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Chapter 9

Concluding remarks and perspective

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1. Conclusions

Natural products were used as a resource to extend the range of ionic liquids and deep eutectic solvents. More than 100 natural ILs and DES (NADES) combinations were prepared, stirring at about 50 °C. NADES are liquid supermolecules composed of natural metabolites as well as water in some cases, that are mixed in certain molar ratios and are characterized by extensive intermolecular interactions e.g. H-bonds or ionic bonds. As solvents, in terms of environmental and economic benefits, they have the following advantages: they are non-volatile, have a low toxicity, are biodegradable, sustainable, cheap, and are made using simple preparation methods. They also show very good physicochemical properties: liquid state even below 0 °C, adjustable viscosity, a broad range of polarity, and high solubilization strength for a wide range of compounds (**chapter 3**). Dilution of NADES with water allows the quantitative adjustment of physicochemical properties, such as their conductivity, polarity, viscosity and density, which facilitate their applications as solvents. It also decreases their viscosity considerably and increases the solubility of some compounds. The optimal water content in NADES – the one that will allow them to reach their highest solubilization capacity –, depends on the composition of NADES and also the polarity of the compounds. It was noted that the addition of 50% of water results in the loss of their supermolecular characteristics (**chapter 4**). NADES improve the stability of phenolic compounds during storage, at high temperature, light exposure and at ambient conditions if compared with water and 40% ethanol solutions. The water content in NADES has a large effect on the stabilizing ability of NADES. Highly viscous sugar-based NADES were efficient in preserving phenolic compounds due to the establishment of strong hydrogen-bonding interactions between solutes and solvent molecules (**Chapter 5**). This is in accordance with the traditional preparation of syrups, jam or marmalades with a high sugar content.

For the application of NADES in extraction, a simple and efficient extraction method was developed for the extraction of anthocyanins that consisted in stirring at 40 °C for 1 hour. The extracts of NADES were easily analyzed by HPLC and no effect of NADES itself on the chromatographic behavior of analytes was observed when extraction solution was diluted with one volume of water. Thus, the compatibility of NADES and reversed phase HPLC was good. In addition, higher stability of cyanidin was observed in some NADES than in acidified ethanol, which facilitates the extraction and analysis process of anthocyanins. With this method, some NADES showed the same ability to extract anthocyanins as acidified methanol, which is used as the conventional extraction method for these compounds (**chapter 6**). Compared with conventional solvents (water, ethanol), some NADES exhibited higher efficiency in extracting a broad range of both polar and less polar compounds. They were more efficient than water in the extraction of polar compounds and more efficient than ethanol in the extraction of less polar compounds under optimized conditions. Phenolic compounds could be recovered from NADES with an open column chromatography using a resin (**chapter 7**). These green, simple, low cost, and efficient methods can be applied for the extraction and isolation of natural products from biomaterials. This holds promise for the further application of NADES in the pharmaceutical, cosmetics, and food industry.

The hypothesis of the existence of NADES in plants is based on the occurrence of the same chemical constituents in all plant cells. The co-existence of NADES and water and their diffusion resulted in solvents with different compositions and properties, which are suitable media for metabolites of diverse polarities in plant cells. NADES may be involved in maintaining the water levels in plants. Furthermore, NADES may exist around cell membranes and play a role in stabilizing the lipid membrane through intermolecular interactions (**Chapter 8**). The attached NADES would be the medium where biosynthetic enzymes may function in an environment where non-water soluble intermediates are dissolved and may be transferred from one enzyme to the next in the sequence of the biosynthesis pathway. The ER could be a metastable NADES phase that interacts with the water phase, again explaining biosynthesis of poorly-water soluble metabolites.

2. Future Perspective

Both deep eutectic solvents and ionic liquids are currently considered to be potential green solvents. However, DES show impressive advantages over synthetic ILs, such as being cheaper and more environmentally friendly. Recently, efforts have been made to explore more DES from natural products for specific applications. Their physicochemical properties and applications as solvents for organic reactions, polymers, biotransformations, or extraction (This

thesis, Carriazo *et al.*, 2012; Ruß and König, 2012; Zhang *et al.*, 2012) have been reviewed. However, there is still little chemical and physical information, and not many applications of NADES have been developed yet. More research on the preparation of NADES is required to make tailor-made NADES for specific applications and to extend the range of NADES to ternary and quaternary mixtures. Their compositional flexibility makes the number of potential NADES unlimited, as does the range of physical properties that NADES can attain. Further research is needed to build up the theory as to how to predict physicochemical properties of NADES, particularly in connection with their potential to dissolve various chemical types of compounds.

The physicochemical properties of ILs depend on the cations and anions. It is possible to design the optimal IL to extract a specific compound by selecting a cation and an anion, which are specific for dissolving a certain type of compound. For example, some ILs with larger π^* values are expected to solubilize particularly π -conjugated organic materials. Secondly, ILs based on simple natural compounds or modified natural products can be synthesized for the extraction of certain types of natural products, such as an amino acid IL that showed enantioselectivity in extracting amino acids (Winkel *et al.*, 2008) or a DES made of glycerol and choline chloride that was used to extract glycerol from biofuel (Hayyan *et al.*, 2010).

