Valorization of expired energy drinks by designed and integrated ionic-liquid-based aqueous biphasic systems

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ABSTRACT

Expired energy drinks are rich in bioactive added-value compounds that can be recovered and reused in order to valorize food waste within a circular economy perspective. However, to accomplish such requirements it is necessary to develop sustainable extraction and recovery processes, which must comprise a decrease in the number of steps required or by developing integrated strategies. In this work, novel aqueous biphasic systems (ABS) composed of ionic liquids (ILs) and a biocompatible polymer polypropylene glycol (400 $g.mol^{-1}$, PPG 400) were studied for the simultaneous extraction and recovery of three added-value compounds, namely caffeine, taurine and niacin, from expired energy drinks. ILs were designed and synthetized in order to have similar anions to the target compounds, thus allowing enhanced selectivity and biological activity, while avoiding an extra step of separation of these high-value compounds from the IL-rich phase. To this end, cholinium-based ILs comprising the anions lactate, pyruvate, taurate and nicotinate were synthesized and their cytotoxicity and ecotoxicity credentials evaluated. Overall, taurine and niacin are majorly enriched in the IL-rich phase, while caffeine preferentially migrates in the majority of the cases towards the PPG-rich phase. However, caffeine also partitions to the IL-rich phase in the ABS formed by cholinium pyruvate and cholinium nicotinate. The ABS formed by cholinium nicotinate and PPG 400 is the best system identified, allowing the almost complete recovery (recovery efficiencies >82%) of all target compounds into the IL-rich phase in one-step. Furthermore, cholinium nicotinate exhibits marginal cytotoxic potential and is harmless from an ecotoxicological point of view. This system is thus a promising platform to simultaneously extract, recover and reuse added-value compounds from expired energy drinks without the need of removing the IL or recovering the target compounds from the IL-rich phase, thus contributing to a sustainable and circular food economy.

INTRODUCTION

In developed countries an increasing amount of food is not consumed and ends up as waste along the food value chain, creating a relevant economic and environmental problem.[1] As disclosed by the Food and Agriculture Organization of the United Nations (FAO), one third of the food produced worldwide for human consumption (1.3 billion tonnes per year) is lost or wasted.[2] In the European Union, for instance, food waste is projected to rise to 126 million tons by 2020.[1] Currently, food waste constitutes a relevant concern, being linked to negative economic, societal and environmental impacts.[3] Accordingly, the valorization of food waste is a priority measure to reduce the carbon footprint of the food production chain[4] and to improve economy as the residues generated may contain high-value compounds.[5] In the framework of circular economy, these evidences reinforce the need on the development of cost-effective and sustainable technologies to recover added-value compounds from food waste.

Among food waste, energy drinks, particularly rich in stimulants and additives, play a significant role. These beverages contain high levels of caffeine (up to 500 mg *per* bottle) and are usually supplemented with taurine, glucuronolactone, and complex B vitamins.[6] Most of these high-value compounds maintain their biological activity, even after the expiration dates of energy drinks. In addition to the environmental concerns generated by this type of drinks, their discharge indirectly has an economic impact by increasing their waste management and production costs. Thus, the recovery and reuse of added-value and bioactive compounds from discharged energy drinks has a relevant environmental and economic influence on this market sector that registered global sales over €38 billion in 2015, being expected to reach €53.4 billion in 2020.[7]

Several methods, including liquid-liquid extraction,[8] nanofiltration[9] and solid-phase extraction[10] have been reported for the recovery of target compounds from diet samples

and beverages. In addition to the extraction step, separation and purification processes are needed, which may comprise numerous drawbacks, including high energy and chemicals consumption.[11, 12] Furthermore, most of these methods are time consuming, labour intensive and use volatile organic solvents, contributing to a relevant environmental impact. Overall, cost-efficient and sustainable techniques able to provide high recovery yields and purity levels of valuable compounds recovered from food waste, ideally combining or integrating several steps, must be developed to meet the current society and environmental standards.

Aqueous biphasic systems (ABS) are downstream processing alternatives acting by liquid-liquid extraction, allowing to recover target products from complex samples.[13] Accordingly, this type of systems can be applied both in the extraction and purification steps of high-value compounds from food waste.[14] Moreover, if properly designed, ABS may be biocompatible and of low cost.[15, 16] Typical ABS consist of two immiscible aqueous-rich phases based on polymer/polymer, polymer/salt or salt/salt combinations. In order to improve their separation performance, ABS formed by ionic liquids (ILs) have been extensively investigated.[15] Besides other relevant properties, their fine-tuning by combining adequate IL cations and anions is one of the most significant.[15, 17, 18]

ILs are able to form ABS in presence of salts, amino acids, carbohydrates or polymers, and have been successfully used in the extraction of phenolic acids, alkaloids, amino acids, proteins, among others.[19-21] However, several concerns have been raised with the most studied ILs in this field, mainly imidazolium-based.[22, 23] This trend is however changing, with novel classes of ILs being proposed for the creation of ABS.[19, 21] Among these, cholinium-based ILs have been the main target since the IL cation is an important micronutrient source belonging to the B-complex vitamins, which may thus overcome the main drawback on the use of ILs in the food industry[24]. Cholinium chloride ([Ch]Cl) is

currently classified as a safe substance by the U.S. Food and Drug Administration (FDA).[25] Previous works reported the synthesis of cholinium-based ILs alongside with their toxicological and biodegradation potential, showing that when combined with appropriate anions, these ILs exhibit low toxicity and high biodegradability.[26]

ABS based on cholinium-based ILs were successfully used in the extraction of flavonoids, polysaccharides, amino acids, proteins and enzymes from aqueous solutions and from complex matrices.[19, 27-29] Some publications can be found in the literature regarding the use of IL-based ABS in the valorization of food waste;[25, 27, 30, 31] yet, the majority of these works focused on ABS formed by imidazolium-based ILs and salts, thus compromising their green credentials, and none envisioned the use of ILs with similar biological features to enhance the biological properties of the recovered compounds, while avoiding the use of an additional step to recover the target compounds from the IL-rich phase. The non-volatile nature of ILs, which is valuable when addressing it from an environmental perspective, is indeed a major drawback when attempting the recovery of target compounds from the IL-rich phase since a simple distillation step cannot be applied. Therefore, and although scarcely considered, the recovery of the target compounds from IL-rich phases has been achieved by the addition of anti-solvents, back-extraction steps with organic solvents or by the use of solid-phase extraction.[32, 33]

Aiming at developing a sustainable and cost-effective process for the recovery of added-value compounds (caffeine, taurine and niacin) from expired energy drinks, we here demonstrate the potential of ABS formed by cholinium-based ILs (4 synthesized ILs and 3 commercial ILs for comparison purposes) and polypropylene glycol with a molecular weight of 400 $g.mol^{-1}$ (PPG 400) to directly extract the target compounds from the real samples. By being extracted to the IL-rich phase, and by using ILs with similar biological features, the additional separation step to recover the target compounds from the IL-rich

phase can be avoided, and may result in an integrated process. The chemical structures and abbreviations of the investigated ILs are depicted in Figure 1.

