Separation of Crocin/Betanin Using Aqueous Two-phase Systems Containing Ionic Liquid and Sorbitol

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Betanin and crocin, two food additives with attractive colors, are bioactive compounds of plants that are widely used in food, pharmaceutical, and medical industries. These bioactive pigments are sensitive to light, heat, organic solvents, and pH. It seems that a benign economic method is needed to extract these biomolecules, especially for industrial applications. The aqueous two-phase system (ATPS) is a liquid-liquid extraction technique that has shown its potential in recent years to extract and separate biomolecules. In this study, an ATPS consisting of carbohydrate (sorbitol) and ionic liquid (tetrabutyl phosphonium bromide) has been proposed as a new separation system with unique properties to study the partition coefficient of crocin and betanin. The results indicated that crocin and betanin had more tendency to the ionic liquid (IL)-rich phase and carbohydrate-rich phase, respectively. The influence of the concentration of IL and sorbitol on the partition coefficient was studied. The results showed that an increase in the tie-line length (concentrations) increased the partition coefficient of crocin and betanin. Enhancement in temperature increased the partition coefficient of crocin. The highest values of crocin recovery (97.55 %) and partition coefficient (39.85) at 308 K show that our proposed ATPS can be considered for crocin separation in one step.

Keywords
ionic liquid, carbohydrate, crocin, betanin, extraction, aqueous two-phase systems

Introduction

Humans have long relied on plants and medicinal plants as a source of food and medicine. Many bioactive compounds of medicinal plants are currently the subject of much research as food additives. Food additives are substances added to food to maintain or improve its safety, increase shelf-life, freshness, taste, texture, or appearance[1]. The use of artificial additives and synthetic colors in food and medicine has been restricted by international and research authorities. It has recently been observed that many additives and synthetic colors can cause numerous side effects such as asthma, urticaria, attention-deficit/hyperactivity disorder, weakened immune system, anaphylaxis (allergic) reactions, sleep disorders, high blood pressure, kidney failures, vitamin deficiency, hormonal imbalance, and incidence of cancer[2]. Therefore, it is time to pay more attention to the extraction of valuable compounds from medicinal plants to replace artificial materials. Extraction of bioactive pigments requires appropriate extraction techniques that maintain the biological activity of pigments extracted from plants.

Betanin and crocin are bioactive pigments of plants with attractive colors that are mainly used in foods or medicine. They are used as high-value biomolecules in the food, pharmaceutical, and medical industries due to their unique medicinal and therapeutic properties. Betanin is a natural red colorant and the most well-known betacyanin in red beetroots. It is utilized in the formulation of food products, such as gelatins, desserts, dairy products, meat products, and sweets[3]. It also has biological active properties, such as antioxidant, anticancer, and antimicrobial[4–6].

Crocin is a glycoside and a water-soluble carotenoid pigment obtained from the saffron plant. Crocin is metabolized in the body and then converted to crocetin[7]. Crocetin has numerous therapeutic uses and is known as a powerful antioxidant and anti-inflammatory agent[7]. It is also characterized as an anticonvulsant[7], antidepressant[8,9], and antitumor agent[10,11]. In this regard, it has been stated that cro-

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cin can control tumors by killing cancer cells or delaying the carcinogenic cell divisions of tumors. Furthermore, it has been reported that crocin reduces the risk of a heart attack in humans. Moreover, this compound has strong antioxidant properties and plays an important role in boosting memory[12].

Crocin and betanin are bioactive pigments that are prone to degradation under unstable conditions. These biomolecules are sensitive to high temperatures, salt concentrations, pH, and organic solvents[13,14]. Methods such as column chromatography, salt/solvent-induced precipitation, and electrophoresis, which are used to separate bioactive pigments such as crocin/betanin, are performed in batch and small-scale processes. These unit operations have scale-up problems and are expensive on a larger scale, and often lead to low product recovery. In addition, bioactive pigment separation techniques require methods that are both economically feasible and do not adversely affect their biological activity. Scaling up laboratory-scale processes is very important for industrial operation. It is necessary to develop simple, efficient, low-cost, environmentally friendly downstream processing methods that can be implemented in continuous mode[15]. Therefore, according to available protocols for food and drug standards and the emphasis on the implementation of appropriate methods in the separation of food and medicine, more efforts should be made to select the best separation method. The use of aqueous two-phase systems (ATPSs) is one of the most effective methods in the separation of biomolecules[16]. ATPS has been largely successful in overcoming the disadvantages of conventional extraction processes. ATPS is a liquid-liquid extraction method and is formed based on water, as the most important constituent of all living organisms and biomolecules. ATPSs are non-flammable, non-toxic, easily scalable, and biocompatible[17–19]. These systems have been applied for the separation and purification of different biological materials such as antibodies, viruses, proteins, antioxidants, as well as for metal ions[20]. Conventional ATPSs of polymers and salts have been employed to separate antioxidants in several studies[21,22]. Ebrahimi and Shahriari investigated ATPSs consisting of polyethylene glycol and sodium sulfate at three different temperatures (25, 30, and 35 °C), in the separation of betanin[21]. In all studied systems, betanin showed a greater tendency to the top phase (polymer-rich phase). Montalvo-Hernandez *et al.* studied the partition behavior of crocin pigments in different types of ATPSs (polymer-polymer, polymer-salt, alcohol-salt, and ionic liquid-salt). ATPS containing ethanol + potassium phosphate showed more potential for use in this particular application[22].

