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To the whole society

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Chapter 1

General Introduction

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Current organic solvents have several problems such as toxicity to humans and the environmental burden caused by their residues and their disposal. In food, pharmaceutical, cosmetic, and chemical industries, organic solvents are widely used for extracting, e.g. flavors, fragrances, medicines and dyes from plants. Plant metabolites differ in many properties such as polarity, stability, boiling point as well as quantity. An extraction of plants can have different goals: 1) complete extraction of single compounds for analysis or industrial production; 2) complete extraction of a group of compounds for analysis or industrial production; 3) extraction of all compounds present for metabolomics analysis. For each of these goals different characteristics of the solvent are required. A universal solvent does not exist. In addition, metabolites have interactions with other cellular components. So, different solvents mixtures are reported to adjust characteristics of solvents such as high cell penetration ability, broad or specific solubilizing capacity, and even cell wall break-down ability. For example, solvents of different polarities are needed in the extraction, separation, purification, and administration of various medicines. Solvents, such as alcohols, chloroform, ethyl acetate, etc. are generally applied for this purpose. However, organic solvents may result in organic impurities in extracts, requiring special assays in quality control of food and drugs. Moreover, organic solvents are often toxic, flammable, explosive or poorly biodegradable. To address these problems, new alternative solvents are required which are biodegradable, and have low toxicity, and at the same time have a broad solubilizing capacity in terms of selectivity and polarity.

With the aim of developing environmentally friendly solvents, ionic liquids (ILS) have attracted great attention because they have negligible vapor pressure at room temperature and features such as polarity and selectivity can be tailor-made for different applications. Compared with conventional organic solvents, ILs are low melting point organic salts ($<100\ ^{o}C$), composed of a cation and an anion, of which at least one is an organic ion. ILs have many attractive physicochemical properties, such as chemical and thermal stability, non-flammability, high conductivity, and good solubility of various compounds. Because of their good physicochemical properties, ILs have been explored in many areas. In biology, they have been applied in the extraction and separation of a wide range of metabolites covering primary and secondary metabolites and macromolecules (e.g.

DNA, proteins and polysaccharides) and as alternative media to enzyme reactions. Particularly, some ILs can dissolve starch and cellulose which are not soluble in water without heating. All those applications are attributed to their special features, forming strong interactions with solutes, e.g. hydrogen or ionic bonds. So far most ILs have been obtained by chemical synthesis. Although ILs overcome some of the problems of organic solvents, their use is limited in food and pharmaceutical applications because of their high costs, the high toxicity of some of the ingredients, and their irritation properties. Another type of solvent with similar physical properties and phase behavior to ILs are deep eutectic solvents (DES). DES are obtained by mixing solid compounds forming a eutectic mixture with a melting point much lower than either of the individual components. For example quaternary ammonium salts, amides, organic acids, polyalcohols, etc. DES show advantages over ILs such as: simple preparation method, high purity, low cost, and low toxicity. These solvents have been used in organic reactions, enzyme reactions, and electrochemistry. However, the high melting point of most reported DES restricts their applications as a green solvent at room temperature.

In order to increase the number of candidates for ILs and DES and to overcome the problems of synthetic ILs and DES, our group explored natural products as a source for such solvents, i.e. primary metabolites, which include organic acids, amino acids, sugars, sugar alcohols, and amines. Natural products are indeed a plentiful and ideal source of ILs and DES due to their enormous chemical diversity, biodegradable properties, sustainability and pharmaceutically acceptable toxicity profile. Our group introduced the term Natural Deep Eutectic Solvents (NADES) for these liquids made from natural products. In addition to being the source of useful ILs and DES, with many potential applications, we hypothesize that the existence of NADES in organisms can explain many biological processes, which cannot be explained by water and lipids as the only liquids in living organisms. For example, the mystery of the biosynthesis, solubilization, storage, and transport of poorly water-soluble metabolites and macromolecules in the aqueous environment of cells may be explained by NADES (Choi, et al., 2011). Also, it may explain the survival of organisms in extreme drought and/or cold conditions. The many advantages of the natural ILs and DES suggests agreat potential for their application in food, cosmetics and pharmaceutical areas, resulting in economical as well as ecological advantages.

Aims of the thesis

Previously, we proposed the hypothesis about the role of NADES in nature. Abundant metabolites in plants might be the source of NADES and those NADES may have diverse biological roles as alternative liquid media to water and lipids in organisms, explaining the biosynthesis of non-water soluble metabolites in living cells and how organisms survive in extreme drought or cold conditions. In this thesis, the range of NADES is extended and these new solvents are explored for their physicochemical properties, their application in metabolite extraction and

food chemistry, and their occurrence and possible function in plants (Fig. 1). In order to address the hypothesis the following objectives for this thesis are formulated.

1 develop a suitable production method for NADES, explore suitable combinations and ratio of ingredients in NADES with different types of primary metabolites;

2 determine the structure, physicochemical properties, and solubilizing capacity of NADES for metabolites in organisms and explore the effect of the water percentage in NADES on their structure and properties;

3 study the stability of compounds in NADES in combination with the effect of temperatures, light or long time storage and identify the major factors that affect the stability of compounds in NADES;

4 develop methods for analysis of extracts prepared with NADES, and explore the effect of NADES on the results;

5 develop extraction methods for phenolic compounds with NADES, compare the extraction ability of NADES with conventional solvents and explore the effect of water percentage in NADES on the extraction efficiency;

6 recover phenolic compounds from NADES;

7 explore the applications of NADES in pharmaceuticals；

8 explore the composition, position and function of NADES in plants and explain the relationship between the composition of NADES and their function in plants.

Outline of the Thesis

This thesis aims to expand the number of NADES, determine their physicochemical properties, explore their applications in metabolite extraction from plants and uncover their possible biological functions. In chapter 2, the synthesis, properties and application of synthetic ionic liquids and deep eutectic solvents in metabolite extraction, separation, and enzyme reactions are reviewed. The analytical methods and the combination of ionic liquids with other technologies are also reviewed.

In chapter 3 and 4, preparation of different combinations of NADES, their structures and physicochemical properties are described. Two preparation methods of NADES are compared. Solubility of poorly water-soluble metabolites and macromolecules is studied. In addition, the effect of the water percentage in NADES on the structure, properties, and solubilizing capacity of NADES is explored. In chapter 5, the stability of metabolites in some NADES are reported, with water and ethanol as references, under different conditions, including heat, light, and long-term storage. The effect of water content on the stability of compounds in NADES is also tested. The mechanism of stabilization ability of phenolic compounds by NADES is discussed.

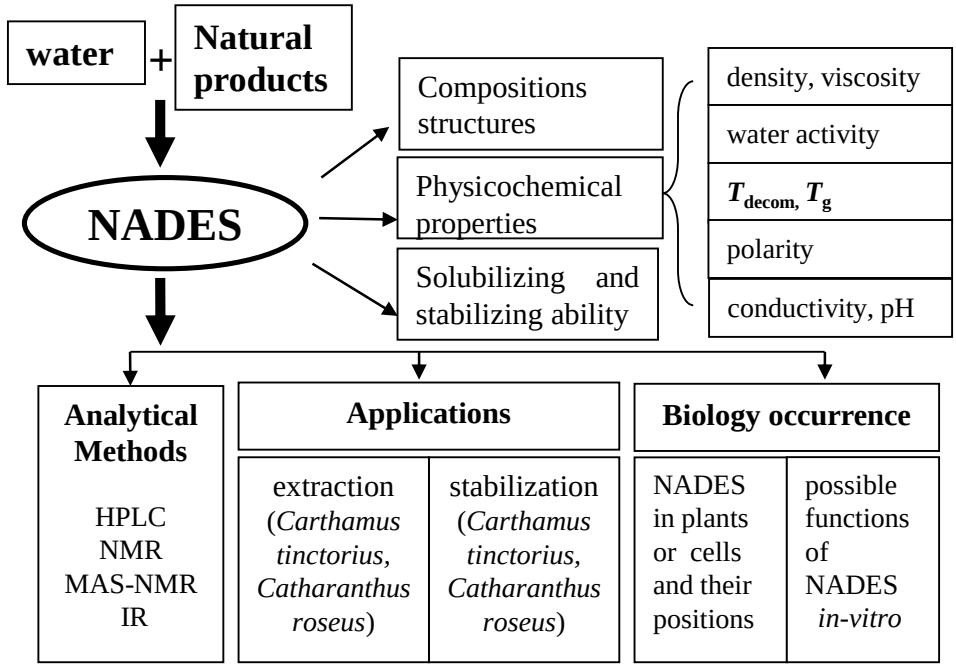

Fig. 1. Schematic diagam of this thesis

The studies in these chapters lay the basis for the application of NADES, which is explored in the following three chapters. In chapter 6 and 7, the application of NADES as solvents in the extraction of phenolic compounds is exemplified with flowers from *Catharanthus* roseus and *Carthamus tinctorius* L.. In chapter 6, the whole process from sample preparation to analysis is reported for anthocyanins from *Catharanthus* roseus, including extraction, analysis, and sample storage methods. Chapter 7 reports the difference in extraction ability of NADES and general solvents for phenolic compounds from *Carthamus tinctorius*, investigated by HPLC-UV based metabolomics. Furthermore, the extraction parameters of high extraction ability of NADES are optimized and the recovery method for phenolic compounds from NADES is studied.

The composition and function of NADES in plants are discussed in chapter 8. The NADES in different plants or plant secretions are analyzed. To explore the functions of NADES in plants, the diffusion between water and NADES, the hygroscopicity of NADES, and barley seeds are investigated. The behavior of liposomes in NADES is tested to explore the function of NADES in the cell membrane. The summary, final conclusion, and the future perspective of the results obtained in this thesis are discussed in chapter 9.

