Applications of natural deep eutectic solvents to extraction and preservation of biomolecules

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C H A P T E R 2

Green solvents from ionic liquids and deep eutectic solvents to natural deep eutectic solvents

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Abstract

Natural deep eutectic solvents (NADESs) are defined as mixtures of certain molar ratios of natural compounds such as sugars, organic acids, amino acids, organic bases that are abundant in organisms. The melting points of these mixtures are considerably lower than those of their individual ingredients and far below ambient temperature. The first publications on the NADESs concept in 2011 created a great expectation regarding their potential as green solvents that could replace conventional organic solvents in a wide range of applications. This was largely because many of the drawbacks of conventional synthetic ionic liquids (ILs) and deep eutectic solvents (DES), particularly their toxicity and environmental hazards, could be solved using NADESs. Throughout the last 7 years, the interest in NADESs has increased enormously as reflected by the exponential growth of the number of related publications. The research on NADESs has rapidly expanded particularly into the evaluation of the feasibility of their application in diverse fields such as the extraction of (targeted) bioactive compounds from natural sources, as media for enzymatic or chemical reactions, preservatives of labile compounds, or as vehicles of non-water-soluble compounds for pharmaceutical purposes. Along with the exploration of these potential applications, there have been a large number of other studies related to their physicochemical features, the search for new NADESs, the research into the interactions between NADESs components or with solutes, the recovery of solutes from NADESs solutions, and the ways of circumventing inherent problems of NADESs such as their high viscosity and the consequent difficulties in handling them. This paper contains a review of the applications of NADESs as extraction solvents, reaction media, and preservative, providing also a perspective of their future.

Keywords*:* Natural deep eutectic solvents, extraction solvents, reaction media, preservation

Introduction

Natural deep eutectic solvents (NADESs) are mixtures of natural compounds, namely, organic acids and bases, amino acids, sugars, sugar alcohols, and polyalcohols that interact through hydrogen bonding and liquefy if combined in specific molar ratios. The concept of NADESs was first presented by Choi et al. in 2011, who speculated on the existence of a third liquid medium in living organisms apart from water and lipids. In their view, NADESs could play a role as an alternative to water and lipids in extreme conditions such as drought and cold resistance, in the desiccation of organisms as they enter a senescence state for long-term survival, for example, seeds, in the resurrection plants and lichens, as media in the biosynthesis of water-insoluble compounds as well as for the transport and storage of non-water soluble metabolites (Choi et al. 2011).

So far, different researchers have proposed more than 150 NADESs combinations (Dai et al. 2013[a]; Paradiso et al. 2016; Bakirtzi et al. 2016; Huang et al. 2017). Sugars, sugar alcohols, polyalcohols, organic acids and bases, and amino acids, have been reported to be good candidates for NADESs. The natural origin of the components (mainly plant primary metabolites which are taken daily from vegetables or fruit), gives NADESs a great edge over synthetic ionic liquids (ILs) and deep eutectic solvents (DES), because they are clearly less toxic and more environmentally friendly. Following the first publications on NADESs they have been actively applied in diverse chemical processes, especially in the extraction of natural ingredients (Dai et al. 2013[b]; Dai et al. 2016; Paradiso et al. 2016; Gonzales et al. 2018), as media for enzymatic or chemical reactions (Zhao et al. 2011; Durand et al. 2012; Yang et al. 2017; Khodaverdian et al. 2018), to solubilize macromolecules such as polysaccharides and lignins (Kumar et al. 2016) or non-water soluble drugs for pharmaceutical purposes (Wikene et al. 2015; Shamseddin et al. 2017; Wikene et al. 2017), to develop cosmetic ingredients (Jeong et al. 2017) and for agrochemical uses (Huang et al. 2018; Zahrina et al. 2018). The publication of the first NADESs report in 2011 was followed by the appearance of more than a thousand scientific articles in the following 5 years reflecting the interest in

NADESs as one of the most types of promising green solvents.

This article provides a review of the applications and most studied topics of NADESs, including their use for the extraction of bioactive metabolites from plants, the isolation of macromolecules from natural resources, their suitability as media for chemical and enzymatic reactions; and for the preservation of labile compounds.

Solid mixtures become liquid: ionic liquids, deep eutectic solvents, and natural deep eutectic solvents

The history of ILs and their applications has been extensively reviewed by Plechkova and Seddon (2008). In 1914, Walden reported that mixing certain solids could change their state to liquid. He based his conclusion on the observation of the physical properties of ethylammonium nitrate salt ($[EtNH_3][NO_3]$) during a thermolysis study (Walden et al. 1914; Plechkova et al. 2008). This resulted in the first patent on ILs in 1934 for the application of salts (e.g., halide and nitrate) of nitrogen-containing bases to dissolve cellulose at temperatures greater than 100 ^{o}C. Furthermore, this IL solution was found to be an efficient medium for diverse chemical reactions (Huckel 1958; Plechkova et al. 2008). The last decades have seen an impressive growth in the field of green chemistry. The range of commercial applications of ILs arising from the close cooperation between academia and industry (Pandey 2006) is remarkable in number and diversity. The prospective uses of ILs has attracted increasing interest among scientists and chemical engineers. Although the full potential of these unique solvents is still unexplored, the initial main applications of ILs have shifted from extraction to synthesis or catalysis (Pandey 2006). Among the numerous applications as reaction media, investigations on the use of synthetic Brønsted acidic ILs as catalysts in esterification and transesterification reactions proved that the use of simple and non-corrosive salts characterized by aliphatic cations associated with an “acidic” anion (in particular, $[HSO_4]^-$) increased in yields . Although a relatively high degree of correlation between the acidity of the IL (H_0) and its catalytic ability was observed, it was considered to be more likely that the hydrophilic nature of the ionic medium was responsible for this

increase in yield (Chiappe et al. 2013). There are also numerous examples of the use of ILs as media for enzymatic reactions. For example, the activity of chloroperoxidase in a hydrophilic IL media has been evaluated using kinetic and stereochemical studies. The activity of the same enzyme in several citrate buffer-IL mixtures was tested with success in chemical synthesis, for the chemo- and stereoselective oxidation of phenyl methyl sulphide (Chiappe et al. 2006). A new L-carnitine/urea (2:3, w/w) mixture was developed and compared to previously reported sugar and sugar alcohol DESs, using several organic reactions as the benchmark. An evaluation of some physicochemical properties of the new L-carnitine-based solvent, including its melting point and polarity with differential scanning calorimetry and solvatochromatic measurements, respectively, showed that this melt displayed a very high polarity. However, although Heck and Sonogashira cross-couplings, Diels–Alder reactions and Cu-catalysed 1,3-dipolar cycloadditions proceeded cleanly in sugar and L-carnitine-based melts, the applicability of L-carnitine melts for standard organic reactions is limited by their lower thermal stability (Ilgen and König, 2009).