Over the past decades, ionic liquid-based aqueous two-phase systems (IL-ATPSs*)* have been greatly expanded due to their potential to design a new technology as a suitable separation process. The use of IL-ATPSs consisting of carbohydrate and ionic liquids (ILs) in the separation of food and medicine is one of the latest approaches of researchers[23]. Therefore, a green approach for sustainable development of IL-ATPS as a new system was proposed by Quental *et al.*[23] The authors suggested a green character ATPS using carbohydrates as a non-toxic and renewable compound, instead of mineral salts with high charge density. The authors also focused on selecting an IL with higher biodegradability, lower toxicity, lower cost, and more stable nature compared to conventional ILS to form ATPS with carbohydrates. By focusing on these features, ATPS consisting of carbohydrate and tetrabutylphosphonium bromide was introduced[23]. Ghasemzadeh *et al.* also utilized this system to separate curcumin. The authors showed that the maximum recovery for curcumin was 99.55 % in the top phase of a one-stage process. Overall, concerning the obtained results, the use of the proposed IL-ATPS can be quite successful in the separation of curcumin[24]. Increasing the performance of IL-ATPS in improving the partitioning of biomolecules is possible by using ionic liquids based on tetrabutylphosphonium bromide due to their unique properties[25]. Phosphonium-based ionic liquids are applied as proper compounds in high-temperature industrial processes due to their availability, relatively low cost, and high resistance to thermal decomposition[26]. Also, it has been reported that IL-ATPS consisting of ionic liquids based on phosphonium and water are very suitable for the separation of short-chain organic acids such as L-lactic, L-malic, and succinic acids[27].

Tetrabutylphosphonium bromide has a lower density than other compounds containing phosphonium-based ionic liquids[26]. This ionic liquid contains mononuclear cations and anions that have relatively small sizes compared to the anions and cations of other ionic liquids, such as imidazolium-based ionic liquids[28]. Therefore, it has a high polarity compared to the aforementioned ionic liquids, which leads to its easier separation of biomolecules. Accordingly, it has been stated that the recoverability of these ionic liquids is high[24]. On the other hand, most research has focused on the application of ATPSs, including mineral salts and ionic liquids[16,21,29–31]. It is important to note that salts can damage sensitive biomolecules and disrupt ionic liquid recycling processes in ATPSs due to the alkalinity of the test medium. For example, betanin decomposes to cyclodopa 5-O-glycoside and betalamic acid in media with pH >8[5,32]. Therefore, alkaline pHs can change the color index of biomolecules such as crocin and betanin, as well as damage their structures. Using carbohydrates instead of salts can overcome these problems.

Sorbitol is a non-volatile polyhydric alcoholic sugar that is formed by the reduction of glucose and is abundantly found in nature. This compound is chemically stable and has a high solubility in water and ionic liquids. Sorbitol is also employed as a sweetener, tissue builder, preservative agent, and non-clotting agent in the food industry. In addition, it is used as a sugar substitute in many foods, including low-calorie and sugar-free foods, as well as oral pharmaceuticals or oral hygiene products, such as toothpaste and chewing gum[33].

In this work, the efficacy of an IL-ATPS containing tetrabutyl phosphonium bromide and sorbitol for the separation of two bioactive pigments such as crocin and betanin was investigated. To determine the best conditions for crocin/betanin separation, the effects of IL weight percentage, sorbitol weight percentage, and different temperatures on the partition coefficient of these pigments have been evaluated.

Materials and methods

All detailed material specifications are provided in Table 1. The distilled water was produced in RO Lab by crossing through a reverse osmosis system using a water distiller (RO-LAB model DW65). Distilled water with a conductivity of 0.053 µS cm^{-1} was applied to prepare the solutions.