Chapter 2

Liquefied mixtures of solids can extract natural products: application of ionic liquids and deep eutectic solvents to natural product research

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Abstract

Mixtures of solid chemicals may become liquids under certain conditions. The liquids are characterized by formation of strong ionic (ionic liquids) or hydrogen bonds (deep eutectic solvents). Due to their extremely low vapor pressure, these liquids are now widely used in polymer chemistry and synthetic organic chemistry, yet little attention has been paid to the extraction capacity of ionic liquids or deep eutectic solvents for natural products. This review summarizes the preparation of ionic liquids and deep eutectic solvents with natural product components, and recent progress in their application to the extractions and analysis of natural products, covering both primary and secondary metabolites, and enzymatic reactions alternative to conventional organic solvents. Additionally, the various factors affecting extraction features of ionic liquids and deep eutectic solvents, as well as potential technologies including microwave and supercritical fluid extraction to increase the extraction efficiency are discussed.

Key words: ionic liquids; deep eutectic solvents; natural products; herbal medicine; extraction; recovery; enzyme activity.

1. Introduction

Ionic liquids (ILs) are low melting point (＜100 ^{o}C) organic salts composed of organic cations and organic or inorganic anions. The well-accepted definition of a room temperature ionic liquid (RTIL) is "a salt that has a melting point lower than ambient temperature" (Welton, 1999). Deep eutectic solvents (DES) are a new class of solvents obtained by mixing solid compounds in certain Molar ratiosinto an eutectic mixture with a melting point that is much lower than either of the individual components (Abbott *et al*., 2004). In most cases, ILs have an asymmetrically substituted cation (e.g., imidazolium, pyrrolidinium, pyridinium, ammonium phosphonium) and a variety of anions, especially halogen-based anions (e.g., [Cl], [Br], [I], [BF_4], [$AlCl_4$], [PF_6]), as summarized in table 1. To overcome the potential toxicity of ILs with halogen-containing anions (Swatloski *et al*., 2003; Garcia-Lorenzo *et al*., 2008), these were replaced by biomaterial-derived products such as organic acids, amino acids, amines or sugars, creating a new safer type of ILs known as "Bio-ILs" or " green ILs" (Tao *et al*., 2006; Fukaya *et al*., 2007; Hu *et al*., 2007). There are also reports of DES made of various natural products that fit into this category (Abbott *et al*., 2003; 2004; Imperato *et al*., 2005; Choi *et al*., 2011).

Interest in ILs stems from this potential application as ''green solvents'' based on perhaps, their most important feature: nearly complete non-volatility at ambient condition. Besides, ILs have many attractive physicochemical properties, such as chemical and thermal stability, non-flammability, high conductivity and a good solubilizing capacity of various organic compounds (Welton, 1999). Both the anion and the length of the *n*-alkyl chain in the cation of the IL affect its physical properties (Zhang *et al*., 2008) and its viscosity and polarity, for example, can be tuned by changing the cation-anion combination. Thus, ILs are termed "tailor-made solvents" and offer a huge potential for practical applications (Visser *et al*., 2001). The same properties are also observed in DES, converting both these types of liquids into ideal substitutes of conventional organic solvents.

Recently, various applications of ILs and DES have been reported. In the case of natural product analysis, ILs have been used as a stationary phase or a mobile phase additive in chromatography (Berthod *et al*., 2008; Herrera-Herrera *et al*., 2009; Tian *et al*., 2010). Ionic liquids have been used for the extraction of compounds of diverse polarity from liquid samples, such as the extraction of polyunsaturated fatty acid methyl esters, organic acids, amino acids, phenols, alkaloids and proteins from aqueous solutions. In the case of solid matrix samples, ILs' applications have been limited, partly due to their high viscosity. However, when applied in combination with other technologies, such as microwave, ultrasonic treatment, or supercritical CO_2 extraction, the extraction process and subsequent separation of the components was greatly facilitated. For example, ILs have been successfully employed to extract different kinds of natural products, such as alkaloids, carbohydrates, polyphenolic compounds, and sesquiterpenes. After extraction, the resulting extracts were generally

diluted with water or organic solvents for further HPLC analysis (Du *et al*., 2007) and volatile compounds were evaporated for GC analysis (Bica *et al*., 2011). The lack of volatility of ILs makes it nearly impossible to directly concentrate and isolate non-volatile compounds from them, but the use of supercritical CO_2 extraction (Kroon *et al*., 2006), with the addition of anti-solvents (Lapkin *et al*., 2006), back extraction (Yu *et al*., 2007) and column separation30 have been successfully used to resolve this problem.

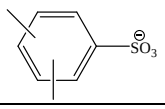
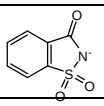
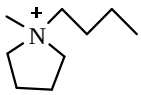
Depending on the structure of the cations and anions, ILs with different physical properties have been obtained. This means that it is possible to design an optimal IL for a specific compound, therefore allowing specific "object-oriented" or "task specific" ILs to be prepared. For example, some ILs with larger π values are expected to solubilize many π conjugated organic substances (Holbrey *et al*., 2008; Du *et al*., 2009). Also, ILs based on simple natural compounds can be synthesized for the extraction of certain kinds of natural products, such as amino acid-based ionic liquids (AAILs) that display enantioselectivity in the extraction of amino acids (Tang *et al*., 2010) and Gly-ChCl, which has been used to extract glycerol from biofuel (Hayyan *et al*., 2010). Similarly, the application of other types of ILs and DESs in the extraction of natural products should be investigated further in terms of their physical properties.

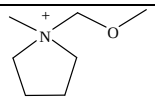
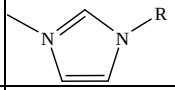
In the case of natural products, these solvents offer a prospect of being novel extraction methods, with a high efficiency and the yield of a greater variety of compounds given the high dissolving power of some ILs for cellulose (Swatloski *et al*., 2002). There are also other great advantages of ILs over conventional organic solvents. For instance, some ILs have been use to extract both essential oils and polar compounds in the same step (Ma *et al*., 2011). However, because of their high viscosity, most ILs are used as diluted aqueous solutions (Du *et al*., 2007). The extraction efficiency of other types of low-viscosity ILs, such as ILs made of natural products, which have been developed requires further exploration, since there are few reports of their applications.

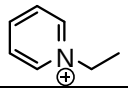
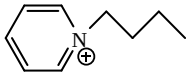
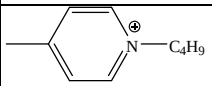
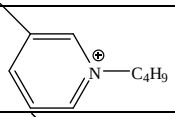
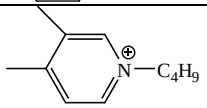
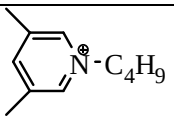
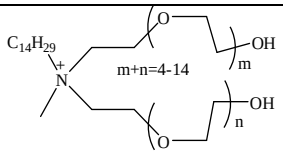
In this paper, all major aspects of ILs and DES concerning natural products will be reviewed, including the synthesis of "green" ILs and DES with natural products and their properties (table 2, 3), and their application to product extraction (table 4, 5), factors affecting the extraction efficiency of ILs, the combined use of ILs with other technologies, methods for the analysis of ILs extracts, and the recovery of compounds from ILs. In addition, enzyme reactions in ILs will also be reviewed (table 6). The problems and challenges that have arisen from the above applications will also be discussed.

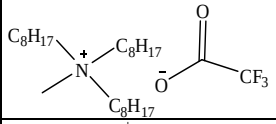
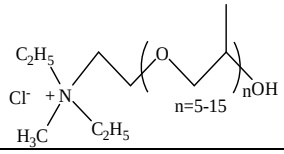
Table 1. The main anions and cations of ionic liquids and deep eutectic solvents.

Components	**Full name**	**Structure or molecular**
anion		

[BF_4]	tetrafluoroborate	BF_4^-
[DAC]	dicyanamide	$(CN)_2N^-$
[SCN]	thiocyanate	SCN^-
[Tf_2N]	bis(trifluoromethanesulfonyl) amide	$(CF_3SO_2)_2N^-$
[OAc]/[ACE]	acetate	CH_3COO^-
[Oct]	octanoate	$CH_3(CH_2)_5\ CH_2COO^-$
[lactate]	lactate	$CH_3CHOHCOO^-$
[salicylate]	2-hydroxybenzoic acid salt	$HOC_6H_5COO^-$
[SO_4]	sulfate	SO_4^{2-}
[$MeSO_4$]/[MS]	methylsulfate	$CH_3\text{-}SO_4^-$
[$EtSO_4$]	ethylsulfate	$C_2H_5\text{-}SO_4^-$
[OSO_4]	octylsulfate	$CH_3\text{-}(CH_2)_7\text{-}SO_4^-$
[MDEGSO4]/ [Meesu]	2-(2-methoxyethoxy) ethylsulfate	$CH_3OC_2H_5OC_2H_4SO_4^-$
[cetyl-PEG10-sulfate]	cetyl-PEG10-sulfate	$C_{16}H_{33}\text{-}PEG_{10}\text{-}SO_4^-$
[MeSO3]	methanesulfonate	$CH_3\text{-}SO_3^-$
[TfO]/[Tf]	trifluoromethane sulfonate	$CF_3\text{-}SO_3^-$
[TSO]	toluolsulfonate	$C_6H_5\text{-}SO_3^-$
[ABS]	alkylbenzenesulfonate	
[XS]	xylenesulfonate	
[H_2PO_4]	dihydrogenphosphate	$H_2PO_4^-$
[$MeHPO_4$]	methylphosphate	$(CH_3)\text{-}HPO_4^-$
[$PHPO_4$]	propylphosphate	$C_3H_7\text{-}HPO_4^-$
[Me_2PO_4]	dimethylphosphate	$(CH_3)_2\text{-}PO_4^-$
[$BHPO_4$]	butylphosphate	$C_4H_9\text{-}\ HPO_4^-$
[Sac]	saccharide	
cation		
[BMP]	1-butyl-1-methylpyrrolidinium	

