Extraction of Phycocyanin from Spirulina (Arthrospira Platensis) And Stability in Eutectic Solvents

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December 2020

Abstract

Phycocyanin (PC), a bright blue pigment derived from the cyanobacteria *Spirulina platensis*, is a promising natural pigment in the food, pharmaceutical and cosmetic industries. This work reassessed the capacity of water as a green extraction solvent for PC extraction from *Spirulina platensis* using maceration method.

The present work described a suitable method for the extraction of PC from the cyanobacteria Spirulina platensis. When yield of 41.886 mg/g (0.1675 mg/ml), purity (P = 1.37) and costs are considered, maceration method (operated for 48 h) was preferred for PC extraction with distilled water at biomass-solvent ratio 4:1 and room temperature. In this PC extraction method, there is opportunity for a scale up due to its simplicity and low cost. The PC is observed to be of reagent grade. Also, *Spirulina platensis* was confirmed to be a good source of PC, to be extracted for diverse purposes.

Established method of heating and stirring at T < 100 ºC for 30-90 mins was adopted to prepare two groups of DES or NADES the two families of DES used in this work, (i) based on Peg-200 as hydrogen bond donor (HBD) and (ii) based on Choline chloride as hydrogen bond acceptors (HBA). In the preparation of Peg-200-based DES, TBPB:Peg-200 (1:2), TBAB:Peg-200 (1:2), and Aliquat-336:Peg-200 (1:2), DESs, were all formed at 70 ºC and remained as transparent and non-viscous liquid at room temperature. The second family of DES prepared include ChCl:Malic acid (1:1), ChCl:Oxalic acid (1:2), which were also formed as transparent liquid but soon became viscous at room temperature, while ChCl:Peg-200 (1:2) formed quickly crystalized at room temperature but readily became non-viscous liquid when the component ratios was changed to 1:3. Also to be mentioned that ChCl:Citric acid (1:1) could not be formed at room temperature without addition of water. At the stoichiometric ratio (1:1:6) with water hence, ChCl:Citric acid:Water DES was formed.

PC has the highest solubility in TBAB:Peg-200 (1:2) and ChCl:Malic acid (1:1) with apparent solubility values of 0.05538 g/g and 0.1325 g/g of DES, respectively. The color observed during solubility tests of the PC in 1 g DES are ChCl:Malic acid (green), ChCl:Oxalic acid (grey), ChCl:Peg-200 (brown) and ChCl:Citric acid (bluish green), respectively. This proved that only ChCl:Malic acid and ChCl:Citric acid have the potential to be used as color preservatives of PC.

Keywords: *Natural Pigments, Spirulina, Phycocyanin, Extraction, Stabilization.*

1. Introduction

The influence of color in human perception and reality is enormous [1]. The organoleptic properties of food impact the sensory organs of the consumers by their color, taste, and smell. This means that properties like freshness, nutritional value, safety, also the aesthetic value of food directly affects the market value of colored food products [2].

Food colorants have been used to mask damaging effects of external conditions, such as light, air, temperature, moisture, and storage conditions, on food color. They are also used to homogenize foodstuffs color, through the correction of color variations and enhancement of naturally occurring food colors. Apart from their direct use as food colorants, they might also be used to contribute to the flavorful assurance, safety, quality, and organoleptic characteristics of foodstuffs as well ensuring consumers satisfaction [3].

Currently, the utilization of food colorants is highly regulated, whether the dye compounds are naturally derived or synthetically produced. The European Food and Safety Authority (EFSA), the U.S. Foods and Drugs Administration (FDA), and The World Health Organization (WHO) [2, 3] represent the most important regulatory organizations empowered to ensure the quality and security of food products, as well as to protect and promote human health. In the USA, the use of food colors is governed by the Code of Federal Regulations (CFR) (Title 21, Part 70-82) [1], while in Europe, the proposed colors and levels of use must comply with EFSA Regulation EC No 1333/2008 [6, 7]. EFSA recognizes food colorants as food additives which are used to add or restore color in food [8].