The synthesized ILs comprise anions derived from natural sources, such as from plant natural acids (lactate and pyruvate), amino acids (taurate) and vitamins (nicotinate). These ILs were designed taking into account the target compounds to recover from expired energy drinks, which could have their biological properties enhanced if combined with IL anions with similar properties. Bearing in mind the potential reuse of the recovered compounds combined with adequate ILs, the ILs cytotoxicity towards human intestinal cell lines and their ecotoxicity by the microtox assay were evaluated. The recovery performance of the investigated ABS for caffeine (alkaloid), taurine (amino acid) and niacin (vitamin B3) from expired energy drinks was finally addressed. The target biomolecules, whose chemical structures are given in Figure 1, are important in the maintenance of the body homeostasis and are widely used by the food, cosmetic and pharmaceutical industries.[34-39]

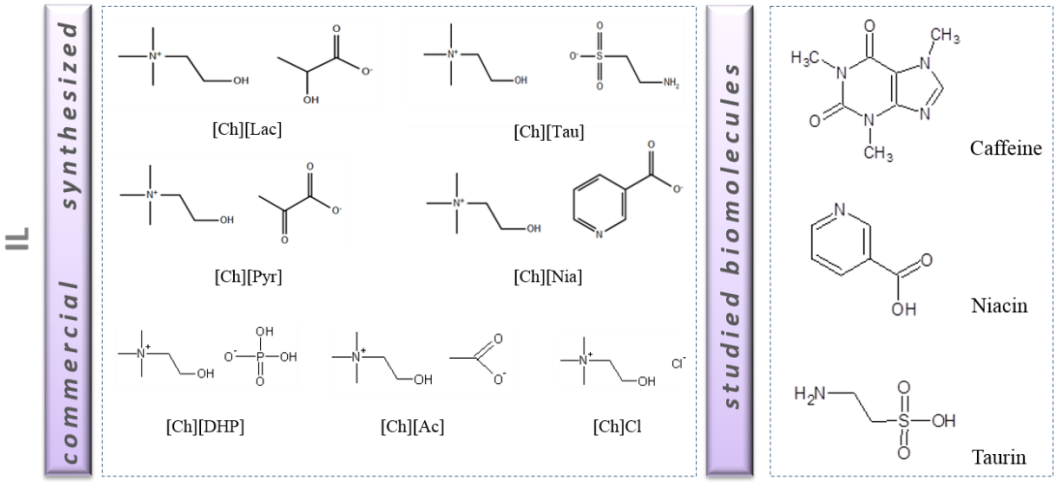


Figure 1. Chemical structures of the investigated ILs and added-value compounds.

EXPERIMENTAL SECTION

Materials. Poly(propylene glycol) with an average molecular weight of 400 $g \cdot mol^{-1}$ (PPG400), cholinium hydroxide ([Ch][OH], 46 wt% in water), taurine (purity ≥99%), lactic acid (purity ≥98%), pyruvic acid (purity ≥98%), nicotinic acid (purity ≥99.5%), 2,4-dinitrofluoro benzene (DNFB purity ≥99%) and dimethyl sulfoxide (DMSO; purity >99.0%) were purchased from Sigma Aldrich (St. Louis, MO, USA). Methanol and acetonitrile (HPLC grade, purity >99.9%) were obtained from Fisher Chemical (Fisher Scientific, USA). Commercial ILs, namely cholinium acetate ([Ch][Ac], >99 wt%) and cholinium dihydrogen phosphate ([Ch][DHP], >98 wt%), were acquired from Iolitec, while cholinium cloride ([Ch]Cl, >98 wt%) was purchased from Acros Organic.

The cell culture medium (Dulbecco's Modified Eagles's Medium – high glucose (HG)) used in the cytotoxicity assays was obtained from Sigma, Fetal Bovine Serum (FBS) from Merck Millipore and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (purity 98%) from ACROS Organics. All chemicals for ecotoxicity assays were purchased from Ambifirst.

The sample of energy drink (sugar free Red Bull®) was acquired from a local market in Aveiro (Portugal); it was used in all experiments with one month after the expiration date. According to the product label it contains caffeine, taurine, B-group vitamins (B3, B5, B6 and B12), aspartame, acesulfame K and water.

ILs Synthesis. Synthesis of cholinium lactate ([Ch][Lac])[40], cholinium nicotinate ([Ch][Nia])[41] and cholinium taurate ([Ch][Tau])[42], were performed according to previously described protocols. Cholinium pyruvate ([Ch][Pyr]) was synthesized in this work for the first time by acid-base titration. The potentiometric acid-base titrations were performed by the slow addition of an aqueous solution of [Ch][OH] (at 1.7524 $mol.L^{-1}$) to lactic, pyruvic, nicotinic and 2-aminoethanesulfonic acids. [Ch][OH] was added in

small excess and then back titrated by adding the acid solution until the desired inflection point is obtained (pH values for [Ch][Lac] = 9.25, [Ch][Pyr] = 8.20, [Ch][Nia] = 7.95 and [Ch][Tau]= 9.02). Excess water was first removed from the synthesized ILs at 70°C using a rotational evaporator, and then under vacuum until constant weight was achieved. ILs were stored with P_2O_5 under vacuum for the following 72 h. The water content in the ILs was determined by Karl-Fisher titration and found to be ≤ 200 ppm in all ILs. The chemical structures and purities of the synthesized ILs, [Ch][Lac] (> 98 wt%), [Ch][Pyr] (>96 wt%), [Ch][Nia] (>99 wt%) and [Ch][Tau] (>99 wt%), were confirmed by ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) and Fourier-transform infrared spectroscopy (FTIR) spectroscopy (Figures S1–S6 in the Supporting Information). NMR spectra were recorded in D_2O at 25°C on a Bruker Advance III 400 MHz spectrometer. Tetramethylsilane was used as internal standard. FTIR spectra were recorded from (4000 to 650) cm^{-1} using a Thermo-Nicolet Nexus 670 spectrometer fitted with a Universal ATR Sampling Accessory. The chemical structures of the investigated ILs are given in Figure 1.