[MMEP]	1-methyl-1-(-2-methoxyethyl) pyrrolidinium	
[Rmim]	1-R-3-methylimidazolium	
[Emim]	1-ethyl-3-methylimidazolium	R=CH_2CH_3
[Bmim]/[C_4mim]	1-butyl-3-methylimidazolium	R=$CH_2CH_2CH_2CH_3$
[Amim]	1-allyl-3-methyl imidazolium	R=$CH_2CH=CH_2$
[Pmim]	1-propyl-3-methylimidazolium	R=$CH_2CH_2CH_3$
[C_2OHmim]	1-hydroxymethyl-3-methylimidazolium	R=CH_2OH
[MOMmim]	1-methoxymethyl-3 -methylimidazolium	R=CH_2OCH_3
[MOEmim]	1-methoxyethyl-3-methylimidazolium	R=$CH_2CH_2OCH_3$
[EOEmim]	1-ethoxyethyl-3-methylimidazolium	R=$CH_2CH_2OCH_2CH_3$
[C_4SO_3mim]	1-(3-sulfopropyl)-3-methylimidazolium	R=$CH_2CH_2CH_2SO_3$
[Hmim]/[C_6mim]	1-hexyl-3-methylimidazolium	R=$(CH_2)_5CH_3$
[Omim]/ [C_8mim]	1-octyl-3-methylimidazolium	R=$(CH_2)_7CH_3$
[Nmim]/ [C_9mim]	1-nonyl-3-methylimidazolium	R=$(CH_2)_8CH_3$
[Bzmim]	1-benzyl-3-methylimidazolium	R=C_6H_5
[Btmsim]	1-butyl-3-trimethylsilyl imidazolium	R_1=$CH_2CH_2CH_2CH_3$ R_3=$(CH_3)_3Si$
[BBim]	1,3-dibutylimidazolium	R_1=R_3= $CH_2CH_2CH_2CH_3$
[C_5O_2mim]	1-[2-(2-methoxy ethoxy)-ethyl]-3-methyl-imidazolium	R= $CH_2CH_2OCH_2CH_2$ OCH_3

[C_2py]/ [Epy]	1-ethylpyridinium	
[Bpy]	1-butylpyridinium	
[BM_4Py]	1-butyl-4-methylpyridinium	
[BM_3Py]	1-butyl-3-methylpyridinium	
[$BdM_{3,4}$Py]	1-butyl-3,4-dimethylpyridinium	
[$BdM_{3,5}$Py]	1-butyl-3,5-dimethylpyridinium	
[TBP/P_{4444}]	*tetra*-*n*-butylphosphonium	$(CH_3CH_2CH_2CH_2)_4P^+$
[TEP/P_{2222}]	*tetra*-ethylphosphonium	$(CH_3CH_2)_4P^+$
[P_{66614}]	trihexyl(tetradecyl) phosphonium	$(C_6H_{13})_3P^+C_{14}H_{29}$
[TAA]	*tetra*-alkylammonium	$(C_nH_{2n+1})_4N^+$
[CPMA]	cocosalkyl pentaethoxy methyl ammonium	
[TMA/N_{1111}]	*tetra*-methylammonium	$(CH_3)_4N^+$
[TEA/ N_{2222}]	*tetra*-ethylammonium	$(CH_3CH_2)_4N^+$
[N_{4444}]	*tetra*-butylammonium	$(CH_3CH_2CH_2CH_2)_4N^+$
[BMOEA]	bis(2-methoxyethyl) ammonium	$(CH_3OCH_2CH_2)_2NH_2^+$
[DMEA]	*N*,*N*-dimethyl ethanolammonium	$(CH_3)_2NH^+CH_2CH_2OH$
[DMMOEA]	*N*,*N*-dimethyl(2-methoxyethyl) ammonium	$(CH_3)_2NH^+ CH_2CH_2OCH_3$
[Ch]/[$N_{111}(C_2OH)$]	choline/(2-hydroxyethyl) trimethylammonium	$HOCH_2CH_2N^+ (CH_3)_3$
anion+cation		

[MTOATFA]	methyltrioctylammonium trifluoroacetate	
[DIMCARB]	*N*'*N*'-dimethyl ammonium *N'N'*-dimethylcarbamate	$(CH_3)_2NH_2^+$ $^-OOCN(CH_3)_2$
[Amim110]	AMMOENGTM 110	
components of DES		
EAC	ethylammonium chloride	$C_2H_5NH_3^+$ Cl^-
ChCl	choline chloride	$(CH_3)_3$ $N^+CH_2CH_2OH$ Cl^-
U	urea	NH_2CONH_2
Gly	glycerol	$(HOCH_2)_2CHOH$
EG	ethyl glycol	$HOCH_2CH_2OH$
Acet	acetamide	CH_3CONH_2

2. Preparation of ionic liquids (ILs) and deep eutectic solvents (DES), their properties

2.1 Preparation of Ionic liquids with natural products

In recent times, there is a growing consensus on the acceptance of ILs as greener alternatives to volatile organic solvents, mainly due to their negligible vapor pressure. However, they fall short of complying with the 12 principles of green chemistry, casting doubt on the legitimacy of this claim (Anastas and Eghbali, 2010). Commonly used ILs based on halogenated anions and their derivatives are low melting point ILs, but their applications are obviously limited by toxicological (Swatloski *et al*., 2003), ecological and economic issues. ILs with the imidazolium cation are synthetic chemicals, even though some of them use amino acids as starting materials (Bao *et al*., 2002). Furthermore, some of these imidazolium-based ILs are also toxic (Garcia-Lorenzo *et al*., 2008).

In order to obtain ‘‘green’’ ILs, the first condition is that the starting materials at least must be non-toxic, whilst for a perfect solution, they should be renewable; secondly, the development of “green” ILs should require relatively low production costs, easy preparation and a green route; another requirement for “green” ILs is that the “designer solvent” properties of ILs be maintained, i.e., that the physicochemical properties of the obtained ILs can be adjusted by e.g. changing the side-chain attached to the main group, and even that the

natural properties of the materials be completely conserved. Bio-renewable natural compounds that have well characterized biodegradable and toxicological properties are ideal materials from both environmental and economical viewpoints. Recently, some kinds of bio-renewable natural products, including organic acids, amino acids, sugars and their derivatives, have been applied to prepare "green" ILs (table 2) and DES (table 3) (Imperato *et al*., 2007; Choi *et al*., 2011).

Table 2. The physical properties of some ionic liquids made of natural products.

ILs	T_m/ °C	T_g/ °C	T_{dec}/	viscosity/cP	Ref.
organic acids					
[Ch][acetate]	51	a	189		Fukaya *et al*., 2007
[Ch][glycolate]	38	-67	220		
[Ch][benzoate]	47	-51	202		
[Ch][propionate]	a	-74	184		
[Ch][succinate]	a	-62	192		
[Ch][malate]	99	-52	212		
[Ch][tartrate]	131	-40	210		
[Ch][maleate]	25	-6	203		
[Ch][fumarate]	80	-72	219		
amino acids					
[Emim][Gly]	b	-65		486	Fukumoto *et al*., 2005
[Emim][Ala]	b	-57			
[Emim][Met]	b	-57			
[Emim][Val]	b	-52			
[Emim][Ile]	b	-52			
[Emim][Leu]	b	-51			
[Emim][Ser]	b	-49			
[Emim][Lys]	b	-47			
[Emim][Thr]	b	-40			
[Emim][Phe]	b	-36			
[Emim][Trp]	b	-31			
[Emim][His]	b	-24			
[Emim][Tyr]	b	-23			
[Emim][Cys]	b	-19			
[Emim][Arg]	b	-18			

[Emim][Asn]	b	-16			
[Emim][Gln]	b	-12			
[Emim][Asp]	b	5			
[Emim][Glu]	b	6			
[Emim][Pro]	b	-48			
$[N_{4444}]$[Ala]	76	a	162		Kagimoto *et al*.,
$[N_{2226}]$[Ala]	a	-40	150		2006
$[N_{1111}]$[L-Ala]	42	a	219	a	Jiang *et al*., 2008
$[N_{2222}]$[L-Ala]	a	-80	185	81	
$[N_{1111}][\beta$-Ala]	a	-82	193	668	
$[N_{2222}][\beta$-Ala]	a	-85	184	132	
$[N_{4444}][\beta$-Ala]	a	-73	180	465	
$[N_{1111}]$[Gly]	a	-68	181	304	
$[N_{4444}]$[Gly]	16	-71	179	214	
$[N_{1111}]$[Val]	40	a	223	c	
$[N_{4444}]$[Val]	25	-69	185	660	
[Ch][Pro]	RT	-	159	-	Hu *et al*., 2007
[Gly][NO_3]	111		192		Tao *et al*., 2005
[Ala][NO_3]	159		168		
$[Ala]_2[SO_4]$	141		193		
[Val][NO_3]	134		169		
[Ile][NO_3]	105		167		
[Thr][NO_3]	b		147		
[Pro][NO_3]	b	-45	138	5140(30 ^oC)	
$[Pro]_2[SO_4]$	92		206		
$[GlyC_1]NO_3]$	44	-26	178	92(70 ^oC)	Tao *et al*., 2005
$[AlaC_1][NO_3]$	61	-34	186	104(80 ^oC)	
$[ValC_1][NO_3]$	74	-33	195	445(80 ^oC)	
$[LeuC_1]NO_3]$	75	-31	210	1550(80 ^oC)	
$[IleC_1][NO_3]$	-14	-36	172	-	
$[PheC_1][NO_3]$	92	-32	224	-	
$[ThrC_1][NO_3]$	-12	-32	156	-	
$[SerC_1][NO_3]$	105	-30	179	-	