Working points and tie-lines

In this study, the weight fraction of each component in the feed (working point) was selected using the phase diagram. The experimental measurement of the phase diagram for [P$_{4444}$] Br + Sorbitol + H_2O was extensively explained in reference[24]. The phase diagram of IL-ATPS composed of [P$_{4444}$] Br + Sorbitol + H_2O was reported in our previous work[25]. The following equation (Eq. 1) was used for determining the tie line length.

$$\mathrm{TLL} = \sqrt{\left([\mathrm{carb}]_\mathrm{T} - [\mathrm{carb}]_\mathrm{B}\right)^2 + \left([\mathrm{IL}]_\mathrm{T} - [\mathrm{IL}]_\mathrm{B}\right)^2} \quad (1)$$

TLL, carb, and IL are tie-line length, carbohydrate, and ionic liquid, respectively. T and B are top and bottom phases, respectively.

Partitioning of biomolecules

The following experimental method was used to measure the partition coefficient of a biomolecule in ATPS, which has been reported elsewhere[24,25]. The IL-ATPSs were prepared by mixing the exact amount of the tetrabutylphosphonium bromide, sorbitol, and an aqueous solution of crocin/betanin in a test tube. The concentrations of aqueous solutions of crocin and betanin were respectively 1 g dm^{-3} and 2 g dm^{-3} in each intended mixture. Since betanin is not stable and its structure undergoes alteration in an acidic medium, it is necessary to control the pH values of IL-ATPSs. It is worth noting that the stability of betanin during separation is a major challenge because temperature changes (above 50 °C), water activity, oxygen, light, and pH activity (stable between pH 3 to 7) can cause degradation. Therefore, the pH values of the system were adjusted with the aid of one molar solution of potassium chloride. The pH measurements were made using a calibrated pH meter (Metrohm-780) at 25 °C.

Each material was weighed with a digital balance (with an accuracy of ±10^{-4} g). The mixture was then completely mixed for 10–15 minutes, and left for 24 hours to reach complete equilibrium. An incubator (Beschichung-loading, Memmert, Germany) with an accuracy of ≤ 0.01 °C was used to control the temperature. After complete phase separation, each of the phases (top and bottom) was carefully separated for further analysis using a glass Pasteur pipette. The weight fractions of crocin and betanin in the top and bottom phases were obtained using a UV/V spectrophotometer (UV/vis Model: sp-2100uv, USA). Betanin and crocin concentrations were measured regarding their maximum absorptions at a wavelength of 529 nm and 441 nm, respectively.

The partition coefficient of biomolecules was obtained according to Eq. 2. It was determined as

Table 1 – *Materials used, suppliers, and general sample characteristics*

Chemical name	Molecular formula	CAS number	Purity (%)	Supplier	Molecular weight (g mol^{-1})	Abbreviation
Tetrabutyl phosphonium bromide	$C_{16}H_{36}BrP$	3115-68-2	98.0	Sigma-Aldrich	339.33	[P$_{4444}$] Br
D (-)-Sorbitol	$C_6H_{14}O_6$	50-70-4	≥99.0	Sigma-Aldrich	182.17	Sorb
Crocin	$C_{44}H_{64}O_{24}$	42553-65-1	≥99.0	Sigma-Aldrich	976.96	Croc
Betanin	$C_{24}H_{26}N_2O$	7659-95-2	≥99.0	Sigma-Aldrich	550.47	Bet
Potassium chloride	KCl	7447-40-7	≥99.0	Merck	74.55	KCl

the ratio of each weight fraction in the top phase (IL-rich phase) to its weight fraction in the bottom phase (carbohydrate-rich phase):

$$K_{\text{biomolecule}} = \frac{[\text{biomolecule}]_{\text{T}}}{[\text{biomolecule}]_{\text{B}}} \tag{2}$$

In this formula, $K_{\text{biomolecular}}$ is the partition coefficient of the biomolecules (crocin/betanin), and $[\text{biomolecular}]_{\text{T}}$, $[\text{biomolecular}]_{\text{B}}$ are the concentration of biomolecule in the top and bottom phases, respectively.