ILs cytotoxicity and ecotoxicity. The cytotoxicity of the synthesized ILs ([Ch][Lac], [Ch][Nia], [Ch][Pyr], [Ch][Tau]) alongside with the commercial ILs [Ch][Ace], [Ch]Cl and [Ch][DHP] was addressed in the human colon epithelial cell line (Caco-2). A stock solution of each IL was prepared in saline aqueous solutions and the test solutions were obtained by successive dilutions of the stock in culture medium, obtaining the final concentrations of 0.1, 1.0, 10, 30, 60, 90 $g.L^{-1}$ of each IL. The epithelial human colon cell line (Caco-2) was grown in high glucose Dulbecco's modified Eagle's medium (DMEM-HG) containing 10% (v/v) fetal bovine serum (FBS), 100 units penicillin, and 50 $\mu g.mL^{-1}$ streptomycin in a humidified atmosphere of 5% CO_2 at 37°C. Cells were plated on polystyrene cell culture dishes at a density of $1x10^4$ cells *per* well in 96 well culture plates.

188 After 16 h, cells were treated with the different concentrations of the target ILs for 24h.
189 Cytotoxicity was evaluated using the 3-(4,5-dimethylthiazol-2-yl)-2,5-
190 diphenyltetrazolium bromide (MTT) assay. This assay is based on the ability of viable
191 cells to convert MTT into a water-insoluble precipitate. Caco-2 cells were incubated with
192 0.5 $mg.dm^{-3}$ of MTT in medium overnight at 37 °C. The precipitate was then dissolved
193 in 10% (w/v) sodium dodecyl sulfate (SDS), and colorimetrically quantified (at 570 nm)
194 using a microplate spectrophotometer. Each concentration was tested in five replicates of
195 three independent experiments (n=3), except for [Ch]Cl for which the values correspond
196 to five replicates of two independent experiments (n=2). In all experiments, a negative
197 control corresponding to untreated cells was always included.
198 The dose response curves and median effective concentration (EC_{50}) calculations were
199 performed using the GraphPad PRISM Software (version 8.0.1). The EC_{50} corresponds
200 to the concentration of IL for which 50% of the cells are viable. Cell viability results are
201 expressed as percentage of the control (i.e. unexposed cells).
202 The ecotoxicity of the synthetized ILs was evaluated using the Standard Microtox liquid-
203 phase assays. This test evaluates the inhibition of the luminescence of the marine bacteria
204 *Vibrio fischeri* and was performed using a range of diluted aqueous solutions (from 0 to
205 81.9%) of IL, where 100% of the compound corresponds to a known concentration of a
206 stock solution (ca. 10 $g.L^{-1}$). After 5, 15, and 30 min of exposure to IL, the light output of
207 the luminescent bacteria was determined and compared with the light output of a blank
208 control sample. The toxicity was evaluated on the basis of the percentage decrease in the
209 bacteria luminescence relative to the blank control. The final output of this test is the EC_{50}
210 parameter, which represents the effective concentration of a given IL that produces 50%
211 of inhibition of light emission. Analyses were performed with the MicrotoxOmni™
212 Software version 4.3.0.1.

213

214 **ABS phase diagrams and recovery studies.** The phase diagrams of the ABS composed
215 of each IL, PPG 400 and water were determined through the cloud point titration method
216 at (25 ± 1) °C and at atmospheric pressure, as previously described.[43-45] Details regarding
217 phase diagram determination are given in the Supporting Information. Initial tests to
218 address the real samples effect in the binodal curves was carried out, with no significant
219 differences (within the experimental error) obtained between the phase diagrams
220 determined with water or with energy drink samples. This fact is due to the low amount
221 of additives present when compared to the amounts of IL and PPG 400 required to create
222 ABS.
223 After addressing the ABS phase diagrams and compositions required to create two-phase
224 systems, their recovery capability for the three biomolecules from expired drinks was
225 evaluated at two mixture compositions: (15 wt% IL + 40 wt% PPG400 + 45 wt% expired
226 energy drink) and (30 wt% IL + 30 wt% PPG400 + 40 wt% expired energy drink). The
227 mixture compositions were chosen taking into account two common mixture points in the
228 biphasic region of all systems, while varying the IL and PPG 400 contents to address their
229 effects. Each biphasic system was prepared in 2 mL micro-centrifuge tubes by adding the
230 appropriate amount of PPG 400, IL and energy drink sample to make up a final weight of
231 1 g. It should be remarked that a liquid energy drink sample is being used directly in the
232 creation of ABS, thus allowing the integration of the extraction and purification steps.
233 Furthermore, by using designed ILs with similar chemical structures to the target
234 compounds, the recovery step can be avoided, allowing to develop and integrated
235 extraction-purification-recovery strategy. All systems were mixed vigorously using a
236 vortex agitator (Reax Top, Heidolph, Germany) and left at 25 °C for 2 h. Each ABS was

then centrifuged for 5 min at 2000 rpm to ensure the complete phase separation. The weights of the top and bottom phases were measured.

Recovery efficiencies of studied molecules (*RE*%) correspond to the percentage ratio between the amount of each biomolecule in a given phase (IL- or PPG-rich) and that in the total mixture, determined according to Eqs (1) and (2):

$$RE_{IL}\ (\%) = \frac{[C]_{IL} \cdot w_{IL}}{m_0} \times 100 \qquad (1)$$

$$RE_{PPG}\ (\%) = \frac{[C]_{PPG} \cdot w_{PPG}}{m_0} \times 100 \qquad (2)$$

where w_{IL}, w_{PPG}, $[C]_{IL}$ and $[C]_{PPG}$ are the weights of the IL- and PPG-rich phases and the concentration of each biomolecule in the IL- and PPG-rich phases, respectively; m_0 corresponds to the mass of each biomolecule present in the overall ABS. At the conditions used in this work, the top phase corresponds to the PPG-rich phase while the bottom phase corresponds to the IL-rich phase.

According to the product label, the used energy drink contains ca. 320 $mg.L^{-1}$ of caffeine, 4000 $mg.L^{-1}$ of taurine and 80 $mg.L^{-1}$ of niacin. The amount of each studied alkaloid (caffeine, niacin and taurine) in Red Bull was confirmed by HPLC using the respective standards and calibration curves. The experimentally determined amounts of these compounds are in accordance with the amounts labelled in the energy drink sample (Figure S9 in the Supporting Information). The stability of caffeine, niacin and taurine in the ABS phases after extraction was also evaluated by HPLC (Figure S10 in the Supporting Information).