[ProC$_1$][NO$_3$]	-16	-67	159	186(30 ^{o}C)	
[ProC$_2$][NO$_3$]	-17	-50	183	213(30 ^{o}C)	
[ProC$_3$][NO$_3$]	6	-71	208	398(30 ^{o}C)	
[ProC$_4$][NO$_3$]	-11	-70	163	275(30 ^{o}C)	
[AlaC$_1$][Sac]	-14	-27	184	1390(80 ^{o}C)	Tao *et al*., 2006
[ValC$_1$][Sac]	-16	-29	185	3040(80 ^{o}C)	
[LeuC$_1$][Sac]	7	-1	223	1050(80 ^{o}C)	
[IleC$_1$][Sac]	7	-8	177	14500(80	
[ThrC$_1$][Sac]	-1	-9	200	55900(80	
[ProC$_1$][Sac]	-19	-29	190	3320(80 ^{o}C)	
[ProC$_2$][Sac]	8	-42	212	3021(50 ^{o}C)	
[P$_{4444}$][Ala]	a	-70	286	344	Tao *et al*., 2006
[P$_{4444}$][Met]	a	-63.5		371	
[P$_{4444}$][Gly]	13.6	-63.7		415	
[P$_{4444}$][Leu]	30	-63.4		389	
[P$_{4444}$][Ile]	b	-60.7		605	
[P$_{4444}$][Ser]	b	-59.9		902	
[P$_{4444}$][Val]	26	-59.1	b	423	
[P$_{4444}$][Lys]	b	-58.8		779	
[P$_{4444}$][Pro]	25.4	-57.5		851	
[P$_{4444}$][Thr]	b	-56.1		965	
[P$_{4444}$][Phe]	8.1	-53.1		927	
[P$_{4444}$][Arg]	30.7	-36		c	
[P$_{4444}$][Trp]	b	-25.6		c	
[P$_{4444}$][Gln]	b	-25		c	
[P$_{4444}$][Glu]	101.7	-23.3		c	
[P$_{4444}$][Gys]	b	-20.7		3029	
[P$_{4444}$][Asp]	b	-7.6		c	
[P$_{4444}$][Tyr]	b	-6.5		c	
[P$_{4444}$][Asn]	83	-3.9		c	
[P$_{4444}$][His]	85.9	a		299	
sugars					
[C$_4$mim][Sac]	RT				Carter *et al*.,
[C$_6$mim][Sac]	RT				

[C_9mim][Sac]	RT				2004
[C_4mim][Ace]	RT				
[C_6mim][Ace]	RT				
[C_9mim][Ace]	RT				
[Ch][Sac]	69			328(70 ^{o}C)	Nockemann *et*
[Ch][Ace]	25			1072(25 ^{o}C)	*al*., 2007

T_m: melting point; T_g: glass transition point; T_{dec}: decomposition temperature.
[a]: not observed; [b]: no melting point; [c]: solid or glass at 25 ^{o}C.

Organic acids A series of ILs composed of choline and organic acids with strong hydrogen bonding characteristics were prepared (Fukaya *et al*., 2007). These ILs were prepared via a two-step anion exchange reaction. On the other hand, mixtures of organic acids and choline chloride (Table 3) were also reported to form DES by heating.

Sugars and sugar substituents were also considered for the preparation of green ILs. Fructose was used as the starting material of a new class of ILs that exhibited tunable solvent properties much like conventional imidazole-based ILs. The process, however, was not simple and included some toxic chemicals even though the starting material was green (Handy *et al*., 2003). Saccharin (Sac) and acesulfame (Ace) are widely used in foodstuffs as non-nutritive sweeteners and they have well-established toxicological profiles, compared with the common anions in ILs, [Cl] and [BF_4]. A series of ILs made of Sac and Ace as anions with common organic cations were prepared via metathesis (Carter *et al*., 2004). Two green and hydrophilic ILs, [Ch][Ace] and [Ch][Sac], were prepared by a silver-free metathesis reaction and ion-exchange. The ecotoxicity of these ILs in aqueous solution was very low in comparison to other types of ILs (Nockemann et al., 2007).

Amino acids (AAs) are molecules with both a carboxylic acid and an amino group. These groups are useful in their ability to introduce functional group(s) and so ILs based on amino acids may be natural "designer solvents". A family of 20 different natural amino acids ILs (AAILs) combined with an imidazolium cation was prepared via a one-step anion exchange reaction and a neutralization method (Fukumoto *et al*., 2005; Ohno and Fukumoto, 2007). Furthermore, as AAs are zwitterions, they can be used as either anions or cations. AAILs with amino acids as cations were synthesized through a one-step acidification without any poisonous by-products (Tao *et al*., 2005). Although 17 products were obtained, half of them were white solids. Fortunately, not only does esterification reduce the amount of hydrogen bounding, resulting in a significant decrease in the melting points of the salts, but it also provides the possibility of adjusting the properties of the resulting ILs, and the "designer solvent" character of ILs is therefore potentially enhanced. Furthermore, the incorporation of an ester group could increase the biodegradability of those

AAILs. A series of ILs with low melting points has been successfully obtained from amino acid esters, through a one- step esterification and the metathesis method. AAILs with other type of cations have been developed, such as [TBP][AA]s made of tetrabutylphosphonium (TBP) and amino acids (Kagimoto *et al*., 2006), and [TAA][AA]s composed of a tetraalkyl ammonium (TAA) cation and an amino acid (Jiang *et al*., 2008). With the objective of increasing the "greenness" level, [Sac] and [NO_3] were used as anions and a family of novel ILs from amino acids esters (AAE) has synthesized applying the metathesis method. The obtained ILs had the same characteristics as conventional imidazolium ILs and the same chirality as natural amino acids (Tao *et al*., 2006). The natural products choline chloride and proline, were applied to synthesize green solvents by a simple and relatively green route (Hu *et al*., 2007). Based on the above studies, choline hydroxide and five different amino acids (glycine, alanine, phenylalanine, threonine and histidine) were used to form natural AAILs through a one-step neutralization method (Moriel *et al*., 2010). Choline, amino acids, [Sac] and [NO_3], are non-toxic, with well-established toxicological profiles and produce pharmaceutically acceptable ions, so that the resulting ILs could qualify as "fully green solvents". In addition, these AAILs are also called "Chiral ionic liquids (CIL)", "functional or task-specific ILs" and "designer solvents" because they have chiral centers, functional groups and properties that can be adjusted by changing their side-chains. In all cases, the stereogenic center present in the amino acid is successfully conserved in the final CILs. Furthermore, AAILs have biodegradable characteristics and high biocompatibility. AAILs have already been used in the field of organic chemistry for asymmetric catalysis with excellent enantioselectivity and good diastereoselectivity (Winkel *et al*., 2008), as well as in the chiral liquid-liquid extraction of amino acids with high enantioselectivity (Tang *et al*., 2010) and in water containing solvents to enhance protease enantioselectivity (Zhao *et al*., 2006).

2.2 The properties of "green" Ionic liquids (Table 2)

The glass transition temperature (T_g), melting temperature (T_m), viscosity, thermal stability, and polarity are important properties for the application of ILs as extraction solvents; they are summarized in Table 2. The properties of AAILs depend greatly on the side groups of the amino acids involved (Ohno and Fukumoto, 2007). These AAILs, composed of an amino acid with some functional groups such as a hydrogen bonding group (-OH, -NH_2, -COOH), a charged group or an aromatic ring, exhibited an increased glass transition temperature (T_g) or melting temperature (T_m) and/or higher viscosity resulting from the additional interactions among ions, such as hydrogen bonding, stacking interaction, and electrostatic interaction. The introduction of -COOH had a much greater effect on the increase of T_m than that of an -OH and -NH_2. [Emim][AA]s and some [TBP][AA]s and [TAA][AA]s have no T_m reported but do have a T_g and most of ILs with amino acid as cations have both known T_g and T_m values. In general, the more symmetric the anion structure, the higher the crystal structure stability, and the higher the T_m. For [Emim][AA]s and

[TBP][AA]s, an increase of the alkyl side-chain length coincides with a gradual increase of T_g due to an increase of the Van der Waals force between alkyl side chains: [Emim][Gly] (-65 ^oC)< [Emim][Ala] (-57 ^oC)<[Emim][Val] (-52 ^oC), whereas the trend of T_g values for AAILs with different functional side groups, is: [Emim][Met] (-47 ^oC)<[Emim][Lys] (-47 ^oC)< [Emim][Gln] (-12 ^oC)< [Emim][Glu] (+6 ^oC), which might due to the difference in the intensity of hydreogen bond. ILs made of choline and organic acids have both T_g (from -6 to -72 °C) and T_m (from 25 to 131 °C) (Fukaya et al., 2007). [AAE][Sac] ILs have a higher T_g, a much higher viscosity and better thermal stability than the corresponding [AAE][NO_3] (Tao *et al*., 2006). There is a linear relationship between the T_g value and ionic conductivity for major amino acid ILs with [Emim] as a cation and the relationship between viscosity and the T_g for [TBP][AA]s is also linear. Viscosities of [AAE][NO_3] are comparable with those of conventional imidazolium ILs. Among AAILs with the same amino acid as the anion, and [Emim], [TBP] or [TAA] as the cation, [TAA]-based AAILs are the least viscous. This can be explained by the lower molecular weight and higher flexibility of the alkyl chain of [TAA] as compared to the [TBP] cation (Jiang *et al*., 2008). The lower viscosity of AAILs with [TAA] than with [Emim] indicates that [TAA]-based AAILs may be promising solvents as extraction media for natural products. As regards to thermal stability, all the obtained AAILs are stable up to 150 or 200 °C, a little below imidazolium based-ILs, but clearly above the minimum 100°C requirement described by Wasserscheid for chiral ILs (Wasserscheid *et al*., 2002). [TBP][AA]s have a higher decomposition temperature (T_m) (>300 ^oC) than [Emim][AA]s (170 to 200 ^oC) and [TAA][AA]s (Kagimoto *et al*., 2006). The properties of AAILs depend greatly on the side groups involved (Ohno and Fukumoto, 2007). The AAILs composed of an amino acid with some functional groups such as a hydrogen-bonding group (-OH, -NH_2, -COOH), a charged group or an aromatic ring, exhibit a high T_g or T_m and higher viscosity resulting from the additional interactions among ions, such as hydrogen bonding, stacking and electrostatic interaction. Esterification reduces the amount of hydrogen bonding, resulting in a significant decrease in the T_m and viscosity of the salts. The T_m decreases to -17 ^oC for [$AlaC_2$][NO_3] from 159 ^oC for [Ala][NO_3] (Tao *et al*., 2005).