Developing new colors for the food industry is a very challenging task, as colorants need to be compatible with a foods flavor, safety, and nutritional value, and ultimately have minimal impact on the price of the product. In addition, a food colorant needs preferably to be a natural rather than synthetic compound [2]. To be mentioned that food legislation has always lagged innovation and product development, sometimes by more than a decade [5]. Natural colors are primarily derived from plants, insects, mineral ores, and microbial sources. Microbial colorants are usually preferred due to their ease of scalability and this potentially lower the cost of production [2]. Natural colors are assumed to be safe if they are non-allergic, non-toxic, non-carcinogenic, and biodegradable, thereby rendering no risk to the human health and environment. Due to the lower risk advantage of natural colors and the perception of consumers to preferably consume natural products, there is an increasing interest in the discovery and research of new natural colors [2].

One of those microbial sources for potential natural pigments that has generated much interest over the years is phycocyanin, produced by the blue-green cyanobacteria *Spirulina platensis*. From this perspective, the cyanobacteria *Spirulina platensis*, a blue-green alga due to the presence of phycocyanin, a pigment-protein complex, is an alternative for obtaining a natural blue pigment. Depending on the purity, the market price for phycocyanin is approximately USD$500 per kg, for

food-grade, to USD$100,000 per kg, for analytical grade [10].

The blue pigments from *Spirulina platensis* have been shown to have promising applications in food and non-food products [11]. The food industry, for example, utilizes blue coloring in several products, especially baby foods, and to obtain other colors, such as purple and violet. It has also been used as a colorant in healthy drinks, beverages, confectionary and cosmetics. Furthermore, phycocyanin has proven to possess therapeutic properties including antioxidant, anti-inflammatory and anti-cancer activities. Small quantities are also used as biochemical tracers in immunoassays due to its fluorescent properties [9, 11].

2. Phycocyanin

Phycocyanin is a protein composed of two kinds of polypeptide subunits – α (20.5 kDa) and β (23.5 kDa), polymerized to form $\alpha_3\beta_3$. There are nine Phycocyanobilins moieties in phycocyanin, one in each α-chain and two in each β-chain, that act as chromophores. A scheme of phycocyanin protein and the chemical structure of the chromophore groups, open chain tetrapyrroles, is depicted in Figure 1 [14]. These tetrapyrrole structures are responsible for the typical blue color of phycocyanin, with absorption maximum at 614 nm, while the protein part confers the stability with respect to pH and temperature.

The major limitation for the extensive application of PC in the food and feed industry is its high sensitivity to heat, which leads to high degradation in protein content and a drastic blue color reduction [15].

In Figure 2, above 40ºC there is a considerable reduction in the relative concentration of PC in solution, for all the pH considered in the study [11].

This instability has encouraged researchers from around the world to search for new technologies applicable to the food and beverage market to obtain new non-toxic colorants that can be used in food or to develop new ways to stabilize the natural colorants in use [2, 9].

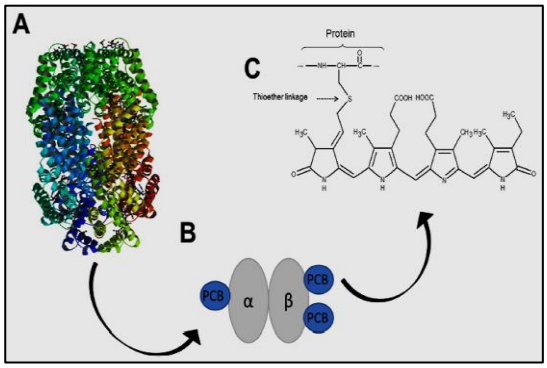

Figure 1: Different structures of PC [14]

Explanation of Figure. 1: *(A) Crystal structure of PC from cyanobacterium S. platensis in form of hexamer. (B) Schematic representation of PC assembly composing of two protein subunits, α and β chains, one phycocyanobilin PCB is bound to the α subunit and two PCBs are bound to the β subunit. (C) Chemical structure of PCB, the chromogen responsible for blue color of PC* ***[14]****.*

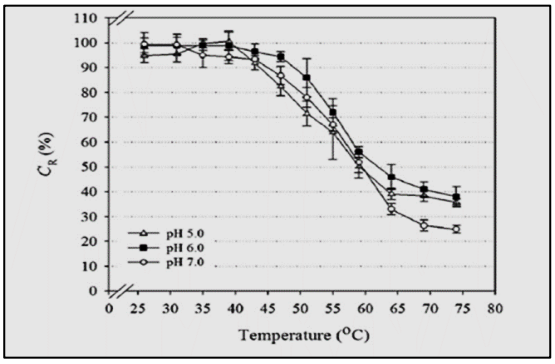

Figure 2: Relative concentration (C_R) of phycocyanin after 30 min incubation at each temperature [11]

3. Experimental Section

Several methods [16], [17] have been used for extraction of biochemicals from microalgae and the process involves disruption of microalgal cell wall with appropriate methods depending on the rigidity of the cell wall and compounds of interest. The ideal extraction method should be selective towards extraction of the target microalgal products and simultaneously minimize the co-extraction of contaminants [16].

