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Separation and purification of phycobiliproteins from *Thermosynechococcus* PCC 6715 by foam fractionation and aqueous two-phase extraction

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Abstract
The use of foam fractionation (FF) followed by aqueous two-phase extraction (ATPE) has emerged as a potential alternative to traditional liquid chromatography, hitherto irreplaceable in the purification of phycobiliproteins. The crude extracts of C–phycocyanin (C–PC) and allophycocyanin were obtained after *Thermosynechococcus* PCC 6715 biomass disintegration. The FF process with air flow of 2.4 $L \cdot h^{-1}$ resulted in purification factors up to 1.47 and partitioning coefficients of about 39, and did not require the addition of surfactants. A temperature of 35 °C allowed for the highest partitioning coefficient of 67.6 and yield of 76%; however, the purity of C–PC in condensate at this temperature was lower than at 25 °C. ATPE was tested in 20 different systems consisting of polyethylene glycol and phosphate or citrate salts, of which PEG1500-citrate gave the highest purification factor value of 2.31. Conversely, partitioning coefficients of 2416 and 1094 were obtained for the PEG1500-phosphate and PEG3000-phosphate systems, respectively. Interestingly, the use of FF condensate in subsequent ATPE step resulted, for the first time, in the separation of the polymer phase into two fractions, one contained C–phycocyanin and the other allophycocyanin. It can be concluded that the use of a two-step system of FF and ATPE is a viable way to separate phycobiliproteins.

Keywords
phycobiliproteins, purification, aqueous two-phase extraction, foam fractionation

1. INTRODUCTION

Phycobiliproteins (PBPs) are protein structures participating in photosynthesis as antenna pigments that harvest wavelengths within 470–650 nm, thus complementing chlorophylls that cannot absorb in this range (Hsieh-Lo et al., 2019). Among PBPs, the only photosynthetic pigments that are water soluble, one can distinguish blue phycocyanin (C–PC), red phycoerythrin (PE) and bluish green allophycocyanin (APC) (Eriksen, 2008). C–PC and PE are used as food dyes, pigments in cosmetics, and as fluorescent reagents in clinical or research laboratories (Spolaore et al., 2006). Moreover, C–PC plays an important role in coping with anaemia, liver disease and is regarded as a nutraceutical which strengthens the immune system having excellent therapeutic and pharmacological properties such as antioxidant or anti-inflammatory activity (Manirafasha et al., 2016). There are also reports on its anticancer properties. Interestingly, it is toxic to cancer cells without exposing any toxicity to normal cells (Jiang et al., 2017). However, the price of C–PC, depending on the purity, ranges from 25 €/mg when partially purified to 200 €/mg for C–PC purity of 3.5 (Fernandes et al., 2023). This is because the concentration and purity of C–PC in a crude extract after biomass disintegration are far from satisfactory for industrial purposes. Therefore, besides cultivation strategy, purifying and concentrating methods are essential in the process of its production. What is more downstream processing, which generally determines even up to 80% of the product cost, in case of C–PC has not been yet satisfactorily investigated and seems to be a key step of their production. The known classical methods used for concentration and purification, such as ultrafiltration and chromatography, are still the most effective, but otherwise unfortunately very expensive and time-consuming. Therefore, the development and utilization of new approaches is still of great interest. To date various methods have been sought for the separation and purification of C–PC from its crude extract. Promising methods currently being investigated are foam fractionation (FF) and aqueous two-phase extraction (ATPE). The first is a bubble separation technique which allows for separation of amphiphilic molecules, such as proteins, from their aqueous solutions (Gerken et al., 2006). FF occurs when a liquid phase is being fed with dispersed gas stream and forms a foam phase (Burghoff, 2012). It is carried out not only under mild conditions for biological molecules, but also suitable for diluted solutions (Uraizee and Narsimhan, 1990). Despite the general simplicity, there is a number of factors influencing the effectiveness of FF, such as column size, initial protein concentration, pH, competing proteins, detergent concentration, liquid and gas flow rates etc. (Brown et al., 1999). Whereas, an aqueous two-phase system (ATPS) consists of two immiscible phases, made by aqueous solutions of specific compounds, such as polymers or salts (Grilo et al., 2016). ATPE can be conducted in a batch mode, by simply mixing the components and separating the

phases. It is an attractive method of biomolecule concentration, due to its mild conditions, relatively low cost, and scale-up potential (Iqbal et al., 2016). Both methods have already been applied to C–PC (Antecka et al., 2022), but not all parameters affecting the processes have been studied. Moreover, despite the advantages described, the results obtained were not on par with chromatographic methods.

The main goal of this work was to find and investigate an efficient method for C–PC purification, which could be an alternative to the hitherto irreplaceable chromatography.

2. MATERIALS AND METHODS

2.1. Crude extract production

The cyanobacteria *Synechococcus* sp. PCC6715 was cultivated in a laboratory helical-tube photobioreactor (Sartorius, Germany) as was earlier described in Gluszcz et al. (2018). Then the biomass was disintegrated by alternating cycles of freezing and thawing of biomass in PBS buffer following the method presented in Klepacz-Smółka et al. (2020). The obtained product called crude extract of phycobiliproteins was stored in a freezer and used for the experiments.