The recovery percentage of crocin in the top phase and betanin in the bottom phase was obtained using Eqs. 3 and 4:

$$R_{\text{T(Croc)}} = \frac{[\text{Croc}(\text{wt.\%})]_{\text{T}}}{[\text{Croc}(\text{wt.\%})]_{\text{T}} + [\text{Croc}(\text{wt.\%})]_{\text{B}}} \tag{3}$$

$$R_{\text{B(Bet)}} = \frac{[\text{Bet}(\text{wt.\%})]_{\text{B}}}{[\text{Bet}(\text{wt.\%})]_{\text{T}} + [\text{Bet}(\text{wt.\%})]_{\text{B}}} \tag{4}$$

where R, [Croc(wt%)], [Bet(wt%)] represents the recovery percentage and the weight percentage of crocin and betanin, respectively. The subscripts T and B are the top phase and bottom phase.

Investigated parameters

The extraction efficiency in an ATPS is highly dependent on the weight percentages of the components in the two adjacent phases. Higher partition coefficients can be achieved by changing the weight percentage of each component in the feed[24]. This study aimed to investigate the parameters affecting the partition coefficients of two bioactive pigments (betanin/crocin) in the IL-ATPS containing sorbitol and tetrabutylphosphonium bromide. To consider the parameters affecting the partition coefficients, repeatable and targeted experiments were planned. Sorbitol with different weight percentages of (30, 32, 34, and 36) wt.%, and tetrabutylphosphonium bromide with different weight percentages of (38, 40, 42, and 44) wt.% were selected as working points. The working points were selected based on the phase behavior of the ATPSs and binodal curve according to our previous works[24,25]. Samples were made with different weight percentages of sorbitol, IL, and water to measure the partitioning of the two bioactive pigments individually. In each sample, by keeping one of the independent variables constant, the effect of the other parameters on the dependent variable (partition coefficient) was determined. The partition coefficient and recovery percentage were measured in each sample. A suitable sample was selected based on the maximum partition coefficient and recovery percentage. In addition, the interaction of variables and the optimal level for maximum response were reported by statistical software MINITAB (v. 18).

Results and discussion

Effect of component concentration and tie-line length on partitioning

The experimental results for the tie line length (TLL) and the weight percentage of the sorbitol and IL in the top and bottom phases are listed in Table 2. The affinity of the biomolecules for each phase can be changed by manipulating TLL (as a function of phase composition). Fig. 1 shows the relation between TLL and the partition coefficient of crocin and betanin. As seen in Fig. 1, increasing tie-line length has a more significant effect on the partition coefficient of crocin. It shows that the effect of tie-line length on the partition coefficient depends on biomolecule nature[34,35]. As the TLL increases, the concentration of IL increases in the top phase, so more IL molecules interact with crocin.

In Table 3, the partition coefficients of crocin are reported in the IL-ATPS containing $[\text{P}_{4444}]\text{Br}$ + Sorbitol + H_2O for different weight percentages of components at 298 K. In this IL-ATPS, the top phase is IL-rich, while the bottom phase is rich in

Table 2 – *Phase composition, tie-line data of the IL-ATPS formed by $[\text{P}_{4444}]$ Br (1) + Sorbitol (2)*

Weight fraction (wt.%)						α	TLL
Overall		Top phase		Bottom phase			
$100 \cdot w_1$	$100 \cdot w_2$	$100 \cdot w_1$	$100 \cdot w_2$	$100 \cdot w_1$	$100 \cdot w_2$		
38.01	35.98	70.60	5.25	1.60	70.31	0.53	94.83
43.97	30.06	67.28	6.26	1.03	73.89	0.65	94.67
37.96	34.00	68.68	5.82	2.41	66.62	0.54	89.94
41.92	30.51	64.97	7.03	1.60	70.32	0.64	89.57
39.94	30.00	64.20	7.31	2.97	64.61	0.60	83.86