Polarity is another important property of ILs, because the solubility of the solute, their miscibility with other solvents and even their extraction ability are influenced by their polarity. Compared to general ILs, AAILs have a stronger hydrogen bond basicity, equivalent hydrogen bond acidity and equivalent dipolarity. The β parameter, hydrogen-bonding basicity, introduced by Kamlet and Taft, has been established as a measure of the hydrogen-bond accepting ability of anions (Chiappe *et al*., 2005). [Emim][AA]s exhibit strong hydrogen-bond basicity (β between 0.88 and 1.38), above [Bmim][Cl] (β=0.95), which is the IL precisely chosen for its high β

values (Ohno and Fukumoto, 2007). ILs made of choline and an organic acid also have a polarity similar to [Bmim][Cl] (Fukaya *et al*., 2007). Thus, [Emim][AA]s and ILs made of organic acid and choline are expected to be polar solvents for scarcely soluble compounds.

2.3 Preparation of DES and their properties

To date, there are far less reports on DES as compared to the large amount of papers on ILs. Different kinds of natural products such as organic acids, sugars and polyalcohols with amine or choline chloride and sugars or amino acids with organic acids have been used to prepare DES in a simple step, heating and stirring (Table 3) (Abbott *et al*., 2003; Imperato *et al*., 2005). Abbott and co-workers reported that mixtures of urea or organic acids with quaternary ammonium salts could become liquid when heated to 80-100 ^{o}C (Abbott *et al*., 2003; 2004). In those liquids, two urea molecules or two carboxylic acid groups were found to be required to complex each chloride ion. With the intention of extending the variety of DES and exploring their application in organic reactions, König's group found different combinations of compounds that formed DES, including sugar-urea/dimethyl urea, polyalcohol-dimethyl urea, organic acid-dimethyl urea (Imperato *et al*., 2005; Gore *et al*., 2011), *l*-carnitine-urea (Ilgen and Koning, 2009), sugar-choline chloride, sugar-malonic acid (Ilgen *et al*., 2009). In addition, salts with a chloride ion, such as $CaCl_2$, NH_4Cl, NaCl could also be used as components in the previously mentioned DES. These DES have a melting point in the 50-100 ^{o}C range, mostly around 70 ^{o}C and some that were found to be stable at 95 ^{o}C for 4 h without any evident decomposition proved to be good solvents for some organic reactions (Imperato *et al*., 2005). DES can also be formed between glycerol and choline chloride in different ratios. Glycerol - choline chloride (3:1) was used as an electrolyte for dye-sensitized solar cells (Jhong *et al*., 2009), glycerol - choline chloride (1:1/2:1) for extracting glycerol from biofuel (Abbott *et al*., 2007) and for enzyme-catalyzed reactions (Gorke *et al*., 2008). All the above-mentioned DES were prepared by heating. Meanwhile, Gutiérrez *et al*. (2009) found that DES could also be made by freeze-drying aqueous solutions, such as urea-choline chloride (2:1) and thiourea-choline chloride (2:1), glycerol-choline chloride (2:1) and ethylene glycol-choline chloride (2:1) (Gutiérrez, *et al*., 2010). The reported freeze-drying method allowed the incorporation of bacteria into the DES in its pure state (Gutiérrez *et al*., 2010), the solubilization of DNA and RNA with secondary structures (Mamajanov *et al*., 2010), and the preparation of nanotubes (Gutiérrez *et al*., 2010).

Table 3. Composition and physical properties of deep eutectic solvents.

Components		**Molar ratio**	T_m/	**Ref.**
organic acid				
adipic acid	choline chloride	1:1	15	Abbott *et al.*,2004
benzoic acid	choline chloride	2:1	12	Abbott *et al.*,2004

citric acid	choline chloride		14	Abbott *et al.*,2004
malonic acid	choline chloride	1:1	13	Abbott *et al.*,2004
oxalic acid	choline chloride	1:1	19	Abbott *et al.*,2004
phenylacetic acid	choline chloride	2:1	77	Abbott *et al.*,2004
phenylpropionic	choline chloride	2:1	48	Abbott *et al.*,2004
succinic acid	choline chloride	1:1	18	Abbott *et al.*,2004
tricarballylic acid	choline chloride		15	Abbott *et al.*,2004
citric acid	dimethylurea	4:6[a]	65	Imperato *et al.*, 2005
acrylic acid	choline chloride	1:2	15	Mota-Morales, 2011
methacrylic acid	choline chloride	1:2		Mota-Morales, 2011
tartaric acid	dimethylurea	3:7[a]	70	Gore *et al.*, 2011
citric acid	choline chloride	1:2;1:3	-[b]	Choi *et al.*, 2011
malic acid	choline chloride	1:1; 1:2;1:3	-[b]	Choi *et al.*, 2011
maleic acid	choline chloride	1:1; 1:2;1:3	-[b]	Choi *et al.*, 2011
aconitic acid	choline chloride	1:1	-[b]	Choi *et al.*, 2011
polyalcohols				
sorbitol	urea+NH_4Cl	7:2:1[a]	67	Imperato *et al.*,2005
sorbitol	dimethylurea	4:6[a]	77	Imperato et al.,2005
glycerol	choline chloride	3:1	20	Jhong *et al.*, 2009
glycerol	choline acetate	2:3	13	Zhao *et al.*, 2011
glycerol	choline chloride	2:1	23	Guti érre *et al.*, 2010
glycerol	choline chloride	2:1		Abbott *et al.*, 2011
glycerol	choline acetate	1:1	20	Zhao *et al.*, 2011
glycerol	choline acetate	1.5:1	13	Zhao *et al.*, 2011
ethylene glycerol	choline acetate	2:1	23	Zhao *et al.*, 2011
sorbitol	urea	5:5[a]	70	Ilgen *et al.*, 2009
sorbitol	choline chloride	4:6[a]	70	Ilgen *et al.*, 2009
sorbitol	imidazole	3:7[a]	80	Ilgen *et al.*, 2009
sorbitol	4-methyl-imidazole	2:8[a]	50	Ilgen *et al.*, 2009
sorbitol	pyrazole	3:7[a]	60	Ilgen *et al.*, 2009
sorbitol	guanidinium HCl		-[b]	Ilgen *et al.*, 2009
sorbitol	dimethylurea+NH_4	7:2:1[a]	67	Gore *et al.*, 2011
sugars				

frutose	urea	6:4[a]	65	Imperato *et al.*, 2005
glucose	urea+$CaCl_2$	5:4:1[a]	75	Imperato *et al.*, 2005
maltose	dimethylurea+NH_4	5:4:1[a]	73	Imperato *et al.*, 2005
mannose	dimethylurea	3:7[a]	75	Imperato *et al.*, 2005
α-cyclodextrin	dimethylurea	3:7[a]	77	Imperato *et al.*, 2005
glucose	choline chloride	4:6[a]	80	Ilgen *et al.*, 2009
mannose	choline chloride	4:6[a]	50	Ilgen *et al.*, 2009
fructose	choline chloride	4:6[a]	70	Ilgen *et al.*, 2009
sucrose	choline chloride	5:5[a]	80	Ilgen *et al.*, 2009
isomaltose	choline chloride	4:6[a]	90	Ilgen *et al.*, 2009
glucosamine	choline chloride	1:9[a]	100	Ilgen *et al.*, 2009
mannose	malonic acid	5:5[a]	90	Ilgen *et al.*, 2009
fructose	malonic acid	7:3[a]	100	Ilgen *et al.*, 2009
sucrose	malonic acid	6:4[a]	80	Ilgen *et al.*, 2009
glucose	dimethylurea	3:7[a]	80	Ilgen *et al.*, 2009
mannose	dimethylurea	4:6[a]	80	Ilgen *et al.*, 2009
fructose	dimethylurea	4:6[a]	70	Ilgen *et al.*, 2009
isomaltose	dimethylurea	4:6[a]	90	Ilgen *et al.*, 2009
glucose	4-methyl-imidazole	2:8[a]	50	Ilgen *et al.*, 2009
mannose	4-methyl-imidazole	2:8[a]	50	Ilgen *et al.*, 2009
fructose	4-methyl-imidazole	2:8[a]	50	Ilgen *et al.*, 2009
sucrose	4-methyl-imidazole	4:6[a]	70	Ilgen *et al.*, 2009
isomaltose	4-methyl-imidazole	3:7[a]	70	Ilgen *et al.*, 2009
glucosamine	4-methyl-imidazole	3:7[a]	50	Ilgen *et al.*, 2009
glucose	pyrazole	5:5[a]	80	Ilgen *et al.*, 2009
mannose	pyrazole	4:6[a]	50	Ilgen *et al.*, 2009
fructose	pyrazole	5:5[a]	70	Ilgen *et al.*, 2009
sucrose	pyrazole	4:6[a]	60	Ilgen *et al.*, 2009
isomaltose	pyrazole	5:5[a]	70	Ilgen *et al.*, 2009
glucosamine	pyrazole	1:9[a]	90	Ilgen *et al.*, 2009
glucose	guanidinium HCl	4:6[a]	70	Ilgen *et al.*, 2009
mannose	guanidinium HCl	4:6[a]	80	Ilgen *et al.*, 2009
fructose	guanidinium HCl	4:6[a]	70	Ilgen *et al.*, 2009
isomaltose	guanidinium HCl	4:6[a]	80	Ilgen *et al.*, 2009

