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Using Ionic Liquids To Tune the Performance of Aqueous Biphasic Systems Based on Pluronic L-35 for the Purification of Naringin and Rutin

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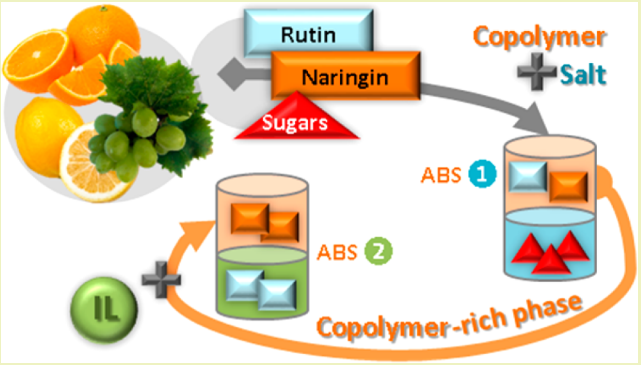
Supporting Information

ABSTRACT: Aqueous biphasic systems (ABS) based on Pluronic L-35, a $(EO)_x$-$(PO)_y$-$(EO)_x$ triblock copolymer, were determined and applied in the separation of two structurally similar flavonoids (naringin and rutin). Two sets of phase formers were paired with Pluronic L-35, one comprising conventional salts/buffer and other including cholinium-based ionic liquids (ILs). It is shown that while the conventional salts induce an unbalanced and strong salting out leading to complete extraction of flavonoids to the same phase in most of the cases ($84.7 \pm 0.6\% \leq R_{NAR} \leq 100\%$ and $53.2 \pm 0.5\% \leq R_{RUT} \leq 99.7 \pm 0.1\%$ with selectivities ranging from 1 to 11.8), the cholinium-based ILs provide an enhanced extractive performance. Indeed, these novel cholinium ILs/Pluronic L-35-based ABS allowed the manipulation of the affinity of both naringin and rutin to opposite phases, thus yielding a selective separation. The best results were achieved for the system using [Ch][Bic] as phase former ($R_{NAR} = 89.6 \pm 0.3$ and $R_{RUT} = 32 \pm 2$ with a selectivity of 18.9). An integrated approach based on the sequential implementation of Na_2SO_4/Pluronic L-35- (step 1) and [Ch][Bic]/Pluronic L-35-based (step 2) ABS was designed to purify the flavonoids from a complex synthetic mixture simulating natural extracts. Remarkably, glucose (the main contaminant) was removed during step 1 with an extraction efficiency of $60 \pm 4\%$ to the Na_2SO_4-rich phase, while step 2 has enabled the efficient separation of naringin from rutin.



KEYWORDS: *Aqueous biphasic systems, Ionic liquids, Copolymer, Purification, Flavonoid, Rutin, Naringin*

INTRODUCTION

Research on ionic liquid-based aqueous biphasic systems (IL-based ABS) has been progressing at a fast pace, and their advantages over other liquid−liquid extraction systems are well marked.[1] It is possible to develop adequate extraction platforms for many biomolecules through the cautious selection of the phase-forming components, which allows one to finely tune their lipophilic−hydrophilic nature and to boost their biocompatibility.[1−3] At this stage, outstanding performances were attained for the extraction and purification of diverse compounds from pharmaceutical, biotechnological, and environmental origins.[1] However, their performance in the separation of structurally similar compounds has seldom been attempted with success.

In the quest for more performant systems aiming at the separation of similar compounds, the introduction of copolymers in the IL-based ABS domain is here envisaged as an approach to create "more tunable" and selective systems. Copolymers are tunable chemicals, since they arise from the simultaneous polymerization of two (or more) types of monomers yielding properties that in some cases are intermediate between those of the homopolymers while in others these may have a completely different behavior and novel properties (e.g., surface activity).[4] Pluronic (trademark registered from BASF, also known as Poloxamer) is an example of a class of triblock copolymers that is currently used, among others, as a pharmaceutical excipient accepted under the United States and British Pharmacopeia criteria.[5] Having their backbones formed by ethylene oxide (EO) blocks at both terminals and propylene oxide (PO) blocks in the middle at distinct EO/PO ratios, Pluronics are often denoted as $(EO)_x$-$(PO)_y$-$(EO)_x$.[6] These have an amphiphilic nature that enables the solubilization of hydrophobic molecules and are thermosensitive, facilitating their recovery and reusability. Playing with their EO/PO composition, Pluronics may be either highly lipophilic or hydrophilic if bearing short EO chains and/or large

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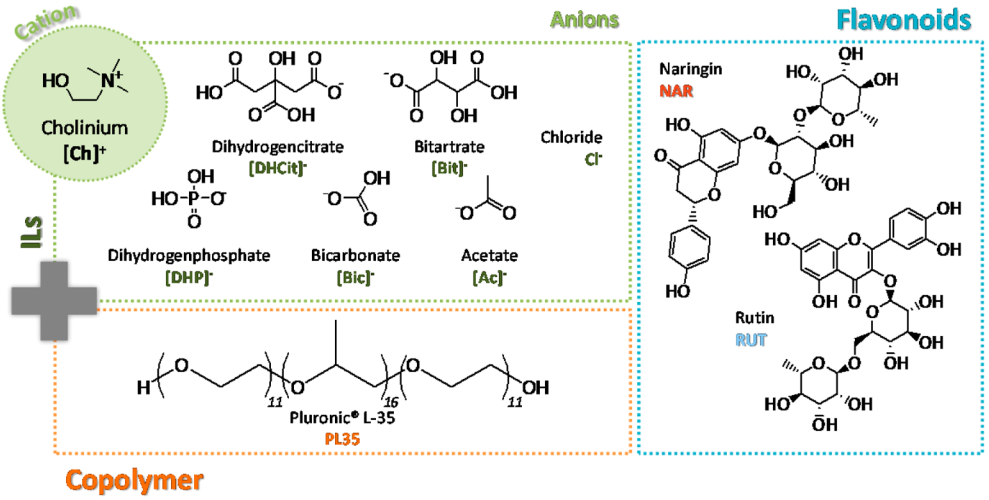

Figure 1. Chemical structures, names, and abbreviations of the cholinium-based ILs and salts, copolymer, and flavonoids studied.

PO chains or vice versa, which means that they can be designed with intermediate degrees of hydrophobicity.[7] For their application in the formulation of ABS, they present convenient properties, namely, (i) their amphiphilic nature that enables the solubilization of hydrophobic molecules,[6] (ii) their thermosensitivity facilitating their recovery and reusability,[8] (iii) their capacity to be designed with intermediate degrees of hydrophobicity,[9] and (iv) their higher biocompatible nature.[10]

The use of $(EO)_x$-$(PO)_y$-$(EO)_x$ copolymers in ABS is not new, and their combination with conventional salts to induce liquid−liquid demixing in aqueous solution is well documented. Their phase separation phenomena were systematically studied considering distinct salts and Pluronics (varying both the molecular weight and EO/PO ratios) at distinct temperatures and pH.[11−16] Scarcer are the studies evaluating the possibility of creating ABS composed of Pluronics and other polymers (e.g., dextran).[17,18] Foreseeing their future application, some authors unveiled the extraction aptitude of Pluronic-based ABS for metallic ions,[19,20] peptides/proteins,[12,13,17] and vitamins,[15] as well as their use for the extractive transformation of steroids.[21] Liu et al.[22] reported the combined use of copolymers and cholinium-based ILs, focusing on the liquid−liquid equilibrium data and the impact of the copolymer nature on the ABS formation. Chakraborty and Sen[23] developed a new ABS composed of an IL and a copolymer for the extraction of zinc−cholesterol complex.