Similarly to most newly developed solvents, ILs were firstly applied to diverse extraction processes and reactions. In one of the first reports, Huddleston et al. (1998) studied the partitioning of substituted benzene derivatives between water and [1-ethyl-3-methylimidazolium (Bmim)][PF6]. Later, using the indicator thymol blue, the same group demonstrated the reversible pH-dependent liquid-liquid partitioning for IL-containing systems (Visser et al. 2000[a]). They also described the possibility of fine-tuning the partitioning process by varying the IL structure. In another case, inorganic ions such as Na^+, Cs^+, and Sr^{2+} were extracted from aqueous solutions with mixtures of 18-crown-6 family crown ethers in [1-alkyl-3-methylimidazolium][PF6] (Visser et al. 2000[b]). The results showed that the efficiency of the extraction generally diminished as the length of the 1-alkyl group of the ILs increased. To further expand the utility of room-temperature ILs in metal ion extraction, the addition of other well-known organic and inorganic extractants was also tested (Visser et al. 2001). The studies proved that the presence of the extractant was

necessary to enhance the metal ion affinity for the hydrophobic phase. Recent applications of ILs as extraction solvents have been reviewed in our previous article (Dai et al. 2013[c]). Initially ILs were mainly applied to the extraction of biopolymers, and only later moved on to secondary metabolites. The targeted biopolymers were mostly polysaccharides and lignin. Lignin is an aromatic polymer composed of phenylpropanoids, and its extraction is dependent on the selection of the anion of the IL. Lignin is highly soluble in polar ILs with anions such as [alkylbenzenesulfonate (ABS)], [Cl], [$MeSO_4$], [trifluoromethylsulfonate], [OA[xylene sulfonate]] but not in less polar ILs with [BF_4] and [PF_6] anions (Lee et al. 2009; Tan et al. 2009). Lignocellulose, a major source of lignin, has been obtained from cellulose with [1-ethyl-3-methylimidazolium (Emim)][OAc] (Lee et al. 2009).

As for polysaccharides, most of them are insoluble in water or organic solvents. However, recent studies have reported that cellulose could be dissolved, without any derivatization, in several hydrophilic ILs such as 1-butyl-3-methylimidazolium chloride ([Bmim][Cl]) and 1-allyl-3-methylimidazolium chloride (Swatloski et al. 2002; Zhu et al. 2006). There is a major interest in scaling up this method to provide a cost-effective and environmentally benign solution for the dissolution and processing of biomass as diverse as cellulose, chitin, and wood (Sun et al. 2009). The mechanism of dissolution of cellulose is anion-dependent, based on the disruption of inter- and intra-hydrogen bonds in cellulose and the formation of new hydrogen bonds between the anions of ILs and the hydroxyl groups of the carbohydrate moiety (Abe et al. 2010).

The IL-based microwave-assisted extraction technique was initially developed to extract different kinds of phenolic compounds such as *trans*-resveratrol (Du et al. 2007), gallic acid, ellagic acid, quercetin (Du et al. 2009), and rutin (Zeng et al. 2010). These studies revealed a strong relationship of the structure of IL components with the efficiency of the extraction, particularly showing that it is anion-dependent. Among the tested ILs, [Bmim][Br] was in general the best choice for phenolic compounds but the presence of an extra aromatic system [Bmim][toluolsulfonate] was found to result in higher yields of

some phenolics (Zeng et al. 2010). In the case of phenolic compounds with fewer hydroxyl groups, such as magnolol and honokiol, the use of [$PF6^-$] as the anion proved to increase the extraction yield when compared with that of [$BF4^-$] (Zhang and Wang, 2010).

A wide range of nitrogen-containing natural products such as alkaloids have also been extracted using ILs. [Bmim] [BF_4] displayed a high efficiency for the extraction of piperine (Cao et al. 2009) and phenolic alkaloids including liensine, isoliensine, neferine, fangchinoline, and tetrandrine (Lu et al. 2008; Zhang et al. 2009). Interestingly, [1-hexyl-3-methylimidazolium][Br] showed a yield almost 40% higher than [Bmim][BF_4] in the case of *N*-nornuciferine, *O*-nornuciferine, and nuciferine (Ma et al. 2010).

Many ILs composed of hydrophobic ingredients have been used for the extraction of flavors, fragrances, and essential oils to replace the conventional steam distillation method. The IL-based method can be advantageous as it shortens the required extraction time significantly (from 2 h to 15 min), particularly when combined with microwave extraction (Zhai et al. 2009). Furthermore, the use of ILs has additional benefits over conventional organic solvents in terms of selectivity and/or high extraction efficiency. For example, [Emim][Meesu] has been used to extract linalool from citrus essential oil (Francisco et al. 2010) and [bis(2-methoxyethyl) ammonium (BMOEA)][bis(trifluoromethanesulfonyl) imide] proved to extract artemisinin with better yields than the commonly used solvents (Lapkin et al. 2006).

