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Application of aqueous biphasic systems based on pluronic copolymer and deep eutectic solvent to achieve purified C phycocyanin

Alireza Ebrahimi[1], Gholamreza Pazuki[1]✉, Mehrdad Mozaffarian[1]✉, Farzaneh Ghazizadeh Ahsaie[1] & Hamed Abedini[2]

The idea of isolating C-phycocyanin from Spirulina platensis microalgae to be used as a valuable protein has a long history, so that today, with the advancement of science and application of various technologies, the isolation of this protein occurred by one of the most important processes. C-phycocyanin has multiple applications in pharmaceutical, cosmetic and food industries as an additive and natural dye. In this research, the aqueous two-phase system (ATPS) based on block copolymers and deep eutectic solvents (DES) was used for C-phycocyanin purification and their phase behavior was evaluated. This method can be used as an efficient and cost-effective method for the extraction and purification of C-phycocyanin compared to other purifying processes such as ion exchange chromatography. After that, the effects of parameters such as copolymer structure, DES type, DES concentration, copolymer concentration and system temperature in the ATPS containing copolymer/DES were studied for C-phycocyanin purification. The results showed that the aqueous biphasic system containing Pluronic 10R5 and ChCl-Glu is the most suitable system for obtaining the food grade C-phycocyanin. The purity index was 2.7 obtained at 25% copolymer concentration, 35% ChCl-Glu concentration and at 35 °C. At these conditions, a 97% C-phycocyanin extraction efficiency was achieved without any loss of stability. After that, the ultrafiltration process was used to increase the purity of C-phycocyanin from 2.7 to 4.8. Pluronic 10R5 was recovered during the extraction process at a temperature above 57 °C. Finally, the purity of C phycocyanin was confirmed by using SDS-PAGE analysis.

Keywords Pluronic copolymers, Deep eutectic solvents, C-phycocyanin, Spirulina platensis, Aqueous two-phase systems, Purification

C-Phycocyanin (CPC) derived from Spirulina platensis is a major phycobiliprotein that has various pharmacological properties, which enables it to offer different benefits namely as antioxidant, neuroprotective, immune enhancer and/or anti-inflammatory agent[1,2]. C-phycocyanin is employed in food additives, cosmetics, fluorescent dye, biotechnology, hepatorenal protection and medicine. CPC is also applied as a nontoxic photosensitizer for the photodynamic therapy (PDT) of tumors[3-6]. Therefore, many methods have been used to extract valuable and economic gains out of CPC, including ion exchange chromatography, ultrafiltration, ammonium sulfate precipitation, ultrasound-assisted separation, electrophoresis and membrane separation[7,8]. Even though these separation and purification methods provide high purity and efficiency, they are not suitable due to their high cost and complexity[9]. Moreover, using water and organic solvents as conventional liquid-liquid extraction systems are not suitable for proteins' purification, because proteins lose their biological activities in organic solvents[10]. However, despite the relatively high purity outcome of these methods, the extraction efficiency of C-phycocyanin was low. Lauceri et al. used the ultrasound-assisted cell lysis method to extract C-phycocyanin[11],but the results showed that the extraction efficiency was only 28%.

[1]Department of Chemical Engineering, Amirkabir University of Technology (Tehran Polytechnic), Tehran 15875-4413, Iran. [2]Mechanical Engineering Department, KU Leuven, Leuven 3000, Belgium. ✉email: ghpazuki@aut.ac.ir; mozaffarian@aut.ac.ir

Recently, aqueous two-phase systems (ATPS) have attracted more research interest as one of the most important procedures for purification of proteins due to their advantages such as environment-friendly, nontoxic, continuous operation, low cost and easy scale up[12-15]. Aqueous two-phase system (ATPS) is a liquid-liquid extraction process that does not use organic solvents. The majority of this system is water and includes two heterogeneous phases with partially immiscible components, which can be more useful for separation of proteins. The aqueous solution splits into two phases above certain concentrations of components. The basis of extraction in these systems is the unequal distribution of biomolecules between two phases due to the difference in physicochemical properties of the phases. The efficient parameters on phase behavior and partitioning of the biomolecules in the ATPS are the interaction between the biomolecule and phase components, phase forming properties, pH, temperature and so on[16].

One of the common ATPSs is the application of ionic liquids (ILs), which were first found in 2003 by Bridges[17]. ILs have various distinctive properties consisting of low vapor pressure, non-flammability, superior solubility, wide liquid range, excellent thermal stability and negligible volatility[18,19]. Additionally, they provide short separation time, low energy consumption and efficient extraction. Based on these characteristics, ionic liquids have considerable applications in the fields of extractions and purification of diverse biomolecules[20,21]. Chang et al. applied different combinations of IL to extract CPC from Spirulina platensis. The results indicated that the separation efficiency for CPC was 99% [22]. IL-ATPSs have been effective for selective separation of proteins[23]. However, the preparation process of ILs is intricate, uneconomical and difficult to purify and even small impurities can adversely affect its performance. Moreover, some ionic liquids like imidazolium ILs have a negative effect on the environment due to their high biotoxicity and negligible biodegradability. These deficiencies have limited their application in large-scale productions[22]. Therefore, it is necessary to find a suitable environmentally friendly alternative such as deep eutectic solvent (DES) for selective separation of protein[24]. Deep eutectic solvents are the eutectic solutions made from the hydrogen bond acceptors (HBA) and the hydrogen bond donors (HBD) with proper molar ratios. They have unique characteristics including low biotoxicity, good biodegradability, inexpensive and simple synthesis[25-28] .So far, fundamental progress has been observed in the field of DES-based ATPS for partitioning of biomolecules such as proteins[29], antibiotics and amino acids[30,31]. Zeng and Xu et al. have applied ChCl-based DESs for extracting of proteins[24,32]. The potential of DES/salt in ATPS for C-phycocyanin purification was recently reported by Wang and co-workers[33]. The purity and recovery of C-phycocyanin obtained in ATPS, based on choline chloride (ChCl) -urea/ K_2HPO_4 were 3.383 and only 65.64%, respectively. Moreover, in another study, Zhuang et al.[34] reported that the extraction efficiency of C-phycocyanin ATPS based on ChCl-Urea/ K_2HPO_4 was 94.2%, but C-phycocyanin purity index was 1.25. Rathnasamy et al.[29] has applied the microwave-assisted biphasic DES system to extract C-phycocyanin with an extraction efficiency of 86%. This was the evidence for the validity of DES as an extractant for C-phycocyanin.