In this work, it is intended to use the tunable and intermediate hydrophilicity−lipophilicity of the thermosensitive $(EO)_x$-$(PO)_y$-$(EO)_x$ copolymers compared to those of the homopolymers for the development of highly selective extraction and/or purification platforms. Two classes of ABS were here studied: one comprising six Pluronic L-35 + cholinium-based ILs + water systems and another with Pluronic L-35 + conventional salts + water systems. In order to gather insights on how to design selective Pluronic-mediated purification processes, the ternary phase diagrams were determined and characterized and the partition of two structurally similar compounds on these systems evaluated. The molecules selected to carry out this study were naringin and rutin, two glycosylated flavonoids belonging to the flavanone and flavonol classes, respectively. Flavonoids are the second most used class of phytonutrients, recognized for their high market value. The valorization forecasted for this market reports a growth from $3.05 billion in 2014 to $4.63 billion in 2020, this being driven by health benefits-related demands and the many applications of these natural products.[24] The use of flavonoids has gained much favor from nutraceutical, cosmetic, and pharmaceutical industries due to their countless health benefits which comprise antioxidant, anti-inflammatory, free radical scavenging, and antimicrobial and anticancer activities, as well as protection against cardiovascular diseases.[25]

Solvent extraction processes with volatile organic solvents (e.g., hexane, chloroform, acetonitrile, benzene, methanol, and ethanol) are nowadays used to extract flavonoids from their biological sources.[26,27] The flavonoids' purification is commonly carried out by applying preparative planar chromatography or column-based chromatography.[26] This later process is more complex and expensive, since it requires it to be coupled to other separation techniques of enhanced resolution power.[26] Aiming at the development of simpler and more performant purification processes, the use of pressurized liquids,[28] supercritical fluids,[29] and ILs[30] was recommended. Herein, a novel and simpler yet efficient process based on the application of Pluronic-based ABS is proposed.

EXPERIMENTAL SECTION

Materials. The copolymer used is a poly(ethylene glycol)-*block*-poly(propylene glycol)-*block*-poly(ethylene glycol), with an average molecular weight of 1900 g mol^{-1} and a EO/PO ratio of 50/50 [commercially known as Pluronic L-35 (PL35), denoted as $(EO)_{11}$-$(PO)_{16}$-$(EO)_{11}$]. It was acquired from Sigma-Aldrich. Cholinium chloride, [Ch]Cl (99 wt % pure), cholinium dihydrogencitrate, [Ch][DHCit] (98 wt % pure), cholinium bitartrate, [Ch][Bit] (98 wt % pure), and cholinium bicarbonate, [Ch][Bic] (80 wt % pure), were supplied by Sigma-Aldrich, while cholinium dihydrogen phosphate, [Ch][DHP] (99 wt % pure), and cholinium acetate, [Ch][Ac] (98 wt % pure) were purchased from Iolitec. Figure 1 displays the chemical structures of the cholinium compounds and the copolymer investigated. Eight salts were used, namely, potassium phosphate monobasic, KH_2PO_4 (99.5 wt % pure), potassium phosphate dibasic trihydrate, $K_2HPO_4{\cdot}3H_2O$ (98−102 wt % pure), potassium phosphate tribasic, K_3PO_4 (≥98 wt % pure), sodium potassium tartrate tetrahydrate, $C_4H_4KNaO_6{\cdot}4H_2O$ (≥99 wt % pure), potassium carbonate, K_2CO_3 (≥99 wt % pure), sodium sulfate, Na_2SO_4 (≥99 wt % pure), sodium succinate hexahydrate, $C_4H_4Na_2O_4{\cdot}6H_2O$ (≥99 wt % pure), and potassium citrate monohydrate, $C_6H_5K_3O_7{\cdot}H_2O$ (99 wt % pure). Apart from potassium citrate

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monohydrate and potassium phosphate dibasic trihydrate, which were, respectively, obtained from Acros Organics and Panreac, all other salts were supplied by Sigma-Aldrich. Citric acid monohydrate, $C_6H_8O_7 \cdot H_2O$ (≥99.5 wt % pure), which was used along with $K_2HPO_4 \cdot 3H_2O$ to prepare a buffer yielding six distinct pH media (3−8), was purchased from Panreac. For the HPLC mobile phase preparation, ultrapure water treated using a Milli-Q 185 water purification apparatus was used as well as methanol (99.99 wt % pure) and phosphoric acid (85 wt % pure) obtained at Fischer-Chemical and Panreac, respectively. Syringe filters (0.45 μm) and membrane filters (0.45 μm) were from Whatman.

Naringin, NAR (97 wt % pure), was supplied by Acros Organics, and rutin hydrate, RUT (98 wt % pure), was obtained at TCI. Their chemical structures are also presented in Figure 1. D-(+)-Glucose anhydrous (extra pure) was acquired at Scharlau.

Determination of Phase Diagrams and Tie-Lines. The ternary phase diagrams were determined by the cloud point titration method (at 25 °C and atmospheric pressure). Briefly, aqueous solutions of cholinium-based compounds at concentrations ranging from 60 to 80 wt % and conventional salts at concentrations between 11 and 50 wt % and of PL35 at 60−70 wt % were initially prepared. Buffers containing $K_2HPO_4 \cdot 3H_2O$ + $C_6H_8O_7 \cdot H_2O$ at 20−60 wt % and distinct pH values (3, 4, 5, 6, 7, and 8) were prepared according to McIlvaine.[31]

The compositions of the solubility curves were determined by weight quantification ($\pm 10^{-4}$ g). The complexed water was taken into account in the salts molality and mass fraction calculations.

To determine the tie-lines, the gravimetric method proposed by Merchuk et al.[32] was adopted. A ternary mixture within the biphasic system of each diagram was gravimetrically prepared within $\pm 10^{-4}$ g, vigorously stirred, and left to equilibrate for at least 12 h. At this point, the phases were separated and weighed for the TLs determination by the lever-arm rule through the relationship between the PL35-rich phase composition and that of the overall system.

More detailed information on the solubility curves correlation (eq S1) and tie-lines calculation (eqs S2−S8) is provided in SI.