fructose	dimethylurea	7:3[a]	71	Gore *et al*., 2011
mannose	dimethylurea	3:7[a]	75	Gore *et al*., 2011
glucose	malic acid	1:1	-[b]	Choi *et al.*, 2011
fructose	malic acid	1:1	-[b]	Choi *et al.*, 2011
sucrose	malic acid	1:1	-[b]	Choi *et al.*, 2011
glucose	citric acid	1:2	-[b]	Choi *et al.*, 2011
sucrose	citric acid	1:1	-[b]	Choi *et al.*, 2011
trehalose	citric acid	2:1	-[b]	Choi *et al.*, 2011
glucose	fructose	1:1:1	-[b]	Choi *et al.*, 2011
amines				
urea	choline chloride	2:1	12	Abbott *et al*., 2003
urea	choline chloride	2:1	25	Morrison *et al*., 2009
urea	Choline acetate	2:1	18	Zhao *et al*., 2011
methyl urea	choline chloride	2:1	29	Abbott *et al*., 2003
1,3 dimethyl urea	choline chloride	2:1	70	Abbott *et al*., 2003
1,1 dimethyl urea	choline chloride	2:1	149	Abbott *et al*., 2003
thiourea	choline chloride	2:1	69	Abbott *et al*., 2003
acetamine	choline chloride	2:1	51	Abbott *et al*., 2003
benamide	choline chloride	2:1	92	Abbott *et al*., 2003
amino acid				
proline	citric acid	1:1;2:1;3:1	-[b]	Choi *et al*., 2011

[a]: ratio of weight; [b]: not detected.

Natural products are indeed a plentiful and ideal source of ILs and DES components due to their enormous chemical diversity, biodegradability, sustainability and pharmaceutically acceptable toxicity profile. Our group introduced the term Natural Deep Eutectic Solvents (NADES) for these liquids, which extends the composition of DES to natural products, such as citric acid and proline (Choi *et al*., 2011). In addition to contributing to the number of ILs and DES, the hypothesis of the existence of NADES in organisms could account for many biological processes that cannot be otherwise explained by the theory that water and lipids are the only media in organisms.

In the case of DES made of urea and quaternary ammonium, it was found that the amides with the greatest ability to form hydrogen bonds (i.e. urea and thiourea) exhibited the largest depression in T_m, e.g., choline chloride-urea (1:2) has a melting point of 12 C, while T_m (urea) =134 ^oC and T_m (choline chloride) >300 ^oC. The freezing point of DES with different choline salts decreased

according to their anion in the order $F^- > NO_3^- > Cl^- > BF_4^-$, suggesting some correlation with hydrogen bond strength. In the case of cations, when the symmetry of the cation decreases so does the T_m of the mixture, similarly to ionic liquids. The charge delocalization that occurs through hydrogen bonding between the halide anion and the hydrogen-donor moiety is responsible for the lower T_m of the mixture, as compared to the T_m of the individual component. Most DES have melting points above 50 ^oC (Table 3). DES made of choline salts with quaternary ammonium are highly conductive, confirming the presence of anionic species in the liquid that can move independently (Abbott *et al*., 2003). The conductivity of DES made of organic acids and choline chloride is in the range of 0.1-10 $mScm^{-1}$ changing with temperature and composition and is is similar to imidazolium-based ILs and DES made of urea and choline chloride. In general, conductivity increases significantly with temperature (Abbott *et al*., 2003; 2004). There is an inverse relationship between the viscosity of DES and temperature, as viscosity decreases with increasing temperature (Abbott *et al*., 2003; 2004). These data and their relationship with temperature are important for their applications.

3. Application of ILS and DES in extraction of natural products

3.1. Application of ILs and DES in liquid-solid extraction of natural products

Diverse ILs, covering the whole range of polarity were tested for their aptitude to extract natural products such as phenolic compounds, alkaloids, essential oils, lignins, and carbohydrates from different materials (Table 4). Most ILs were used in the form of aqueous solution because of their high viscosity in pure state.

Phenolic compounds The ILs-based microwave assisted extraction (IL-MAE) technique was first developed to extract different kinds of polyphenolic compounds. The structure of ILs components plays an important role in extraction. In the case of phenolic compounds, the efficiency has proven to be anion-dependant, with [Bmim][Br] being the best choice as compared to the other ILs tested (Du *et al*., 2007; 2009; Zeng *et al*., 2010). This can be attributed to its strong solvatation power and its multiple interactions, especially H-bonding, polarity, π-π, n-π and ionic/charge-charge (Anderson *et al*., 2002; Guo *et al*., 2007). On the other hand, for phenolic compounds with less hydroxyl groups, such as magnolol, honokiol, quercetin and *trans*-resveratrol, ILs with $[BF_4]$ showed a high extraction ability and [Bmim][TSO] also exhibited a high extraction efficiency because of the extra aromatic system.

The parameters that are generally used in an extraction process involving an IL are the following: its concentration (2.0-3.0 mol/L for liquids and 1:30-1:20 for solid/liquid ratio) and a temperature range of 60-70 ^oC. Additionally, the pH value of the solution may also affect the extraction efficiency (Zhang and wang, 2010).

Alkaloids The extraction of alkaloids is structure dependent and interactions such as H-bonding, *n–π*, ionic/charge-charge, are the driving forces for the

extraction. [Bmim][BF_4] showed efficient extraction ability for piperine (Cao, *et al*., 2009) and phenolic alkaloids (liensine, isoliensine, neferine, fangchinoline and tetrandrine) (Lu, *et al*., 2008; Zhang, *et al*., 2009), while [Hmim][Br] showed ca 40% higher extraction ability than [Bmim][BF_4] for *N*-nornuciferine, *O*-nornuciferine, and nuciferine (Ma, *et al*., 2010). The extraction of alkaloids thus depends on both the kind of IL and the structure of the alkaloids and should also take the extraction method into account. For example, [Hmim][BF_4] and [Bmim][BF_4] can reach the same extraction efficiency for the three phenolic alkaloids in IL-MAE (Ma, *et al*., 2010), while the extraction ability of [Bmim][BF_4] was 80% higher than that of [Hmim][BF_4] with ionic liquids based ultrasonic-assisted extraction (IL-UAE) (Cao, *et al*., 2009). This means that in IL-UAE, the viscosity of ILs has a big influence on the extraction efficiency, while the high temperature in IL-MAE decreases the viscosity differences between ILs caused by the alkyl chain length. As for the extraction parameters, the important and generally measured ones are the concentration of ILs (1.0-2.0 mol/L) and solid/liquid (1:30-1:10) and an extraction time of 60-90 s for MAE and 30 min for UAE.

Essential oils Most essential oil components are sensitive to high temperatures and will degrade causing undesirable effects in the properties of the oil. Shortening the general distillation time is a good way of avoiding the damage caused by high temperature. IL-MAE can provide a great advantage as it shortens the required extraction time significantly (from 2 h to 15 min). This is because the microwave absorption performance of ILs ([Bmim][PF_6]) is better than water, and this reduced exposure of the essential oils decreases the amount of oxy-compounds ratio in the fraction obtained with MAE as opposed to that obtained by distillation (Zhai, *et al*., 2009). Furthermore, ILs have advantages in terms of selectivity and high extraction efficiency over normal organic solvents. [Emim][Meesu], for example, demonstrated a high selectivity and efficiency (extraction ratio close to 1) in the extraction of linalool from citrus essential oil (Francisco, *et al*., 2010) and in the case of artemisinin, if compared to hexane extraction, [DMEA][Oct] gives a similar efficiency at a faster rate, while, [BMOEA][Tf_2N] is more efficient at the same rate (Lapkin, *et al*., 2006). Again, the selection of ILs for the extraction of essential oils depends on the extraction method. Some ILs with a long alkyl chain or with lower polarity anions such as [Tf_2N] were used directly in liquid-solid extraction, while a [Bmim][PF_6] aqueous solution with a relative higher polarity also proved to be highly efficient with IL-MAE.

Lignin Lignin is an aromatic polymer composed of phenylpropanoids and its extraction is anion-dependent. It is highly soluble in polar ILs with anions such as [XS], [ABS], [Cl], [MeSO4], [TfO], [OAc], while they are not soluble in less polar ILs with [BF_4], [PF_6] anions (Lee, *et al*., 2009; Tan, *et al*., 2009). [Bmim][PF_6] extracted the phenylpropanoids magnolol and honokiol very efficiently (Zhang and wang, 2010), but could not solubilize phenylpropanoid

polymers. Lignocellulose is a major source of lignin and a highly concentrated solution of chemically unmodified lignin was obtained from lignocelluloses with [Emim][OAc] (Lee, *et al*., 2009).

Carbohydrates Carbohydrates are only sparingly soluble in common organic solvents. However, ILs containing the dicyanamide anion ([dca]) are able to dissolve glucose in high concentrations (>100 gL^{-1}) (MacFarlane, *et al*., 2001) and di- and trisaccharides in considerable but unspecified amounts (Forsyth, *et al*., 2002; Forsyth and McFarlane, 2003). Sheldon proved [dca] based ILs to be highly effective, non-protic solvents, capable of dissolving carbohydrates from glucose to starch and even cellulose in large amounts (Liu, *et al*., 2005). Fort, *et al*. (2006) found that banana pulp at any stage can be completely dissolved in [Bmim][Cl] or a [Bmim][Cl]/DMSO-d_6 mixture while neat DMSO-d_6 under the same conditions led only to partial dissolution of the samples. Another example is the solubiliization of up to 10% (w/w) of starch in [Bmim][dca] or [Bmim][Cl] at 80 ^{o}C (Biswas, *et al*., 2006).