It is worth noting that other ATPSs have been employed in the separation field, including polymer/salt ATPS[35,36], polymer/polymer ATPS[37], copolymer/polymer[38] and PEG-PPG copolymers/salt ATPS[39]. Unlike other ATPSs, one of the salient features of copolymers as smart polymers is that their hydrophilicity decreases with increasing temperature, which leads to their recovery by heating[40,41]. Ebrahimi et al. investigated the separation and purification of C-phycocyanin from contaminates by copolymer-based ATPS[42]. Haraguchi et al. reported the influence of copolymer hydrophobicity on phase behavior of insulin in an ATPS based on thermoseparating copolymer[43]. They realized that hydrophobicity of copolymer had higher efficiency on insulin partitioning. Furthermore, many papers have systematically indicated the application of ATPS based on tri-block copolymers and DESs to isolate different biomolecules[44,45,46].

As far as we know, there was no report on purifying C-phycocyanin using DES-copolymer ATPS. So, in this study, the extraction and purification of C-phycocyanin from Spirulina platensis by an ATPS based on DESs and PEG-PPG copolymers (Pluronic 10R5 and L35) as a green and efficient method will be a first attempt. Herein, a series of DESs based on choline chloride with different HBD (fructose, glucose, Ethylene glycol and glycerol) have been successfully synthesized to purify this protein in copolymer-DES ATPSs. For this purpose, the complete binodal curves were developed at room temperature for the above ATPSs. The effects of temperature, types of DES and copolymer, DES concentration and copolymer concentration on partition coefficient, extraction efficiency and purification of C-phycocyanin in optimal ATPS were investigated. The recovery and reuse of copolymer in aqueous two-phase systems was explored. Finally, the ultrafiltration process was selected to achieve a higher purity index.

Materials and methods

Materials

Materials used in this study are reported in Table 1. Samples were prepared in double distilled water with a conductivity of 0.08 $\frac{\mu s}{cm}$.

Methods

Preparation of DESs

In this study, four different DESs, which consisted of one hydrogen-bond acceptor (Choline chloride) and hydrogen bonding donors (fructose, glucose, Ethylene glycol and glycerol) were prepared using a stirring-heating step at 80 ℃ and 200 rpm for 4 h until a transparent liquid was observed[32,47].

Solid-liquid extraction (SLE)

The first step to obtain protein in microalgae involves separation of solid from liquid, and in most cases, the biomolecules were released by breaking algae cells[48,49]. In this way, the microalgae cells were broken up by a chemical method (decomposition by a chemical), and all constituents such as carbohydrates, fatty acids, lipids, proteins, chlorophyll, etc. were removed along with the cell residues. In this work, sodium phosphate buffer

Chemical Name	Molecular formula	M_w (gr/mol)	Purity (wt%)	Source	CAS number
Choline chloride	$C_5H_{14}ClNO$	139.62	≥99%	Sigma Aldrich	67-48-1
Fructose	$C_6H_{12}O_6$	180.16	≥99.5%	Scharlau (Spain)	57-48-7
D- (+)-Glucose	$C_6H_{12}O_6$	180.16	≥99.5%	Sigma Aldrich	50-99-7
Ethylene glycol	$C_2H_6O_2$	62.07	≥99%	Sigma Aldrich	107-21-11
Glycerol	$C_3H_8O_3$	92.09	≥99.5%	Synth	56-81-5
L35 copolymer	$(EO)_{11}$-$(PO)_{16}$-$(EO)_{11}$	1900	≥99%	Sigma Aldrich	9003-11-6
10R5 copolymer	$(PO)_{22}$-$(EO)_8$-$(PO)_{22}$	2000	≥99%	Sigma Aldrich	9003-11-6
Spirulina Platensis	70–110 kDa	-	-	Spiru (Iran)	

Table 1. Molecular formula, molecular weight (Mw), purity, CAS number and source for materials.

(NaPB) was used as an aqueous solution at 20mM and pH = 7.4 in accordance with the method applied by Sarada et al.[50]. Therefore, NaPB and the fresh biomass were blended with a solid-liquid ratio of 0.1. Then the mixture was placed in a thermomixer at 25 °C and operated for 60 min at a speed of 1500 rpm. It should be noted that the mixture should not be exposed to light for the duration of all extraction stages due to the high sensitivity of pigments to light. Finally, the samples were centrifuged at 12,000 rpm for 10 min. Then, the cell debris was separated, and the crude extract containing more C-phycocyanin was collected, and its amount is measured by UV spectrophotometer at 620 nm. This solution was kept at 4 °C for further experiments.

Determination of phase diagrams and Tie-lines

To apply the binodal curve, the cloud point titration method was used. In this method, an aqueous solution is prepared from both components of the system with a known concentration[42]. The concentration of aqueous solution made from each component is different and depends on the ability of that component to form two phases. In most of the studied systems, copolymer solution was used as the base solution. This solution with a known mass fraction was provided gravimetrically and then, a DES solution of known mass fraction was added to the base solution dropwise until the base mixture became cloudy. Then, the composition of this solution was calculated. To determine the next point of the binodal curve, a drop of deionized water was added to the base solution and the weight of added water was recorded (clear and single-phase solution was obtained) and this method was repeated. It should be noted that each point of binodal curve was determined by quantifying the weight. The composition percentage of each component can be calculated for each point of the curve. The experimental binodal curves were adjusted by the following three-parameter Eq. 5[1]:

$$[COP] = A \times \exp\left[\left(B \times [DES]^{0.5}\right) - \left(C \times [DES]^3\right)\right] \tag{1}$$

Where [COP] and [DES] are mass fraction percentage of the copolymer and DES, respectively, and A, B and C are fitting parameters obtained by experimental data regression. Merchuk et al. first introduced a gravimetric method for determination of ATPS Tie-lines (TLs)[51]. In this method, the compositions of the Top and Bottom phases are determined by the lever-arm law:

$$[COP]_{Top} = A \times \exp\left[\left(B \times [DES]_{Top}^{0.5}\right) - \left(C \times [DES]_{Top}^3\right)\right] \tag{2}$$

$$[COP]_{Bot} = A \times \exp\left[\left(B \times [DES]_{Bot}^{0.5}\right) - \left(C \times [DES]_{Bot}^3\right)\right] \tag{3}$$

$$[DES]_{Top} = \frac{[DES]_F}{\alpha} - \frac{1-\alpha}{\alpha}[DES]_{Bot} \tag{4}$$

$$[X]_{Top} = \frac{[X]_F}{\alpha} - \frac{1-\alpha}{\alpha}[X]_{Bot} \tag{5}$$

The subtitles F, Top, and Bot indicate the feed, the top phase, and the bottom phase, respectively. Also, the parameter α shows the weight ratio of top phase to feed.