Flavonoids Partition Studies Using Pluronic L-35-Based ABS. Aimed at evaluating the extraction ability of the ABS under study, partition studies for both flavonoids (rutin and naringin) were carried by fixing mixture points at the biphasic zone of the ternary phase diagrams. Taking into account the distinct aptitudes to undergo phase separation, the following ternary mixture compositions were selected: 35 wt % of PL35 + 30 wt % of cholinium-based compounds + 35 wt % of water, 23 wt % of PL35 + 10 wt % of salt + 67 wt % of water, and 35 wt % of PL35 + 16 wt % of $K_2HPO_4/C_6H_8O_7$ + 49 wt % of water. Around 2 mg of naringin or rutin was added to each ternary mixture of 3 g total mass (initial concentration of flavonoid around 0.67 $mg_{Flav} \cdot g_{ABS}^{-1}$) in independent assays. Vigorous agitation was used, and at least 12 h of equilibration time was allowed. After separating the phases, they were weighed and the flavonoids content in each phase assayed by UV spectroscopy using a Shimadzu UV-1800 spectrophotometer at wavelengths of circa λ_{NAR} = 282 nm and λ_{RUT} = 255 nm. At least three replicates were prepared and further analyzed (two analysis per each aqueous phase) aimed at determining the recoveries of each flavonoid and the respective standard deviations. Blank controls, i.e., the same ternary mixtures without the addition of flavonoid, were systematically tested to eliminate possible interferences of PL35 and cholinium-based compounds or salts.

Three parameters were determined to fully characterize the extraction performance of Pluronic-based ABS, namely, the flavonoids recovery (R_{Flav}) to the PL35-rich phase, the partition coefficient (K_{Flav}), and the selectivity (S), estimated by eqs 1, 2, and 3

$$R_{Flav}(\%) = \frac{Abs_{Flav}^{PL35} \times w_{PL35} \times f_{PL35}}{Abs_{Flav}^{PL35} \times w_{PL35} \times f_{PL35} + Abs_{Flav}^{salt} \times w_{salt} \times f_{salt}} \times 100 \quad (1)$$

$$K_{Flav} = \frac{Abs_{Flav}^{PL35} \times f_{PL35}}{Abs_{Flav}^{salt} \times f_{salt}} \quad (2)$$

$$S = \frac{K_{Flav_1}}{K_{Flav_2}} \quad (3)$$

where Abs_{Flav}^{PL35} and Abs_{Flav}^{salt} are the absorbance of the target flavonoid in the PL35-rich and cholinium-based compound/salts-rich phases (considering the respective dilution factors, f_{PL35} and f_{salt}) and w_{PL35} and w_{salt} are the weights of PL35-rich and cholinium-based compound/salts-rich phases, respectively. For naringin and rutin, the general notation Flav is replaced by NAR and RUT. K_{Flav1} and K_{Flav2} stand for the partition coefficients of either naringin or rutin in which $K_{Flav1} \geq K_{Flav2}$ so that $S \geq 1$.

Purification of Flavonoids from a Complex (Synthetic) Mixture by Applying Pluronic L-35-Based ABS. The separation of flavonoids and sugars, here considered as the main contaminants, was carried by sequentially implementing two distinct types of Pluronic L-35-based ABS in two sequential steps. During step 1, ternary mixtures of 23 wt % of PL35 + 10 wt % of Na_2SO_4 + 67 wt % of water were prepared. A powder mixture of rutin, naringin, and glucose in a mass proportion of 1:1:1 was then added in the same amounts indicated in the partition studies. The glucose content was determined by a colorimetric method using dinitrosalicylic acid (DNS).[33] Briefly, after adding 1 mL of a DNS solution to 1 mL of ABS phase properly diluted, the resulting mixture was vigorously agitated and placed in a water bath at 100 °C for 5 min to react. After this period, the reaction was stopped by immersing the samples on ice for brief minutes and diluting them with 10 mL of distilled water. The reaction product (3-amino-5-nitrosalicylic acid) was quantified at a wavelength of 540 nm using a Shimadzu UV-1800 spectrophotometer, based on a calibration curve previously established. Blank controls composed of flavonoids and without glucose were applied to eliminate the flavonoids interference within the colorimetric method. Three replicates were prepared with two analyses per sample aiming to calculate the extraction efficiencies of glucose and the respective standard deviations.

The extraction efficiency of glucose ($EE_{Glucose}$) was determined according to eq 4

$$EE_{Glucose}(\%) = \frac{m_{Glucose}^{Na2SO4}}{m_{Glucose}^{0}} \times 100 \quad (4)$$

where $m_{Glucose}^{Na_2SO_4}$ and $m_{Glucose}^{0}$ denote, respectively, the mass of glucose in the Na_2SO_4-rich phase and the sugar amount initially added in the ABS.

In this part of the work, the partition of the flavonoids was assessed through HPLC-DAD using a liquid chromatograph HPLC Elite LaChrom (VWR Hitachi) equipped with a diode array detector (DAD) l-2455, column oven l-2300, autosampler l-2200, and pump l-2130. The analytical column used was from Merck, and it was a 5 μm, 250 mm × 4 mm i.d. C_{18} reversed-phase column (LiChrospher 100 RP-18) linked to a 5 μm, 4 mm × 4 mm guard column with the same stationary phase. The method used was adapted from Nogata et al.[34] with modifications. The column oven and the autosampler were operated at controlled temperatures of 40 and 25 °C, respectively. The separation was performed with a mobile phase composed of 30% of methanol as the organic phase and 70% of phosphoric acid (0.008 M) as the aqueous phase under isocratic conditions at a flow rate of 0.6 $mL.min^{-1}$ and using an injection volume of 10 μL. DAD was set to measure naringin at 284 nm and rutin at 258 nm. Calibration curves were prepared in methanol. Naringin and rutin display retention times of around 35.7 and 40.1 min, respectively. Step 1 was conducted by preparing a mixture containing 23 wt % of PL35 + 10 wt % of Na_2SO_4 + 67 wt % of a synthetic mixture prepared in water containing a concentration of 0.01 $g \cdot L^{-1}$ of each flavonoid. The flavonoids-enriched (top) phase (PL35 rich) was then used in the preparation of a new ABS, this constituted by 35 wt % of PL35 + 30 wt % of [Ch][Bic]. After the phases' separation, each phase was properly diluted in the mobile phase and analyzed at least twice. The concentration of each flavonoid was assessed using the calibration curves previously established. These results were further applied on the calculation of R_{Flav} and log K_{Flav} by using eqs 1 and 2.

