THE GLYCEROL/WATER MIXTURE IS AN EFFECTIVE DEEP EUTECTIC SOLVENT FOR THE EXTRACTIONS FROM FOOD WASTE MATRIXES

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ABSTRACT

Deep Eutectic Solvents (DESs) are a novel class of organic liquids that are becoming increasingly important in the literature for their green and catalytic properties and for their peculiar structural features. Because of these, they are finding fruitful applications in many different topics. One of the most explored one is their use as green effective extracting agents from food matrices and food waste matrices of valuable bioactive compounds, thanks to their affinity with aromatic and phenolic compounds.

In this work, the mixture glycerol/water at 1/2.55 molar ratio is demonstrated to be a DES thanks to the comparison of the experimental melting points with the theoretical curves. This approach is used with both liquid starting components for the first time to our knowledge. This DES was then tested as extracting agent on different food waste matrixes: onion, tomato and apple peels; spent coffee grounds and grape pomace.

The results were compared with those obtained with the water and glycerol only components, showing a superior capability of the DES in the extractions, as well as superior antioxidant properties of the extracts. This promotes the use of this mixture at the eutectic point as a DES and opens for a novel approach in which the extract could be directly used without further purification procedures considering the food grade of the DES components.

KEYWORDS

Deep Eutectic Solvents; Food Waste; Glycerol; Glycerol/water mixtures; Extractions; Antioxidants.

INTRODUCTION

Deep Eutectic Solvents (DESs) are a class of solvents that are rapidly increasing in their relevance in the literature thanks to their environmentally-friendly features, as well as to their peculiar structural features and to their catalytic capabilities(Khandelwal, Tailor, & Kumar, 2016)(Buzolic, Li, Aman, Warr, & Atkin, 2022)(Ünlü, Arıkaya, & Takaç, 2019). These liquids are formed via weak interactions, mainly H-bonds, between two species: a hydrogen bond donor (HBD) and a hydrogen bond acceptor (HBA). The interactions occurring between the two different species (HBD-HBA), as well as the ones occurring between the same species (HBA-HBA and HBD-HBD) lead to an impossible regular crystal lattice formation, therefore to a substantial lowering in the melting points(Araujo et al., 2017)(Stephens & Smith, 2022)(Kaur, Kumari, & Kashyap, 2020). The environmentally-friendly features of this class of liquids relies on their low or absent toxicity; their low or absent vapor pressure; their realization in absence of any other solvent; their easy recycle capabilities and so on(Gomez, Espino, Fernández, & Silva, 2018; Hayyan et al., 2013; Wen, Chen, Tang, Wang, & Yang, 2015). The needing for reduction of the human being impact on the environment for the climate emergency is pushing forward for the application of DESs in many different areas as substitutes to common volatile organic compounds (VOCs). In organic synthesis, DESs can also have acid, basic, reductive, organocatalytic catalytic properties and in the extraction/preconcentration of valuable molecules from food or vegetal matrixes they show better results than VOCs(Duan, Dou, Guo, Li, & Liu, 2016; Rollo et al., 2023). In particular in the latter, DESs are finding excellent results because of their affinity with aromatic, in particular phenolic, molecules(Giofrè, Celesti, Mistretta, & Tiecco, 2023). Thanks to the richness of this class of valuable molecules in food and vegetal matrixes, to the effectiveness of this class of liquids far superior by far to the VOCs, and to their environmental friendly features, DESs can be clearly identified as a winning choice in this subject(Zainal-Abidin, Hayyan, Hayyan, & Jayakumar, 2017).

In order to clearly recognize the DES' identity of a liquid mixture, a comparison of the theoretical melting curves with the experimental data at different molar fractions is mandatory to define a liquid as a DES and to identify its eutectic point(Abranches & Coutinho, 2023; Martin & Shipman, 2023; Martins, Pinho, & Coutinho, 2019). The experimental curves of a DES, in fact, show an experimental eutectic point with a lower melting point and a shift in the molar fraction compared to the theoretical one. Also, the activity coefficients of the components show a non-ideal behavior. This is important considering that whenever a liquid is formed by mixing two solids, it is clear that a non-ideal system is formed and interactions are occurring between the two species; in the case one

of the components is already a liquid, it is impossible to define it as a simple solution rather than a DES without the use of these quantitative curves. With this approach, recently *aquo*DESs were realized, characterized and identified as DESs(Picciolini et al., 2023; Tiecco et al., 2022). These are mixtures in which water, thanks to its widely known capabilities as acceptor and donator of H-bonds, is part of a binary mixture. However, nothing has been reported yet in the literature about possible DESs mixtures realized with both components being liquids at room temperature. From a practical point of view, this could appear redundant as the resulting system is obviously a liquid, but the properties of a DES, even if are relatable to the ones of the components, are different from the formers and, in many cases, better.