3.1. Microwave Assisted Extraction Method

This is an alternative green method for cell disruption and extraction of compounds from natural matrices, in this case microalgae. It provides a possibility of extraction of biochemical components including lipids, pigments, carbohydrates, vitamins, and proteins, individually or as an extract. Some of the advantages of application of microwave-assisted extraction are the reduction of working time and higher yield and purity of the extracted products [16]. It has been critically evaluated for industrial-scale applications, revealing effective cell wall disruption with relatively low energy input, a rapid treatment time and the avoidance of the utilization of hazardous substances [16].

3.1.1. Materials

Spirulina (*Arthrospira platensis*) was the cyanobacteria selected for this research work. A sachet of dried Spirulina powder, ISWARI brand (100% biological), was bought at Celeiro. A microwave (Discover CEM SP-D QLab O and Sorvall centrifuge GS-3 rotor), a spectrophotometer (Thermo Scientific Genesys 10uv), a magnetic stirrer with thermometer, 200 ml capped bottle and a measuring scale, Microwave (Discover CEM, SP-D, Q Lab O) capable of operating at input power of 1000 W and frequency of 2450 MHz, and a centrifuge (Hettich Zentrifigen Mikro 220R) were used.

3.1.2. Method

20 mg of *Spirulina platensis* was weighed into the 20 mL test tube plus 5 mL of distilled water. The mixture was then placed inside a microwave for 30 s, at 100 ºC and a power input of 100 W. The resulting mixture was divided into two samples and centrifuged at 3140xg for 5 min [12] to separate the biomass residue and the supernatant. The biomass residue was discarded. The supernatant was then analyzed to determine the concentration and purity of PC through absorbance measurements with the spectrophotometer as shown in Table 1.

Table 1. Absorbance of PC extract from Spirulina Platensis using the microwave method.

Absorbance					
	Wavelength (nm)	Sample 1	Sample 2	Average	Standard Deviation
1.	620	0.695	0.774	0.74	0.06
2.	652	0.542	0.591	0.57	0.04
3.	280	0.666	0.716	0.69	0.04

3.2. Maceration Extraction Method

Maceration is a solvent extraction method that is applied by soaking the dried biomass materials in solvent (distilled water). This method has some drawback like use of large amount of solvent, long extraction time and generally low yield, and is being replaced by more environmentally friendly, functional, fast methods.

Two sets of extractions were made, the first using an equal amount of PC powder (20 mg) to that used in the microwave method to compare the two methods; and the second one of 600 mg of PC powder, to produce larger quantity of extracted PC for further solubility experiments.

3.2.1. Materials

Three 200 mL bottles with caps, magnetic stirrers, clamps, spatulas, measuring scale balance, Petri-dish, Aluminum foil paper, dried spirulina powder and stop-watch.

3.2.2. Method

20 gram of dried powdered *Spirulina platensis* was weighed inside 200 mL test tube and 5 mL of the distilled water were added. Then, the mixture was put inside a rotary shaker for one cycle of 48 h at room temperature. The mixture was centrifuged at 6000 rpm for 10 mins and the supernatant UV-vis spectra was drawn. The spectrophotometer readings are presented in Table 2. The mixture was divided 2 and 3 parts for the sake of centrifuging for both maceration I and II, respectively.

This work reassessed and confirmed published paper of Mari Carmen Ruiz-Dominguez *et al.* [12] that water, being a green solvent proof to be a good extraction solvent for phycocyanin using maceration method. It also observed the solubility of extracted phycocyanin in water and the prepared DES.

According to Chaiklahan *et. al.* [16], the total concentration of PC is calculated considering the total amount of PC, that absorb light at 620 nm, and allophycocyanin, that absorb light at 652 nm. Bennette and Bogorad [18] used the following equations 1, 2 and 3 to calculate the total concentration of PC.