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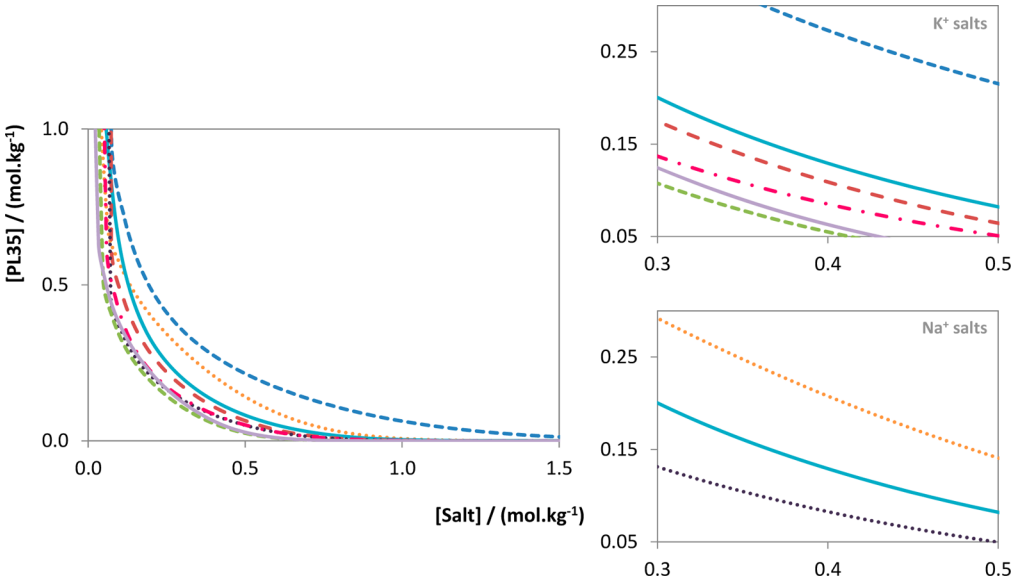

Figure 2. Binodal curves obtained for each ABS formed by Pluronic L-35 + salts + water at 25 °C and atmospheric pressure: K_3PO_4 (green dashed line), $C_6H_5K_3O_7$ (purple solid line), Na_2SO_4 (purple dotted line), K_2HPO_4 (red dashed−dotted line), K_2CO_3 (red dashed line), $C_4H_4KNaO_6$ (blue solid line), $C_4H_4Na_2O_4$ (orange dotted line), and KH_2PO_4 (blue dashed line).

pH and Conductivity Determination. Aqueous phases pH (±0.02), a central parameter in any extraction process, and conductivities, the property herein adopted to identify the top and bottom layers as being PL35 rich or salt/[Ch]X rich, were determined using a Mettler Toledo S47 SevenMulti dual meter pH/conductivity.

RESULTS AND DISCUSSION

Aiming at the creation of more tunable and selective purification processes based on ABS, ILs and the triblock copolymer Pluronic L-35 were combined as phase formers. Moreover, the use of common salts in the preparation of ABS was also explored and the results compared with those obtained by ILs. In this regard, the phase diagrams and partitions of the two flavonoids in each class of ABS were investigated. Using all results here gathered, in particular, the distinct partition behaviors displayed by the flavonoids for each class of ABS, an integrated process for the extraction and purification of flavonoids from natural extracts is designed.

Pluronic L-35 + Salts ABS. *Phase Diagrams.* The first set of ABS here explored combines the use of Pluronic L-35 with common salts of both inorganic and organic nature, including buffers. Ternary phase diagrams for each Pluronic L-35 + salt/buffer + water at 25 °C and atmospheric pressure were determined. All data are provided in SI, namely, the mass fraction compositions experimentally obtained (Tables S1−S14), the Merchuk equation parameters (Table S15), and the detailed characterization of the phases based on the mixture compositions, pH, and conductivity (Table S16). In these systems, as further inferred from the conductivity measurements, the bottom layer is salt rich whereas the top layer is copolymer rich.

Among the eight salts used to prepare the ABS reported in Figure 2, $Na_2C_4H_4O_4$[11] and Na_2SO_4[14,20] were previously reported to form ABS with Pluronic L-35 (at 25 °C), and the data here obtained are in good agreement with that reported by other authors.[11,14,20]

To eliminate the effects induced by the different molecular weights of salts upon phase formation behavior, the binodal curves are presented in Figures 2 (inorganic and organic salts) and 3 ($K_2HPO_4/C_6H_8O_7$ buffer) in molality units. The larger the biphasic region (placed above the solubility curve), the higher the salt ABS formation capacity and, accordingly, the salts aptitude at promoting liquid−liquid demixing, which can be ranked as follows (at [salt] = [PL35] = 0.35 mol.kg^{-1}): K_3PO_4 > $C_6H_5K_3O_7$ > Na_2SO_4 ≈ K_2HPO_4 > K_2CO_3 > $C_4H_4KNaO_6$ > $C_4H_4Na_2O_4$ > KH_2PO_4. This trend follows the Hofmeister series. Stronger salting-out agents, i.e., those of higher valence, are better at inducing two-phase separation due to their higher affinity for the water molecules and consequent ability to expel the copolymer toward a second phase. This mechanism is well established in the literature and consistent with observations for systems made of polyethylene glycol + salts[35] and ILs + salts.[36]

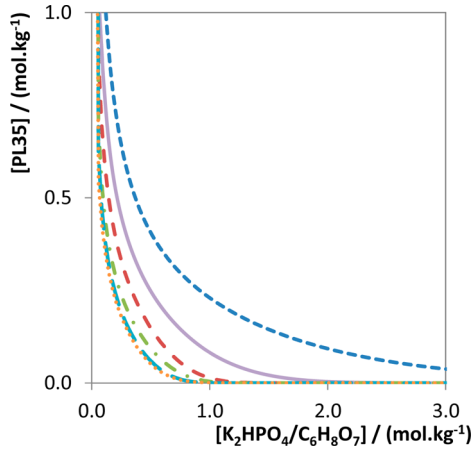

Figure 3. Binodal curves obtained for each ABS formed by Pluronic L-35 + $K_2HPO_4/C_6H_8O_7$ buffer + water at 25 °C, atmospheric pressure and distinct pH: pH 8 (orange dotted line), 7 (blue dashed line), 6 (green dashed−dotted line), 5 (red dashed line), 4 (purple solid line), and 3 (dark blue dashed line).

A buffer composed of K_2HPO_4 and $C_6H_8O_7$ was selected to prepare ABS at controlled pH, within a range from 3 to 8.

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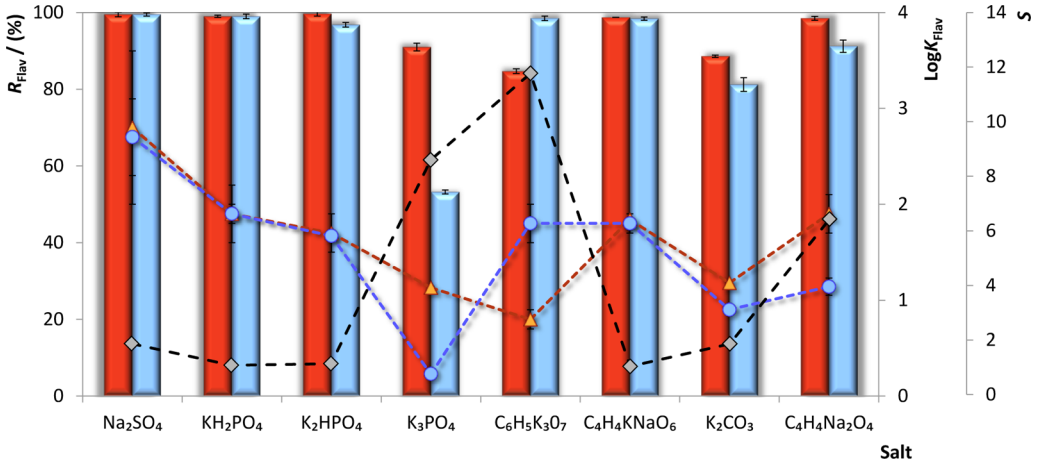

Figure 4. Recoveries (R_{Flav}, bars) and partition coefficients (log K_{Flav}, symbols) of naringin (orange bars and triangles) and rutin (blue bars and circles) along with the selectivity data (S, black dashed line) regarding the application of ABS composed of 23 wt % of PL35 + 10 wt % of salt +67 wt % of water.