In this work the mixture glycerol/water in 1/2.55 molar ratio (GLY/H_2O) was undoubtedly defined as a deep eutectic solvent thanks to the comparison of the theoretical melting curves with the experimental ones and to the analysis of the experimental activity coefficients. Glycerol/water mixture is widely characterized in literature in its chemical-physical properties in all the composition ranges; still nothing has been reported with this novel approach considering it as a DES(Takamura, Fischer, & Morrow, 2012)(Lane, 1925). This mixture is a well-known industrial product, widely produced and used in a high number of different applications such as: antifreeze agent; liquid for electronic cigarettes; liquid for cryopreservation of living cells; preservation of proteins; artificial smoke production and so on (Chaveiro, Liu, Engel, Critser, & Woelders, 2006; Meryman, 2007; Pan et al., 2018; Zhang, Kleinstreuer, & Hyun, 2012). Because of the safety of the components ((ANS) et al., 2017) and because of the above-mentioned advantageous properties with food or vegetal products of the DESs, GLY/H_2O DES was tested in extraction procedures in five different food waste matrixes: onion, tomato and apple peels; spent coffee grounds and grape pomace. The superior capabilities of this DES were demonstrated by comparing the results with the ones of the single components water and glycerol in terms of extracted polyphenols amounts and of antioxidant activity of the extracts, underlining the importance of the DES' identity of this liquid.

EXPERIMENTAL

Reagents, food matrixes, DES realization

Glycerol (10% w/w water content) and 1,2-propanediol (>99%) were purchased from Merck and Carlo Erba suppliers. Folin–Ciocolteu reagent and sodium carbonate (Na_2CO_3) were purchased from Sigma-Aldrich (St. Louis, MO, USA). DPPH (2,2-diphenyl-1-picrylhydrazyl) was bought from

Glentham Life Sciences (Corsham, UK). Water was bidistilled grade. Metrohm 684 KF Coulometer Karl Fischer titrator was used to determine the glycerol starting water amount.

DESs was realized adding the proper amount of bidistilled water to the glycerol (considering the starting amount of water in the glycerol) in closed screw-capped vials then shaking with vortex and gently heating with a heating gun the samples. With the same procedure the 1,2-propanediol/water mixtures were realized at the different molar fractions.

Onions, tomatoes and apples were purchased from local market and spent coffee grounds was taken from local coffee producers. Grape pomace wastes were provided by a regional winery located in Morro D'Alba (AN, Italy).

Theoretical/experimental melting curves

The experimental melting points of 1,2-propanediol/water mixture were obtained by measuring the melting points of the different samples at the different molar ratios with a thermometer via immersion of the samples in liquid nitrogen in a Dewar. The melting temperatures were evaluated in triplicate to avoid any kinetic effect on the melting of the mixtures and they showed a standard deviation <1 °C in the triplicates.

The solid-liquid theoretical curves were determined by using the following Formula (1) that represents the solid-liquid equilibrium curve in a eutectic mixture:

$$ln(\chi_i \cdot \gamma_i) = \frac{\Delta_m h_i}{R} \cdot \left(\frac{1}{T_{m,i}} - \frac{1}{T}\right) + \frac{\Delta_m Cp_i}{R} \cdot \left(\frac{T_{m,i}}{T} - ln\frac{T_{m,i}}{T} - 1\right) \qquad (1)$$

where χ_i is the molar fraction of component i, γ_i is its activity coefficient in the liquid phase, $\Delta_m h_i$ and $T_{m,i}$ are its melting enthalpy and temperature, respectively, $\Delta_m Cp_i$ is its heat capacity change upon melting, R is the ideal gas constant, and T is the absolute temperature of the system. Glycerol $\Delta_m h$ = 18280 J/mol, T_m = 290.9 K; 1,2-propanediol $\Delta_m h$ = 8400 J/mol, T_m = 213.15 K; water $\Delta_m h$ = 6007 J/mol, T_m = 273.15 K(Haynes, 2016). This equation can be simplified by considering the heat capacity change upon the melting of a substance as negligible, therefore equation (2) was used:

$$ln(\chi_i \cdot \gamma_i) = \frac{\Delta_m h_i}{R} \cdot \left(\frac{1}{T_{m,i}} - \frac{1}{T}\right) \qquad (2)$$

The theoretical melting temperatures were determined from the theoretical curves by considering the activity coefficients γ_i = 1. The eutectic points were defined as the minimum in the experimental curves and they were compared to the theoretical ones.