Standard uncertainties: u(wt.%) = ±0.3; u(TLL) = ±0.3

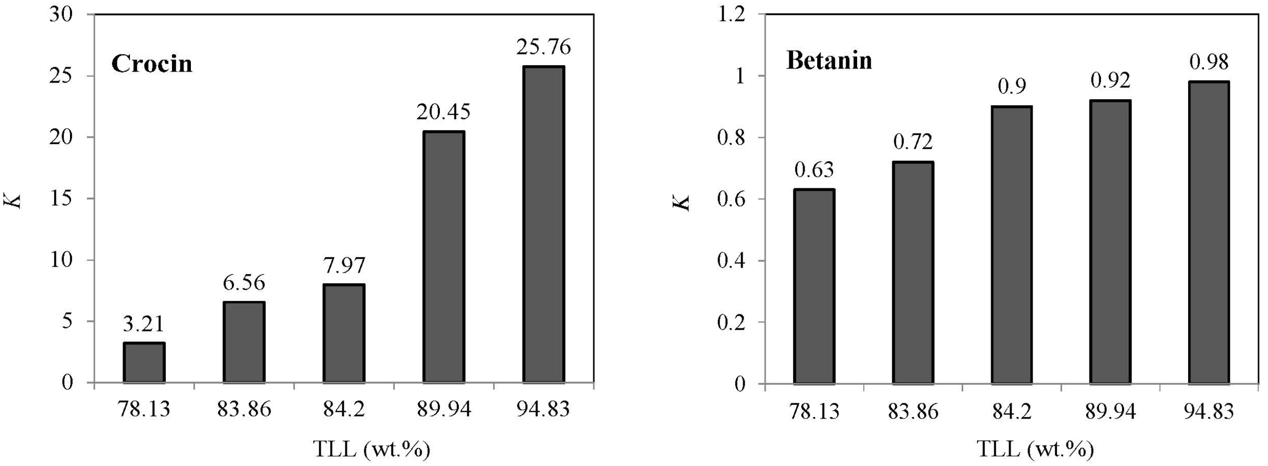

Fig. 1 – *Effect of tie-line length (TLL) on partition coefficient (K) crocin / betanin of the ATPS containing sorbitol + [P_{4444}] Br + Sorbitol + H_2O at 298 K*

sorbitol[24,25]. The partition coefficient of crocin is greater than 1 (K>1), indicating that crocin has more tendency to the top phase. The reason why crocin tends towards the IL-rich phase is the favorable interactions between IL and crocin. The results indicate that the weight percentage of components has a significant effect on the crocin partitioning so that, with increasing the concentration of IL or sorbitol, the partition coefficient of crocin increases. It can be inferred that the solubility of crocin in the IL-rich phase is much better than in the sorbitol-rich phase. The highest partition coefficient of crocin (25.76) was obtained in an IL-ATPS composed of [P_{4444}] Br (38 wt.%) + Sorb (36 wt.%) + H_2O with the highest tie-line length.

The effect of changes in the IL and sorbitol concentrations on the partition coefficient of betanin is given in Table 3. Betanin is a compound containing an aromatic ring bearing one or more hydroxyl groups. It is water soluble/hydrophilic and combined with sugars in glycosidic form. Hence, it showed more affinity toward the sorbitol-rich phase and its partition coefficient was less than one. The results showed that increases in the concentration of sorbitol or IL caused an increase in the partition coefficient of betanin. In most cases, it was expected to have a more one-sided partition by increasing TLL, but the partition coefficient of betanin (K<1) increased by increasing IL or sorbitol concentration (TLL). This trend can be justified by the charge of betanin. The isoelectric pH (pI) of betanin is located in 1.5–2. In all studied systems, pH values of top and bottom phases are greater than pI, so betanin is negatively charged. Table 3 shows that increasing the concentration of components causes a decrease in pH values and betanin negative charge, so the interaction between betanin and sorbitol is decreased leading to an enhancement in the betanin partition coefficient. Similar results were reported by Ghasemzadeh and Oliveira[24,36]. The results showed that both hydrophobic-hydrophilic and electrostatic interactions had affected the partition coefficient of betanin[36].

Recovery percentages of biomolecules

In recent years, one of the most suitable methods for recycling biomolecules in the downstream process is the use of ATPS. The recovery of biomolecules is a function of parameters such as temperature, phase components, and weight percentage of the components in each phase.

Table 3 presents the recovery percentages of crocin in the top phase of the IL-ATPS at a temperature of 298 K. It was observed that by increasing the weight percentage of IL and sorbitol in the feed, the recovery percentages of crocin in the top phase increased. The maximum recovery of crocin was 96.8 % in the top phase IL-ATPS containing 38 wt.% of [P_{4444}] Br +36 wt.% of sorbitol. The recovery percentages of betanin in the bottom phase of the IL-ATPS at temperature 298 K are given in Table 3. In addition, the effects of IL and sorbitol concentrations on the betanin recovery can be seen in this table. The maximum amount of betanin recovery was 61.38 % at 38 wt.% of [P_{4444}] Br + 30 wt.% of sorbitol. The percentage of recovery of betanin and crocin changed as the concentration of the components changed.