Table 2. Absorbance of PC extract from Spirulina Platensis using the microwave method.

a)	Absorbance				
	wavelength (nm)	Sample 1	Sample 2	Average	Standard Deviation
1.	620	1.426	1.418	**1.422**	**0.006**
2.	652	1.115	1.110	**1.113**	**0.004**
3.	280	1.156	1.153	**1.155**	**0.002**

b)	Absorbance					
	wavelength (nm)	Sample 1	Sample 2	Sample 3	Average	Standard Deviation
1.	620	2.275	2.278	1.662	**2.0**	**0.4**
2.	652	1.532	1.445	1.332	**1.4**	**0.1**
3.	280	1.685	1.369	1.498	**1.5**	**0.2**

$$C_{PC}\left(\frac{mg}{mL}\right) = \frac{A_{620} - 0.474A_{652}}{5.34} \qquad Equation\ (1)$$

$$C_{PC}\left(\frac{mg}{g}\right) = \frac{(PC_{conc.}) \times V}{dw} \qquad Equation\ (2)$$

$$Purity\ (P) = \frac{A_{620}}{A_{280}} \qquad Equation\ (3)$$

where C_{PC} is the concentration of phycocyanin in mg/mL, A_{620} and A_{652} represent the absorbance at 620 nm and 652 nm, V is the volume of solvent used in mL and dw represents the dry biomass used in g.

3.3. Comparison of the Extraction Methods

Using Bennette and Bogorad [18] equations, equations (1), (2) and (3) above, the concentration of PC (in mg/mL and in mg/g) and the purity of extracted PC using both the Microwave and the Maceration Methods were calculated and are listed in Table 3. The values used for these calculations are the average value reported in Table 1 and 2.

It can be observed that a higher concentration of PC in the extract and a higher purity was observed for the maceration method (48 h) in comparison with the microwave method (30 s).

Table 3. PC concentration and purity of the two extraction methods used in this work.

Method	C_{PC} (mg/mL)	C_{PC} (mg/g)	P
Microwave	0.087	21.815	1.063
Maceration I	0.168	41.886	1.232
Maceration II	0.260	65.115	1.375

Thus, apart from the length of time required, maceration method is preferred when PC yield , purity and set-up cost is considered. The extracted PC is reagent grade since value of purity obtained from both methods is between 0.7 and 3.9. It is also interesting to notice that in the 2nd maceration procedure a higher concentration and purity of PC was obtained in comparison to the 1st extraction performed using the same methodology and biomass-solvent ratios. This can be because a higher mass of Spirulina was used and the fact that no heating was involved in the extraction process. Also, the experimental procedure was not fully established in the lab and two experiments were not enough to obtain fully reproducible results.

4. DES for stabilizing PC from Spirulina platensis

4.1. Preparation of Deep Eutectic Solvents

Two different sets of DES or NADES were prepared: in the first set the same HBD, Polyethylene glycol-200 (Peg-200), was used with four different HBA; in the second set the same HBA, Choline Chloride (ChCl), was used with another four different set of HBD. To be mentioned that a chemical compound can act as HBD or HBA depending on the other DES component. Stirring and heating was the preferred method for DES preparation.

4.1.1. Materials and Methods

The HBA, HBD chemicals, distilled water, capped vials, spatula, magnetic stirrer with temperature measuring devices, and measuring balance. All the HBA and HBD, their suppliers and purities are listed in Table 4. Only Malic Acid was supplied by ACROS, New Jersey, USA.

4.1.2. Preparation of Peg-200 based DES

Peg-200 DES were prepared by mixing and heating at stoichiometric ratios, previously determined, and published in the literature by other authors, of Peg-200 and each one of the HBA. These ratios were carefully chosen so that a homogeneous liquid would be obtained from the mixture of the two compounds. The composition of the DES prepared and the HBA are listed in Table 4.

Table 4: Stoichiometric ratios of the Peg-200 DES components

	HBD component	HBA components	Supplier	Stoichiometric ratios
1.	Peg-200 99% purity Supplier: Sigma Aldrich	Tetramethylammonium bromide (TMAB - $C_4H_{12}BrN$ – 99% purity)	Sigma Aldrich Germany	1:2
2.		Tetra-n-Phosphonium bromide (TBPB - $C_{16}H_{36}BrP$ – 99% purity)	Sigma Aldrich Germany	1:2
3.		Tetrabutylammonium bromide, (TBAB - $C_{16}H_{36}BrN$ – 99% purity)	Sigma Aldrich Germany	1:2
4.		Aliquat 336 ($C_{25}H_{54}ClN$ – 99% purity)	Sigma Aldrich Germany	1:2

The actual amount in grams (g) of each component was calculated and weighed out with

the use of scale type OHAUS (*e = 0.01 g, d = 0.001 g*). Then, the mixtures were heated at 70 ºC and magnetically stirred at 405 rpm for about 30-90 minutes until uniform liquids were formed as shown in Figure 3.