Caffeine and niacin in each ABS were quantified by HPLC-DAD. The HPLC analysis was performed using an Agilent 1100 liquid chromatograph (USA) with a Zorbax XDB-C18 column (4.6 mm × 250 mm, 3.5 μm particle size). The mobile phase was composed of 18% methanol and 82% water, at a flow rate of 1.5 $mL.min^{-1}$, with an injection volume of 20 μL and temperature oven at 25°C. Quantification was carried out at 273 nm for caffeine and at 261 nm for niacin. The system was controlled by the Chemstation software. The taurine concentration was determined by HPLC including a pre-column derivatization with DNFB due to low absorption of taurine in the UV-Vis region. The procedure of derivatization is described elsewhere.[46, 47] Briefly, 100 μL of the sample, 200 μL of the 0.01 M carbonate buffer (pH 9), 500 μL of DMSO and 10 μL of DNFB were added into an Eppendorf tube, vigorously mixed using a vortex agitator at 2500 rpm for 30 s and placed in a water bath at 40 °C for 15 min. Then, 650 μL of 0.01 M phosphate buffer (pH 6) was added. The mobile phase was a mixture of 0.01 M phosphate buffer pH 6 (A) and acetonitrile (B) and the following gradient profile was run: 0–10 min, 90% A and 10% B; 10–15 min, 75% A and 25% B; 15–19 min, 50% A and 50% B; from 19 min, 90% A and 10% B at flow rate of 1 mL min^{-1} with a sample injection volume of 20 μL and detection wavelength at 360 nm.

RESULTS AND DISCUSSION

In this work, cholinium-based ILs with anions presenting similar characteristics to the ones of the target high-value compounds, to recover from expired energetic drinks, were synthetized and characterized. The ILs were then applied in the creation of ABS to simultaneously extract and recover these compounds directly from the real samples. The ILs selection was based on the premise that the IL-rich phase will have a superior selectivity to the target compounds and that the activity of the recovered compounds could

285 be enhanced, while avoiding a final step to recover these target compounds from the IL-
286 rich phase. The ILs correspond to [Ch][Lac], [Ch][Nia], [Ch][Tau] and [Ch][Pyr]. Three
287 commercial ILs, namely, [Ch][DHP], [Ch][Ac], [Ch]Cl, were also used for comparison
288 purposes. The chemical structures of the investigated ILs and target added-value
289 compounds are depicted in Figure 1.
290
291 **ILs toxicity**
292 Given the potential applications of the recovered ABS phases containing the target
293 bioactive compounds, it is crucial to evaluate the ILs cytotoxicity. For this purpose, the
294 epithelial human colon cell line (Caco-2) was used to study the intestinal cytotoxic effects
295 of the investigated ILs. Figure 2 depicts the cytotoxic profile for the newly synthetized
296 ([Ch][Lac], [Ch][Nia], [Ch][Pyr], [Ch][Tau]) and the commercial ([Ch][Ace], [Ch]Cl and
297 [Ch][DHP]) ILs. The respective dose-response curves and the EC_{50} values are shown in
298 the Supporting Information. Overall, the ILs investigated exhibit EC_{50} values in the $g.L^{-1}$
299 range. [Ch][Lac] has the lowest toxicity (EC_{50}: 96.64 $g.L^{-1}$; 95% Confidence Interval
300 (CI): 74.95-118.3 $g.L^{-1}$), followed by [Ch][Pyr] (EC_{50}: 62.61 $g.L^{-1}$; 95% CI: 54.15-71.08
301 $g.L^{-1}$) and [Ch][Nia] (EC_{50}: 57.59 $g.L^{-1}$; 95% CI: 53.25-62.32 $g.L^{-1}$). Similar results were
302 obtained for the commercial IL [Ch][Ac] with an EC_{50} of 65.11 $g.L^{-1}$ (95% CI: 56.29-
303 73.93 $g.L^{-1}$). [Ch][Tau] exhibits the highest cytotoxicity amongst the synthetized ILs
304 (EC_{50}: 30.05 $g.L^{-1}$; 95% CI: 29.67-30.43 $g.L^{-1}$), being comparable with the ones obtained
305 for the commercial ILs [Ch]Cl (EC_{50}: 30.61 $g.L^{-1}$; 95% CI: 29.85-31.36 $g.L^{-1}$) and
306 [Ch][DHP] (EC_{50}: 28.97 $g.L^{-1}$; 95% CI: 28.50-29.44 $g.L^{-1}$). The toxicity of [Ch][Tau] is
307 approximately twice as high as [Ch][Pyr], [Ch][Nia] or the commercial [Ch][Ac]. A
308 similar profile was obtained for the ecotoxicity tests, with [Ch][Tau] showing the highest
309 toxicity (results and discussion below).

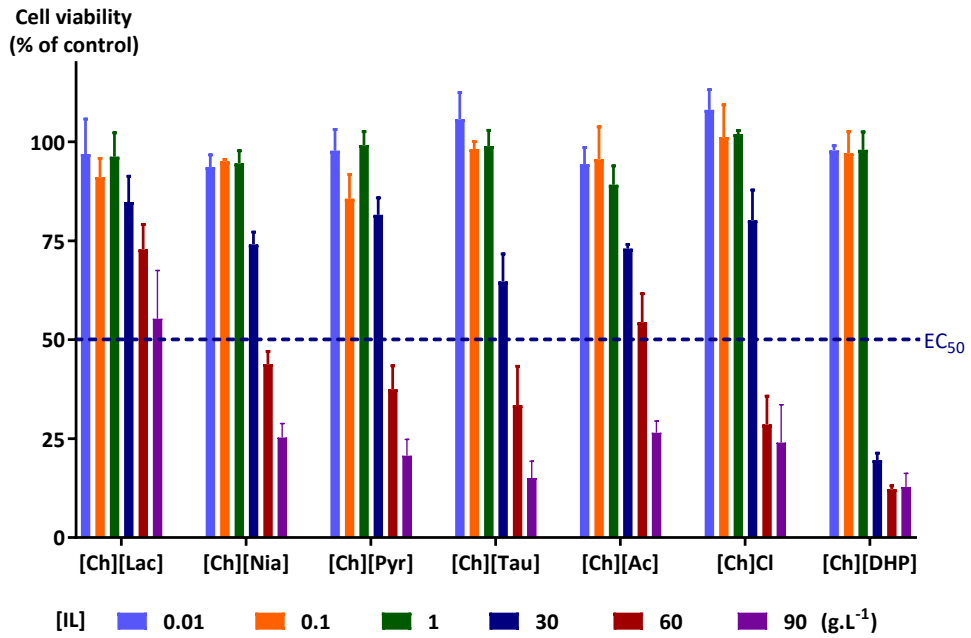


Figure 2. Caco-2 cell viability after 24 h of exposure to the ILs (average values with respective standard error). The dashed line corresponds to the EC_{50}.