Cellulose is insoluble in water and most common organic liquids. Recent studies found, however, that cellulose could be dissolved, without derivitization, in some hydrophilic ILs such as [Bmim][Cl] and [Amim][Cl] (Swatloski, *et al*., 2002; Zhu, *et al*., 2006). Unfortunately, these ILs are toxic and have a high melting point, and viscosity, requiring energy for the dissolution. To overcome the above drawbacks, a series of ILs with alkylimidazolium carboxylates, such as acetate and formate salts with lower viscosity were applied to dissolve cellulose from wood biomass in high concentrations (Sun, *et al*., 2009). These ILs with carboxylates are not particularly thermally stable, however, which limits their use. In a further step, alkylimidazolium cations coupled with alkylphosphates, [Emim][H_2PO_4], [Emim][$MeHPO_4$], were found to be good solvents for cellulose, with low viscosity and thermal stability (Abe, *et al*., 2010). In general, ILs with various anions, such as [Cl], carboxylates or akylphosphates provide good solubility for cellulose.

Dissolution of cellulose is anion-dependent and is based on the disruption of inter- and intra-hydrogen bonding of cellulose and formation of new hydrogen bonds between the anions of ILs and the hydroxyl groups of carbohydrates (Resming, *et al*., 2006). Highly polar ILs from [Bmim][dca], [Bmim][Cl], [Bmim][OAC] to [Emim][H_2PO_4] in order of increasing polarity have a good solubilizing power of different carbohydrates, Further studies showed that the dissolution of cellulose increases with the hydrogen bond-accepting ability (hydrogen bonding basicity β) of its anions (Xu, *et al*., 2010) and among polar ILs with similar β values, the extraction ability of ILs for polysaccharides in a limited time is dependent on the viscosity of ILs (Abe, *et al*., 2010). Another important factor that affects the solubility of cellulose is the temperature. Cellulose did not dissolve in ILs under ambient conditions, but when heated to 100-110 ^{o}C dissolution occured at a slow rate in 3-methylimidazolium based ILs containing [Cl], [Br] or [SCN]. Moreover, microwave heating significantly accelerated the dissolution process, increasing the solubility of cellulose in [Bmim][Cl] up to a 25% (Swatloski, *et al*., 2002).

Cellulose can dissolve in [Emim][H_2PO_4] at room temperature but its solubility increases three-fold (up to 42%) when the temperature increases to 50 ^oC.

From the extraction studies mentioned above, it is clear that ILs are excellent solvents for all those types of compounds, showing faster or at least an equivalent rate of extraction and solubilizing ability for different kinds of target compounds when compared to classical solvents. Another aspect of extraction is the selectivity of solvents. Some studies have revealed differences between the selectivity of ILs and common organic solvents. For example, obvious differences in the components and in the content of one same compound have been observed in the chromatograms of essential oils extracted by distillation or ILMAE-([Bmim][PF_6]) (Zhai, *et al*., 2009). Furthermore, the latter also showed significant activities (Cieniecka-Roslonkiewicz, *et al*., 2007).

In all, if compared with the current industrial extraction methods based on volatile organic solvents, ILs are promising solvents for the extraction of active metabolites in terms of environmental, economicals and pharmaceutical aspects.

Table 4. Application of ionic liquids (ILs) and deep eutectic solvents (DES) in the liquid-solid extraction of different types of natural products.

extractants	ILs/DES	conclusions	Ref.
phenolic compounds			
trans-resveratrol	[Bmim][Cl], [Bmim][Br] [Bmim][BF_4]	1.Under optimized conditions the extraction efficiency of *trans*-resveratrol was 92.8% in a single-step extraction; 2.With the same cation, the extraction efficiency follows the trend: [Br](47.9%)>[Cl](35.8%)>[BF_4] (17.9%); 3.A comparison between the extraction behavior of [Bmim]Br and methanol showed higher extraction yield for [Bmim]Br than methanol (92.8% vs 88%) and these two solvents had different selectivity for the substances in the sample.	Du *et al*., 2007
gallic acid ellagic acid quercetin *trans*-resveratrol	[Bmim][Br] [Emim][Br] [Hmim][Br] [Bmim][Cl] [Bmim][BF_4] [Emim][BF_4] [Bmim][dca], [Bmim]$_2$[SO_4], [Bmim][H_2PO_4]	1. [Bmim]Br was the optimal IL; 2.The cations and anions influenced the extraction yields. For the [Bmim] based ILs, [H_2PO_4] and [Br] were more efficient than other anions for polyphenolic compounds from P. *guajava* leaves, and [Br] and [BF_4] were more efficient for *trans*-resveratrol and quercetin from S. *china* tubers. [Bmim]Br was more efficient than [Emim]Br, and [Hmim]Br in the MAE of polyphenolic compounds; ③The extraction efficiency follows the trends: [bPy]Cl>[Bmim]Cl>$(CH_3)_4$NCl because cations with electron-rich aromatic π-systems produced more interactions; 3.The extraction ratio increased with the increase in concentration of [Bmim]Br below 3 M ml^{-1}.	Du *et al.*, 2009

	[bPy][Cl] $[(CH_3)_4N][Cl]$	Important parameters to be optimized are sample size (0.45-0.5 mm for leaves and 0.9-0.45 for tubers), temperature (70 ^oC for leaves and 60 ^oC for tubers) and liquid/solid (20:1) ratio.	
rutin	[Bmim]Cl, [Bmim]Br, $[Bmim][BF_4]$, [Bmim][TSO],	1.With the same cations, the extraction ratio follows the trend: [TSO]=[Br] > Cl >$[BF_4]$; 2.Optimal conditions: for Flos Sophorae and Saururus, the concentration of [Bmim]Br (2.0 mol/L) and solid/liquid (1:30) are the same. The extraction temperature is 60 ^oC for Flos Sophorae and 70 ^oC for Saururus; 3. the extraction efficiency obtained by [Bmim]Br was quite close to to that obtained by methanol.	Zeng *et al*., 2010
magnolol and honokiol (IL-UAE[1])	$[Bmim][BF_4]$ $[Bmim][PF_6]$	1.An aqueous solution of $[Bmim][PF_6]$ was more efficient than the $[Bmim][BF_4]$ aqueous solution (48.6% and 45.9% higher, respectively) and the traditional ethanol reflux extraction (16.2% and 13.3% above, respectively) with a short extraction time; 2.The optimized conditions were 2.0 M $[Bmim][PF_6]$, sonication power: 200W for 30 min; 3.The change of pH had no effect on the extraction efficiency.	Zhang and wang, 2010
hydrolysable tannin	*N'N*'-dimethylammonium *N'N*'-dimethylcarbamate (DIMCARB)	1.The extraction efficiency was significantly higher compared to traditional extraction methods with water; 2.The ILs method was more selective for ellagic acid and total tannins; 3.The ILs extracted materials possessed good shelf life and fungal growth resistance.	Chowdhury *et al*., 2010
lignins			
lignin from wood	[Mmim][$MeSO_4$] [Emim][OAC] [Amim]Cl [Bmim] with anions ([Cl], $[BF_4]$, $[PF_6]$,][TfO]) [Bzmim]Cl	1. For lignin solubility [Mmim][$MeSO_4$], [Bmim][TfO] > [Emim][OAC], [Amim]Cl > [Bmim]Cl , [Bzmim]Cl; 2. For wood flour solubility: [Amim]Cl,[Bmim]Cl>[Bzmim]Cl>[Emim][OAC], while [Mmim],[$MeSO_4$] could not dissolve wood; 3.[Emim][OAC] selectively extracted lignin from wood flour and was easily reused, resulting in a highly concentrated solution of chemically unmodified lignin; 4.$[Bmim][BF_4]$ and $[Bmim][PF_6]$ were not effective at dissolving either lignin or cellulose.	Lee *et al*., 2009
lignin from sugarcane plant waste	[Emim][ABS], [Emim][XS],	1. An extraction yield exceeding 93% was attained at 170-190 ^oC; 2. The regenerated lignin showed a good preservation of structure and properties and the ILs could be recycled.	Tan *et al*., 2009
alkaloids			
phenolic alkaloids (liensine, isoliensine, neferine)	$[Emim][BF_4]$ [Bmim] with anions ([Cl], $[BF_4]$, [Br], $[PF_6]$)	1.The new method reduced extraction time (from 2 h to 90 s) and had remarkably higher efficiency (20-50% more) than the conventional heat-reflux extraction and regular MAE; 2.The anion had apparent effects on the overall extraction efficiency. [BF4] was more efficient than others for the target analytes. Increasing the alkyl chain length had no significant effect on the extraction	Lu *et al*., 2008