The experimental γ_i values were determined via equation (3) by using the experimentally observed melting temperatures:

$$\gamma_i = \frac{\exp\left[\frac{\Delta_m h_i}{R}\left(\frac{1}{T_{m,i}} - \frac{1}{T}\right)\right]}{\chi_i} \qquad (3)$$

Extraction procedures

The extractions were made via heating/ultrasonication procedures at 59 kHz (FALC ultrasonic bath, Treviglio, Italy) following protocols already optimized and published in the literature. The matrixes were put in 3 mL Eppendorf tubes containing the extracting liquids and the shredded and dried (40 °C for 48 hours) solid matrixes. The detailed extraction procedures are reported in Table 1:

Table 1: Food matrices analyzed; references for the procedures adopted, correspondent solvent/solid ratios (mg/ml), Temperatures (°C) and times (mins) used.

Matrix	Reference	Solid/Solvent ratio, mg/ml	Temperature, °C	Time, minutes
Onion peels	(Ciardi et al., 2021)	1/30	50	45
Tomato peels	(Vorobyova, Skiba, & Vasyliev, 2022)	1/20	65	60
Grape pomace	(Abouelenein et al., 2023)	1/10	45	30
Spent coffee ground	(Jeong, Han, Kim, Jin, & Lee, 2018)	1/38	60	10
Apple peels	(Rashid, Wani, Manzoor, Masoodi, & Dar, 2023)	1/30	40	40

After heating/sonication, the samples were centrifuged for 20 minutes at 5000 rpm (Thermo Scientific IEC CL10 Centrifuge, Thermo Electron Industries SAS) and the supernatants were diluted in water for the subsequent UV-Vis analysis (see further). The dilutions were made in order to have the maximum of the absorbances between 0.1 and 1 A.U., proper values for TPC and DPPH analysis (dilutions spanning from 1/1 to 1/10 of supernatant/water). The same procedures with the same matrixes were replicated with GLY/H$_2$O DES, water and glycerol respectively. All the measures were made in triplicates with standard deviations determined via these replicates.

Determination of total phenolic contents (TPC)

The Total Phenolic Content (TPC) was determined spectrophotometrically using an Agilent Cary 8454 UV-Vis spectrophotometer, following the procedure described by Santanatoglia et al. (Santanatoglia et al., 2023), with some modifications: 0.5 mL of each water diluted sample was added, followed by 2.5 mL of Folin–Denis reagent and 7 mL of a 7.5% w/w Na_2CO_3 water solution. The reaction mixture was left in the dark for 2 hours at room temperature, then absorbance was measured at 765 nm. TPC was quantified using a gallic acid calibration curve and expressed as mg gallic acid equivalents (GAE) per g of food matrix.

DPPH Radical Scavenging Activity

The antioxidant activity was assessed via the DPPH method, which involves spectrophotometric measurement of the reduction of the 2,2-DiPhenyl-1-Picrylhydrazyl radical by antioxidant compounds. An Agilent Cary 8454 UV-Vis spectrophotometer was used for the measures. This evaluation was performed by adapting the methodology outlined by Santanatoglia et al. (Santanatoglia et al., 2023) 0.5 mL of each water-diluted sample was mixed with 4.5 mL of a 0.1 mM ethanolic DPPH solution. The mixture was left in the dark for 30 minutes at room temperature. The decrease in DPPH radical concentration was then measured at 517 nm. Trolox as the reference antioxidant, and results were expressed in terms of mg Trolox equivalents (TE) per g of food matrix.