In addition to the cytotoxicity, the ecotoxicity of the synthesised ILs was addressed using the bioluminescent bacteria *Vibrio fischeri*. The experimental EC_{50} values were determined by Microtox® bioassays for 5, 15 and 30 min of IL exposure. The detailed results are provided in the Supporting Information. According to the Passino's classification[48], the obtained results reveal that [Ch][Nia] is harmless (EC_{50} > 1000 $mg.L^{-1}$), [Ch][Lac] is practically harmless (100 $mg.L^{-1}$ < EC_{50} > 1000 $mg.L^{-1}$) and [Ch][Tau] is moderately toxic (10 $mg.L^{-1}$ < EC_{50} > 100 $mg.L^{-1}$). [Ch][Pyr] has non-toxic character to the addressed bacteria given that at 60 $mg.L^{-1}$ concentration it was not possible to calculate the EC_{50} value. These results suggest that the investigated anions have a relevant role in defining the cholinium-based ILs toxicity, in agreement with the findings of Ventura et al.[49] showing that [Ch][Ac], [Ch]Cl and [Ch][DHP] are "practically harmless" with EC_{50} values after 30 min of exposure of 673.21 $mg.L^{-1}$ for [Ch][Ac], 469.34 $mg.L^{-1}$ for [Ch]Cl and 572.72 $mg.L^{-1}$ for [Ch][DHP]. [Ch][Tau] is more toxic than these

commercial ILs. However, [Ch][Lac] is less toxic than [Ch]Cl and has a similar toxicity to [Ch][DHP] and [Ch][Ac]. [Ch][Nia] and [Ch][Pyr] are less toxic than all commercial ILs investigated. Overall, most of the studied ILs have a low environmental impact, where [Ch][Nia] and [Ch][Pyr] stand out as less toxic cholinium-based ILs than those commercially available. This feature is even more relevant if considering the largely investigated imidazolium-based ILs in ABS for separation purposes.[15]

ABS phase diagrams and extraction and recovery of added-value compounds from expired energy drinks

By using expired energy drinks, which are liquid samples, to directly create ABS, it is possible to integrate the extraction and recovery steps of the target added-value compounds. With this goal in mind, the ABS phase diagrams were first determined at 25°C and atmospheric pressure. The binodal curves experimental data for the ABS composed of each IL, PPG 400 and water are given in the Supporting Information. The respective phase diagrams, given in weight fraction, are shown in Figure 3. Each binodal curve represents the minimum concentration of the system constituents required for the formation of two aqueous phases at 25°C and atmospheric pressure. The binodal curve data were fitted according to the equation proposed by Merchuk et al.,[50] being given in Figure 3. Fitting parameters obtained from the correlation of experimental data and corresponding correlation coefficients are provided in the Supporting Information.

Considering that PPG 400 and the cholinium cation are common species to all investigated ABS, the two-phase forming ability of each system is a main result of the IL anion chemical structure. The closer the binodal curve is to the origin of the phase diagram, the lower the concentrations of phase-forming components required for the formation of ABS. The phase-forming ability of the studied ILs in ABS formation, at ca.

25 wt% of PPG 400, follows the order: [Ch][DHP] > [Ch][Ac] > [Ch]Cl > [Ch][Tau] > [Ch][Lac] > [Ch][Pyr] > [Ch][Nia]. [Ch][DHP] shows the highest ability to form ABS, whereas [Ch][Nia] exhibits the lowest.

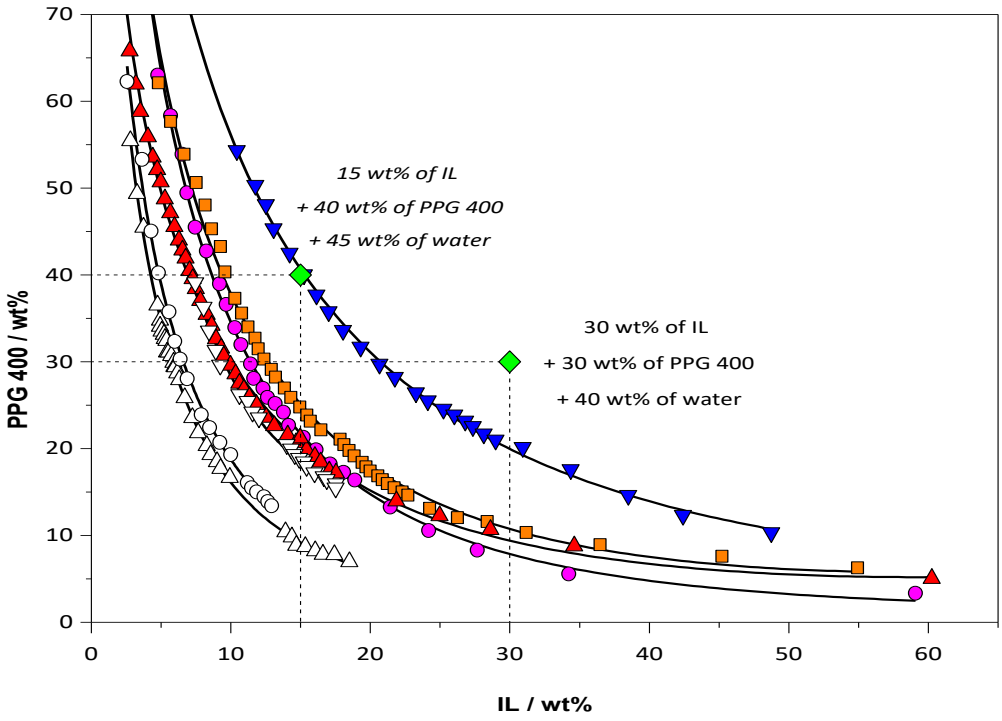


Figure 3. Ternary phase diagrams of the studied ABS (IL + PPG400 + H_2O) at 25°C and atmospheric pressure: △, [Ch][DHP]; ○, [Ch][Ac]; ▽, [Ch]Cl; ▲, [Ch][Tau]; ●, [Ch][Lac]; ■, [Ch][Pyr] and ▼, [Ch][Nia]. ◆, ABS compositions used in the extraction studies; continuous line, fitting of the experimental binodal data.

According to the given trend, it seems that the cholinium-based ILs act as the salting-out species over the moderately hydrophobic PPG 400. The salting-out aptitude of ILs is directly related to the hydration capacity of their ions.[32, 51] Since all ILs share a common cation, cholinium, more hydrophilic anions with a higher charge density, such as $[DHP]^-$, have a higher hydration ability and display stronger salting-out effects. However, it should be taken into account that the mechanisms behind the formation of ABS containing