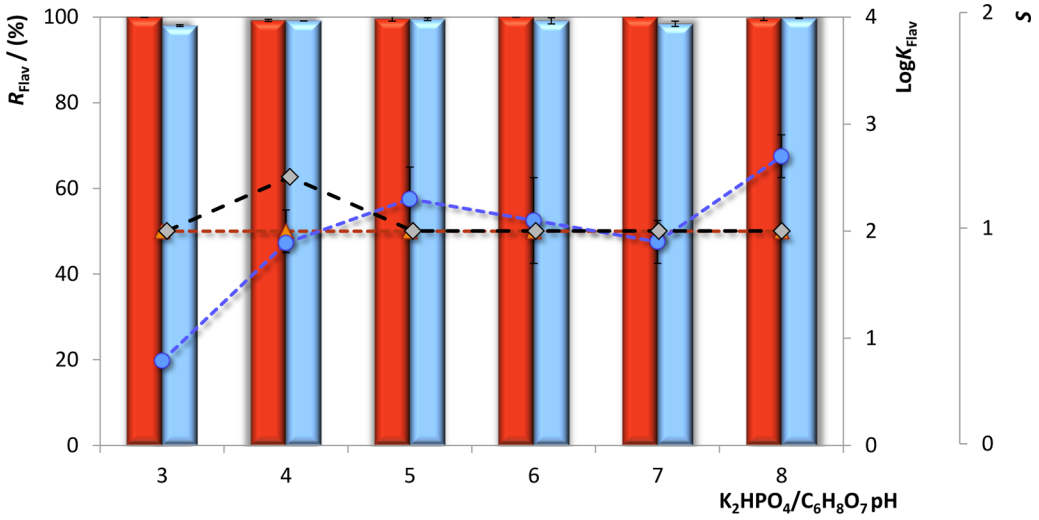

Figure 5. Recoveries (R_{Flav}, bars) and partition coefficients (log K_{Flav}, symbols) of naringin (orange bars and triangles) and rutin (blue bars and circles) along with the selectivity data (S, dashed black line) considering the application of ABS composed of 35 wt % of PL35 + 16 wt % of $K_2HPO_4/C_6H_8O_7$ + 49 wt % of water.

Although Pluronic-based ABS have already been studied at distinct pH values (e.g., K_2HPO_4/KH_2PO_4 at pH 7 and K_2HPO_4/KOH at pH 12),[16,37] the pH window previously available was quite narrow, limiting the purification/fractionation of biomolecules. The buffer here used affords a wider pH range and also offers a more benign nature due to the presence of the natural organic acid $C_6H_8O_7$. The body of results gathered is represented in Figure 3 and reveals that the higher the pH, the larger the immiscibility region. This enhanced aptitude of forming ABS under more alkaline conditions is explained again in light of the salt salting-out power and ions valence. The HPO_4 and $C_6H_8O_7$ ions are, respectively, present as divalent and trivalent ions at pH 8, valences higher than the non-, single-, and double-charged species exhibited at lower pH.[38]

Partition Studies of Flavonoids. Having established the phase diagrams, the following ternary mixtures were selected to carry out the studies on the flavonoids partition: 23 wt % of PL35 + 10 wt % of salt +67 wt % of water and 35 wt % of PL35 + 16 wt % of $K_2HPO_4/C_6H_8O_7$ + 49 wt % of water. Figures 4 and 5 show the extraction parameters obtained in this study. The detailed data is reported in SI (Table S17).

With the exception of K_3PO_4-containing ABS, in which rutin is partitioned between the two phases (R_{RUT} = 53.2 ± 0.5% and log K_{RUT} = 0.23 ± 0.06), on the other systems the flavonoids completely partition to the copolymer-rich phase. This is patent in the large positive values of log K_{Flav} (0.8 ± 0.1 ≤ log K_{NAR} ≤ 2.8 ± 0.8 and 0.23 ± 0.06 ≤ log K_{RUT} ≤ 2.7 ± 0.4) and the large R_{Flav} values obtained (84.7 ± 0.6% ≤ R_{NAR} ≤ 100% and 81 ± 2% ≤ R_{RUT} ≤ 99.7 ± 0.1%).

Although the behavior observed seems to be almost independent from the salt used or the pH, the analysis of Figure 4 shows that the impact of the salt nature upon the flavonoids' recoveries is the following

NAR: Na_2SO_4 > KH_2PO_4 > $C_4H_4KNaO_6$ > $C_4H_4Na_2O_4$ > K_2HPO_4 > K_3PO_4 > K_2CO_3 > $C_6H_5K_3O_7$

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RUT: Na_2SO_4 > KH_2PO_4 > $C_6H_5K_3O_7$ > $C_4H_4KNaO_6$ > K_2HPO_4 > $C_4H_4Na_2O_4$ > K_2CO_3 > K_3PO_4

This pattern indicates that the pH induced by the salts is the parameter ruling the partition of the flavonoids to the Pluronic L-35-rich phase. Salts providing more acidic environments (e.g., Na_2SO_4, KH_2PO_4, and $C_4H_4KNaO_6$) are more efficient at completely extracting naringin and rutin, whereas those of alkaline pH (e.g., K_2CO_3 and K_3PO_4) limit the complete partition of the flavonoids toward one of the phases. With the pH, both naringin and rutin suffer from changes on their charge due to the deprotonation of their hydroxyl groups (Figures S1 and S2 in SI).[38] Under acidic pH conditions, the flavonoids are present in their neutral form, thus preferentially partitioning toward the copolymer-rich phase, while the presence of charged species at alkaline pH allows the flavonoids to also establish interactions with the salt-rich phase.[38] This phenomenon is more relevant for rutin, which has a higher valence than naringin in alkaline conditions (cf. Figures S1 and S2 in SI),[38] in particular for the K_3PO_4-containing systems. Hence, among the salt/buffer-based ABS screened, this is the most selective system (S = 8.6) since significant partition of naringin and rutin to opposite phases is observed (R_{NAR} = 91 ± 1% vs R_{RUT} = 53.2 ± 0.5%). Similar partition patterns of rutin were found in alcohol−salt-based ABS, wherein replacing a phosphate buffer by K_3PO_4 allowed changing the partitioning from the alcohol-rich to the salt-rich phase.[39,40]

It should be noted that the log K_{Flav} values in some cases were established as >2 as an easier way to show the complete partition of the biomolecules to the copolymer-rich phase. In most cases, this was observed for both flavonoids, and the selectivity values were thus fixed at ∼1 (Table S17 in SI). The picture emerging from these results suggests that although the salting out by the salt plays the most important role on the partition of these flavonoids, the pH may also be used to tune their migration.