2.2. Foam fractionation

The experimental set-up for foam fractionation was previously described in Antecka et al. (2022). In this work the influence of air flow rate, temperature and pH value, as well as the addition of surfactants were thoroughly investigated. The three values of air flow rates were examined: 2.4; 3.5 and 6.0 $L \cdot h^{-1}$, the pH was changed in the range from 3 to 10 and temperature from 15 to 50 °C. Furthermore, for the chosen air flow rate of 2.4 $L \cdot h^{-1}$ the addition of two surfactants was examined, there were the cationic detergent cetyltrimethylammonium bromide (CTAB) (Sigma-Aldrich) in a concentration of 0.2 mM and non-ionic Polysorbate 80 (Fluka) in a three concentrations of 0.5; 1.0 and 2.0 mM. In each case a sample of 120 mL of the crude PBPs extract was poured into the column, the air flow was fixed to determined values and carried out until the foam was no longer able to reach the top of the column before collapsing. Temperature control was provided by a special heating jacket filled with water at a set temperature. After the system reached steady state operation all samples were taken, the volumes of the foamate and the retentate were measured and they were spectrophotometrically analyzed. All experiments were performed in triplicates.

2.3. Aqueous two-phase extraction

The experiments were performed in specially designed extraction vessels similar to those presented by Prinz et al. (2012). First, the stock solutions of polyethylene glycols (PEGs) and salts as well as the crude extract of PBPs were transferred into the mixing parts of the vessels and complemented with deionized water like in Antecka et al. (2022). Then the vessels with all components were put on a magnetic stirrer and stirred for an hour at 300 rpm to achieve phase equilibrium between the phases. After the mixing, the flasks were carefully flipped upside-down, to introduce the mixture into the burette part, and stored in this position for twenty-four hours in order to separate the phases. Finally, the phases were collected into different vessels and weighed. All the extraction experiments were performed in triplicates, under temperature control set to 25 °C. In the described experiments three different molecular weights of PEG (1500, 3000, 6000) and two salts phosphate and citrate with a different phase compositions were investigated. The phase compositions (mixing points) were chosen to test different ratios of phase forming components, polymer phase to salt phase. Choosing such points allowed us to see how phycocyanin behaves at different polymer and salt ratios and how this affects its purity. Meanwhile, the concentrations of the phase forming components were chosen so that all systems were in the biphasic region. As a result, 20 different systems were tested, as shown in the table (Table 1). The total mass fraction of the phase forming components in each system calculated as in Blatkiewicz et al. (2018) was in the range of 19–37% w/w. The weight of the crude PBP extract was 4 g, and the final weight of each experimental mixture supplemented with deionized water as needed was 15 g.

Table 1. The phase forming component concentrations of 20 tested aqueous two-phase systems.

No.	System/stock solution		Phase composition (w/w, %)	
	PEG	Salt	PEG	Salt
1	1500	citrate	5	17
2	1500	citrate	4.2	18.8
3	1500	citrate	20	9
4	1500	phosphate	5	17
5	1500	phosphate	16.3	9
6	3000	citrate	5	17.2
7	3000	citrate	4.2	18.8
8	3000	citrate	16	11.5
9	3000	citrate	18	10
10	3000	phosphate	4.2	18.8
11	3000	phosphate	16.8	13.2
12	3000	phosphate	14	11
13	6000	citrate	5	14.8
14	6000	citrate n	5	18.3
15	6000	citrate	15	11.5
16	6000	citrate	22	8.5
17	6000	phosphate	5	18.3
18	6000	phosphate	6.5	17.5
19	6000	phosphate	16.3	8.2
20	6000	phosphate	20.5	5.5

2.4. Foam fractionation followed by aqueous two-phase extraction

To intensify the purification process both studied methods were used as a two-stage process. In the way that the condensate after foam fractionation called foamate was used as a substrate for aqueous two-phase extraction. The FF was run at the same system as was described above, with the air flow rate of 2.4 L·h^{-1}, room temperature, no regulation of pH and without addition of detergent. The ATPE was conducted in systems for which the best results were obtained. The phase-forming component concentrations of 7 systems tested are presented in the table (Table 2).

Table 2. The phase-forming component concentrations of 7 tested aqueous two-phase systems after foam fractionation process.

No.	System		Phase composition [w/w, %]	
	PEG	Salt	PEG	Salt
21	1500	citrate	4.2	18.8
22	1500	citrate	13.8	11.8
23	3000	citrate	5	17.2
24	3000	citrate	13.8	11.8
25	6000	citrate	15	11.5
26	6000	phosphate	5	18.3
27	6000	phosphate	16.3	8.2

2.5. Target parameter calculation

The concentrations of C–PC and APC were determined spectrophotometrically using EPOCH 2 microplate spectrophotometer (Agilent BioTek) and then calculated using the following equations by Bennett and Bogorad (1973).

$$C_{C-PC} = \frac{A_{615} - 0.474 \cdot A_{652}}{5.34} \left[\frac{mg}{mL}\right] \quad (1)$$

$$C_{APC} = \frac{A_{652} - 0.208 \cdot A_{615}}{5.09} \left[\frac{mg}{mL}\right] \quad (2)$$

where A is the absorbance in a given wavelength.