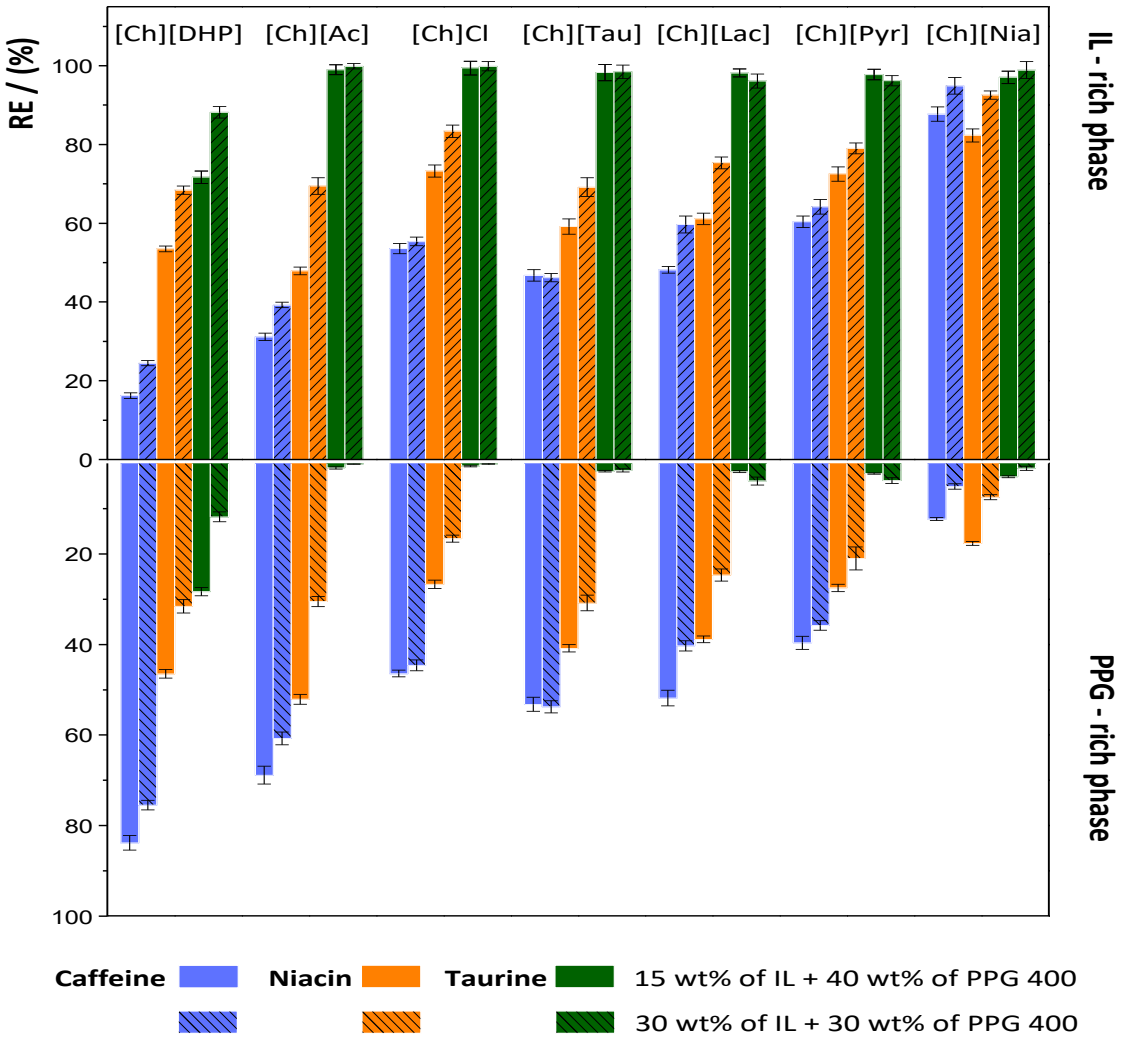
ILs and polymers are far more complex than those observed in ABS formed by salts and ILs.[45] It was previously reported that the ABS formation in systems comprising higher melting temperature cholinium-based ILs, e.g. [Ch][DHP], [Ch][Ac] and [Ch]Cl, is mainly governed by their affinity for water.[52] A higher affinity for water implies a higher ability to promote phase separation when dealing with systems involving polymers, where the ILs acts as the salting-out species. In the case of ABS based on ILs with lower melting temperatures (which will be the case of [Ch][Tau], [Ch][Lac], [Ch][Pyr] and [Ch][Nia]) and polymers, the two-phase formation ability is not a main result of the IL ions to create hydration complexes, but yet the IL-polymer interactions play a significant role.[53]

The anions hydrophobicity may be appraised by the logarithmic values of their octanol-water partition coefficients (logK_{ow}); for conjugated acids of the studied anions they are in the range from –2.77 to 0.22 (detailed values given in the Supporting Information). The most hydrophilic anion is $[Tau]^-$, justifying its higher affinity for water and salting-out ability. On the other hand, the most hydrophobic anion is $[Nia]^-$, which shows the lowest ability to form ABS. However, the obtained trend of ABS formation does not straight follow the logK_{ow} values; according to this rank, [Ch][Pyr] (logK_{ow}= - 1.24) should be a stronger salting-out agent than [Ch][Lac] (logK_{ow}= - 0.70). On the other hand, the trend of low melting cholinium-based ILs to induce ABS follows the decrease in the anion polar surface, which is the surface sum over all polar atoms (80.39, 60.36, 57.20 and 50.19 Å^2 for [Ch][Tau], [Ch][Lac], [Ch][Pyr] and [Ch][Nia], respectively).[54] These results suggest that the ABS formation of these low melting ILs with PPG is governed by their ability to be solvated by water to act as salting-out species and by specific interactions with the polymer, being in agreement with the literature.[52] For instance, [Ch][Nia] contains π electrons able to establish strong hydrogen bond interactions with

391 the ether oxygen atoms of PPG, and this could be the major factor for the lower ability of
392 [Ch][Nia] to promote the creation of ABS with PPG.
393 Tie-line (TL) compositions, tie-lines length (TLL) and slope were additionally
394 determined for ABS formed with the synthesized ILs, being given in the Supporting
395 Information. TL data are particularly relevant to have information on the compositions of
396 the phases at which the extractions are carried out.
397 After addressing the phase-forming components compositions required to create ABS,
398 the potential of these new ABS formed by cholinium-based ILs and PPG 400 to recover
399 added-value compounds from expired energy drinks, namely caffeine, taurine and niacin
400 (Vitamin B_3), was investigated. The synthesized ILs were designed to have similar anions
401 to the target products, which could be beneficial to improve selectivity and biological
402 activity while ideally avoiding the need of the products recovery from the ABS phases.
403 The commercial energy drink contains aspartame, acesulfame K (110 $g.L^{-1}$), caffeine (320
404 $mg.L^{-1}$), taurine (4 $g.L^{-1}$), vitamin B_3 - niacin (80 $mg.L^{-1}$) and other B complex vitamins
405 (Figure S9 in the Supporting Information). The properties of the targeted compounds
406 which can influence the partitioning in the proposed ABS and their stability in the ABS
407 phases are given in the Supporting Information (Table S6 and Figure S10, respectively).
408 All studied bioactive compounds are stable in the ABS' phases and ILs/PPG 400 aqueous
409 solutions. According to the chromatograms given in Figure S10 in the Supporting
410 Information, it is shown that the retention times of taurine, niacin and caffeine do not
411 change when compared to the standards and that no new peaks appear in addition to those
412 already existing in the energy drink sample.
413 The extraction of the target compounds from the energy drink was performed at two
414 ternary system compositions (identified in Figure 3): (A) 15 wt% of IL, 40 wt% of PPG
415 400 and 45 wt% of expired energy drink, and (B) 30 wt% of IL, 30 wt% of PPG 400 and

40 wt% of expired energy drink. The composition of the ternary mixtures was selected based on the phase diagrams previously determined, and close to the binodal curve of the ABS with lowest ability to undergo liquid-liquid demising aiming at improving their sustainability, i.e. requiring lower amounts of the phase-forming components (IL and PPG 400). The obtained recovery efficiencies (*RE*%) in the IL-rich and PPG-rich phases for niacin, caffeine and taurine are depicted in Figure 4 (detailed results are provided in the Supporting Information).