In addition to these applications of ILs for extraction, in the late 1990s they were also reported to be used in chromatography and preanalytical extractions as well as for electroanalytical purposes as reviewed by Pandey (2006). ILs have been used as stationary phases in many chromatographic methods, showing that some characteristic properties of these solvents provide potential benefits to many areas of separation sciences. Evaluating the use of ILs as a stationary phase in GC, Armstrong et al. (1999) tested the behavior of two common ILs, [Bmim][PF6] and [Bmim][Cl] coated onto fused silica capillary columns comparing their performance with that of commercial polysiloxane stationary

phases. They found that ILs acted as low-polarity stationary phases for nonpolar compounds, but more interestingly that solutes containing strong proton-donor groups were also efficiently retained. Some ILs have also been applied for chiral separations, perhaps the most attractive topic currently in chromatography separation science. For example, [Bmim][Cl] has been used to dissolve per- and dimethylated β-cyclodextrins to prepare stationary phases for capillary columns in GC (Berthod et al. 2001), and recently, Ding et al. (2004) have presented the first enantiomeric separations using chiral IL stationary phases in GC.

The applications of ILs in high-performance liquid chromatography (HPLC) have been extensively reviewed (He et al. 2003; Stalcup et al. 2004; Pandey 2006). As an initial attempt, the chromatographic behavior of ephedrine on a C18 column with different concentrations of [Bmim][BF4] as an eluent at pH 3.0 was investigated (He et al. 2003). The addition of ILs resulted in decreased peak tailing, reduced peak broadening, and improved resolution. Similar studies were reported with 1-alkyl-3-methylimidazolium and *N*-butylpyridinium like [BM_3Py] salts as new mobile phase additives for the separation of catecholamines in reversed-phase HPLC (Zhang et al. 2003). Efficient separations were achieved for both additives. These researchers also reported the separation of amines using the same ILs as additives in the mobile phase (Zhang et al. 2003).

ILs have also been used in other chromatographic techniques such as thin-layer chromatography. In this case, it was observed that the addition of 0.5-1.5% (v/v) of ILs of the [imidazolium][BF4] with silica as a stationary phase blocked the more active silanol groups eliminating their deleterious effect on tailing, for example, especially in separations of strongly retained basic drugs (Kaliszan et al. 2004). The application of [Bmim][PF6] as a novel solvent in countercurrent chromatography (CCC) has been extensively reviewed by Berthod and Carda-Broch (2003). Investigating the partition of 38 aromatic derivatives possessing acidic, basic, or neutral moieties between [Bmim][PF6] and water, Berthod and Carda-Broch (2004) concluded that the high viscosity inherent to neat [Bmim][PF6] limits its use as a solvent in CCC.

The ionic features of ILs have allowed their successful use as room temperature buffer electrolytes in non-aqueous capillary electrophoresis, for the separation of water insoluble compounds. As an example, a mixture of dyes that was impossible to separate with conventional capillary electrophoresis (CE) was achieved using ILs (Vaher et al. 2001). It was concluded that the anionic part of the ILs selectively changed the general electrophoretic mobility of the analytes in the system, allowing the separation of the dyes.

Thus, for over 20 years, ILs were considered to be green solvents, attracting the attention of scientists due to features such as their nonvolatility at ambient conditions, their chemical and thermal stability, nonflammability, high conductivity, and high solubilizing capacity. Even potential drawbacks such as their high viscosity and polarity could be tailored by changing the cation-anion combination (Welton 1999; Dai et al. 2013[c]). However, their toxicity and the poor degradability of IL components resulted in their exclusion from the category of green solvents. As a response to this, a new generation of ILs was developed, the DESs, also known as deep eutectic ILs, low-melting mixtures, or low transition temperature mixtures.

The first examples of DESs were obtained by mixing a quaternary ammonium salt with hydrogen bond donors such as organic acids, urea, or glycerol that have the ability to form a complex with the halide anion of the quaternary ammonium salt. The physical properties of DESs are similar to those of ILs, except that these DESs are made of generally nontoxic, easy, accessible, cheap sustainable compounds, and include also nonionogenic compounds (Abbot et al. 2004; Jhong et al. 2009). The safety of these solvents, however, needs further study because some pure DESs could be harmful for living organisms (Hayyan et al. 2013; Ventura et al. 2014; Wen et al. 2015).

Solvents occupy a strategic place in the green chemistry world. A solvent has to meet strict requirements regarding their nontoxicity, biodegradability, recyclability, sustainability, availability, and low price to qualify as a green medium. To date, the number of available DESs is still rather limited. Similarly to ILs, DESs are mixtures of two or three solid components in certain molar ratios, which are capable of interacting through hydrogen bonding to form a

eutectic mixture (Abbot et al. 2004). The resulting DESs are characterized by a melting point that is lower than that of each individual component. ILs and DESs have similar physical-chemical characteristics. Both have very low melting points as compared to their individual components, extremely low vapor pressure resulting in a very high flash point, high dissolving power, and high viscosity. Most DESs are also biodegradable, nonreactive with water, but unlike most ILs, these solvents usually have acceptable toxicity profiles. In addition, although the preparation of most ILs is complex as they include complex synthetic compounds, DESs usually contain components that are available as bulk chemicals (Abbot et al. 2004; Zhang et al. 2012; Dai et al. 2013[a]). Generally, DESs are characterized by a very large depression of the freezing point and are liquid at temperatures less than 150 ^{o}C (del Monte et al. 2014), but most applications use DESs that are liquids at ambient temperature.

One of the major applications of DESs lies in the field of catalysis and organic synthesis, allowing eco-efficient processes, for example, providing the possibility of selectively and conveniently extracting products of the reaction from the DES phase, dissolving not only organic and inorganic salts but also transition-metal-derived complexes or nanoparticles (Zhang et al. 2012). In material chemistry applications, ILs can apparently be advantageously replaced by cheap and safe DESs for the ionothermal synthesis of a wide range of inorganic materials with different textures and structures. Basically, DESs have similar applications to ILs but their cheaper and safer ingredients have allowed them to be used in food and pharmaceuticals. Currently, DESs are applied in diverse fields, such as polymerization (del Monte et al. 2014), biomass processing (Francisco et al. 2012; Xia et al. 2014), materials preparation (Carriazo et al. 2012; Wagle et al. 2014), biodiesel synthesis (Zhao et al. 2013), enzyme-catalyzed reactions (Durand et al. 2013; Weiz et al. 2016), carbon dioxide adsorption (Sze et al. 2014), electrochemistry (Hillman et al. 2014), extraction (Oliveira et al. 2013), nanotechnology (Abo-Hamad et al. 2015), and organic synthesis (Ailing et al. 2014), all of which have been extensively reviewed in Zhang et al. (2012) and Liu et al. (2015).