Tie line length (TLL) and its slope (STL) are determined by Eqs. (6) and (7)[52]:

$$TLL = \sqrt{\left([DES]_{Top} - [DES]_{Bot}\right)^2 + \left([COP]_{Top} - [COP]_{Bot}\right)^2} \tag{6}$$

$$STL = \frac{[COP]_{Top} - [COP]_{Bot}}{[DES]_{Top} - [DES]_{Bot}} \tag{7}$$

C-phycocyanin separation

Aqueous biphasic systems consisting of copolymer, DES and water were prepared by weight method. 1 gram of crude extract containing c-phycocyanin was added to 9 g of each mixture point (10% by weight). The mixture was stirred and centrifuged at 2700 rpm for 15 min, then incubated at 25 °C for 24 h. The phases rich in copolymer (top phase) and rich in salt (bottom phase) were carefully separated and the mass and volume of these phases

were measured. The C-phycocyanin's amount was measured by UV-vis spectrophotometer (Shimadzu, Japan, model UV-200 S) at a wavelength of 615 nm, and its concentration was calculated by the following Eq. 5[3]:

$$CPC\left(\frac{mg}{ml}\right) = \frac{[OD]_{615} - 0.474\,[OD]_{652}}{5.34} \tag{8}$$

Where, OD_{652} and OD_{615} represent the absorbance at 652 nm and 615 nm, respectively.

C-phycocyanin partition coefficient (K), top and bottom phases' extraction efficiency (E) and purity index obtained from the ATPS were determined using Eqs. (9 through 12)[54]:

$$K_P = \frac{[CPC^{Top}]}{[CPC^{Bottom}]} \tag{9}$$

$$E_{Top} = \frac{K_P V_R}{K_P V_R + 1} \tag{10}$$

$$E_{Bottom} = \frac{1}{K_P V_R + 1} \tag{11}$$

$$Purity\ index = \frac{A_{620}}{A_{280}} \tag{12}$$

Where, V_R represents the volumetric ratio of top phase to bottom phase. To measure the volume of the phases, a 10–100 µl sampler was used. It should be stated that all experiments were carried out in three independent trials and the presented results are the average of obtained partition coefficient, recovery and purity index.

Results and discussion

Solid-Liquid extraction

The optimal conditions for extracting C-phycocyanin from Spirulina platensis algae were at 25 °C, solid-liquid ratio of 0.1 (1 g of fresh biomass is added to 10 ml of buffer), sodium phosphate buffer at pH = 7, 1500 rpm and extraction time of 60 min, so the achieved extraction yield and purity index of C-phycocyanin were 84 $\frac{\text{mg of C-phycocyanin}}{\text{g of fresh biomass}}$ and 0.43, respectively at the end of the extraction process.

Phase behavior

In this study, binodal curves of the ATPSs consisting of Pluronic 10R5, Pluronic L35 thermo-separating tri-block copolymers and DES including choline chloride with hydrogen bond donor compounds (HBD) including ethylene glycol (ChCl-EG), glucose (ChCl-Glu), glycerin (ChCl-Gl) and fructose (ChCl-Fr) are shown in Figs. 1 and 2. The adjustment parameters obtained by fitting the experimental data are presented in Table 2.

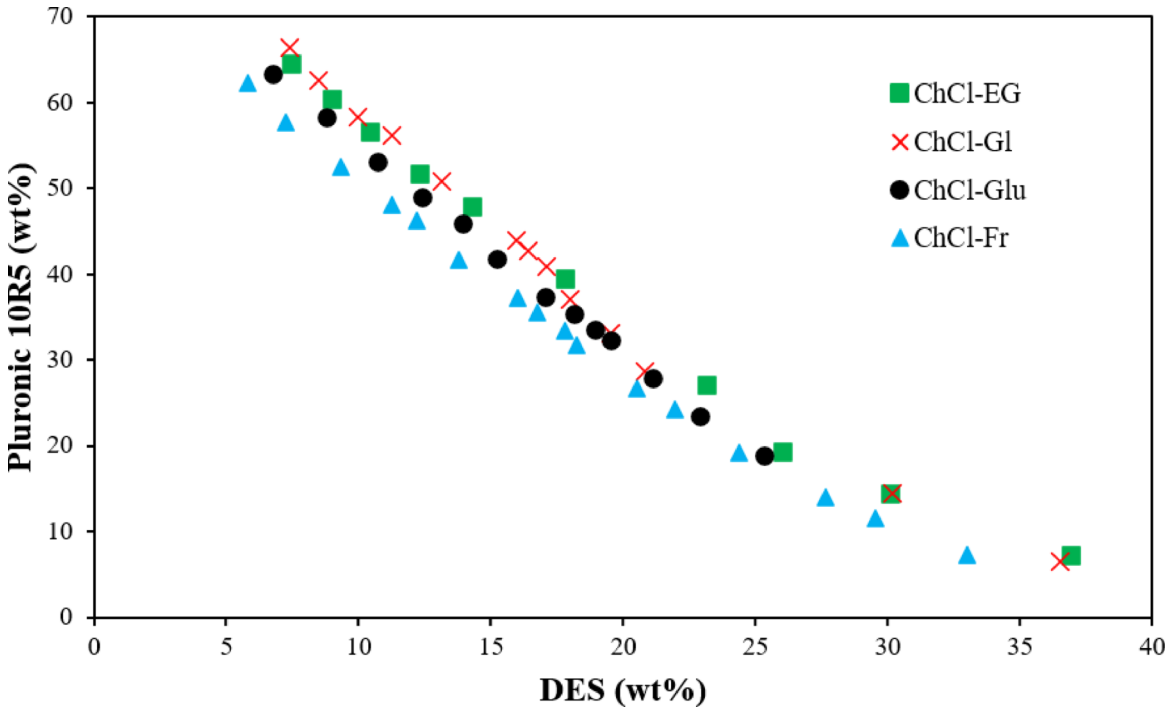


Fig. 1. Binodal curves of ATPS including different DES and 10R5 copolymers.