However, despite the remarkable extraction ability revealed by the buffered systems, these do not allow one to fine tune the partition of the flavonoids. With these systems, highly effective extraction systems can be created in which the flavonoids are concentrated in the same phase; yet, the development of an efficient separation process for flavonoids is constrained by the poor selectivities achieved.

Pluronic L-35 + Cholinium-Based ILs ABS. *Phase Diagrams.* In the search for an efficient system allowing the selective separation of the glycosylated flavonoids, the substitution of the conventional salts by ILs was here attempted.

ILs are more advantageous for this purpose due to their *designer solvent* characteristic. Resorting to a strategy similar to that outlined for the study of Pluronic L-35-salts-based ABS, the first step in the development of novel Pluronic L-35/cholinium-based ABS was the measurement of the ternary phase diagrams. Again, the SI reports all of the detailed information on the experimental mass fraction compositions (Tables S18−S23), Merchuk equation parameters (Table S24), and tie-lines, along with the pH and conductivity data for both phases (Table S25). In most systems and analogously to what was observed for the Pluronic L-35/salt-based ABS, the top and bottom phases are, respectively, copolymer and IL enriched. The exceptions are the [Ch][Ac] and [Ch]Cl for which inversions in the phases densities are observed. Molality units were again adopted when plotting the binodal curves presented in Figure 6. The binodal curves follow the trend $[DHP]^-$ > $[Bit]^-$ > $[DHCit]^-$ > $[Bic]^-$ > $[Ac]^-$ > Cl^-. Previous works have demonstrated that cholinium-based ILs are able to form ABS in aqueous solutions of polymers, viz. PEG and PPG,[41,42] and of K_3PO_4.[43] The molecular mechanism behind the formation of ABS formed by cholinium and inorganic salts is well understood, and it is based on the salting out of the salt (e.g., K_3PO_4) over the cholinium-based salts and ILs.[1,43] In turn, in PPG-based systems, cholinium-based compounds are the salting-out agents as discussed by Quental et al.,[42] although recent evidence suggests that for other ILs the PPG−ILs interactions may also play an important role in the ABS creation.[44] In ABS composed of PEG and cholinium-based ILs, Pereira et al. evidenced different mechanisms between the cholinium-based ILs, for which the molecular mechanisms are more complex and specific interactions implying $[Ch]^+$, $[anion]^-$, PEG, and water must be considered, and the cholinium-based salts (with higher melting points), for which the salting out seems also to be the dominant mechanism.[41]

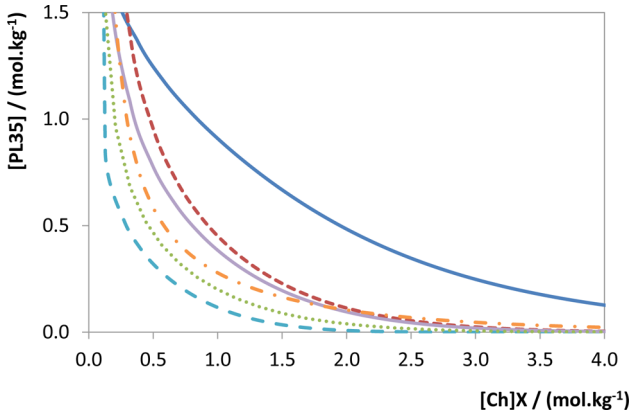

Figure 6. Binodal curves obtained for each ABS formed by Pluronic L-35 + [Ch]X + water at 25 °C and atmospheric pressure: [Ch][DHP] (blue dashed line), [Ch][Bit] (green dotted line), [Ch][DHCit] (orange dashed−dotted line), [Ch][Bic] (purple solid line), [Ch][Ac] (red dashed line), and [Ch]Cl (dark blue solid line).

Anion polar surface (surface sum over all polar ions, including oxygen and their attached hydrogen atoms) and lipophilicity (represented by the octanol−water partition coefficients−log $K_{o/w}$) of ILs are well documented as being correlated with the ABS formation capability. The ABS-inducing ability seems to correlate well with increasing anion polar surfaces and decreasing lipophilicities, as described below.

Anion polar surface: $[DHP]^-$ (90.40 A^2) > $[Bit]^-$ (82.06 A^2) > $[DHCit]^-$ (134.96 A^2) > $[Bic]^-$ (60.36 A^2) > $[Ac]^-$ (40.13 A^2) > Cl^- (0 A^2);

Octanol−water partition coefficients: $[DHP]^-$ (log $K_{o/w}$ = −3.70) > $[Bit]^-$ (log $K_{o/w}$ = −1.43) > $[DHCit]^-$ (log $K_{o/w}$ = −1.32) > $[Bic]^-$ (log $K_{o/w}$ = −3.70) > $[Ac]^-$ (log $K_{o/w}$ = −4.66) > Cl^- (log $K_{o/w}$ = −3.70).

The scenario here reported resembles that discussed by Pereira et al. for ABS made of cholinium-based derivatives and PEG.[41] The odd behavior of [Ch][DHCit] here observed (i.e., a lower ability to form ABS than predicted by the descriptors used) was also previously observed for other polymer-based ABS, and it has been justified in the light of $[anion]^-$/$[anion]^-$ interactions due to the $[DHCit]^-$ self-aggregation.[41,42,45] Also, [Ch][Ac] was expected to perform worst at promoting ABS

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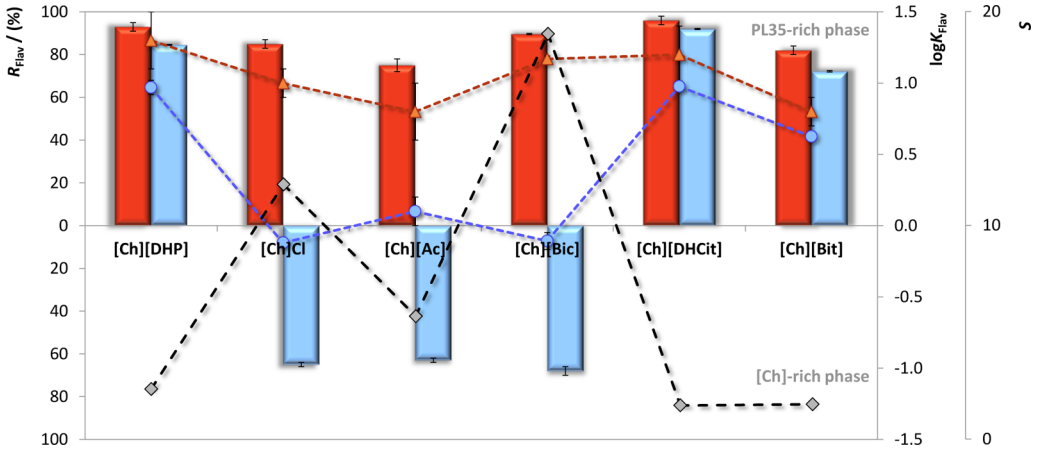