Deep eutectic solvents were also used as a medium to freeze-dry bacteria

for their preservation (Gutiérrez et al. 2010). The bacterial integrity and viability were efficiently conserved. This application opened new perspectives for the application of green solvents in biotechnology as nonaqueous media for bio-catalytic processes.

As a vehicle in drug-delivery systems, some DESs have been applied to solubilize poorly soluble drugs increasing the concentration range for enhanced bioavailability in early drug development such as toxicology studies. Morrison et al. (2009) reported a 5- to 22,000-fold increase in the solubility of five model drugs in urea-choline chloride and malonic acid-choline chloride eutectic systems as compared to water. Salsalate, an inducer of brown adipose tissue (BAT) also dissolved in higher concentrations in 1,2 propanediol-choline-water (Rozema et al. 2015). Thus, DESs can be a promising vehicle for poorly soluble compounds for preclinical studies, increasing the range of tested concentrations in dose-response curves.

Selectivity of DESs has been increased using the novel magnetic DESs molecularly imprinted polymers for the selective recognition and separation of bovine haemoglobin (Liu et al. 2016). A DES composed of methacrylic acid and choline chloride integrated with magnetic particles such as Fe_3O_4@AA was successively applied to the separation of protein from bovine haemoglobin.

In the field of electrochemistry, apart from conventional applications, a DES composed of choline chloride-urea (1:2) has been used for thin-film solar cells for electrodeposition of metal precursors such as $CuIn_{(1-x)}Ga_{(x)}Se_2$ (Malaquias et al. 2015).

A recent application of DESs for the extraction of targeted bioactive principles from natural products is an example of their ease to be customized to suit the compound and its matrix, one of the greatest advantages of DESs. In this case, artemisinin was extracted from *Artemisia annua* leaves with the tailor-made hydrophobic DESs composed of methyl trioctyl ammonium chloride (N81Cl) and a wide range of alcohols with ratios of 1:2 to 1:4 (molar ratio). Of the tested DESs, the combination of N81Cl and 1-butanol showed the highest extraction yield of artemisinin (Cao et al. 2017).

ILs are undeniably attractive solvents, particularly because of their low

vapor pressure. However, the toxicity of IL components prompted scientists to investigate other solvents. The results of this, DES, have become increasingly attractive in recent years because of their interesting properties and benefits such as the low cost of their components, low toxicity, and increased sustainability, enabling large-scale processes (Francisco et al. 2013; Liu et al. 2015). However, more recently, and as a further development, scientists turned to new sources, considering the natural origin of the potential ingredients of these solvents (Fukaya et al. 2007; Moriel et al. 2010; Chen et al. 2014).

Concept of NADESs and their applications

DESs were an improvement on some negative features of ILs such as their lower toxicity and cost. However, concerns regarding their safety still prevailed and scientists turned to natural sources in the search of alternative ingredients. Fukaya et al. (2007) composed ILs based on common natural compounds such as a choline mixed with various organic acids. As another approach, Moriel et al. (2010) synthesized new room-temperature ILs using several amino acids (alanine, glycine, histidine, phenylalanine, and threonine) and choline from feedstocks. They were applied to a reaction, Knoevenagel condensation of benzaldehyde with activated methylene compounds, finding them to be highly selective at room temperature. Natural DESs or ILs, known as NADESs, have been proposed as a different alternative for two reasons: their ingredients are sourced from nature and they are believed to have a real physiological or biological role in nature. Our group published an article with the concept of NADESs in 2011, containing many examples of NADESs made with different combinations of natural ingredients and suggesting their hypothetical roles in nature and possible applications to life sciences (Choi et al. 2011). Since then, many scientists have reported a wide array of NADESs in nature and further applications (Berthod et al. 2001; Ruß et al. 2012; Paiva et al. 2014). At present, many terms similar to NADESs are being used, as mentioned before. However, what distinguishes NADESs from other DESs made of natural ingredients, such as low-melting mixtures, low transition temperature mixtures, and bio-ILs, is

that the latter do not necessarily have a biological and physiological role in living organisms. Thus, the term NADESs goes beyond the reference to a simple deep eutectic liquid, because it has a biological significance. Among other features this implies, in our view, the presence of different levels of water in NADESs, which strongly affect the physical-chemical properties of the NADESs.

NADESs are made with common naturally occurring ingredients that are individually usually present in food. Although this should guarantee a low or even insignificant level of toxicity, this must be assessed in the mixture of ingredients before any use in food, pharmaceuticals, and cosmetics. Three choline chloride-based DESs combined with glucose, glycerol, and oxalic acid as hydrogen bond donors were evaluated for in vitro toxicity using fish and human cell lines, for their phytotoxicity on wheat and for biodegradability in waste water using microorganisms with the closed-bottle test. The data obtained with the in vitro toxicity test on cell lines indicated a low toxicity for choline chloride:glucose and choline chloride:glycerol (EC_{50} >10 mM for both cell lines), whereas moderate cytotoxicity was observed for choline chloride:oxalic acid (EC_{50} value 1.64 and 4.19mM for fish and human cell line, respectively). The phytotoxicity tests showed that the tested NADESs were nontoxic for seed germination (EC_{50} >5000 mg/L) (Radošević et al. 2015). The cytotoxicity of a few tailor-made NADESs was also tested with in vitro assays using two human cell lines (MCF-7 and HeLa). The tested NADESs exhibited low cytotoxicity, making them good candidates for green extraction solvents in novel applications in the food, cosmetics and pharmaceutical industry (Radošević et al. 2016).

Applications of NADESs have been developed in the same manner as ILs or DESs. They were firstly used to extract plant secondary metabolites and in the pharmaceutical and food industry. Hydrogen bonding is the basis of the dissolving power of NADESs (Zhang et al. 2012). Considering this, phenolic compounds were chosen among natural products as a starting point to investigate the properties of NADESs as an extraction solvent. In 2013, our group reported the extraction of safflower (*Carthamus tinctorius* L.) with

several NADESs combinations comparing their yields with those obtained with water and ethanol. The extracts were analyzed by HPLC, NMR, and LCMS. The results showed both quantitative and qualitative advantages in the use of NADESs. Not only were yields higher than those obtained with water and ethanol, but NADESs proved to be more effective in extracting both polar and nonpolar compounds. In addition, NADESs also enhanced the stability of carthamin (Dai et al. 2013[b]; Dai et al. 2014).