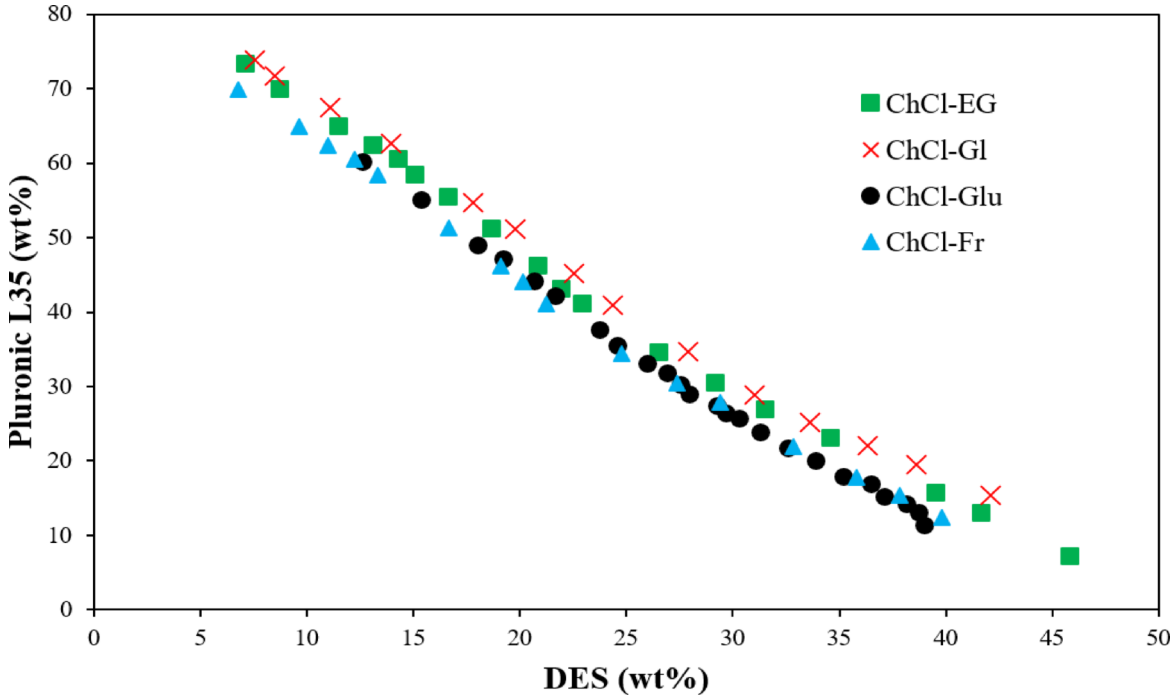


Fig. 2. Binodal curves of ATPS including different DES and L35 copolymers.

Copolymer	DES	A ± σ	B ± σ	C ± σ
Pluronic 10R5	ChCl-EG	119.81 ± 4.33	-0.220 ± 0.031	0.034 ± 0.005
	ChCl-Gl	134.76 ± 5.12	-0.253 ± 0.027	0.033 ± 0.004
	ChCl-Fr	109.03 ± 2.21	-0.229 ± 0.020	0.041 ± 0.003
	ChCl-Glu	106.31 ± 1.89	-0.194 ± 0.042	0.047 ± 0.003
Pluronic L35	ChCl-EG	118.53 ± 6.10	-0.172 ± 0.033	0.016 ± 0.006
	ChCl-Gl	120.36 ± 5.84	-0.171 ± 0.068	0.014 ± 0.005
	ChCl-Fr	114.46 ± 2.65	-0.179 ± 0.046	0.018 ± 0.005
	ChCl-Glu	129.41 ± 3.52	-0.204 ± 0.038	0.018 ± 0.007

Table 2. Merchuk parameters (A, B and C) obtained by fitting the experimental data related to the binodal curve (Eq. (1)).

It is clear that the types of DES shown in these figures will not significantly change the phase equilibria, and as a result, the formation of the two-phase region. The effect of any HBD type on formation of ATPS depends on its level of hydrophilicity/hydrophobicity, so that the higher the hydrophilicity of HBDs, the smaller the two-phase region[55]. Table 3 shows the logarithms of the water-octanol distribution coefficient (log KOW) for different HBDs with their structures. (Log KOW) shows the amount of hydrophilicity/hydrophobicity of a molecule in such a way that the smaller this parameter is, the more hydrophilic the substance is. This explanation can also be deduced from the number of hydroxide functional groups of each HBA. The more hydrophilic molecule acts exclusively as an auxiliary to regulate the properties and characteristics of the phase. The effect of these compounds on the formation of the ATPS is minor and Independent of HBD concentration. Therefore, the presence of ethylene glycol, glucose, fructose and glycerin as HBDs did not have much effect on the binodal curve due to their high hydrophilicity.

Unlike the DESs mentioned above, the copolymer structure has an important effect on the binodal curve. As shown in Fig. 3, in all discussed ATPSs, the tendency of 10R5 copolymer for phase separation is better than that of L35 copolymer, and it has a higher potential in forming an ATPS. The reason for this superiority is due to the greater hydrophobicity of 10R5 copolymer due to its reverse structure. In other words, Pluronic 10R5 with a PPG–PEG–PPG sequence, is known as a reverse copolymer, and the presence of PPG at both ends of this copolymer increases its overall hydrophobicity compared to Pluronic L35, which is a normal copolymer with repetitive PEG–PPG–PEG units. Wu et al. also showed that reverse copolymers are more hydrophobic than normal copolymers[56]. Like our work, the influence of copolymer structure on partitioning of other proteins such as cytochrome c and azocasein was examined by Vicente et al.[44].

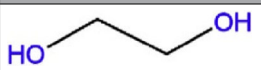
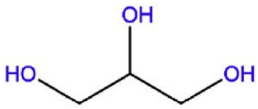
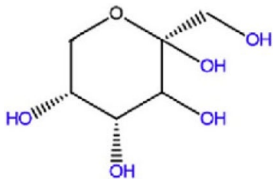
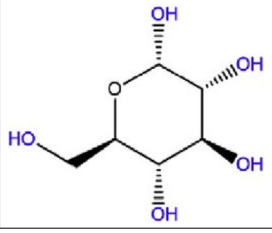
HBD Type	log (KOW)	Structure
Ethylene glycol	-1.21	
Glycerin	-1.84	
Fructose	-2.76	
Glucose	-2.93	

Table 3. Log (KOW) for different hbds[55].