Figure 7. Recoveries (R_{Flav}, bars) and partition coefficients (Log K_{Flav}, symbols) of naringin (orange bars and triangles) and rutin (blue bars and circles) along with the selectivity data (S, black dashed line) considering the use of ABS composed of 35 wt % of PL35 + 30 wt % of [Ch]X + 35 wt % of water.

formation as it is the least lipophilic compound tested (reflected by its negative log $K_{o/w}$ of −4.66). Still, this is the only IL under investigation (melting point below 100 °C) for which the interactions involved in the ABS formation with PEG are different from those occurring when higher melting temperature cholinium salts are used.[41]

Concerning the dehydration of the $(EO)_x$-$(PO)_y$-$(EO)_x$ copolymer by the IL in aqueous solution, a recent study on the critical micellization temperature of aqueous solutions of Pluronic F-108 provided new insights.[46] Fluorescence studies show that both [Ch][Bit] and [Ch][DHCit] are more prone to dehydrate the copolymer than [Ch][Ac] and [Ch]Cl,[46] in accordance with their relative position in the ABS formation ability ranking.

Overall, comparing the trends (in molality units) elsewhere disclosed for ABS formed by the same group of cholinium-based compounds and either PEG or PPG (cf. Figure S3) a clear image emerges: Pluronic L-35, despite having a 50/50 EO/PO ratio, behaves closer to PEG than to PPG in the formation of ABS with cholinium-based salts.

Partition Studies of Flavonoids. To conduct the partition studies of naringin and rutin in systems containing cholinium-based compounds, ternary mixtures with fixed compositions of 35 wt % of Pluronic L-35 + 30 wt % of [Ch]X + 35 wt % of water were used. A detailed overview of the extraction results obtained is reported in SI, Table S26, and graphically represented in Figure 7.

Unlike to the flavonoids partition pattern afforded by the ABS composed of conventional salts and Pluronic L-35, where an extensive partition toward the copolymer-rich phase is observed, the cholinium-based systems produced a more diversified scenario. Even though naringin preferentially partitions toward the copolymer-rich phase in all systems studied ($75 \pm 3\% \leq R_{NAR} \leq 96 \pm 2\%$ and $\log K_{NAR} > 0$), as for conventional salt systems, the use of [Ch][Ac], [Ch]Cl, and primarily [Ch][Bic] (defined as the most selective system reported) allowed fine tuning of the separation of rutin for the [Ch]-rich phase ($R_{RUT} = 37 \pm 1\%$, $35 \pm 1\%$, and $32 \pm 2\%$ and $\log K_{RUT} = 0.1 \pm 0.1$, -0.12 ± 0.02, and -0.11 ± 0.06, respectively). If the preferential partition of naringin for the copolymer-rich phase seems to be dominated by a combined effect of a salting out exerted by the cholinium-based ILs and the aptitude of the copolymer for hydrophobic interactions, the rutin partition toward the [Ch]-rich phase is dominated by electrostatic/polar interactions. These interactions result from the degree of protonation exhibited by the flavonoids under distinct pH as gathered from observation of Figures S1 and S2 in SI. Within the pH range of these ABS (pH = 5−9), naringin is mostly present in its neutral form between pH 5 and 8 (>78.67%), adopting a more complex speciation at pH 9 (neutral ≈ 23%, single charged ≈ 52%, and double charged ≈ 18%).[38] For rutin, neutral species are present at pH 5−6, whereas between pH 7 and 9 single-, double-, and triple-charged species are prevalent.[38] In ABS composed of [Ch][DHP], [Ch][DHCit], and [Ch][Bit], which present pH values around 5 (Table S25 in SI), at which both flavonoids are neutral, the partition of the flavonoids is preferential toward the copolymer-rich phase. However, with [Ch]Cl, [Ch][Ac], and [Ch][Bic] (pH 6.5−9, Table S25 in SI), while naringin is always present in its neutral form, charged species of rutin are predominant, thus displaying opposite partition tendencies.[38] This allows one to separate naringin from rutin, with the maximal selectivity of 18.9 attained with [Ch][Bic]. The body of results herein collected points toward the importance of cautiously designing an IL, in this case by the proper selection of the anion, to achieve the separation of structurally similar compounds by applying ABS.

Conceptual Design of an Integrated Process to Purify the Flavonoids from a Synthetic Mixture. After the optimization step in which the flavonoids' partition was determined for each ABS type, the development of an integrated process to handle with real extracts rich in the flavonoids is of high demand. In this context, citrus juices represent a convenient source of flavonoids due to their widespread availability and well-established chemical composition.[47] The flavonoids content profile depends upon several factors, e.g., species and geographical origin, and encompasses an impressive structural diversity.[47,48] In addition to being the major flavonoid in grapefruits, naringin is ubiquitous among other types of citrus, including orange and lemon.[47,48] Rutin is also found in grapefruit, although to a less extent than naringin, and in orange juice.[48] Carbohydrates, in particular sucrose, fructose, and glucose (considered in this work as the model contaminant) are considered as one of the principal constituents of the citrus juice.

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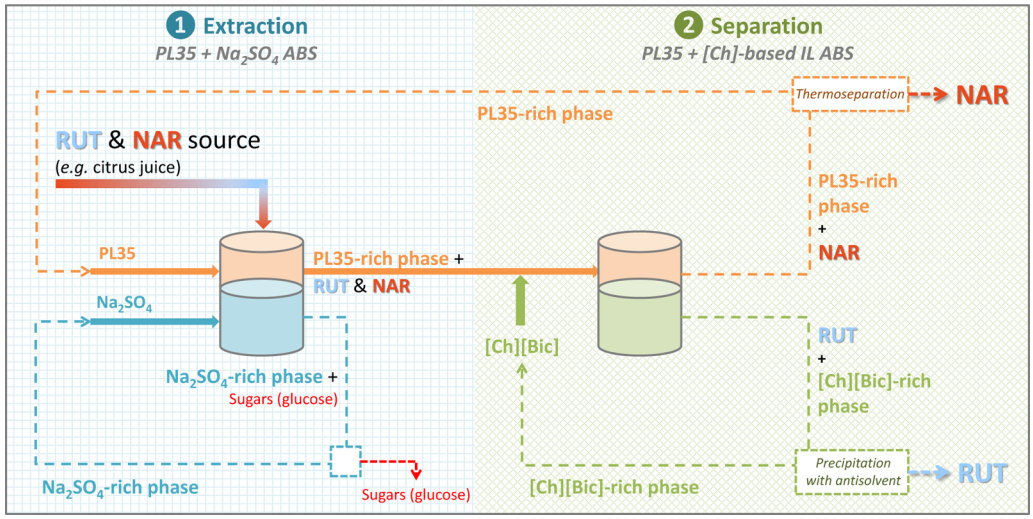

Figure 8. Schematic representation of the integrated two-step process of extraction and separation of naringin and rutin by the sequential implementation of Pluronic L-35-based ABS.