Effect of temperature on partitioning

In Table 4 presents the results of the effect of three different temperatures (298, 303, and 308 K) on the partition coefficient and recovery percentage of crocin in IL-ATPS containing [P_{4444}] Br + Sorbitol + H_2O. Increasing the temperature caused an en-

Table 3 – *Weight percentage composition of each mixture point, partition coefficients, recovery percentages of crocin and betanin in the system of [P_{4444}] Br + Sorbitol + water at 298 K*

Biomolecule	IL (wt%)	SOR (wt%)	$K \pm \sigma^a$	$R_T \pm \sigma^a$	pH_{Top}	pH_{Bot}
Crocin	38 %	30 %	3.21 ± 0.22	76.20 ± 1.22	2.98	2.76
		32 %	7.97 ± 0.13	88.85 ± 0.16	2.82	2.56
		34 %	20.45 ± 0.30	96.27 ± 1.38	2.80	2.51
		36 %	25.76 ± 0.73	96.81 ± 0.67	2.77	2.54
	38 %	30 %	3.21 ± 0.22	76.20 ± 1.22	2.98	2.76
	40 %		6.56 ± 0.24	86.78 ± 0.42	2.79	2.61
	42 %		8.37 ± 0.79	89.29 ± 0.91	2.70	2.41
	44 %		12.17 ± 0.65	92.34 ± 0.39	2.61	2.52
Betanin	38 %	30 %	0.63 ± 0.14	61.38 ± 5.04	2.97	3.16
		32 %	0.90 ± 0.11	52.83 ± 3.11	2.87	3.16
		34 %	0.92 ± 0.10	52.08 ± 2.80	2.76	3.05
		36 %	0.96 ± 0.02	51.15 ± 0.56	2.67	3.04
	38 %	30 %	0.63 ± 0.14	61.38 ± 5.04	2.97	3.16
	40 %		0.72 ± 0.00	57.99 ± 0.06	2.87	3.50
	42 %		0.88 ± 0.01	53.19 ± 0.40	2.81	3.08
	44 %		0.92 ± 0.12	52.04 ± 3.31	2.75	3.01

[a] Standard uncertainties: $u(R_T)$= 0.90; $u(P)$= 10 kPa; $u(T)$ = 0.5 K.

Table 4 – *Effect of temperature on the partition coefficient and recovery of crocin in IL+ sorbitol ATPS and the values of ΔG_m^0 of partitioning of crocin*

IL (wt.%)	SOR (wt.%)	$K \pm \sigma^a$			$R \pm \sigma^a$			ΔG_m^0		
		Temperature (K)								
		298	303	308	298	303	308	298	303	308
38 %	30 %	3.21 ± 0.22	3.21 ± 0.50	3.33 ± 0.07	76.20 ± 1.22	76.22 ± 1.22	74.42 ± 0.99	–2.89	–2.94	–3.08
	32 %	7.97 ± 0.13	8.34 ± 0.10	8.52 ± 0.84	88.85 ± 0.16	89.29 ± 0.11	89.77 ± 0.93	–5.14	–5.34	–5.49
	34 %	20.45 ± 0.30	21.02 ± 0.60	35.44 ± 0.27	96.27 ± 1.38	95.46 ± 0.12	97.26 ± 0.02	–7.48	–7.67	–9.14
	36 %	25.76 ± 0.73	27.27 ± 0.19	35.80 ± 0.05	96.81 ± 0.67	96.46 ± 0.02	97.28 ± 0.00	–8.05	–8.32	–9.16
38 %	30 %	3.21 ± 0.22	3.21 ± 0.50	3.33 ± 0.07	76.20 ± 1.22	76.22 ± 1.22	74.42 ± 0.99	–2.89	–2.94	–3.08
40 %		6.56 ± 0.24	5.85 ± 0.41	8.92 ± 0.51	86.78 ± 0.42	85.38 ± 0.88	89.92 ± 0.49	–4.66	–4.45	–5.60
42 %		8.37 ± 0.79	10.39 ± 0.92	16.88 ± 0.28	89.29 ± 0.91	91.20 ± 0.72	94.41 ± 0.09	–5.26	–5.90	–7.24
44 %		12.17 ± 0.65	11.81 ± 0.66	39.85 ± 0.99	92.34 ± 0.39	92.2 ± 0.40	97.55 ± 0.06	–6.19	–6.22	–9.44

hancement in partition coefficient in all studied systems. At higher concentrations of IL and sorbitol (higher TLL), the temperature had a more significant effect. Increasing temperature led to more hydrophobicity of [P_{4444}] Br[37]. The maximum partition coefficient of crocin was 39.85 in IL-ATPS containing 44 wt.% of [P_{4444}] Br +30 wt.% of sorbitol at T = 308 K. This can be explained by the fact that the simultaneous effect of increasing the temperature and weight percentage of ionic liquid reduced the viscosity of the ionic liquid. Decreased viscosity in the IL-rich phase led to increased biomolecule mobility and increased biomolecule affinity for the top phase[27].