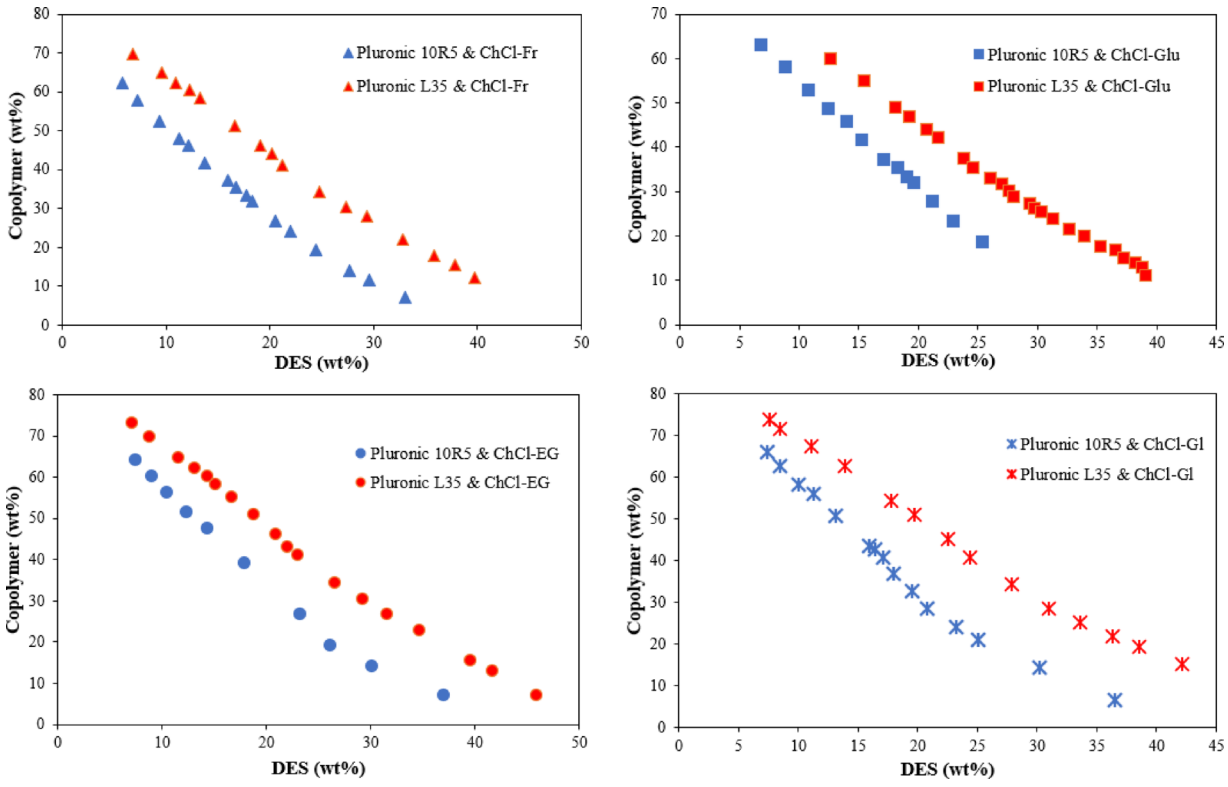


Fig. 3. Comparison of binodal curves for ATPS of 10R5 and L35 copolymers with different DESs.

Partitioning and purification of C-phycocyanin protein in the ATPSs based on DES-copolymer

After obtaining the binodal curve related to the system consisting of DES-copolymer, the effects of different hydrogen donors of DES on the separation of C-phycocyanin protein were investigated. Thus, in order to determine the partition coefficient of C-phycocyanin in an ATPS, a mixture containing 30% by weight of copolymer and 25% by weight of DES was prepared. This high composition percentage has been chosen due

to the smallness of the two-phase area in the mentioned systems. In the next step, to purify C-phycocyanin, the liquid extract obtained from the SLE process (contains C-phycocyanin and other contaminants such as other proteins and chlorophyll) was added to the copolymer-DES ATPS. After reaching the equilibria, the partition coefficient, extraction efficiency and purity of C-phycocyanin were determined in these systems and are presented in Table 4. It is clear that C-phycocyanin has migrated to the rich phase of DES solvent in the ATPS that includes copolymer-DES. C-phycocyanin is a hydrophilic biomolecule, and has a great tendency to go to the phase with high hydrophilicity (DES-rich phase). In all the investigated ATPSs based on copolymer-DES (except ChCl-EG), the protein partition coefficient was less than one and C-phycocyanin transfers to the bottom phase (DES-rich phase). It should be noted that in copolymer-DES ATPS that includes HBD of ethylene glycol, the partition coefficient is higher than 1 and as a result, C-phycocyanin has migrated to the top phase. The important point is that the top phase in this system is the DES-rich phase, and only the phases are reversed. The phenomenon of inversion of phases occurs due to the difference in density of phases in different concentrations. As seen in Table 4, the highest purity index was obtained for the ATPS consisting of Pluronic 10R5 copolymer and ChCl-Glu DES. Considering that the salting-out phenomenon for the mentioned HBDs does not have a significant effect on isolation and partitioning of C-phycocyanin, therefore, other factors such as the level of hydrophilicity can be decisive in isolation of this protein. The hydrophilicities of fructose and glucose are higher than other HBDs, which has led to better partitioning of this protein in the bottom phase (85% and 88%). Therefore, C-phycocyanin is expected to have a better purity index in the system including these two HBDs and Pluronic 10R5 compared to the systems containing other DESs. However, it is clear that fructose has the lowest purity index among all HBDs. This means that in the ATPS consisting of fructose, other contaminants also move to the bottom phase, and as a result, the purity index is greatly reduced. Therefore, the highest C-phycocyanin purity index and extraction efficiency are related to the ATPS based on ChCl-Glu (2.3 and 88%, respectively), which will be used in the following sections of this paper.

Effect of DES concentration on purification of CPC

After the binodal curves and tie lines were determined for each ATPS, the effect of DES concentration on separation of C-phycocyanin from other contaminants was studied. As seen in Fig. 4, increasing the concentration of ChCl-Glu leads to a decrease in the volume ratio of the top to bottom phases. In other words, with the increase in DES concentration, the hydrophilicity of the bottom phase increases, and more water is transferred from the copolymer phase to the DES-rich phase, leading to an increase in the volume of the bottom phase (and thus a decrease in V_R).

After adding the protein to this system, the partition coefficient, the purity index and the extraction efficiency of C-phycocyanin in the bottom phase are shown in Table 5. The results show that when the concentration of DES increases from 17 to 35%, the extraction efficiency increases and reaches 93%, but the efficiency starts to decline with any further increase in concentration. The explanation for this change is that DES and protein accumulate in the top phase. That is, with the increase of DES concentration, the number and size of DES micelles gradually increase, and therefore more C-phycocyanin are accumulated by DES micelles. Now, by further increasing DES concentration, the viscosity of the bottom phase increases and this prevents the presence of C-phycocyanin in the bottom phase, and as a result, the extraction efficiency decreases. Similar results have been obtained to investigate the effect of DES concentration on protein separation. It should be noted that according to Table 5, this trend does not apply to the C-phycocyanin purity index and this parameter has remained almost constant. The reason for this can be attributed to the simultaneous presence of contaminants along with C-phycocyanin in the bottom phase by increasing the concentration of DES. Therefore, 35% wt of DES is selected for application in the following parts of this paper.

Effect of copolymer concentration on purification of CPC

In order to investigate the effect of copolymer concentration on the selected ATPS at a fixed concentration of DES (35%), the copolymer concentration was changed from 10 to 45%. According to Fig. 5, the extraction efficiency enhances by increasing the copolymer concentration to 25%. In this way, the hydrophobicity of 10R5 copolymer increases with the rise of copolymer concentration, and as a result, the protein solubility in the top phase is

Copolymer	DES	Concentration-CPC (mg/l)		K-CPC	Extraction Efficiency	Purity $_{\text{DES–rich phase, CPC}}$
		Top	Bottom			
Pluronic L35	$ChCl-EG$	2.54	1.24	2.05	85%	1.70
	$ChCl-Gl$	1.03	2.01	0.51	74%	1.53
	$ChCl-Fr$	0.49	1.25	0.39	85%	0.98
	$ChCl-Glu$	0.87	2.71	0.32	86%	2.02
Pluronic 10R5	$ChCl-EG$	2.68	1.15	2.33	84%	1.88
	$ChCl-Gl$	0.95	2.16	0.44	79%	1.66
	$ChCl-Fr$	0.31	1.01	0.31	85%	1.02
	$ChCl-Glu$	0.70	3.03	0.23	88%	2.30

Table 4. Partition coefficient, extraction efficiency and purity index of C-phycocyanin in ATPS based on DES and copolymer.