The purity ratio which indicates amount of C–PC to other contaminating proteins was calculated based on spectroscopic absorbance in two wavelengths as a ratio of absorbance in A_{616} to A_{280} (Liu et al., 2005).

All the spectrophotometric measurements of each sample were performed in triplicates.

The purification factor (PF) indicating the increase in purity in a given process was calculated from the formula:

$$PF = \frac{P_p}{P_c} \quad (3)$$

where P_p is the purity of the extract after the purification process and P_c is the purity of the crude extract.

The recovery yield (R), which informs about the recovery of the C–PC or APC in a given method, was calculated from the formula:

$$R = \frac{C_p \cdot V_p}{C_c \cdot V_c} \cdot 100\% \quad (4)$$

where: C stands for concentration of C–PC/APC, V stands for volume, the index p refers to the phase, where C–PC/APC is separated (the concentration of C–PC/APC is higher) and the index c refers to the crude extract introduced to the system.

The foam enrichment coefficient (E), calculated as the ratio of the protein concentration in the foam condensate (foamate), to the protein concentration in the crude extract.

$$E = \frac{C_p}{C_c} \quad (5)$$

The partitioning coefficient (K), which informs about the ratio of C–PC concentrations between the phases:

$$K = \frac{C_p}{C_s} \quad (6)$$

where the index p refers to the phase with concentrated C–PC and the index s refers to the second phase of the analyzed system.

3. RESULTS AND DISCUSSION

3.1. Foam fractionation

Based on the literature, the parameters, such as air flow rate, temperature and pH values have significant influence on the foam fractionation of protein (Merz et al., 2011). Therefore, their effect in case of C–phycocyanin separation was fully investigated. From three tested values of air flows the highest value of purity and foam enrichment coefficient was achieved in the lowest values of 2.4 L·h^{-1}. Therefore, the experiments addressing the addition of surfactants were examined with this value. For the 7 studied systems the purity of C–PC in foamate, retentate and in crude extract with and without the addition of surfactants as well as the obtained enrichment coefficients and recovery yields are presented in the figure (Fig. 1). In each experiment foam fractionation resulted in concentration of C–PC and grow in its purity. However, the highest value of purification factor 1.47 was achieved at a lowest air flow rate of 2.4 L·h^{-1} and in the system without the addition of surfactants.

Regarding the foam enrichment coefficient, its value decreases as gas flow rate is increased. It is because high levels of gas flow rate cause greater amounts of liquid to be taken into the foam, and the residence time of foam in the column is reduced (Brown et al., 1999). Both these factors result in an increase in foam flow rate, which leads to a reduction in protein concentration in the foam and hence, to lower enrichment

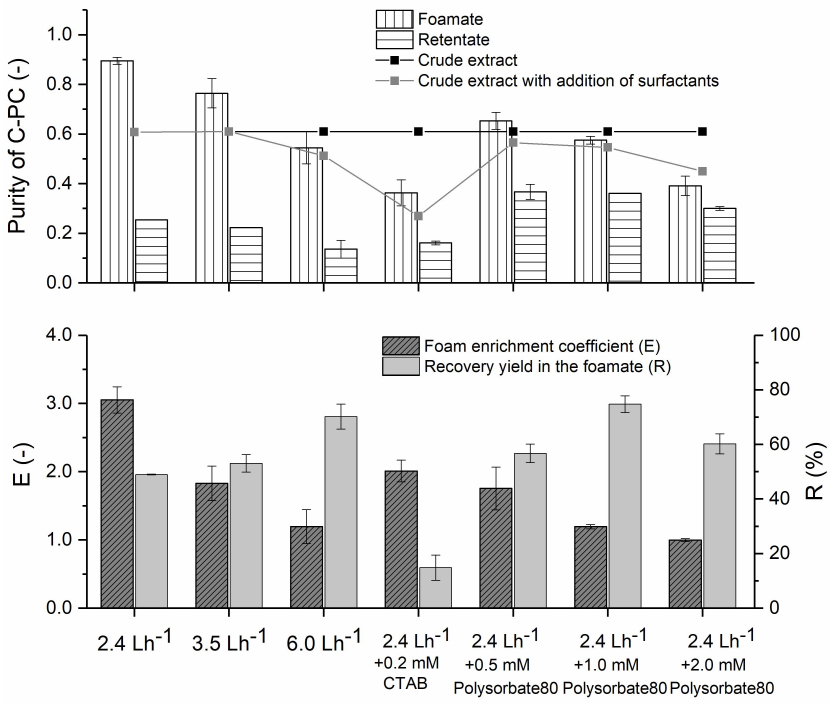

Figure 1. The purity of C–PC in foamate and retentate after FF process and in crude extract with and without the addition of surfactants (above); the enrichment coefficient and recovery yield in the foamates (below) in the systems with different values of air flow rates and with the addition of surfactants.