Another experiment on the extraction ability of phenolics with diverse NADESs was conducted by Gonzalez et al. (2018). In this study, vanillin was extracted from vanilla pods using 14 different NADESs combinations. All of these proved to be more efficient than the conventional organic solvents used for this type of extraction. Moreover, a vanilla NADESs solution suitable for flavoring was also produced. Applications of NADESs to natural product extractions are listed in Table 1.

Processes involving the use of NADESs have been developed to recycle waste products of the wine industry. Wine lees can be upgraded as a source of phenolic compounds, especially anthocyanins, and is cheaper than grape seeds and skin. Anthocyanins were extracted more efficiently using choline chloride-based NADESs with malic acid than with conventional solvents. It can thus be concluded that the use of NADESs as a green solvent is a good choice when designing eco-friendly extraction methods of phenolic compounds from different sources (Bosiljkov et al. 2017).

NADESs have also been used in the field of analytical chemistry, specifically in electrochemical determinations. For example, the sensitivity of quercetin to electrochemical detection was found to be 380% higher when different NADESs based on glucose, fructose, citric acid, and lactic acid were added as solvents. The same group that reported this finding also reviewed the development of NADESs as natural designer solvents with outstanding advantages for analytical applications (Gomez et al. 2016; Espino et al. 2016).

Table 1. Application of NADESs in natural product extraction.

NADESs (mole ratio)	Source of natural products	Target Compounds	References
Proline–malic acid (1:1) Sucrose–choline chloride (4:1)	*Carthamus tinctorius*	Carthamin	Dai et al. 2013[b]; Dai et al. 2014
Lactic acid–fructose (5:1) Choline chloride–citric acid–water (1:1:6)	Vanilla pod	Vanillin	González et al. 2018
Lactic acid–glycine–water (3:1:3)	*Origanum dictamnus*; *Foeniculum vulgare*; *Origanum majorana*	Polyphenols	Bakirtzi et al. 2016
Lactic acid–glucose–water (6:1:6)	Virgin olive oil	Phenolics	Paradiso et al. 2016
Choline chloride–glycerol (1:1)	*Fagopyrum tataricum* hull	Rutin	Huang et al. 2017
Lactic acid–glucose (5:1) 1,2-Propanediol–choline chloride (1:1)	*Catharanthus roseus*	Anthocyanins	Dai et al. 2016
Choline chloride–malic acid (1:1)	Wine lees	Anthocyanins	Bosiljkov et al. 2017
Fructose–citric acid (1:1)	-	Wheat gluten	Lores et al. 2017
Proline–glycerol (2:5).	*Flos sophorae immaturus*	Quercetin, kaempferol, isorhamnetin and their glycosides	Nam et al. 2015
Choline chloride–oxalic acid (1:1)	Grape skin	Phenolics	Bubalo et al. 2016
Choline chloride–maltose (1:2).	*Cajanus cajan*	Phenolics	Wei et al. 2015

NADESs as media for enzymatic processes

As hypothesized in a previous article (Choi et al. 2011), NADESs could be a third liquid phase (apart from water and lipids) in cells, playing important physiological and chemical roles. These roles could include their participation

in enzymatic processes (Choi et al. 2011; Dai et al. 2013a). Although some NADESs may contain compounds that can break down proteins per se, several studies have been carried out to investigate the role of NADESs as a reaction media using protease, laccase, and cellulase as examples.

Kumar et al. (2016) studied the pretreatment of rice straw biomass with NADESs. The pretreatment of this abundant lignocellulosic residue aimed at the removal of lignin from cellulose and hemicellulose (holocellulose) biomass to avoid interference with the subsequent break down by cellulase. The NADESs made of lactic acid-choline chloride (5:1) separated high quality lignin (purity >90%) from holocellulose (total polysaccharide fraction of wood or straw after removing lignin) in a single step. Quantitative analysis showed that nearly 60 ± 5% (w/w) of total lignin was extracted from the rice straw. The subsequent enzymatic hydrolysis of this biomass showed a saccharification efficiency of 36.0 ± 3.2% as evaluated by both FTIR and HPLC analysis.

Other applications of NADESs in enzymatic processes have exploited its capacity for increasing the solubility of the substrates. One example of this is the catalyzed oxidation of inorganic and aromatic compounds, especially phenolics with laccases, multi-copper proteins. The substrates of laccase are scarcely soluble in water or buffers but much more soluble in certain NADESs. To investigate this option, laccase activity and stability were tested using betaine and choline-based NADESs. The results showed that choline-based NADESs produced a sudden drop in enzyme activity, whereas betaine-based NADESs improved laccase activity and stability. Among these NADESs, the highest laccase activity was observed with glycerol-betaine (2:1), whereas sorbitol-betaine-water (1:1:1) provided the highest stability. Activity was monitored by fluorescence (Khodaverdian et al. 2018).

NADESs can be used as a cosolvent in the transformation of isoeugenol to vanillin catalyzed by *Lysinibacillus fusiformis*. Vanillin is the most widely used flavor in the world, and its demand is impossible to meet with the extraction from natural sources and chemical synthesis. A possible approach to solve this is the use of a whole cell catalyst, such as the microorganism *L. fusiformis*, in the biotransformation of vanillin from isoeugenol. With NADESs as a

cosolvent, the production yield of vanillin was increased by 142%. The authors suggested that the benefits of using NADESs as a cosolvent in whole-cell biocatalysis could derive from an increase in cellular membrane permeability (Yang et al. 2017. Table 2 summarizes some applications of NADESs as media for enzymatic reactions.

Choline chloride, betaine, sugar alcohol, and sugars are the NADESs components that are usually used in enzymatic reactions because they increase the yield and the enzyme stability, as compared to other components such as organic acids (Yang et al. 2017). However, as in the previous example, better results have been achieved with betaine than with choline chloride-based NADESs (Khodaverdian et al. 2018).