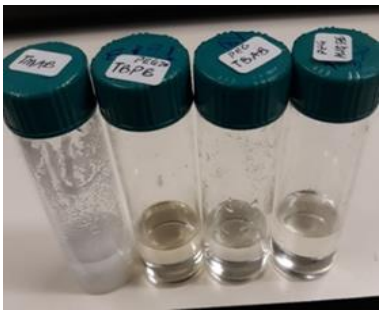

Figure 3. Photograpgh of the Peg-200 based DES prepared in this work.

As it can be seen from Figure 3, all the DES are liquid at room temperature, except for TMAB:Peg-200 DES which was obtained as a liquid with a solid in suspension. A possible explanation for this fact is that the amount weighted was not accurate enough and the composition prepared fall inside this region of the phase diagram.

4.1.3. Choline Chloride based DES

The preparation of this set of choline chloride-based DES followed the same procedure as those based on Peg-200, using the mixing and heating procedure. Choline chloride was mixed with each one of the HBD listed in Table 5. Again, the stoichiometric ratios were taken from literature. DES prepared are presented in Figure 4. Again, two of the DESs, ChCl:Citric Acid, (1:1) and ChCl:Peg-200 (1:2) did not yield a homogeneous liquid solution and thus were discarded. Nevertheless, there are some authors in literature that add water to DES, to make them a completely homogeneous solution. This was done to ChCl:Citric Acid DES with the addition of water at molar ratio (1:1:6), while ChCl:Peg-200 NADES at (1:3) was prepared and since it is a homogeneous liquid it was used in the PC apparent solubility tests as reported in Table 5 .

Table 5: Stoichiometric ratios of the Choline Chloride DES components

	HBA component	HBD components	Supplier	Stoichiometric ratios
1.	Choline Chloride Purity – 99+% Supplier: Sigma-Aldrich Germany	Oxalic acid - $(COOH)_2$ purity 99+%	Sigma-Aldrich Germany	1:2
2.		Malic acid - $C_4H_6O_5$ Purity – 99+%	ACROS New Jersey, USA	1:1
3.		Peg-200 - $H(OCH_2CH_2)_nOH$ Purity – 99%	Sigma-Aldrich Germany	1:2
				1:3
4.		Citric acid - $C_6H_8O_7$ Purity – 99.5%	Sigma-Aldrich Germany	1:1
5		Citric acid + water		1:1:6

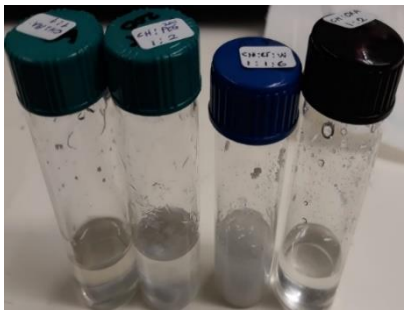

Figure 4. Choline Chloride-based DES prepared.

5. Apparent Solubility determination

To better evaluate which DES should be used in the extraction of PC from *Spirulina platensis,* the solubilities of PC in the prepared DES could yield valuable information about the interactions between PC and the solvent. Since it was not possible to prepare a calibration curve, the apparent solubility was determined. Apparent solubilities of PC in the prepared DES were determined by adding a known small quantity of PC to 1 g of DES in a capped vial with continuous stirring at 50 ºC, until PC precipitation was observed. The last addition of PC that is still

soluble in DES is taken as the apparent solubility. Due to the high viscosity of the prepared DES at room temperature, the apparent solubility was measured at 50ºC.

5.1. Solubility of PC in Peg-200 based DES

The apparent solubility of PC in 1 g of Peg-200-based DES is listed in Table 6. The extracted PC was found to be much less soluble in the ALIQ366:Peg-200 DES than in the other two DESs, which are based on TBAB and TBPB salts. Also, the PC solubility in these other two DES is very similar which is probably due to the similarity between the salts, just changing the ammonium core center for a phosphonium core center. Aliquat 366 is a more hydrophobic salts than these other two salts, with longer hydrocarbon chains (methyltrioctyl ammonium chloride) and this is probably the explanation for the low PC solubility in DES containing this salt.