Natural deep eutectic solvents are of great importance for applications in all areas in which general organic solvents are used. For natural products, they show high efficiency and yield a great variety of compounds. This feature allows NADES to be considered as a new kind of solvent capable of extracting all metabolites for total metabolomics. To be applied in metabolomics, NADES that are made of components that are replaced with a deuterium or ^{13}C label are required.

The combination of DES with supercritical CO_2 has a great potential for various applications, such as separation and extraction (Zhang *et al.*, 2012). Water affects the solubility of CO_2 in DES. The solubility of CO_2 in ChCl /urea decreases with an increasing water content (Su *et al.*, 2009). Thus, the combination of water, CO_2 and DES may be a good system for the extraction and separation of natural products especially for thermally unstable compounds.

As green solvents, NADES have very promising application in food, pharmaceuticals and cosmetics. The components of NADES are abundant in our daily diet; therefore the extracts in NADES might be used for preparation of final products without a need for isolation from NADES. The effect of the active compounds and particularly their bioavailability in NADES solutions should be evaluated.

NADES might be involved in the biosynthesis and storage of various non-water soluble metabolites (e.g. secondary metabolites and macromolecules) in

cells. They may play an important role in protecting organisms from extreme drought, cold, high salinity, in the germination of a seed, among others. In harsh conditions, NADES may protect the integrity of the cell membranes as well as stabilize macromolecules. NADES may also have the function of adjusting the oxygen/carbon dioxide content in plant cells. In our view, NADES are attached to cell membranes, like being caught on an ion-exchanger, but in a dynamic equilibrium with the water phase. In fact this hypothesis would explain many biological phenomena at the level of the cell, the tissues and the whole organism. Nature has probably already invented ILs and DES in ancient times, if not already in the very beginning, developing self-organizing fluids as the start of life.

It seems that with the NADES we are at the beginning of something very fundamental. On one hand, this may explain many basic biological cellular processes and on the other hand it will generate many applications in the field of extractions and enzymatic reactions. For example, the biosynthesis of poor water-soluble compounds may occur in NADES in which both substrates and enzymes are dissolved. Thus characteristics of the enzyme might be quite different from what has been measured in water. In certain NADES, enzymes might be more active than in the classical solvents because of much higher solubility of the substrates compared to water, and the biosynthetic process also may be controlled by e.g. water content in the NADES. The green chemistry has opened a real 'Pandora's box'.

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Summary

In the past decade, ionic liquids (ILs) and deep eutectic solvents (DES) have received great attention due to their environmentally friendly and non-toxic features caused by their negligible vapor pressure. Recently, ILs and DES made with bio-renewable natural products as starting materials were reported in order to reduce the potential toxic and production cost, and increase biocompatibility. In view of the physicochemical properties, effort was also put in making ILs or DES with low viscosity, low melting point, high thermal stability and high selectivity for desired chemicals. They have been used in the extraction and separation of different kinds of natural products as well as enzyme reactions. These synthetic solvents showed better solubilization of a broad range of compounds including macromolecules such as lignin and cellulose than the classical organic solvents. The main driving forces in the extraction are molecular interactions (such as hydrogen-bonding and ionic interactions) between ILs and solutes. This is greatly affected by the molecular structures of the ILs components, water content, viscosity, pH, and salting-out effects. These factors also play an important role in enzyme reactions in ILs or DES. The extracts can be diluted and analyzed with conventional analytical tools such as HPLC, NMR, IR, CD and SEM (scanning electron microscope). In addition, the extracted compounds can be isolated and the ILs can be reused (**chapter 2**). All the previous extraction processes using ILs or DES mainly focus on imidazolium-based ILs, which, however, are toxic. In addition, due to their high viscosity or solid state at ambient temperature, the use of high temperature and diluted ILs aqueous solution are applied in most reported extractions.

Another type of solvents - natural deep eutectic solvents (NADES) covering ILs and DES - were proposed by our group to extend the range of ILs and DES, particularly to develop cheap, nontoxic, and low viscosity green solvents, and to apply them in health-related fields. They are liquid supramolecules composed of common metabolites in certain molar ratios, including some water in some cases, which are characterized by extensive intermolecular interactions e.g. hydrogen bonds and ionic bonds. More than 100 combinations have been found and they can be classified into five groups: ionic liquids with an acid and a base, sugar-based NADES with only neutral compounds, sugar-based NADES with bases, sugar-based NADES with acids and sugar-based NADES with amino acids. These NADES are prepared simply by stirring a mixture of the compounds at 50 °C. The physical properties of some typical NADES showed that they have a higher density than water, a high viscosity, low glass transition point keeping liquid state even below 0 °C, high thermal stability, and broad polarity from more polar than water to the same as methanol. Concerning applications, NADES proved to be excellent solvents for a wide range of natural

products of low to medium polarity that are non-soluble or poorly soluble in water. Macromolecules such as DNA, proteins and polysaccharides are also soluble in NADES (**chapter 3**). Their physicochemical properties and high solubilizing ability make them of great interest for their application in pharmaceutical, food, and cosmetic industry.