RESULTS AND DISCUSSION

The first step of this work was the demonstration of the DES identity of the glycerol/water mixture. With this purpose, the theoretical melting points curves of the two components were drawn according to Eq. 2 and compared with the experimental melting points taken from literature data(Lane, 1925). The graphs, comprehensive also of the activity coefficient profiles calculated with Eq. 3, are reported in Figure 1.
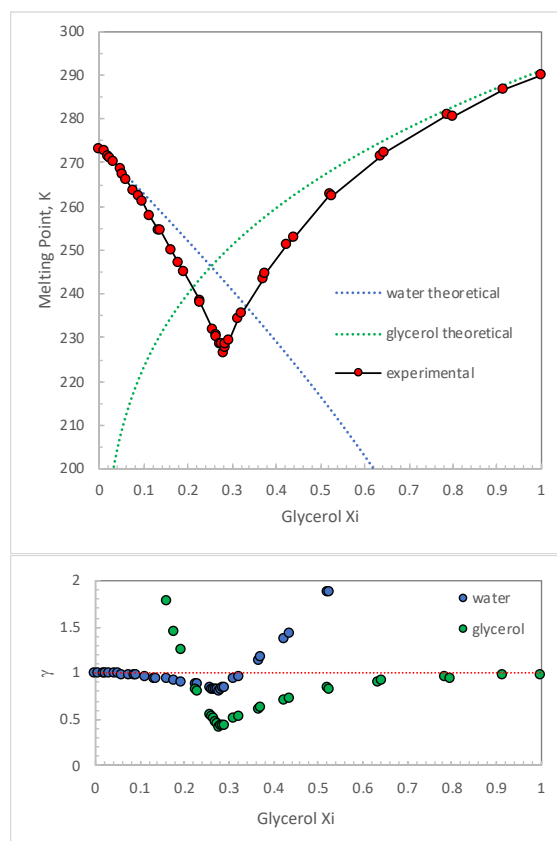
Figure 1: DES identity of glycerol/water mixture: UPPER PART comparison of theoretical melting curves of water (blue dashed line) and glycerol (green dashed line) with experimental melting points (red dots)(Lane, 1925); LOWER PART activity coefficients of water (blue dots) and of glycerol (green dots).

As it can be clearly seen from Figure 1, the glycerol/water mixture is a deep eutectic solvent. The mixture shows a eutectic point at glycerol/water 1/2.55 molar ratio and a melting point of -46.5 °C (226.65 K). This point shifts from the crossing point of the theoretical melting point curves of the two components both in terms of the melting point value (that is lowered by about 20 degrees) and of the molar ratio, that is theoretically observed at about -27 °C and at 1/3 GLY/H_2O molar ratio. From these melting points, the activity coefficients of both components were calculated and reported in the same Figure 1. The values are almost all below the value of 1, they both reach a minimum at the experimental eutectic point then rapidly increase over 1 overpassing the minimum at the eutectic. According to the literature, these are clear and sufficient evidences of the DES' identity of this mixture. Glycerol itself is a really hygroscopic liquid that can easily absorb water from the air, so we measured the water content of our batch in order to see how behaves under this point of view. The starting water content of the glycerol (measured to be 10% w/w, corresponding to $X_{glycerol}$ = 0.64) puts this liquid in a part of the curve where it behaves almost as an ideal mixture as it can be easily seen also from the activity coefficients, slightly below 1 in this region. The non-

ideal behavior of this mixture is more significant in the range of $X_{glycerol}$ from 0.25 to 0.45, closer to the eutectic point.

To strengthen these data, another water mixture was realized by mixing 1,2-propanediol with water and measuring the melting points with a thermometer in a liquid nitrogen bath. This diol has a structure very similar to the glycerol (lacks of just one -OH group compared to glycerol which has three). The theoretical/experimental melting points graphs and the activity coefficients are reported in Figure 2.
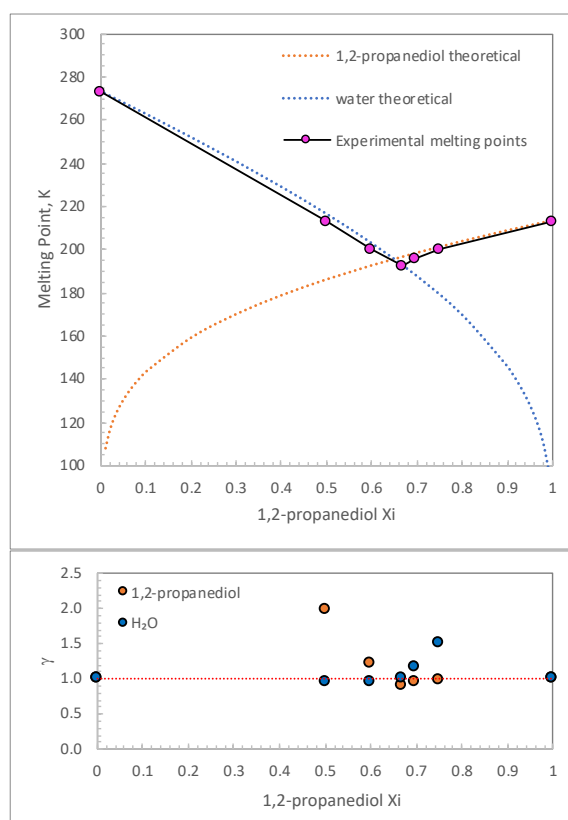