Table 6. Apparent solubility of PC in Peg-200 based DES (g/g).

HBA	PC Solubility (g/g)
TBAB	0.0554
ALIQ. 336	0.0380
TBPB	0.0516

5.2. Apparent Solubility of PC in ChCl-based DES

The total amount of PC that dissolved in 1 g of ChCl-based DES is listed in Table 7. The extracted PC was found to be more soluble in ChCl : Malic acid DES than the other two DESs as shown in Figure 13.

Table 7. Apparent solubility of PC in ChCl based DES (g/g).

HBD	PC Solubility (g/g)
Malic acid	0.1325
Oxalic acid	0.0859
Peg-200	0.0808
Citric acid + water	0.0202

From the results of Table 7, it can be concluded that PC has the highest solubility in the ChCl:Malic Acid DES, followed by ChCl:Oxalic Acid and ChCl:Peg-200 and finally, ChCl:Citric Acid presents the lowest solubility. A comparison between the apparent solubilities for the two classes of DES indicates that there is not much of a difference in the PC apparent solubility when ChCl, TBAB and TBPB are used, in combination with small chain organic acids or alcohols, such as Peg-200. The very low apparent PC solubility for ChCl:Citric Acid:Water DES is probably due to the water presence and more stronger hydrogen bond. It is also interesting to observe the PC color in these DES: green in ChCl:Malic Acid DES, greenish brownish in ChCl:Peg-200 DES and blueish in ChCl:Citric Acid:Water NADES. Also, to be mentioned that PC precipitated in ChCl:Oxalic Acid DES with a grey color as shown in Figure 5.

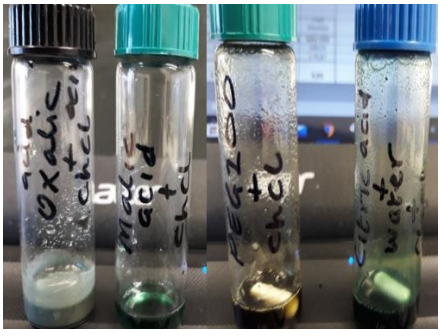

Figure 5. PC solubilized in ChCl based DES

6. Results and Discussions

Both the spirulina powder and the extracted phycocyanin were carefully shielded with aluminum foil paper to prevent them from sunlight oxidation since phycocyanin is known to be highly sensitive to environmental conditions like light, temperature, etc., [20]. The color observed during solubility of the PC in the prepared NADES proved that only ChCl:Malic Acid and ChCl:Citric Acid have potential to be used for maintaining the blue-green color of the PC. The bluish green color produced when extracted PC was dissolved in ChCl:Citric Acid can be attributed to chelating effect of citric acid [16] and its ability to stabilize protein fraction conformation of PC solution. The other two DESs. ChCl:Peg-200 and ChCl:Oxalic acid did not show any potential as stabilizing agent for PC.

7. CONCLUSIONS

When yield of 41.886 mg/g (0.1675 mg/ml), purity 1.37 and costs are considered, maceration method (48 h) was preferred for PC extraction with distilled water at biomass-solvent ratio 4:1 and room temperature. The PC is observed to be of reagent grade. Also, *Spirulina platensis* was confirmed to be a good source of PC, to be extracted for diverse purposes.

Established method of heating and stirring at temperatures below 100 ºC for 30-90 mins was adopted to prepare two groups of DES or NADES using Peg-200 and ChCl, respectively.

Apparent solubility of PC in the prepared DES was determined and both Peg-200:TBAB and ChCl:Malic Acid were observed to have the highest apparent solubilities of 0.0554 g/g of DES and 0.1326 g/g of NADES, respectively.

PC showed a blueish green color when in solution with this solvent, while in ChCl:Malic Acid it acquired a green color. This shows that the two NADES possess the potential to be used as PC stabilizers. The low solubility of PC in these DES or NADES is probably due to their high viscosity. Consequently, this property needs to be further tuned for better results.

PC high solubility in distilled water and its subsequent color stabilization in edible preservatives like citric acid and malic acid, confirm PC as a suitable pigment of high economic consideration and safety for food industry.

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