ratios. Conversely, at lower gas flow rates, enrichment is greater because less liquid is taken into the foam, and the residence time of foam in the column is longer, allowing more time for drainage, and, consequently increasing coalescence (Brown et al., 1999). On the other hand, an increase in the air flow rate is accompanied by an increase in the recovery yield, where maximum value of 70% was obtained at 6.0 $L \cdot h^{-1}$ and as air flow rate decreased to 2.4 $L \cdot h^{-1}$ the recovery yield decreased to approximately 50%. Among the two surfactants tested, only Polysorbate 80 appears to be worth considering, as the addition of CTAB caused rapid denaturation of C–PC, which was evident immediately upon its addition to the crude extract. Concentrations of 2 mM of Polysorbate 80 also caused partial denaturation of C–PC and the results obtained were not satisfactory. At lower concentrations of the surfactant, the parameter values were also not more favourable than without its addition. Generally, as it was earlier acknowledged (Burghoff, 2012) the enrichment coefficient and recovery yield act contrarily in foam fractionation applications. This can be seen also in case of PBPs, where, depending on combinations of the process parameters either high enrichment or high recovery were obtained. However, further experiments showed that high protein enrichment and high protein recovery can both be reached under the same conditions.

The results from the experiment with different pH values are presented in the figure (Fig. 2). It was noticed that the FF process and C–PC is stable in pH from 5 to 8.5. Acidic (pH = 4) and alkaline pH above 9 causes the denaturation of C–PC which was seen as drop in concentration and purity value.

As mentioned above, for the pH from 4 to 6 the highest enrichment values obtained are accompanied by the lowest recoveries and conversely for the highest values of E, the R have the lowest values. However, for the pH from 6.5 to 8 both parameters have satisfactory values, allowing to conclude that this is the optimal range for the process.

Finally, the temperature dependence of the process was investigated in the range from 15 to 50 °C. The results of purity of C–PC as well as enrichment and recovery rates are presented in the figure (Fig. 3). As C–PC is derived from a thermophilic strain, temperatures up to 50 °C did not cause its denaturation. However, enrichment and recovery are relatively low at the highest process temperature. The purities of the obtained foamates had comparable values regardless of the process temperature, although the highest purity was achieved at a room temperature of 25 °C. Interestingly, lowering the process temperature to 15 °C caused an apparent decrease in purity of the foamate and increase in purity of retentate. This is also visible by the low value of the enrichment coefficient obtained at this temperature. Further with increasing temperature up to 45 °C, the value of the enrichment coefficient increases, although again, its value is the highest at 25 °C. As far as recovery efficiency is concerned, its value increases with increasing temperature in the range up to 35 °C. This may be due to the reduction in the surface tension of the thin liquid film lamellae with increasing temperature, which can increase foam formation (Kumpabooth et al., 1999). As temperature increases, foam formation is generally enhanced, but foam stability once formed is generally decreased, so at higher temperatures the recovery values are already lower.

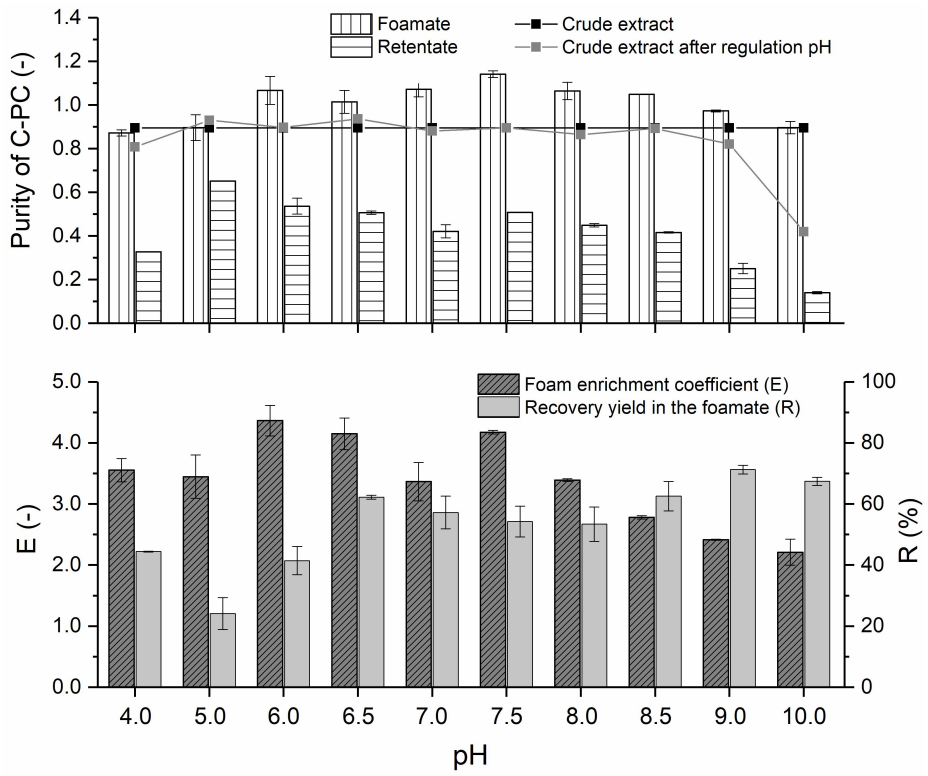

Figure 2. The influence of pH values on: purity of C–PC in the foamate, retentate and crude extract before and after the regulation of pH (above); enrichment coefficient and recovery yield (below).