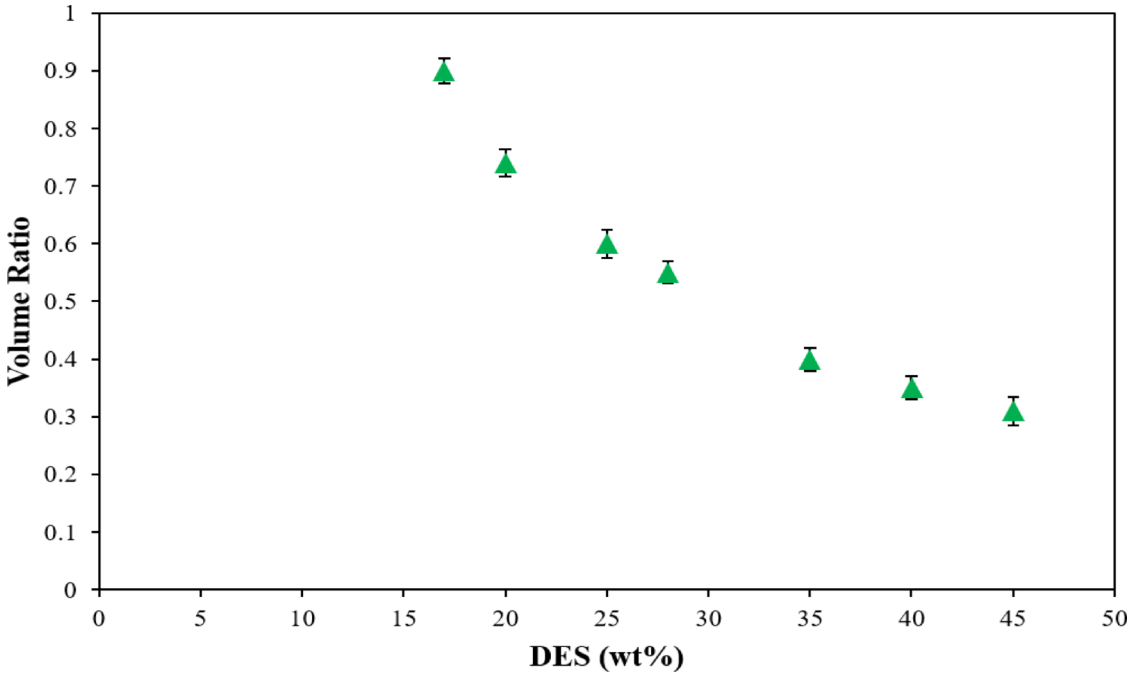


Fig. 4. The effect of ChCl-Glu concentration on the volume ratio of phases in the ATPS based on 10R5 copolymer and DES.

DES Conc (wt%)	K_{CPC}	Extraction Efficiency %	Purity
17	0.45	71%	2.21
20	0.36	79%	2.25
25	0.23	88%	2.3
28	0.21	90%	2.27
35	0.19	93%	2.28
40	0.3	90%	2.3
45	0.38	89%	2.33

Table 5. Partition coefficient, extraction efficiency and purity index of C-phycocyanin in ATPS based on DES and copolymer in different DES concentrations.

reduced and a larger amount of C-phycocyanin is transferred to the bottom phase. Therefore, the extraction efficiency increases up to 96%. In addition, as the copolymer concentration increases, the extraction efficiency decreases gradually. The reason for this is the reduction of water content in the DES-rich phase.

On the other hand, the highest purity of C-phycocyanin occurs in the bottom phase at a copolymer concentration of 25% (equal to 2.51). It indicates that the amount of contaminants transferred to the bottom phase at this concentration is less than that of C-phycocyanin.

Effect of temperature on purification of CPC

Temperature is one of the factors affecting the separation of C-phycocyanin. In order to optimize the temperature, the extraction process was carried out in the ATPS in the temperature range of 10 to 45 °C. The purity index and extraction efficiency in the ATPS including 35% of ChCl-Glu and 25% of 10R5 copolymer can be seen in Fig. 6. As the extraction temperature increases, the hydrophobicity of the copolymer in the top phase enhances and the viscosity of DES decreases. These two factors lead to movement of water molecules from the copolymer-rich phase to the DES-rich phase, which makes the bottom phase more hydrophilic and facilitates the transfer of protein to this phase. Therefore, increasing the temperature to 35 °C will increase the efficiency of C-phycocyanin to 97% and the purity index to 2.7. However, any further increase in temperature destroys the hydrogen bonding interaction between the remaining amino acid and the surface water of the protein and hinders the extraction of protein. In addition, at high temperatures, decomposition rate of C-phycocyanin increases significantly and changes its structure. The reason for the reduction of the purity index above 35 °C is due to stability of the protein at high temperatures. Chaiklahan et al. also showed that decomposition of C-phycocyanin occurs more rapidly at temperatures higher than 40 °C[57]. Consequently, 35 °C is the best temperature for C-phycocyanin extraction by the ATPS consisting of ChCl-Glu and Pluronic 10R5.