Table 2. Application of NADESs as media in enzymatic reactions

NADESs (mole ratio)	Enzyme	Substrate	References
Lactic acid–choline chloride (5:1)	Cellulase	Lignocellulose	Kumar et al. 2016
Gycerol–betaine (2:1)	Laccase	Protein	Khodaverdian et al. 2018
Choline chloride–glycerol (1:2)	Subtilisin; α-chymotrypsin	Chitosan	Zhao et al. 2011
Choline chloride–tartaric acid (1:1) Choline chloride–sorbitol (5:2) Choline chloride–lactose (4:1)	Whole cell *Lysinibacillus fusiformis*	Isoeugenol	Yang et al. 2017
Choline chloride–urea (1:2) Choline chloride–glycerol (1:2)	*Candida antarctica* lipase B	Vinyl laurate	Durand et al. 2012
Choline chloride–glycerol (1:2) Choline chloride–ethane diol (1:2)	Epoxide hydrolase	(1,2)-*trans*-2-Methylstyrene oxide	Lindberg et al. 2010
Choline chloride–glycerol (1:2)	Lipase	Soybean oil	Zhao et al. 2013

Application of NADESs to solubilize macromolecules

Studies on the solubilizing capacity of NADESs have not been restricted to small organic molecules because macromolecules such as proteins and polysaccharides that are generally difficult to solubilize, have also been tested. Lores et al. (2017) investigated the solubility of gluten in NADESs, monitoring the process with electrophoresis and molecular fluorescence. The results demonstrated that gluten was best solubilized in dilute fructose:citric acid with the additional advantage that citric acid could prevent gluten oxidation. The authors also suggested that this method could replace the sample preparation recommended in the AgraQuant Gluten G12 Assay kit (2 h of magnetic stirring with a hydroalcoholic solution of 2-mercaptoethanol) with 15 min of sonication with diluted NADESs that provided comparable results in real samples.

The nucleic acids DNA and RNA are prone to degradation with heat and in the presence of certain chemical substances, requiring special environmental conditions to keep the molecules stable. The abundant sources of NADESs components in living cells suggest that they may dissolve and stabilize these macromolecules intracellularly. However, not much information is available to date about the use of NADESs in this field. Mondal et al. (2013) studied the extractability of DNA from salmon testes in NADESs composed of choline chloride and levulinic acid, glycerol, ethylene glycol, sorbitol, or resorcinol. Of the tested NADESs, combinations of choline chloride and glycerol or ethylene glycol showed higher extraction yields and stability of DNA. These NADESs could be recycled. The extraction of RNA with aqueous biphasic systems (ABS) is considered to be more effective than liquid-liquid separation techniques. The use of DESs in ABS has attracted a great deal of attention and a lot of work has been put into the search for the most suitable composition. Zhang et al. (2017) demonstrated that RNA extraction could be achieved using NADESs. Application of NADESs to macromolecules is displayed in Table 3.

Table 3. Application of NADESs to solubilize macromolecules

NADESs (mole ratio)	Source of natural products	Macromolecule	References
Fructose–citric acid (1:1)	-	Gluten	Lores et al. 2017
Malic acid–proline (1:1)	Salmon	DNA (solubility)	Choi et al. 2011
Choline chloride–ethylene glycol (1:2)	Salmon testes	DNA (preservation)	Mondal et al. 2013
Choline chloride–glycine (1:2)	-	DNA and RNA solution (stability)	Mamajanov et al. 2010
PEG–salts	-	RNA (extraction)	Zhang et al. 2017

Application of NADESs to pharmaceuticals, cosmetics, and food

The applications of NADESs have not been limited to their use as extraction solvents. The feasibility of their incorporation into formulations as solubilizing media of non-water-soluble natural compounds has also been studied. As mentioned above, the fact that NADESs components are safe, nontoxic, and even edible, allows NADESs to be considered for pharmaceutical, cosmetic, and food applications, as NADESs can easily comply with the stringent requirements for use in these applications.

Faggian et al. (2016) reported that NADESs composed of proline-glutamic acid (2:1) and proline-choline chloride (1:1) efficiently dissolved rutin, a flavonoid used as a nutraceutical for its health-promoting activity. They also reported a pharmacokinetic study in mice that revealed that the administration of an NADES solution of rutin resulted in higher and more persistent levels of rutin in plasma as measured by LCMS/MS, which indicates that this increased bioavailability of rutin is due to the NADESs. The solubility in several NADESs of berberine, a natural product with poor bioavailability, has been evaluated and the pharmacokinetic properties of the resulting NADESs solutions were measured in mouse plasma. The results indicated that berberine solubility in proline-malic acid (2:1), proline-urea (2:1), and proline-malic acid-lactic acid-water (1:0.2:0.3:0.5, w/w) was higher than in water and ethanol. The berberine plasma levels achieved with NADESs formulations were also significantly higher than those of an aqueous suspension (Sut et al. 2017).

These reports confirm the high solubility of compounds in NADESs has a positive effect on bioavailability.

Among the applications of NADESs in medicine, its inclusion in preparations of neutral porphyrins for antibacterial photodynamic therapy has proven highly promising. The activity of neutral porphyrins, although less cytotoxic than other photosensitizers, is hampered by solubility issues in aqueous media. However, a novel hydrophilic formulation of the neutral porphyrin 5,10,15,20-tetrakis(4-hydroxyphenyl)-porphyrin (THPP) in certain NADESs achieved a total photoinactivation of *Escherichia coli* using only nanomolar amounts of THPP. In addition, the photostability of this compound formulated in undiluted NADESs was higher than in conventional solvents (Wikene et al. 2015).

Another application was directed at increasing the range of concentrations of scarcely soluble drugs for clinical trials. Investigating the solubility of salsalate, an inducer of BAT in diverse NADESs, Rozema et al. (2015) found that a combination of 1,2-propanediol-choline chloride-water increased the solubility of salsalate in cell culture medium (T37i) allowing the assay of a full dose-response curve. This opens the way for clinical tests of poorly water-soluble drug candidates.