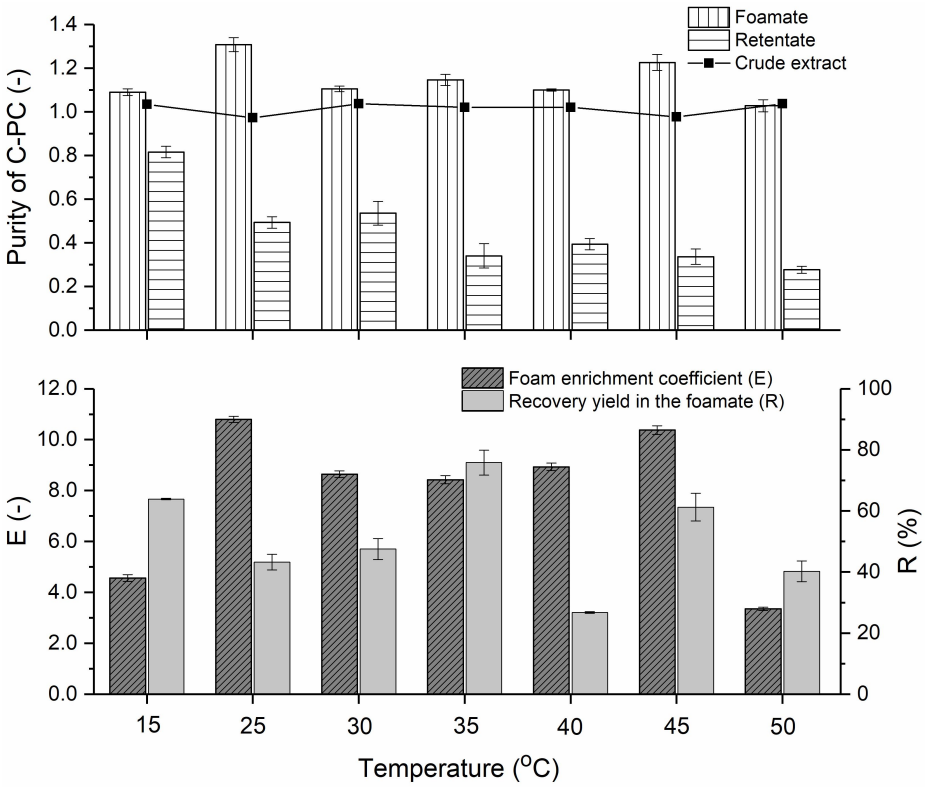

Figure 3. The influence of temperature on: purity of C–PC in the foamate, retentate and crude extract before and after the regulation of pH (above); enrichment coefficient and recovery yield (below).

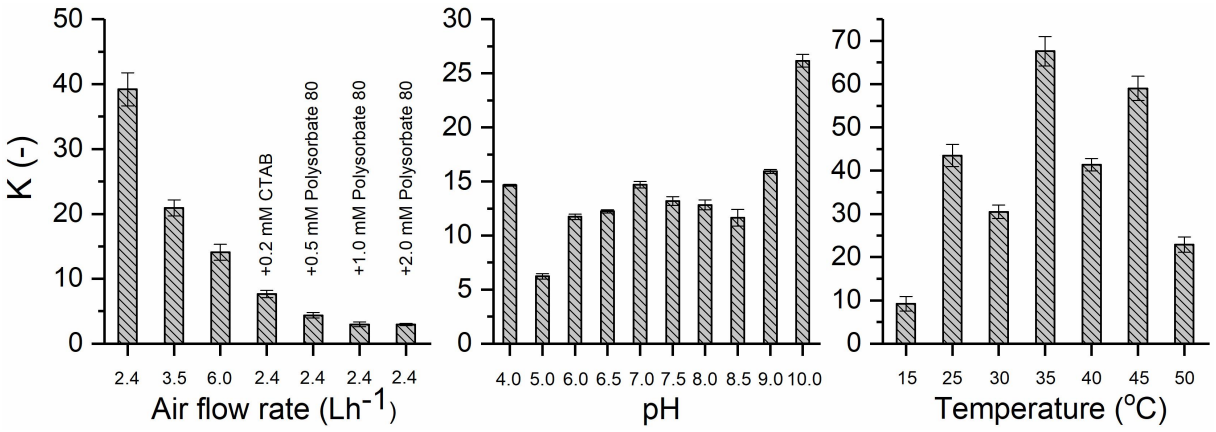

Figure 4. The partitioning coefficients of all tested systems in the process of foam fractionation.

Although enrichment and recovery are standardly sufficient to describe the foam fractionation process (Gerken et al., 2006), in this work the partitioning coefficients for all the systems studied were also determined. The calculated values are presented in the figure (Fig. 4). The partitioning coefficient definitely decreased with the decreasing values of air flow rates. Also the addition of surfactants caused its significant drop relative to its value for crude extract. The process with air flow of 2.4 $L \cdot h^{-1}$ resulted in partitioning coefficients of about 39, which is three times higher than previously reported for the same system (Antecka et al., 2022). With regard to pH, for most of the systems studied, the value of the partitioning coefficient was around 15, with an increase observed for pH 10 and a decrease for pH 5. Interestingly, a temperature of 35 °C allowed for the highest partitioning coefficient of 67.6; 45 °C of around 60 and 25 °C of 43, respectively. The low value of the partitioning coefficient for 15 °C, equal to 9, confirms that the process is not efficient at this temperature.

