4.476	3.645	2.736	3.286	3.837
10	37.5	15	60	2.891	2.977	2.702	2.694	2.752	3.055	2.216	1.918	1.536	1.019	2.893	2.982	2.702	2.702	2.880
11	50	37.5	60	5.143	3.752	5.271	3.950	4.659	4.942	3.693	5.108	3.815	4.429	5.143	3.754	5.264	3.961	4.659
12	37.5	37.5	40	5.798	4.779	5.442	5.798	6.419	6.193	4.794	5.479	5.502	6.247	6.434	4.726	5.497	5.516	6.254
13	25	37.5	20	5.663	4.349	5.481	5.353	5.888	5.864	4.407	5.644	5.487	6.117	5.663	4.358	5.481	5.352	5.888
14	50	37.5	20	6.516	4.988	6.132	5.884	6.942	6.375	4.826	5.965	5.594	6.764	6.558	4.988	6.132	5.886	7.365
15	37.5	37.5	40	6.124	4.581	5.395	5.543	6.221	6.193	4.794	5.479	5.502	6.247	6.434	4.726	5.497	5.516	6.254
16	37.5	37.5	40	6.314	4.740	5.353	5.659	5.233	6.193	4.794	5.479	5.502	6.247	6.434	4.726	5.497	5.516	6.254
17	37.5	60	20	8.182	6.279	6.663	8.027	8.174	8.019	7.040	7.446	9.185	9.907	8.182	6.279	6.662	8.026	8.174
R <sup>2</sup>									0.981	0.857	0.895	0.865	0.755	0.984	0.992	0.981	0.986	0.956
RMSE									0.779	2.174	2.222	3.258	5.169	0.732	0.351	0.717	0.660	1.313
SEP(%)									13.368	48.211	44.482	63.405	92.619	12.556	7.783	14.357	12.841	23.533
RPD(%)									3.104	9.595	10.056	14.069	21.036	1.233	0.965	1.741	2.019	2.994

**Table 3:** Optimal conditions predicted by RSM for each NaDES

Optimale conditions				
NaDES	T (°C)	Time (min)	Water ratio (%)	PC (mg/g DB)
G:G	34.55	60	29.48	8.12
G:B	37.04	59.78	26.28	6.30
G:P	27.33	57.29	21.33	6.73
G:G:B	28.56	59.78	22.74	8.03
G:G:P	36.90	59.4	20.21	8.18

**Table 4:** ANOVA for the RSM model.

Source	Sum of squares					df	Mean of squares					F-value					P-value				
	G:G	G:B	G:P	G:G:B	G:G:P		G:G	G:B	G:P	G:G:B	G:G:P	G:G	G:B	G:P	G:G:B	G:G:P	G:G	G:B	G:P	G:G:B	G:G:P
Model	32.631	14.930	24.850	36.930	37.890	9	3.630	1.660	2.760	4.100	4.210	40.550	53.050	67.310	40.240	16.140	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.0007
A-T°C	0.231	0.108	0.420	0.003	0.069	1	0.231	0.108	0.420	0.003	0.069	2.590	3.460	10.240	0.033	0.263	0.1518	0.1052	0.0151	0.8621	0.6241
B-Time	27.403	11.630	18.550	29.540	25.630	1	27.400	11.630	18.550	29.540	25.630	306.450	372.050	452.180	289.670	98.230	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001
C-ratio	3.188	1.790	1.980	5.330	7.020	1	3.190	1.790	1.980	5.330	7.020	35.650	57.320	48.190	52.230	26.900	0.0006	0.0001	0.0002	0.0002	0.0013
AB	0.479	0.239	0.246	0.525	1.310	1	0.479	0.239	0.246	0.525	1.310	5.350	7.630	6.000	5.150	5.030	0.0539	0.0280	0.0441	0.0575	0.0599
AC	0.029	0.035	0.019	0.022	0.213	1	0.029	0.035	0.019	0.022	0.213	0.325	1.110	0.462	0.213	0.816	0.5863	0.3277	0.5187	0.6586	0.3965
BC	0.107	0.140	0.284	0.150	0.276	1	0.107	0.140	0.284	0.150	0.276	1.200	4.470	6.920	1.470	1.060	0.3096	0.0722	0.0339	0.2642	0.3380
A <sup>2</sup>	0.329	0.268	0.005	0.800	0.573	1	0.329	0.268	0.005	0.800	0.573	3.680	8.550	0.131	7.840	2.200	0.0965	0.0222	0.7286	0.0265	0.1820
B <sup>2</sup>	0.019	0.009	3.080	0.000	1.900	1	0.019	0.009	3.080	0.000	1.900	0.214	0.278	75.170	0.002	7.290	0.6576	0.6142	< 0.0001	0.9703	0.0306
C <sup>2</sup>	0.761	0.668	0.181	0.489	0.591	1	0.761	0.668	0.181	0.489	0.591	8.510	21.370	4.420	4.800	2.260	0.0225	0.0024	0.0736	0.0646	0.1760
Residual	0.626	0.219	0.287	0.714	1.830	7	0.089	0.031	0.041	0.102	0.261										
Lack of Fit	0.382	0.124	0.230	0.409	0.351	3	0.128	0.041	0.077	0.136	0.117	2.090	1.730	5.310	1.790	0.318	0.2437	0.2984	0.0703	0.2889	0.8134
Pure Error	0.244	0.095	0.058	0.305	1.470	4	0.061	0.024	0.014	0.076	0.369										
Cor Total	33.257	15.150	25.140	37.640	39.720	16															