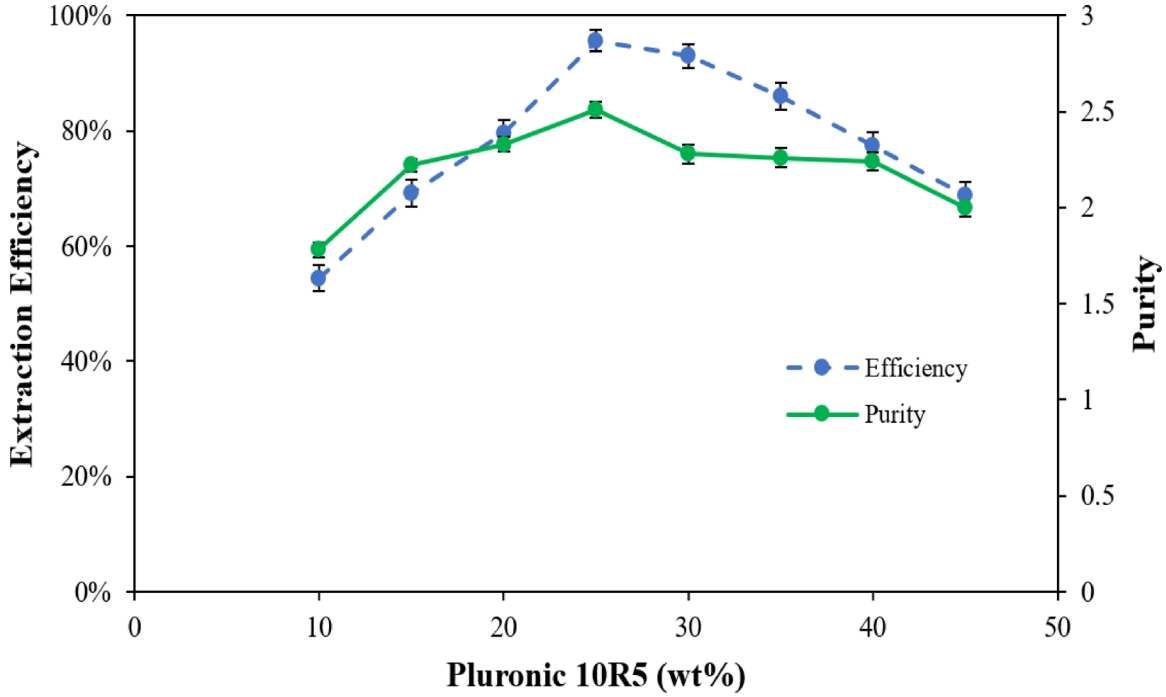


Fig. 5. The effect of copolymer concentration on extraction efficiency and purity index of C-phycocyanin in ATPS based on ChCl-Glu and 10R5 copolymer.

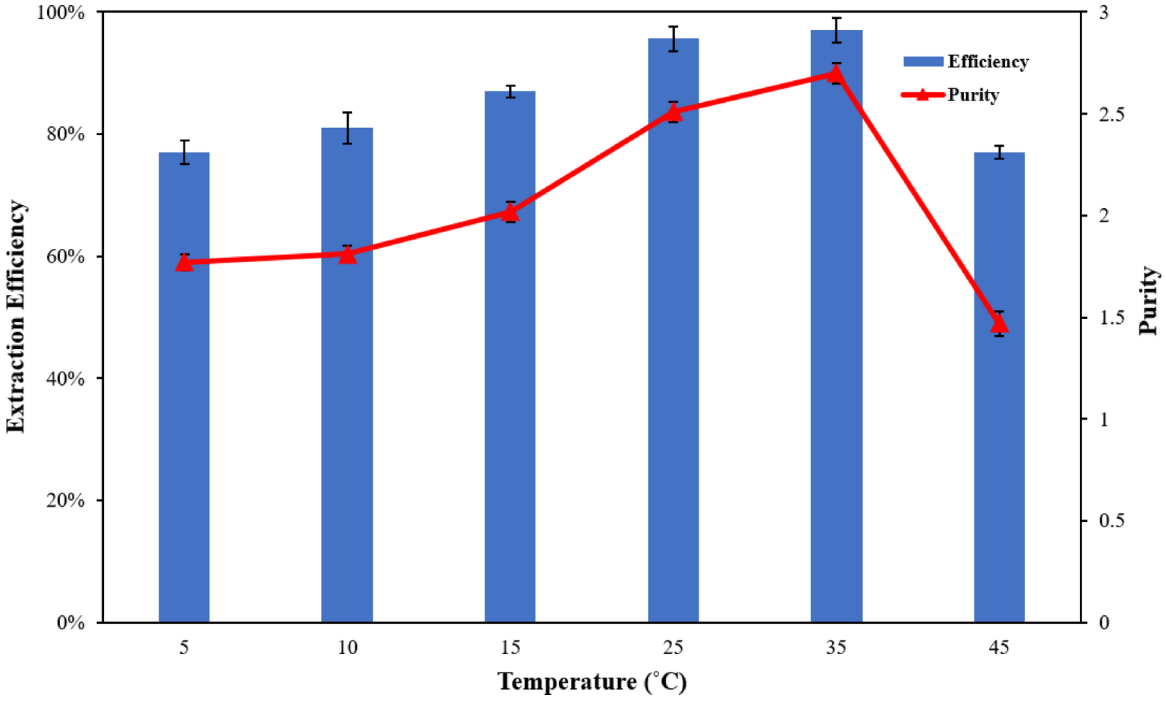


Fig. 6. The effect of temperature on the extraction efficiency and purity of C-phycocyanin in ATPS based on ChCl-Glu and 10R5 copolymer.

Copolymer recovery and reuse capability in aqueous two-phase system

One of the special capabilities of smart copolymers is that as the temperature increases, their hydrophobicity increases, leading to the formation of a two-phase system and ultimately their separation. However, as the temperature increases and exceeds the low critical solution temperature (LCST) of the copolymer, the hydrophilic interactions are weakened, and the hydrophobic interactions are strengthened, which leads to the

aggregation of solute molecules and formation of a two-phase system. Therefore, the separation of Pluronic 10R5 from water can be achieved by simply adjusting the temperature at 57℃, and 10R5 copolymer and water will be distributed in the top and bottom phases, respectively. Finally, a new optimal ATPS containing separated 10R5 copolymer, fresh 10R5 copolymer and DES was created to evaluate the purity and extraction efficiency of C phycocyanin. The results showed that the purity and extraction efficiency remained almost constant (2.8 and 97.6%, respectively), indicating that the copolymer can be effectively reused during the ATPS process. This can significantly reduce the costs of separation and purification of C-phycocyanin.

Ultrafiltration process

A separation process was applied using an Amicon Ultra Centrifugal Filter device to enhance the purification of C-phycocyanin. Afterwards, the bottom phase including the ChCl-Glu and C-phycocyanin solution was passed through ultrafiltration with a 30 kDa cut-off membrane, and the achieved purity index of C-phycocyanin was 4.8. The operating process diagram is shown in Fig. 7.

SDS-PAGE analysis of the purified C-PC

The Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) profile with original blots is shown in Fig. S1 (Supplementary Information) which presents α and β subunits with molecular weights of 18.0 and 21.0 kDa for C-phycocyanin, respectively. Lane A represents the standard molecular weight marker, Lane B indicates the crude extract that contains the C-phycocyanin and contaminants, Lane C is the purified C-phycocyanin in the bottom phase after ATPS application, and Lane D shows C-phycocyanin after the ultrafiltration process. As can be seen in the Fig. 8, reducing the number of bands in Lane C and Lane D compared to the crude extract (Lane B) indicates that most of the contaminants present in the crude extract have migrated to the top phase during ATPS and ultra-filtration, resulting in increased C-phycocyanin purity.