3.2. Aqueous two-phase extraction

In all systems tested, phycocyanin and other phycobiliproteins diffused into the top phase, which means that they show affinity to the polymer phase. The obtained results concerning purity, partitioning coefficient and recovery yields are presented in the graph (Fig. 5).

The highest purity of C–PC in the polymer phase of 2.72 and the highest purification factor equal to 2.31 were obtained in the PEG 1500 – citrate system (system No. 1). Regarding the PEG – phosphate systems, the most favourable was the system with the PEG 1500 (No. 4) with a recovery of about 80%, a partitioning coefficient of 2416 and a purification factor of about 2. This is in contrast to what has been described so far (Antecka et al., 2022), where the PEG 6000 – phosphate system was recommended. The explanation is that in this system the PEG phase has split into two phases, so an additional intermediate phase has appeared, which was located between the polymer and the salt phase. Spectrophotometric measurements of this phase made for systems 17-20 showed the presence of APC in it. In PEG – citrate systems, this phenomenon was also observed. Systems with lower molecular weight of PEG (1500, 3000) were characterized by apparently higher recoveries and partitioning coefficients than systems with PEG 6000. In terms of different mixing points, it was noted that regardless of the salt used, higher purity values were obtained with a lower proportion of PEG to salt. Then, the smallest phase volume ratio occurs, and the smaller the PEG/salt ratio, the higher the purity of the C–phycocyanin and the more concentrated it is in the polymer phase. The obtained C–PC had a purity reaching 2.3, which qualifies it as a food and cosmetic dye. The results obtained allow to conclude that the ATPE process itself yields such satisfactory results (recoveries of up to 95%, purification factors of about 2) that it can be successfully used as an alternative to chromatographic processes when analytical grade purity is not required.

In order to examine the phenomenon of polymer phase partitioning, the three obtained phases were separately collected and the C–PC and APC concentrations were determined. The results for PEG 6000 – citrate system (No. 15) are shown in the graph (Fig. 6). Even organoleptic analysis indicated a separation of phycobiliproteins, where the blue colour of the upper polymer phase indicated a predominance of C–PC, and the lower greenish polymer phase of APC. The results of spectrophotometric analysis confirmed this conjecture and showed that in the upper polymer phase (UPP) the recovery of C–PC is above 82%, of APC only 3.7% and in the lower polymer phase (LPP) the recovery of C–PC is only 3.4% and APC is above 33%. Interestingly, a quite high APC recovery of almost 10% was demonstrated in the salt phase, while the C–PC concentration was there negligible (1% of recovery). To the best of our knowledge, the observed phenomenon has not yet been reported in the literature. Admittedly, Suarez Ruiz et al. (2020) had demonstrated the presence of the interface between the aqueous phases, but it was treated as a third phase, that mainly contains debris generated by cell disruption method, not considered part of any of the aqueous phases.