Table 1. Extraction and Separation Performance of the Two-Step Process Developed

system	$EE_{Glucose} \pm \sigma$ (%)	$R_{NAR} \pm \sigma$ (%)	log $K_{NAR} \pm \sigma$	$R_{RUT} \pm \sigma$ (%)	log $K_{RUT} \pm \sigma$	S
step 1: 23 wt % of PL35 + 10 wt % of Na_2SO_4	60 ± 4	100	>2	100	>2	~1
step 2: 35 wt % of PL35 + 30 wt % of [Ch][Bic]		86.8 ± 0.6	1.09 ± 0.01	38.8 ± 0.7	0.07 ± 0.05	10.4

As previously discussed, distinct profiles of extraction and separation of Pluronic L-35-based ABS containing either conventional salts or cholinium-based ILs were hitherto reported. The ABS developed can thus be sequentially implemented to separate both flavonoids from sugars (one of the main classes of contaminants identified) by applying PL35 + conventional salts/buffer ABS, followed by the use of the PL35 + [Ch]-based ABS to finally separate the flavonoids toward opposite phases. In this sense, an integrated process was conceptualized in this work, as depicted in Figure 8.

Among the 20 ABS screened during the optimization studies, those composed of Pluronic L-35 + Na_2SO_4 (R_{NAR} = 99.5 ± 0.6% and R_{RUT} = 99.5 ± 0.4%) were anticipated as ideal to extract the flavonoids from the juice, separating them from the major contaminants, i.e., sugars (step 1). Moreover, the system based in Pluronic L-35 + [Ch][Bic] (S = 18.9) was applied on the separation of rutin from naringin (step 2), thus creating two distinct extracts enriched in each one of the flavonoids. During step 1, flavonoids are completely extracted into the PL35-rich phase, as shown in Table 1. On the contrary, glucose, a highly polar compound (used here in representation of the sugars class), mostly remains in the Na_2SO_4-rich phase, with $EE_{Glucose}$ = 60 ± 4% (Table 1). The partition behavior of glucose [toward the more hydrophilic (salt-rich) phase] is in agreement with the observations of Wang et al.,[49] which have used, among other systems, ABS formed by a Pluronic-like block copolymer and salts.[50,51]

After the separation of the flavonoids from glucose, the fractionation of the flavonoids was achieved by applying the Pluronic L-35 + [Ch][Bic]-based ABS (step 2) with a selectivity of 10.4 (Table 1).

Although outside of the scope of the experimental study here reported, some suggestions to recycle and reuse the phase formers are also provided in Figure 8 (dashed lines) as a way of reinforcing the sustainable character of the process. Pluronic L-35 presents a lower critical solution temperature (LCST), above which it could be separated, releasing naringin in aqueous solution,[8] and thus leading to the naringin recovery and the copolymer recycling. ILs are hydrotropes, i.e., solubility enhancers in aqueous milieu, for biocompounds including flavonoids. Rutin could thus be recovered from the [Ch][Bic]-rich phase by precipitation with an antisolvent (e.g., water) as used in previous works.[30,52]

The process proposed in Figure 8 allows significant progress in the development of new, more sustainable, and efficient technologies to extract and separate flavonoids from their natural sources.[27] The conventional process for flavonoids production includes liquid−liquid extractions conducted with organic solvents, followed by chromatographic separations, where the quantities of hazardous solvents, low product recoveries, and poor selectivity over distinct flavonoids remain as the main drawbacks. As previously highlighted, alternative purification methods have been developed; however, ABS still seem to be the simplest. A review of the literature on the theme reveals a couple of works addressing the use of polar organic solvents (e.g., ethanol) and inorganic salts (e.g., NaH_2PO_4 and K_2HPO_4) to purify flavonoids from solid biological matrices.[40,53] In these works,[40,53] the extraction of flavonoids was carried out using ethanol (pure state or in aqueous solution), coupled ultrasounds, or microwaves. Moreover, the ABS step in these works allows only the separation of the flavonoids from the main contaminants and the concentration of the flavonoids in a single phase. Although useful to obtain enriched extracts with applicability in the fields of cosmetics and nutraceutics, these are not selective at separating distinct flavonoids.[40,53] Recently, alcohol-based ABS were also studied; however, only the extraction of rutin from acerola wastes was attempted, and the purification was not investigated.[39]

Meanwhile, the lack of selectivity for distinct flavonoids and the poor hydrophobic−hydrophilic balance between the alcohol-rich and salt-rich phases may constrain ABS application. Moreover, some authors have implemented the use of IL-ABS

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to purify flavonoids, namely, those based in imidazolium ILs[54] and, more recently, ILs based in carboxylated[30] and aminoate anions or cholinium cations.[55] The powerful status of ILs-based ABS at extracting and purifying flavonoids is patent in these works,[30,55] but the selectivity toward distinct flavonoids remains unexploited. The process herein proposed offers a greener, more efficient, and selective alternative to the current techniques for the separation of naringin and rutin that may be with little effort extended to other valuable natural compounds if a similar process design is carried.

CONCLUSIONS

Motivated by the tunable nature of both ILs and Pluronic copolymers, this work reports novel ABS composed of cholinium-based ILs and Pluronic L-35 and discloses their ability to separate two glycosylated flavonoids, viz. naringin and rutin. Initially, the ternary phase diagrams were determined at 25 °C and used to study the ability to selectively separate naringin and rutin. Good results were achieved by the proper choice of the IL anion, in particular with $[Bic]^-$ (S = 18.9). As compared to ABS constituted by more conventional salts, cholinium-based ILs/Pluronic L-35-based systems performed better at separating naringin and rutin, notwithstanding being less effective in their extraction. With the conventional salts, the salting-out effect dominated both the liquid−liquid demixing phenomenon and the partition of the flavonoids toward the copolymer-rich phase. Given the extraction pattern achieved using the distinct classes of ABS studied, these were sequentially implemented in an integrated approach, according to the two-step process designed in Figure 8. It comprises two main operations: (i) step 1 regarding the separation of flavonoids from glucose (representative of sugars as the main contaminants in the system) using PL35 + Na_2SO_4 ABS and (ii) step 2 considering the separation of both flavonoids using PL35 + [Ch][Bic] ABS (most selective system). In summary, this work proposes a new and selective process to separate narigin and rutin from their natural liquid sources for use in nutraceutical, cosmetic, and pharmaceutical applications.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssuschemeng.7b00178.

Experimental data of each ternary phase diagram, Merchuk equation correlation parameters, tie-lines, weight fraction composition and extraction parameters of the systems applied in the partitioning studies, speciation curves of naringin and rutin(PDF)

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Notes

The authors declare no competing financial interest.

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