Conclusion

In this work, first, the SLE method was used to release C-phycocyanin and other contaminants from Spirulina platensis. After that, the aqueous two-phase systems including copolymers and types of DESs were selected to investigate the extraction efficiency of C-phycocyanin and its purification. At first, the binodal curves of ATPSs composed of two copolymers and different DESs were obtained experimentally, and then they were compared. It was observed that in the case of all used DESs, the salting-out phenomenon did not have a significant effect on partitioning and separation process, so other parameters such as hydrophilicity and hydrophobicity were investigated. Pluronic 10R5 was chosen for C-phycocyanin purification, because its hydrophobicity was higher than that of Pluronic L35, which caused the C-phycocyanin to move to the DES-rich phase. Moreover, the results showed that among all the DESs, the highest purity index and extraction efficiency are related to ChCl-Glu solvent in the ATPS consisting of 10R5/ChCl-Glu. However, the effects of DES concentration, copolymer

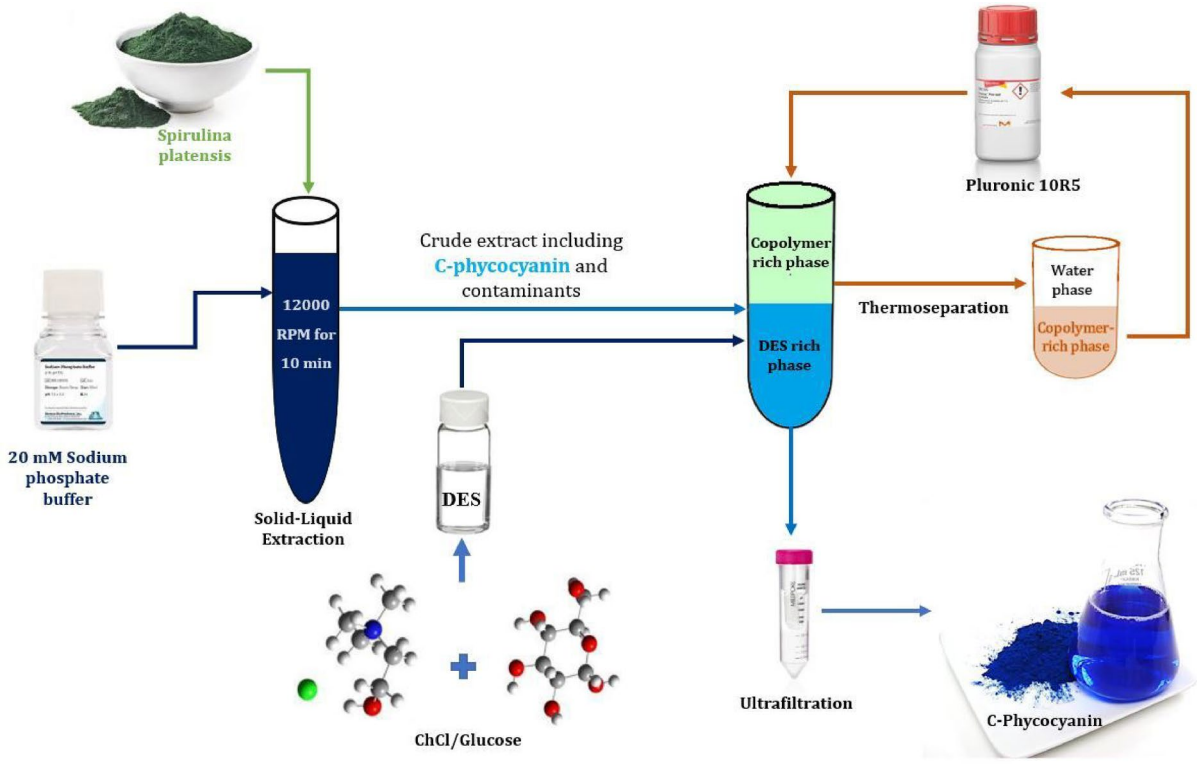


Fig. 7. The operating process diagram for purification of C-phycocyanin by copolymer/DES ATPS.

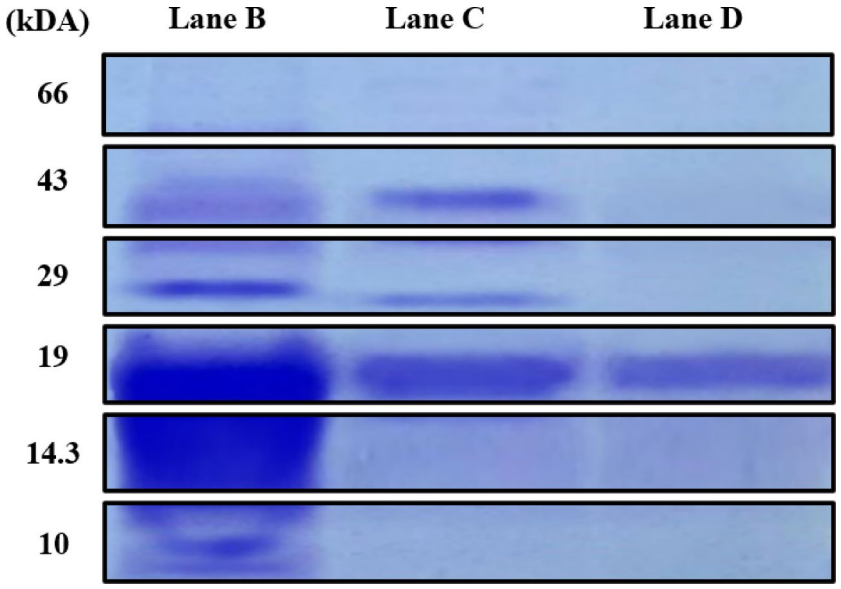


Fig. 8. SDS PAGE of C-phycocyanin, Lane A: Molecular Marker; Lane B: Crude extract; Lane C: C-phycocyanin after ATPS (bottom phase), Lane D: C-phycocyanin after ultrafiltration process.

concentration and temperature were studied in this system. The highest efficiency of 97% and purity index of 2.7 were achieved by the 10R5/ChCl-Glu ATPS that had 35% of DES, 25% of copolymer, and was processed at a temperature of 35 °C. Note that other technologies such as microwave and ultrasound-assisted cell lysis do not have significant C-phycocyanin extraction capabilities. Finally, the Pluronic copolymer was recovered at 57 °C to be used in a new ATPS, and an ultrafiltration process was performed to achieve a C-phycocyanin purity index of 4.8.

Data availability

The datasets used during the current study are available from the corresponding author upon reasonable request.

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Author contributions
(A.E.): conceptualization, methodology, investigation, formal analysis, writing–original draft. (G.P.): investigation, writing–review and editing, supervision. (M. M.): writing–review and editing, supervision. (F.G.A.): methodology, investigation, writing–review and editing. (H. A.): investigation, supervision.

Declarations

Competing interests
The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to G.P. or M.M.

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