At the conditions investigated, taurine and niacin from the expired drink preferentially migrate to the IL-rich phase in all ABS, whereas caffeine shows an opposite pattern in most of the systems investigated (preferential migration towards the PPG-rich phase, with the exception of [Ch][Pyr]-, [Ch][Nia]- and [Ch]Cl-based ABS) – *cf.* Figure 4. Recovery efficiencies varying from 47.89 to 92.55% for niacin and from 71.68 to 99.87% for taurine to the IL-rich phase, and varying between 5.12 to 83.81% for caffeine to the PPG-rich phase, were obtained. These values support the ILs designer aptitude since the almost complete extraction of the three target compounds is achieved in one-step into the IL-rich phase with the ABS formed by [Ch][Nia], whereas the ABS formed by [Ch][DHP] allows to separate niacin and taurine from caffeine (by their partitioning to opposite phases).



433

434 **Figure 4.** Recovery efficiencies of the caffeine, niacin and taurine in the studied ABS at

435 two mixture compositions at 25°C.

436

437 Caffeine can be used as an effective and valuable probe to characterize the relative

438 hydrophobicity of a series of ABS, particularly when hydrophobic type interactions are

439 predominant, since this alkaloid does not suffer speciation in a large range of pH values

440 (pK_{a1}/pK_{a2} = 0.12 / 10.5) while being moderately hydrophilic ($\log K_{ow}$ = –0.63).[55]

441 According to the *RE*% values, caffeine preferentially partitions to the PPG-rich phase at

442 both compositions, except in the case of the ABS composed of [Ch][Nia] and [Ch][Pyr]

where caffeine preferentially migrates to the IL-rich phase (Figure 4). Recovery efficiencies of caffeine in the range from 16.19 to 55.38% toward the IL-rich phase and from 5.12 to 83.81% to the PPG-rich phase were obtained, meaning that the enrichment of caffeine in a given phase can be tailored by changing the IL chemical structure. In ABS composed of [Ch][DHP], [Ch]Cl and [Ch][Ac], caffeine is majorly enriched in the PPG-rich phase. On the other hand, when more hydrophobic ILs are applied, such as [Ch][Pyr] and [Ch][Nia], caffeine preferentially partitions to the IL-rich phase. It should be noted that the water content in the PPG400-rich phase in the ABS based on [Ch][Pyr] and [Ch][Nia] is significantly low (3.13-20.52 wt% and 1.85-15.85 wt%, respectively) compared to ABS based on [Ch][Tau] (20.04-33.79%) and [Ch][DHP][56] (17.16-35.87%) (cf. the Supporting Information, tie-line data), thus justifying the inversion on the caffeine partitioning among the coexisting phases, and in agreement with the use of caffeine as a valuable probe to characterize the relative hydrophobicity of ABS phases.[49]

Niacin (pKa=4.80)[57], unlike caffeine, is negatively charged in the pH range of the studied ABS (5.8-10.1). Moreover, niacin is the most hydrophilic compound (logK_{ow} values ranging from –2.03 to –2.93 in the pH range 5.8-10.1) and contains an acidic (–COOH) and a basic group (N from the pyridine core. In the same line, taurine (pKa=-1.49 /9.06)[57] has a high hydrophilic nature (logK_{ow} values ranging from –5.27 to –6.23 in the pH range 5.8-10.1), and exists as a zwitterion or as negatively charged species at the working pH. The results disclosed in Figure 4 show that niacin and taurine preferentially migrate to the IL-rich phase, which is the most hydrophilic phase in the ABS phases, mainly due to their hydrophilic character.

In summary, the ABS evaluated are able to remarkably recover added-value compounds directly from expired energy drinks. Furthermore, caffeine, taurine and niacin can be recovered in one phase or caffeine can be separated from the remaining target compounds

only by changing the IL chemical structure. For the first strategy, the best ABS is formed by [Ch][Nia] that allows to recover all compounds in one step with *RE*% higher than 82.32%, whereas the best ABS able to separate caffeine from taurine and niacin is composed of the commercial IL [Ch][DHP]. [Ch][Nia] exibits low citotoxicity and low ecotoxicity, while PPG 400 is approved as a food additive by FDA.[58] Given the enviosioned target applications and assuming that no separation of caffeine from taurine and niacin is aimed, the results obtained reveal that the ABS formed by [Ch][Nia] and PPG 400 is the most efficient system to recover added-value compounds from expired energy drinks, which could be then reused in diverse industrial applications without the need of removing the IL or recovering the target compounds from the IL-rich phase. For instance, these compounds can be used together in food supplements and in cosmetic or pharmaceutical formulations. Despite our promising results, it should be stressed that a thorough risk assessment is required in order to demonstrate the safety use of the IL-rich phase directly by these industries. This assessment includes further *in vitro* and *in vivo* tests in order to evaluate the acute and chronic toxicity towards mammals and towards other test organisms from different trophic levels to guarantee that there are no hazards related with these products.

A schematic overview of the developed integrated platform, and within a circular economy perspective, to extract and recover added-value compounds from expired energy drinks without the need of recovering them from the IL-rich phase is shown in Figure 5. This strategy envisions the direct use of the IL-rich fraction with enhanced biological activity by the food, cosmetics or pharmaceutical industries, although additional tests are required as highlighted above. The reuse of the PPG-rich phase is also envisioned, particularly in the creation of a new ABS with similar samples, at least up to saturation or losses of separation performance.