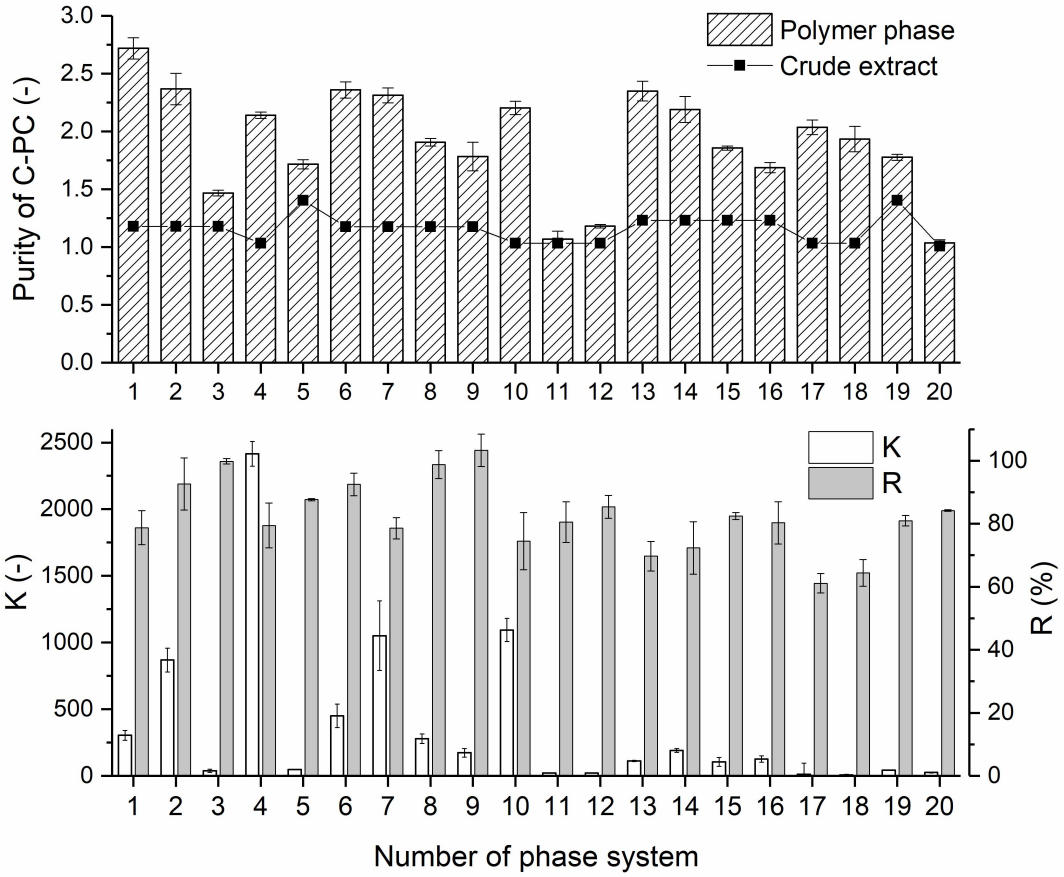

Figure 5. The purity of C–PC in the polymer phase and in crude extract (above); the partitioning coefficient and recovery yield in the polymer phase (below) in the 20 tested systems.

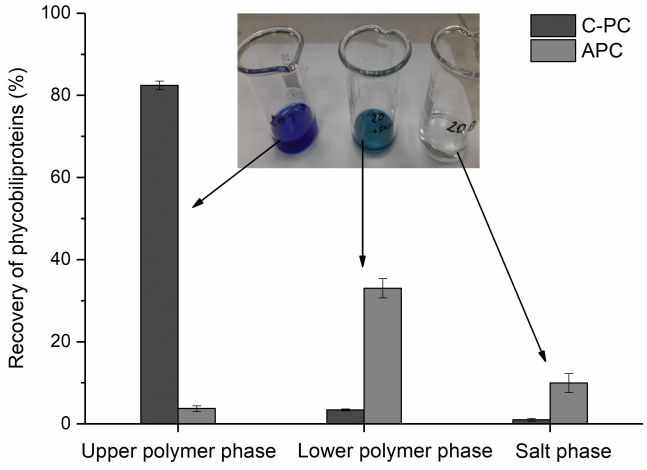

Figure 6. Recovery of C–PC and APC in the three obtained phases in the system PEG 6000 – citrate (No. 15) with a photo of three separately collected phases.

3.3. Foam fractionation followed by aqueous two-phase extraction

Further studies have been conducted to investigate the two-stage process of foam fractionation followed by aqueous two-phase extraction. Based on the earlier results seven systems were selected and the results of purity, partitioning coefficient and recovery of C–PC in the polymer phase are presented in the figure (Fig. 7).

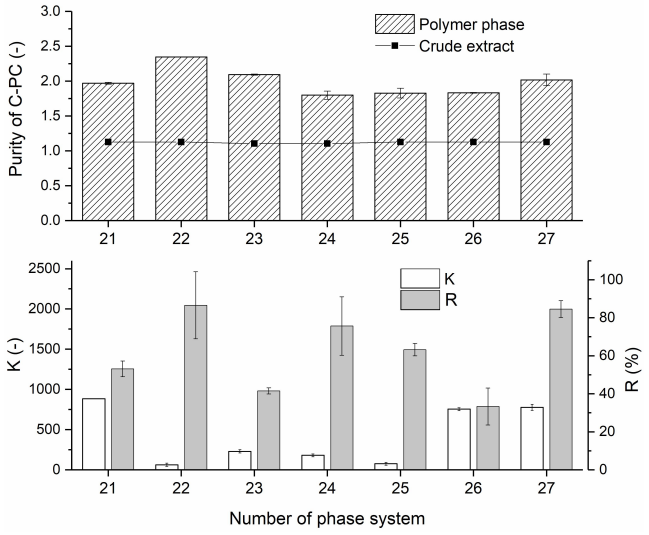

Figure 7. The purity of C–PC in the polymer phase and in crude extract (above); the partitioning coefficient and recovery yield of C–PC in the polymer phase (below) in the 7 tested systems.

The application of FF process prior to ATPE resulted in a significant volume reduction and increase of concentration of the C–PC in the tested solutions. However, the use of two-stage process did not result in an increase of purity and purification factor (Fig. 7) compared to the results of the ATPE alone. This was due to the previously mentioned division of the polymer phase into two parts: an intensely blue upper polymer phase (UPP) and greenish lower polymer phase (LPP), which is visible in the picture (Fig. 8), as well as the lowest salt phase, which is colourless. In each of the 7 studied systems, the appearance of an intermediate phase was observed, in which the presence of APC dominated. The phenomenon was particularly evident in the systems with PEG 6000 regardless of the salt phase used. Furthermore, it was found that in systems where the ratio of the polymeric phase to the salt phase was close to 1, this partitioning of the polymer phase was more apparent and APC recoveries were higher. However, the highest APC recoveries were observed in PEG 6000 systems regardless of the salt used, while the highest recovery and degree of purification of C–PC was again obtained in the PEG 1500 – citrate system (No. 22) (Fig. 7).