Figure 2: 1,2-propanediol/water mixture: UPPER PART comparison of theoretical melting curves of water (blue dashed line) and 1,2-propanediol (orange dashed line) with experimental melting points (purple dots); LOWER PART activity coefficients of water (blue dots) and of 1,2-propanediol (orange dots).

1,2-propanediol/water mixture behaves in an ideal way in all the composition range, showing an almost perfect overlap of the theoretical melting curves, except of a really slight lowering at 1/1 molar ratio and at the eutectic point, maybe due to the slight uncertainties of the measures via thermometer. The activity coefficients calculated from the experimental melting points are very close to 1, then show an increase passing over the eutectic in the parts where the counterpart is the major part. This indicates that even if these two molecules are reciprocally soluble, the interactions occurring between them are not so strong to determine a DES with the presence of HBD-HBA, HBA-

HBA and HBD-HBD domains responsible of the disorganization of the crystal lattice of the mixture. The difference of only one hydroxyl group of 1,2-propanediol compared to the glycerol underlines again the singularity of GLY/H_2O mixture.

The next step of this work was the demonstration of the GLY/H_2O DES validity by applying it in food matrices extractions, considering the suitability of glycerol and, of course, of water in food applications(Azelee et al., 2019). In this experimental design, the results coming from the DES extractions were compared with the ones made in same identical conditions with the same food batches made with water and with glycerol only. The food matrixes analyzed were onion, tomato and apple peels; spent coffee grounds and grape pomace. The extraction capabilities were determined by measuring the total polyphenolic contents (TPC), avoiding any chromatographic analysis of the extract such as HPLC. This was made because the TPC value can give, with a single number, an evaluation of the overall capacity of extraction of a solvent for this class of compounds. Any chromatographic study can lead to difficult comparisons of different molecules amounts and they also need standard calibrations of their chromatograms. Moreover, in this paper the overall efficiency of the GLY/H_2O DES was made by analyzing five different food matrixes, therefore increasing the number of experiments, standards and so on by much. In Figure 3 the histograms of the TPC values observed for the extractions on the five matrixes of the DES compared to the water and to the glycerol components are reported.
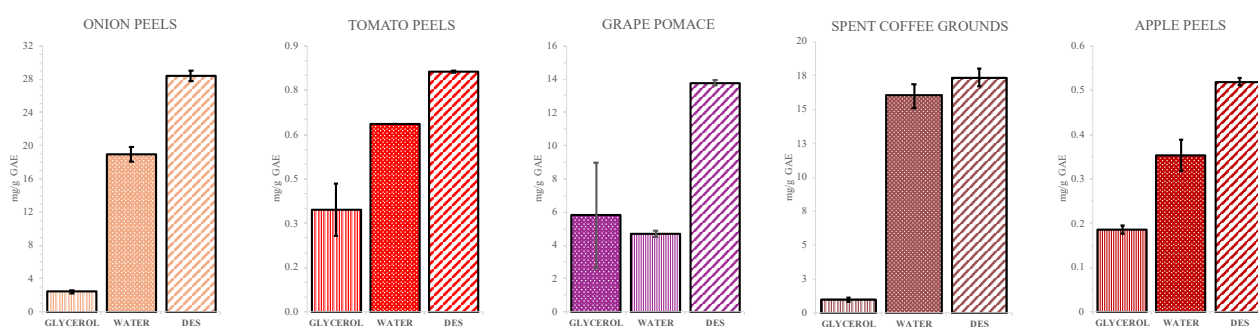

Figure 3: Total polyphenolic contents (TPC, mg Gallic Acid Equivalent (GAE)/g) of the extracts form onion peels, tomato peels, grape pomace, spent coffee grounds and apple peels with glycerol, water and glycerol/water DES respectively.