Molar Gibbs free energy of transformation of crocin was determined by Eq. 5:

$$\Delta G_m^{\ 0} = -RT \ln K_{\text{crocin}} \qquad (5)$$

T, R and K_{crocin} represent temperature, gas constant, and crocin partition coefficient, respectively. The values of $\Delta G_m^{\ 0}$ are reported in Table 4. The results showed that partitioning of crocin was a spontaneous process due to the negative $\Delta G_m^{\ 0}$. The value of $\Delta G_m^{\ 0}$ became more negative with an increase in temperature. The maximum recovery of crocin was 97.55 % in IL-ATPS containing 44 wt.% of $[P_{4444}]$ Br + 30 wt.% of sorbitol at T = 308 K. This shows that the separation of crocin using green IL-ATPS is possible in a one-step process.

A counter-plot for the partition coefficients of crocin, as well as the weight percentage of the ionic liquid and sorbitol is presented in Fig. 2. In this plot, the response of the partition coefficient (response variable) to the simultaneous changes of IL and sorbitol (control variable) at 308 K was observed. The contour plot shows a three-dimensional surface on a two-dimensional plane. In this plot, the interaction of variables and the optimal level for maximum response are well comprehensible. According to Fig. 2, it is clear that the concentration of sorbitol and IL had a significant impact on crocin partitioning. An increase in the weight percentage of the sorbitol and IL caused the partition coefficient of crocin to increase.

Comparison of the measured partition coefficient with literature values

The results found in the literature for the partition coefficient of betanin and crocin using different ATPS at the temperature of 298 K are reported in Table 5[21,38,39]. The partition coefficients of betanin show that betanin tends toward the top phase (PEG-rich phase) in ATPS consisting of polyethylene glycol and salt[21]. Nevertheless, betanin in the ATPSs containing tetrahydrofuran (THF) and two different salts, tended to migrate to the bottom phase[38]. The results demonstrate that the partition coefficient of betanin in ATPS containing THF + Na_2CO_3 / $Na_3C_6H_5O_7$ + H_2O is lower than those obtained by our system ($[P_{4444}]$ Br + sorbitol+ H_2O). The application of salts in ATPS alkalizes the system so that it can cause damage to the structures of pH-sensitive pigments. The use of carbohydrates with ILs increases the efficiency and solubility of ATPS.

Recently, the results on the partitioning of crocin in ATPSs composed of water, deep eutectic solvents (DESs), and acetonitrile (ACN) were published by our team (Table 5). Two DESs composed of choline chloride-urea (reline) and choline chloride-ethylene glycol (ethaline) were considered[39]. The authors defined the partition coefficient of crocin as the ratio of crocin concentration in the bottom phase to crocin concentration in the top phase. Therefore, the partition coefficients of crocin show that crocin tends toward the bottom phase