To facilitate and expand the applications of NADES, the structure and properties of mixtures of NADES and water were examined (**chapter 4**). With water dilution hydrogen-bond interactions between the components of NADES are weakened and then disappear with the addition of 50% of water. Not only the structure but also the physicochemical properties of NADES are affected by the water content in NADES. Conductivity of NADES increases by up to 100 times with water dilution. Water activity and density of NADES showed a quantitative relationship with the water content in NADES. Dilution of NADES with a certain amount of water dramatically decreases their viscosity, changes their polarity, and affects the solubility of some compounds. All these effects of water on the structure and characteristics of NADES provide a basis for modulating NADES to meet the demands for potential applications.

The stabilizing ability is an important aspect to consider when designing an application of NADES. The stability of compounds in NADES was investigated using the unstable pigment carthamin as a model. Higher stability of carthamin was observed in sugar-based NADES compared with water or 40% ethanol solution under all tested conditions including high temperature, light exposure, and storage time. Even in crude extracts, carthamin together with hydroxysafflor yellow A and cartormin exhibited improved stability in two sugar-based NADES at ambient conditions in sunlight. The hydrogen bond interactions formed between solutes and molecules of NADES contribute to their high stabilizing ability. This bonding restricts the movement of solutes thus avoiding contact with oxygen from the gas phase at the interface with the air and reduces the speed of degradation because of low solubility of oxygen in NADES. In view of the viscosity of NADES, the stabilizing ability of NADES increases with their increasing viscosity and decreases with increasing water content (**chapter 5**). Thus, sugar-based NADES with high viscosity are efficient in preserving phenolic compounds.

For the application of NADES in the extraction of phenolic compounds, the compatibility of NADES with reversed phase HPLC and the stability of extracts were investigated with anthocyanins as an example. Three extraction methods were compared including sonication at ambient conditions, sonication at 40 °C, and stirring at 40 °C. Stirring at 40 °C for 1 hour proved to be most effective. The compatibility of NADES with reversed phase HPLC was good and no effect from the NADES on the chromatographic behavior of anthocyanins was observed. The dilution of extracts in NADES, however, with one volume of water is necessary for sample injection. Stability experiments showed that

cyanidin is more stable in some NADES than in acidified ethanol, which facilitates the preparation, analysis and isolation of anthocyanins. With the above methods, different NADES were applied to extract anthocyanins and HPLC/UV- based metabolomics was used for the analysis of the anthocyanins in flower petals of *Catharanthus roseus*. Multivariate data analysis showed that LGH, and 1,2-propanediol-choline chloride (PCH) show the same extraction yield of anthocyanins as acidified methanol (**chapter 6**).

The extraction of phenolic compounds with NADES was compared with conventional solvents. Safflower was selected because its aromatic pigments cover a wide range of polarity, including polar phenolic compounds (such as hydroxysafflor yellow A (HSYA) and cartormin) and less polar compounds (like carthamin and tri-*p*-coumaroyl spermidines). With the above-established methods, extracts made with eight NADES, water, and ethanol were analyzed with HPLC-UV. Multivariate data analysis demonstrated that NADES gave a higher yield and broader spectrum of both polar and less polar compounds than the conventional solvents. The extract yield of phenolic compounds was greatly affected by the water content in NADES. Using resin (HP-20) column chromatography, most major phenolic compounds were recovered from NADES with a yield of 75% - 97% (**chapter 7**). This study shows a green, low cost, and efficient approach to extract and recover natural products from biomass, which can be applied for the extraction and isolation of natural products from biomaterials in the pharmaceutical, cosmetics, and food industry.

The existence of NADES and their possible functions in plants were explored (**chapter 8**). Comparatively higher amount of ingredients of NADES were observed (dry moss compared with fresh one and in the episperm of barley compared with embryo). High amounts of sugars, sugar alcohols, choline, or glycine betaine dominate in plants secretions such as sap and nectar. Besides having a similar composition, NADES showed similar physicochemical properties to those in the cytoplasm in plants, which provides evidence for the existence of NADES in plants. NADES showed similar hygroscopicity as plant materials, implying a water content controlling role of NADES in plants. The diffusion between NADES and water resulted in metastable liquids with different compositions and properties, which provide ideal solvents to dissolve metabolites of diverse polarities in plants. Furthermore, NADES may accumulate around the membranes and stabilize them, as revealed in experiments with liposomes. Further studies on the biological roles of NADES in cells and organisms are required, as this will possibly revolutionize our views on the cell physiology, as well as the physiology of whole organisms. For example in plants, both drought and cold resistance has been extensively reported to involve compounds that we have found to form NADES.