The use of NADESs to dissolve antioxidants has been investigated by Durand et al. (2017). A combination of 1,2-propanediol-choline chloride-water (1:1:1) was used to dissolve several antioxidants, including decyl rosmarinate, sinapine, CR-6, a vitamin E analogue and CR-6 palmitate, and bis-ethylhexyl hydroxydimethoxy benzylmalonate (Bis-EHBm). When dissolved in NADESs, the antioxidants had a higher reactive oxygen species inhibition activity, especially for those that had not shown good short-term efficiency. Shamseddin et al. (2017) revealed the potential activity of NADESs as an adjuvant for resveratrol formulations. Resveratrol is a plant polyphenol used to decrease inflammation processes related to gelatinolytic metalloproteases (MPP-9) activity. The results showed that resveratrol was highly soluble in the tested NADESs, and that the hormetic mode of action of resveratrol, consisting in a decrease of MPP-9 activity, was enhanced, increasing its anti-inflammatory

activity.

Table 4. NADESs application to pharmaceuticals, cosmetics, and agricultures

NADESs (mole ratio)	Target Compounds	Applications	References
Proline-glutamic acid (2:1) Proline-choline chloride (1:1)	Rutin	Increased solubility and high bioavailability	Faggian et al. 2016
Proline-malic acid (1:1) Proline-urea (1:1) Lactic acid-proline-malic acid-water (0.3:1:0.2:0.5)	Berberine	increased solubility and bioavailability	Sut et al. 2017
Citric acid–sucrose (1:1) Malic acid–fructose–glucose (1:1:1)	Porphyrin (THPP)	Antibacterial photodynamic therapy	Wikene et al. 2015; 2017
1,2-propanediol–choline chloride – water (1:1:1)	Salsalate	Inducer (BAT)	Rozema et al. 2015
Choline chloride–malic acid (1:1)	Phenolic, anthocyanin	Antioxidant, antitumor,	Radošević et al. 2016
Betaine–glycerol–glucose (4:20:1)	Catechin from green tea extract	Antioxidant	Jeong et al. 2017
1,2-Propanediol–choline chloride–water (1:1:1)	Decyl rosmarinate, sinapine, Bis-EHBm, CR-6, CR-6 palmitate	Antioxidant	Durand et al. 2017
1,2-Propanediol–choline chloride–water (1:1:1)	Resveratrol	Anti-inflammatory	Shamseddin et al. 2017
Choline chloride–tartaric acid–water (1:1:2) Choline chloride–sorbose–water (5:2:5) + saponin	Removing Cadmium from rice flour	Washing solvents	Huang et al. 2018
Betaine monohydrate–glycerol (1:8)	Palm oil	Deacidification	Zahrina et al. 2018

There are also reports of interesting applications in agriculture. Among these, the use of NADESs to remove cadmium (Cd) from rice flour was very important because rice is the staple food of many countries in the world. In this experiment, 20 choline chloride and glycerol-based NADESs were used as a solvent to wash and remove Cd from contaminated rice. A saponin was also added as a surfactant in combination with NADESs to increase Cd removal. Choline chloride-based NADESs proved to be the most efficient, ranging from

51% to 96% of the total Cd, whereas the combination with the saponin increased the efficiency of the removal to 99%. It is important to note that the washing process with NADESs did not affect the chemical components or the structure of the rice flour (Huang et al. 2018).

Another interesting application of NADESs in the agrochemical area is the deacidification of palm oil using betaine-based NADESs. The combination of betaine monohydrate-glycerol (1:8) was the most selective with a distribution coefficient of palmitic acid of 0.52 $(g/L)(g/L)^{-1}$ and the lowest distribution coefficient of antioxidants, as observed by HPLC and NMR (Zahrina et al. 2018).

The applications of NADESs in these fields are displayed in Table 4.

Occurrence of NADESs in nature

Choi et al. (2011) introduced the hypothesis of NADESs as a third liquid phase in cells of all organisms, presenting indirect evidence for their presence in plants. This evidence was based on ^{1}H NMR metabolomic analyses that showed the presence of common metabolites like sucrose, glucose, fructose, malic acid, lactic acid, choline, and betaine at high levels and with similar intensity signals in all kinds of the biological material. This raised the question about the presence of ILs in plants, which was supported by the fact that all of the advantages reported for ILs in chemistry would also be advantageous in a biological context, such as the biosynthesis of non-water-soluble metabolites, in a wide range of molecular masses, for example, from terpenoids to cellulose and lignin. The first experiment consisting in mixing equimolar amounts of malic acid and choline chloride was successful. After this, we identified a large number of natural ILs and DESs, which we named NADESs. To date, the evidence of their presence in nature that has been reported is still indirect. In one case, the analysis of the aleurone tissue of barley seeds (*Hordeum vulgare*) by ^{1}H NMR spectroscopy showed that the major low mass molecules were sucrose and choline and that they were present in approximately equimolar amounts. The analysis of a plant sap, maple syrup (*Acer* spp.), showed that it consists mainly of sucrose and malic acid, and an NMR- based metabolomic

study of the nectar of *Cleome hassleoriana* showed the presence of sucrose, glucose, and fructose as major components in practically equimolar ratios. However, although the direct evidence for the in situ presence of NADESs in cells is still missing, the amount of indirect evidence is increasing. For example, drought, desiccation, and cold resistance are always reported to be connected with a high level of osmolytes. Osmolytes encompass all the compounds found to form NADESs (Choi et al. 2011). Not only is it necessary to find proof of their localization in the cells, but also it is important to consider the dynamic equilibrium that is believed to exist between the aqueous phases of the different cell compartments and the potential presence of NADESs attached to membranes or in vesicles. The problem in this case is that it is possible that within the same cellular space there may be different NADESs attached to different membranes or in different compartments, implying the importance of water in their composition. This is in line with the experimental data obtained related to the water content of NADESs that showed its significant effect on their solubilizing capacity and the reduction of their viscosity (Dai et al. 2013[b]; 2014). The protective role of NADESs on proteins has also been proved (Choi et al. 2011) as well as the activation of enzymes in an NADES solution by dilution with water (Choi et al. 2011; Khodaverdian et al. 2018).