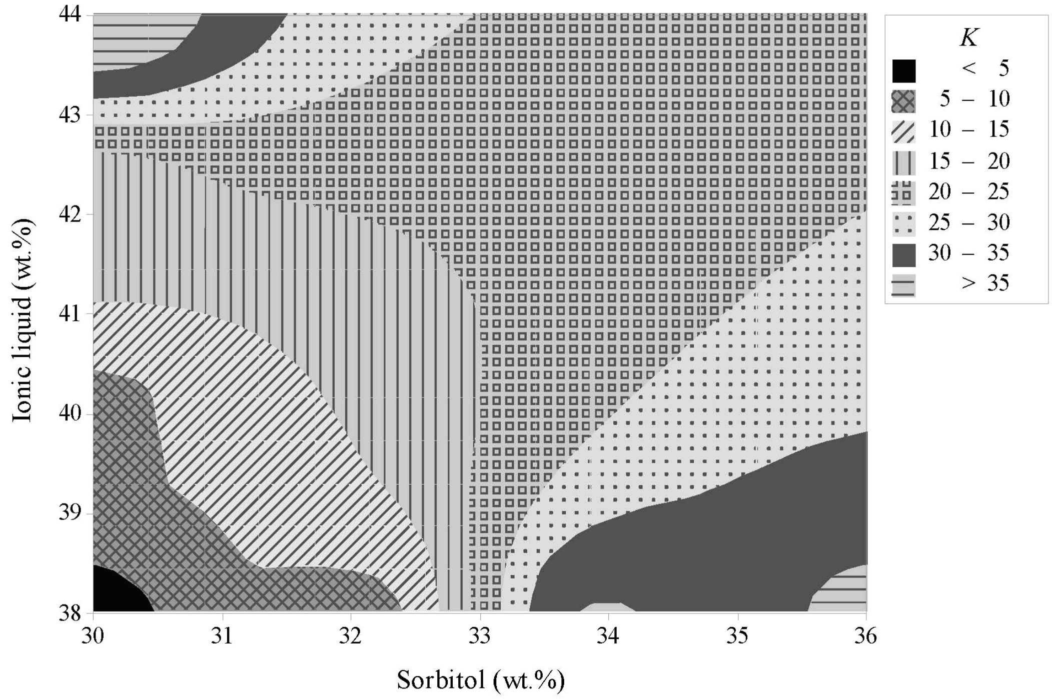

Fig. 2 – *Counter plot on the partition coefficients of crocin with the combined effects of weight percentage of the IL and sorbitol of the ATPS containing sorbitol +* $[P_{4444}]$ *Br + Sorbitol +* H_2O *at 308 K*

Table 5 – *Comparison of the reported maximum partition coefficient (K_{max}) of betanin/crocin with literature values at 298 K and 101 kPa in different ATPSs*

Betanin		
System	$K_{\max}=\frac{[\text{betanin}]_T}{[\text{betanin}]_B}$	Reference
Polyethylene glycol 4000 + Na_2SO_4 +H_2O	2.421	21
Polyethylene glycol 10000 + Na_2SO_4 + H_2O	2.059	21
System	$K_{\max}=\frac{[\text{betanin}]_T}{[\text{betanin}]_B}$	Reference
Tetrahydrofuran + Na_2CO_3 + H_2O	0.048	38
Tetrahydrofuran + $Na_3C_6H_5O_7$ + H_2O	0.117	38
Crocin		
System	$K_{\max}=\frac{[\text{crocin}]_B}{[\text{crocin}]_T}$	Reference
Ethaline +Acetonitrile + H_2O	36.53	39
Reline +Acetonitrile + H_2O	113.44	39

(DES-rich phase). As reported, the maximum value of crocin partition coefficients is presented as 36.53 and 113.44 for ethaline-based ATPS and reline-based ATPS, respectively[39]. However, the maximum partition coefficient of crocin in the ATPS containing [P_{4444}] Br + sorbitol+ H_2O was 25.76 at 298 K.

In recent years, ATPS has become a simple, selective, and low-cost tool that is promising in separation technology. The easy scalability of ATPS allows the industry to be used for downstream processing. In fact, determining the partition coefficient of bioactive pigments in different ATPS makes it easier to choose an efficient system. New types of ATPS and more knowledge about phase components lead to more advanced applications. Therefore, we used a new ATPS (containing [P_{4444}] Br and sorbitol) to evaluate the partitioning of crocin and betanin in this study. The main point of our research was based on our previous study that this ATPS has a high potential for a sensitive pigment such as curcumin (Kcur. = 79.33; RT % = 99.15)[24]. Since the partitioning mechanism in ATPS is still unknown, the authors believe that they can improve the partitioning of bioactive pigments by manipulating the main factors influencing partition behavior in ATPS in the future.

Conclusions

Aqueous two-phase systems based on ILs are currently receiving a great deal of attention. In this study, to use a powerful biocompatible method for the separation of crocin and betanin, the green IL-ATPS containing [P_{4444}] Br and sorbitol were investigated. The working points of IL-ATPS were determined with the help of the phase diagram at 298 K and ambient pressure. Assessing the potential of a benign IL-ATPS for the extraction of sensitive bioactive pigments could open new horizons for biological research and real applications at the industrial level. To evaluate the efficiency of the system, the partition coefficients and the recovery percentage of crocin and betanin were measured. In addition, some independent variables affecting the biomolecule partitioning such as concentrations of IL / carbohydrate, and temperature were examined. The experimental data showed that crocin tended toward the IL-rich phase and betanin tended toward the carbohydrate-rich phase. The results indicated that increasing the temperature increased the crocin partitioning in the top phase. The selected IL-ATPS composition for crocin was 44 wt.% of [P_{4444}] Br + 30 wt.% of sorbitol at T = 308 K. The maximum recovery percentage of crocin for a one-stage process was 97.55% in the top phase. The maximum amount of betanin recovery was 61.38 % for a one-stage process in IL-ATPS containing [P_{4444}] Br (38 wt.%) + sorbitol (30 wt.%).

DECLARATIONS

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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