As it can be clearly seen from the histograms in Figure 3, GLY/H_2O DES allowed a greater extractability of phenolic from all tested matrixes compared to the use of only glycerol and water components alone. Based on the literature data, the mainly phenolics compounds belong to phenolic acids and flavonoids classes (Azelee et al., 2019). In the spent coffee matrix, a slightly better extraction capability was observed with water as the extraction solvent. However, this may be due

to the matrix is realized for water extraction, and some target compounds are hydrophilic (Gil-Martín et al., 2022).This data underlines the importance of the eutectic composition of the DES in these processes, showing results that are not related to the ones of the components of the DES mixture.

The DESs are known for the antioxidant activity of extracts obtained from different matrixes(Ozturk, Parkinson, & Gonzalez-Miquel, 2018), this is particularly relevant for the extraction of polyphenols. Therefore, we also tested the antioxidant activity of the DESs extracts with the DPPH radical scavenging method, once again comparing the data with those obtained with the same extracts in water and in glycerol (Figure 4).
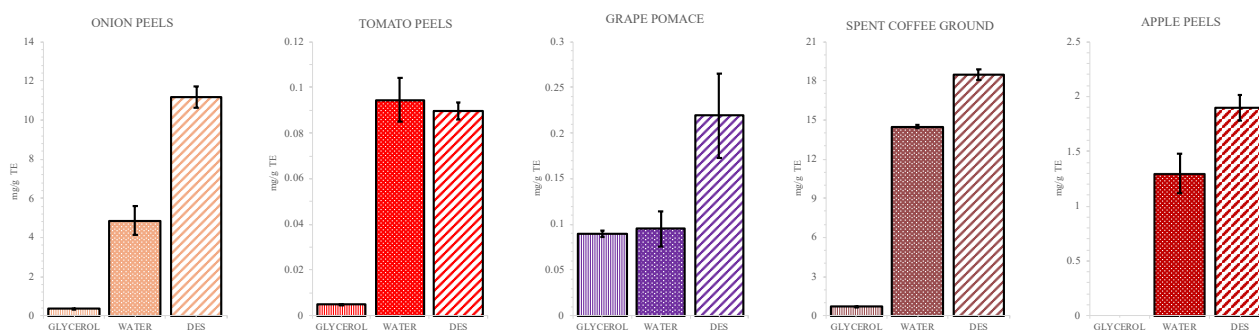

Figure 4: Radical scavenging method (DPPH, mg Trolox Equivalent (TE)/g) of the extracts form onion peels, tomato peels, grape pomace, spent coffee grounds and apple peels with glycerol, water and glycerol/water DES respectively.

In general, the highest extraction of bioactive compounds with the DPPH method was observed in all matrices' extracts obtained by using DES. Water showed a moderate antiradical activity in all extracts, except for the ones from grape pomace wastes. Indeed, the antioxidant activity results were well confirmed by the TPC, which highlight a similar trend for all extracts.

CONCLUSIONS

Glycerol/water mixture is widely produced in the industry for various applications and purposes. Still because of the lack of applications of the theoretical/experimental melting curves comparison, it was not yet demonstrated to be a deep eutectic solvent. In this work it is demonstrated to be a DES thanks to quantitative approach. GLY/H_2O (1/2.55 molar ratio) DES was applied for the extraction of polyphenols from five different food matrixes, showing superior results compared to its components, underlining the validity of this mixture at the eutectic point. The antioxidant activity

of DES extracts showed better results also for the preservation from oxidation compared to the water and to the glycerol.

Because of the food-grade, the safety and the suitability for food applications of the components in all the concentrations, GLY/H$_2$O DES represents an extremely promising mixture for the possible direct application of the extracts in food and food supplements applications, opening an innovative approach to the extraction procedures avoiding further purifications. Undoubtedly, cytotoxicity studies are needed, and they are currently undergoing in our laboratories. These will define any synergic/antisynergic effect between the components that could impact on their toxicity in the same way they impacted on their non-ideal melting points. All of the above-mentioned studies are mandatory to develop a new regulation that could consider using GLY/H$_2$O DES with this approach.

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