The work of Chen et al. (2014) showed that the presence of NADESs is not restricted to plants. They reported the possible formation of a viscous IL by the ant species *Nylanderia fulva* after being attacked by *Solenopsis invicta* that spray them with an alkaloid-based venom. The attacked *N. fulva* ant detoxifies the venom by grooming itself with formic acid, causing the formation of a viscous IL with the piperidine alkaloids of the *S. invicta* venom. The principle of NADESs can thus be used to explain a diverse number of biological processes, although the actual direct proof remains the major challenge for further research.

Conclusions and perspectives

This review provides a summary of the development of NADESs, starting from the initial ILs, through the DESs and ending with NADESs as a new

generation of versatile green solvents, which are cheap, safe, nontoxic, environmentally friendly, and sustainable. The diverse applications of NADESs include extraction, enzymatic reaction media, dissolution of non-water-soluble compounds, and a delivery system or media for drugs and cosmetics, among others, will provide a platform for future research on NADESs as prominent solvents to be applied at a larger scale for industrial purposes.

Current research trends in NADESs are very much in agreement with those of other green solvent technologies, that is, to increase the number of NADESs candidates and the diversity of fields of applications to learn more about the ways to develop tailor-made NADESs for specific applications. For large-scale processes, the challenge is how to deal with highly viscous nonvolatile solutions of pure compounds or extracts. In the case of extraction, the advantage could be the direct use of the NADESs preparation in food, pharmaceutical, or cosmetic formulations. If pure compounds are to be isolated from NADESs extracts, in some cases it is necessary to find novel methods to eliminate the solvent as it cannot be evaporated. Combinations with supercritical extraction, liquid-solid or liquid-liquid extraction, are potential solutions to this problem. The direct application of the NADESs extracts in CCC also seems an interesting option.

Regarding the search for new NADESs candidates, the compounds of choice are primarily abundant natural products from plants, animals, microorganisms, and marine organisms. Moreover, adopting an observation-based systemic approach to study diverse organisms may lead to the discovery of novel NADESs candidates, following the hypothesis of their presence as an alternative media in living organisms. The search for potential ingredients should not only be limited to increasing the number of studied organisms, but should also include biological or physiological processes in organisms that may provide ideas or act as models to emulate. For example, when plants are submitted to cold stress, some metabolites such as proline and trehalose are known to accumulate. These are thus candidates for new NADESs components. It is also important to bear in mind that although most reported NADESs consist of only two compounds, there is no reason to discard the option of more

constituents, adding further opportunities to optimize NADESs for certain applications. In addition, specific parts of organisms such as plant saps, organs, or glandular hairs could be a target for further investigations. Thus, the number of potential NADESs is almost infinite.

All NADESs are made of hydrophilic or polar ingredients, a fact that should theoretically limit their application to hydrophilic compounds. Consequently, NADESs applications have been restricted mainly to the extraction of polar metabolites. It is thus worthwhile to develop nonpolar NADESs, extending their applications. The mentioned drawbacks of ILs and DESs, that is, high viscosity and virtually no vapor pressure prevail in NADESs, resulting in some challenges for the analysis of NADESs extracts in certain cases. Regarding their high viscosity, this can be solved in several ways, such as adding water, working at higher temperatures, or using mechanical force (Zhang et al. 2009; Dai et al. 2014).

So far, NADESs extracts have been mainly analyzed using HPLC-DAD, a method that allows only the detection of compounds with UV/vis chromophore because other available HPLC detectors could be incompatible with NADESs components. To solve this problem, our group has found an interesting alternative in the use of high performance thin-layer chromatography that might be a promising tool for the assessment of compounds in NADESs extracts such as ginsenosides of ginkgolides that do not have an easily detected UV-chromophore (Liu et al. 2018).

When compared to conventional ILs or DESs, NADESs have a great advantage when the applications demand the use of totally nontoxic ingredients. For example, as drug delivery systems, Stott et al. (1998) described a case in which the target drug was itself an ingredient of the NADESs. The formation of eutectic systems between ibuprofen and seven terpene skin-penetration enhancers and the effects of these eutectic systems on the melting point depression of the delivery system for transdermal delivery were investigated.

However, before considering NADESs as drug delivery systems, it is important to bear in mind that they might dissociate in contact with biofluids. Thus, even if a compound was totally dissolved in an NADESs, it could

precipitate when the NADESs dissociates in the organism. Other pharmaceutical applications of NADESs might be found among traditional medicines such as traditional Chinese medicines and Indian ayurvedic medicines. There are many examples that seem to bear a great similarity with NADESs such as mixing sugar-rich plants (*Glycyrrhiza glabra* roots or honey) with bioactive plants. This would imply that inadvertently, the NADESs concept has been used for a long time in traditional medicine systems. Another direction of research in NADESs might be for preservation or stabilization. The reversible thermal unfolding/refolding and long period stabilization against aggregation and hydrolysis of concentrated solutions of lysozyme (>200 mg/mL) in IL-rich, ice-avoiding, solvents have been reported (Byrne et al. 2007). In this sense, it could be expected that NADESs might be a good or even better solvent than ILs for the preservation of labile materials considering the low reactivity of NADESs compared to that of ILs.

Summarizing the properties of NADESs, the advantages are their selectivity as solvents for all kinds of compounds, particularly of medium polarity, which are often difficult to dissolve; a virtual zero vapor pressure, which means no losses through evaporation, and low flammability; low toxicity, which allows application in food, cosmetics, agrochemicals, and medicines; increased stability of compounds; and environmentally safe. Their high viscosity and nonvolatility are constraints, which require the development of novel technologies that may turn these disadvantages into advantages.

Finally, considering the distinctive characteristics of NADESs, that is, their biological role in nature, their potential for a large number of applications in biotechnology or biological engineering is extremely promising. Among them, the possibility of using NADESs to preserve bacteria opens interesting perspectives for their use in biocatalytic processes carried out in nonaqueous solvents. Similarly, the cryopreservation of cells might become possible by adding certain NADESs. This option is supported by the fact that at least in the case of plant cell cryopreservation it is common practice to add various cryoprotectants, all of which are potential NADESs ingredients (Mustafa et al. 2011).

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