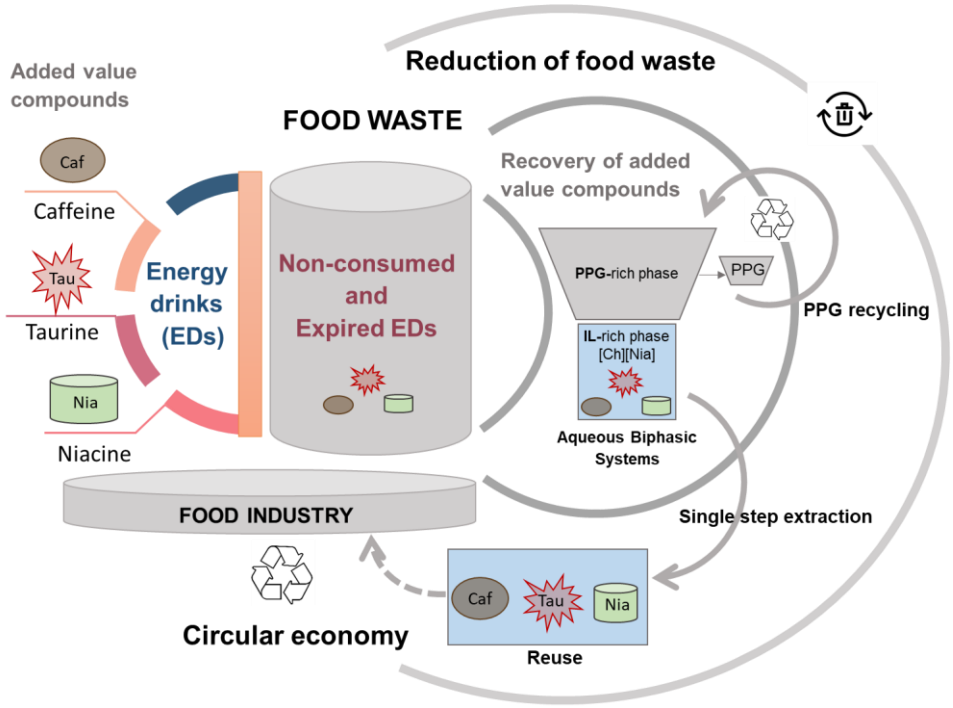


Figure 5. Schematic representation of the proposed integrated platform to extract and recover added-value compounds from expired energy drinks using ABS composed of cholinium-based ILs and PPG 400.

CONCLUSIONS

In this work, we proposed an integrated approach to extract and recover high-value and bioactive compounds from expired energy drinks using aqueous biphasic systems composed of cholinium-based ILs and PPG 400. A set of cholinium-based ILs was synthesized, characterized and used. [Ch][Lac] exhibits the lowest toxicity towards the human epithelial colon cell line (Caco-2), followed by [Ch][Pyr] and [Ch][Nia]. [Ch][Tau] exhibits the highest cytotoxic character, being comparable to the commercial ILs [Ch]Cl and [Ch][DHP]. A similar profile with [Ch][Tau] exhibiting the highest

toxicity was obtained for the ecotoxicity tests, with this IL being considered moderately toxic. [Ch][Nia] is harmless to the marine bacteria *V. fischeri*, whereas [Ch][Lac] and [Ch][Pyr] were considered as practically harmless.

The ABS phase diagrams were determined at 25°C and atmospheric pressure, in which the ILs in ABS formation followed the order: [Ch][DHP] > [Ch][Ac] > [Ch]Cl > [Ch][Tau] > [Ch][Lac] > [Ch][Pyr] > [Ch][Nia]. In the studied ABS, taurine and niacin preferentially migrate to the IL-rich phase, while caffeine migrates in the majority of the cases towards the PPG-rich phase. However, an opposite behaviour on the partition of caffeine was achieved with ABS formed by [Ch][Pyr] or [Ch][Nia], in which caffeine also partitions to the IL-rich phase. The system composed of [Ch][Nia] and PPG 400 allows the almost complete recovery (*RE*% >82.32%) of all the target compounds into the IL-rich phase in one-step. Given that [Ch][Nia] exhibits lower cytotoxic potential and is considered harmless from an ecotoxicological point of view, this system can be considered a promising platform to simultaneously extract, recover and reuse added-value compounds from expired energy drinks and thus contribute to a more sustainable and circular food economy.

ASSOCIATED CONTENT

Supporting Information. Supplementary Information available: [NMR and FTIR spectra; information on the determination of the ABS phase diagrams; Caco-2 cytotoxicity dose response curves; Microtox toxicity results; Ternary phase diagrams and binodal weight fraction data of ABS, TLs and TLLs; HPLC chromatograms; Recovery efficiencies].

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Notes

There are no conflicts to declare.

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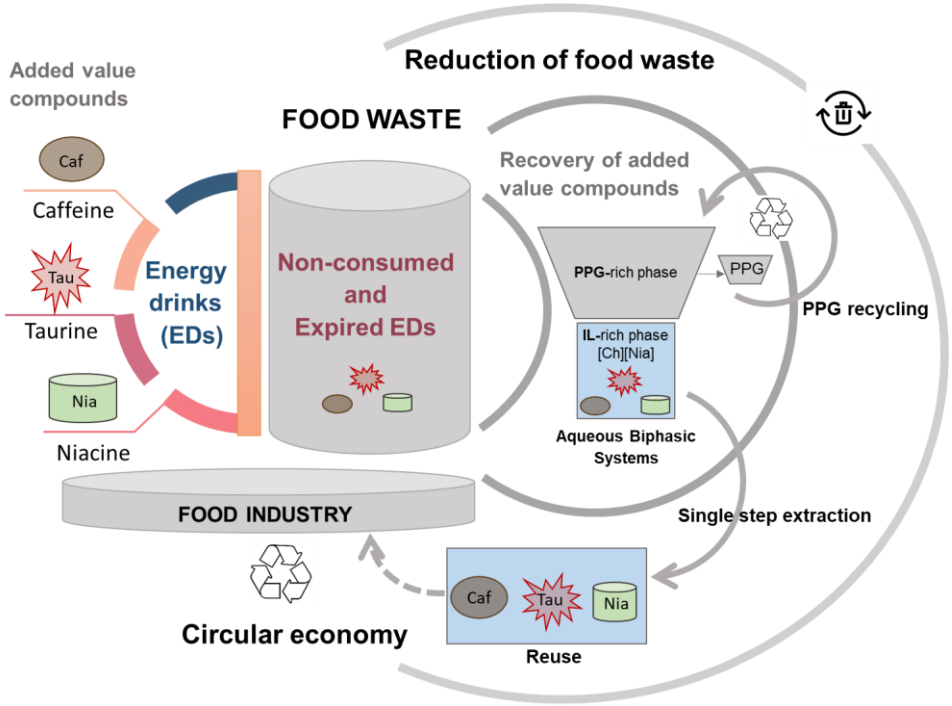
SYNOPSIS



Ionic-liquid-based aqueous biphasic systems are effective platforms to simultaneously extract, recover and reuse added-value compounds from expired energy drinks, contributing to a sustainable and circular food economy.

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