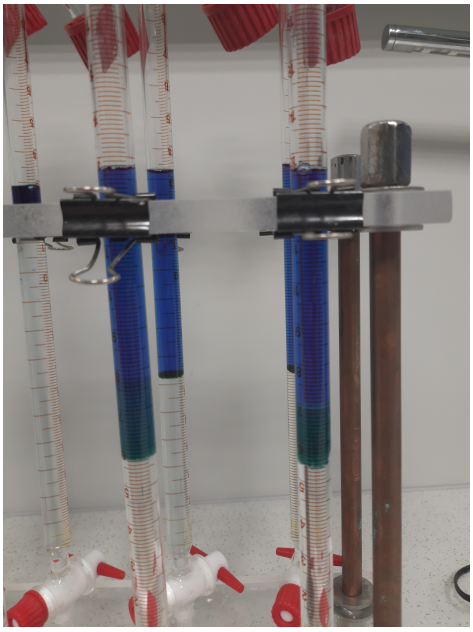

Figure 8. Photo of the experimental vessels with two polymer phases after partitioning.

The graph (Fig. 9) shows the recoveries of C–PC and APC in the three selected phases for two PEG 6000 systems with phosphate and citrate salt. A comparison of the two systems presented shows that the PEG 6000 – phosphate salt system is more favourable in terms of the recovery values obtained. In this system the recovery of C–PC in the UPP is 85% and there is almost no APC, while in LPP there were about 15% of C–PC and 90% of APC. This demonstrates the almost complete separation of phycobiliproteins in the method used which makes it an alternative to chromatographic ones.

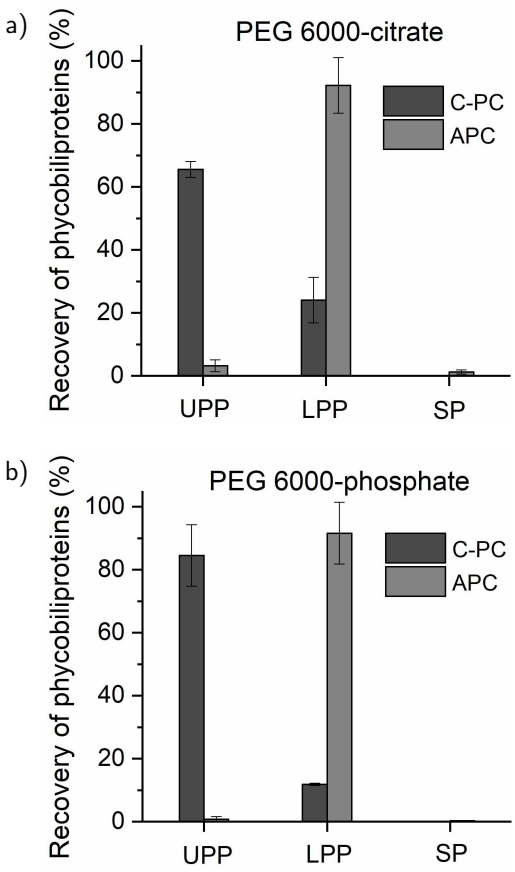

Figure 9. Recovery of C–PC and APC in the three obtained phases in the system a) PEG 6000 – citrate (No. 25); b) PEG 6000 – phosphate (No. 27).

3.4. CONCLUSIONS

The results obtained confirm that both studied FF and ATPE methods can be used for C–PC purification and to some extent can substitute other traditionally applicable methods, like ultrafiltration, precipitation or chromatography.

The advantages of FF process are its low price, no need to add any chemicals, pH or temperature regulation and low air flow rates, which reduces the environmental impact. However, the recovery is only about 50%, enrichment of 3, partitioning coefficient of 39 and purification factor of 1.47, so the resulting C–PC is classified as a food dye (purity of 0.5–1.5).

In contrast to FF, ATPE results in higher values of purification degrees and partitioning coefficients. The best results obtained for PEG 1500-citrate system are: recovery of 79%, partitioning coefficient of 304, purification factor of 2.31 and for PEG 1500-phosphate system: recovery of 80%, partitioning coefficient of 2416, purification factor of 2.07; the obtained C–PC is classified as a cosmetic dye (purity 1.5–2.5). However, all of the polymer-salt systems tested resulted in a concentration of C–PC in the polymer phase, which requires additional separation of this organic phase from the C–PC and may be a hindrance to further processes or applications.

Interestingly, it was discovered that when combining the two methods, applying ATPE to the condensate after FF, separation of the phycobiliproteins is achieved, i.e. APC is concentrated in an additional lower polymeric phase, which can be easily separated from the polymeric upper phase where C–PC is concentrated. This means that the combination of FF and ATPE gives separation of phycobiliproteins and makes it an effective, economical alternative to chromatographic methods.

ACKNOWLEDGMENTS

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SYMBOLS

A_{280}	absorbance at 280 nm, –
A_{615}	absorbance at 615 nm, –
A_{652}	absorbance at 652 nm, –
C_{C-PC}	concentration of C–PC, mg·mL^{-1}
C_{APC}	concentration of APC, mg·mL^{-1}
C_c	concentration of C–PC/APC in the crude extract, mg·mL^{-1}
C_p	concentration of C–PC/APC in the phase, mg·mL^{-1}
C_s	concentration of C–PC in the second phase, mg·mL^{-1}
E	enrichment coefficient, –
K	partitioning coefficient, –
PF	purification factor, –
P_p	purity after the purification process, –
P_c	purity of the crude extract, –
R	recovery yield, %
V_p	volume of the phase, mL
V_c	volume of crude extract, mL

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