(IL-MAE[2])	[Hmim][BF_4] [Omim][BF_4]	efficiency; 3. Optimized conditions: 1.5 M [Bmim][BF_4] or 1.0 M [Hmim][BF_4], liquid/solid ratio 15:1for [Bmim][BF_4] and 10:1for [Hmim][BF_4], irradiation power 280 W, extraction time 90 s.	
piperine (IL-UAE[1])	[Hmim][BF_4] [C_4SO_3mim] [Bmim] with anions ([BF_4], [Br], [PF_6], [H_2PO_4])	1.Both the characteristics of anions and cations have remarkable effects on the extraction efficiency. The extraction ability of [Bmim][BF_4] was 60% higher than that of [Bmim][Br] and 80% than [Hmim][BF_4]; 2.With [Bmim] based ILs, the extraction ability followed the order: [BF_4]> [Br]> [H_2PO_4]> [PF_6]; 3.Optimized conditions: extraction time 30 min, 2.0 M [Bmim][BF_4], solid/liquid ratio 1:15.	Cao *et al*., 2009
N-nornucife rine, *O*-nornucife rine, nuciferine (IL-MAE[2])	[Omim][Br][Hmim][Br][E mim][Br] [Bmim] with anions ([Cl], [BF_4], [Br], [PF_6])	1.For extraction efficiency, the influence of anions was much greater than cations. The extraction efficiency of [Bmim]Br was improved by 80% compared with that of [Bmim][PF_6], whereas the difference between different cations was 30%; 2.With [Bmim] based ILs, the extraction ability followed the order: [Br]>[BF_4]>[Cl]>[PF_6]; 3.[Hmim][Br] solution has much stronger multi-interactions including H-bonding, n–π, ionic/charge-charge with alkaloids; 4.Optimized conditions: extraction time 60 s, 1.0 M [Hmim][Br], solid/liquid ratio 1:30, irradiation power 280W.	Ma *et al*., 2010
fangchino line and tetrandrin e (IL-UAE[1])	[Bmim][BF_4]	1.The proposed methods showed higher efficiency than ethanol- based UAE (ca 30% improved) and reflux ethanol extraction (ca 15% increased) in a shorter time; 2.Optimized conditions:1.5 M [Bmim][BF_4], sonication power 150 W for 40 min, pH 9.8.	Zhang *et al*., 2009
carbohydrates			
mono-, di- and poly-saccharides	[Bmim] with anionas([BF_4], [PF_6][dca]) [MOMmim] with anions ([Tf_2N], [BF_4], [dca], [TfO]) [MOEmim] with anions ([Tf_2N], [PF_6], [BF_4], [dca],], [TfO]) [EOEmim] with anions ([Tf_2N], [PF_6], [BF_4], [dca])	1.The solubility of glucose was influenced much more by the nature of the anion than that of the cation although the positive effect of the oxygenated side-chains could be observed: with the same anion, the solubility decreased in the order [dca]> [TfO]≥[BF_4]> [PF_6]> [Tf_2N]; 2.[dca] anion is highly effective, dissolving carbohydrates from glucose to starch and even cellulose in large amounts, attributed to the H-bond acceptor properties of the [dca] anion; 3. The solubility of sucrose in [dca] containing ILs was better than glucose; 4.The solubility of glucose in those ILs increased by a factor of 2.5-5 when the temperature increased from 40 ^oC (211 g L^{-1}) to 75 ^oC (405 g L^{-1}); 5. The solubility of glucose in ILs was much higher than in *tert*-butyl alcohol.	Liu *et al*., 2005

banana pulps	[Bmim]Cl	1. A clear 5 %(w/w) solution containing polyglucan, starch, amylopectin, sucrose, glucose and fructose in banana was obtained; 2.[Bmin]Cl showed higher solubility than Neat DMSO-d_6.	Fort *et al*., 2006
starch	[Bmim][Cl] [Bmim][dca], NH_4Cl-Urea, $CaCl_2$-Urea, DES made of ChCl (Urea, succinic acid, maleic acid, citric acid, oxalic acid, phenylacetic acid, $ZnCl_2$)	1.Starch was soluble at 80 ^oC in [Bmim]Cl, [Bmim][dca] in concentrations up to 10% (w/w); 2.Starch was also soluble in the following DES ChCl-oxalic acid, ChCl-citric acid, ChCl-$ZnCl_2$, ChCl-Urea and $CaCl_2$-Urea.	Biswas *et al*., 2006
cellulose	[Hmim]Cl [Omim]Cl [Bmim] with different anions(Cl, Br, [SCN], $[BF_4]$, $[PF_6]$)	1.On heating to 100-110 ^oC, cellulose slowly dissolved in the $[Cl]^-$, $[Br]^-$,and $[SCN]^-$containing ILs; 2.Microwave heating significantly accelerated the dissolution process, reaching a solubility of up to 25% in [Bmim]Cl;3. The solubility of cellulose was influenced by the nature of anions, in [Bmim] based ILs, the solubililizing power followed the trend: [Cl]>[Br], [SCN]	Swatloski *et al*., 2002
wood from southern yellow pine and red oak	[Emim][OAC] [Bmim][Cl] [Amim][Cl]	1.Both types of soft wood were completely dissolved in [Emim][OAC] by heating in an oil bath after mild grinding; 2.[Emim][OAC] is a better solvent than [Bmim][Cl] for dissolving wood; 3.Microwave and ultrasound can accelerate the dissolution. Variables such as type of wood, initial wood load, particle size, affect dissolution; 4.Carbohydrate-free lignin and cellulose-rich materials can be obtained by using proper reconstituted solvents.	Sun *et al*., 2009
microcrystalline cellulose	[Bmim] with different anions ($[CH_3COO]$ $[HSCH_2COO]$ $[HCOO]$ $[C_6H_5COO]$ $[H_2NCH_2COO]$ $[HOCH_2COO]$ $[CH_3CHOHCOO]$ $[N(CN)_2]$)	1.The solubility of cellulose in [Bmim] based ILs decreased in the order: $[CH_3COO]$> $[HSCH_2COO]$>$[HCOO]$>$[C_6H_5COO]$>$[H_2NCH_2COO]$>$[HOCH_2COO]$>$[CH_3CHOHCOO]$>$[N(CN)_2]$. Cellulose could dissolve in $[Bmim][CH_3COO]$ at 40 ^oC, but not in [Bmim][$N(CN)_2$] even at 70 ^oC;2. The addition of lithium salts increased the solubility of cellulose; 3.Cellulose was regenerated by adding water or ethanol and the regenerated cellulose had good thermal stability; 4. The solubility test was performed at 40-70 ^oC with stirring under argon atmosphere.	Xu *et al*., 2010
cellulose from bran	$[MeHPO_4]$ with cations ([Emim], [Amim], [Pmim],	1.The extraction efficiency of polysaccharides varied according to the cation (19% difference) and anion structures (23% difference); 2.The extraction efficiency depended on the viscosity of ILs within a limited time span, following the order: [Emim]>[allylmim]>[pmim]>[Bmim]	Abe *et al*., 2010

	[Bmin]) [Emim] with anions ([$EtHPO_4$] [$EtHPO_4$] [$pHPO_4$][$bHPO_4$][H_2PO_4])	([Me_2PO_4] as the anion); [PO_4]>[Et_2PO_4]> [pPO_4]> [bPO_4] ([Emim] as the cation) (the β values of those ILs were similar); 3.[Emim][H_2PO_4] with the lowest viscosity, extracted a series of polysaccharides including cellulose, hemicelluloses, and residual starch from bran without heating with a 40% extraction ratio within 10 min.	
wool keratin fibers	[C_4mim]with anions([Cl], [Br],[BF_4], [PF_6]) [C_2mim][Cl]	1. Temperature has a strong effect on the solubility; 2.Fibers showed a better solubility in ILs with [Cl] than [Br] and were not soluble in ILs with non-coordinating anions, such as [BF_4], [PF_6].	Xie *et al*., 2005
essential oils and sesquiterpenes			
essential oil from dried fruits	[Bmim][PF_6]	1. IL-MAE showed higher efficiency (15 min at the microwave power of 440 W), a lower ratio of oxidized compounds (decreasing from 57% in distillation method to 29%), compared with the water distillation method; 2.Obvious differences were observed in the content of identical compounds and in the type of constituents obtained with the two methods.	Zhai *et al*., 2009
linalool from citrus essential oil	[Emim][Meesu]	[Emim][Meesu] exhibited high selectivity and efficiency (extraction ratio close to 1).	Francisco *et al*., 2010
artemisinin	[DMEA][Oct] [BMOEA][Tf_2N] [DMMOEA][Pro]	1.[DMEA][Oct] and [BMOEA][Tf_2N] showed the best performance; 2.Optimized conditions: liquid/solid ratio 6.3:1 (w/w) for [DMEA][Oct] and 0.9:1 for [BMOEA][Tf_2N] at 25 ^oC in 30 min.	Lapkin *et al*., 2006
others			
active extracts	[Mmim][lactate](1); 1-methyl-3[(pentyloxy) methyl]-1Hmimidazol-3-ium[BF_4](2); 1,3-bis (butoxymethyl)-1H-imidazol-3-ium[BF_4](3); 1-[(nonyloxy)methyl]-1H-imidazol-3-ium	1.The type of ILs played a significant role in the yield and composition of the extract and the protic ionic liquid (4) was the most efficient extracting agent, being superior to classical solvents such as EtOAc or hexane; 2.The extracts showed high insecticidal activities of similar potency as the standard pesticides and significant activity against bacteria, yeast and moulds.	Cieniecka-Roslonkiewicz *et al*., 2007

3.2. Application of ILs and DES in liquid-liquid extraction of natural products

The use of solvents for liquid-liquid extraction depends on their physical properties, such as viscosity, density and miscibility. It is convenient to select solvents with low viscosity to facilitate mixing but with a large density

difference for the separation process (Huddleston, *et al*., 2001). The hydrophilicity of ILs is an important factor determining its water miscibility and extraction efficiency. ILs can be hydrophobic or hydrophilic depending on the structure of cations and anions, though the anion seems to be more important. Those based on lower H-bond accepting strength anions ($[PF_6]$, $[Tf_2N]$ and $[BF_4]$) are normally water immiscible, therefore, they are the solvents of choice for forming biphasic systems in most IL extraction applications (Wang, *et al*., 2005; Soto, *et al*., 2005; Katase, *et al*., 2007). However, hydrophilic ILs such as with [Cl] or [I], can be induced to form aqueous biphasic systems when in contact with concentrated solutions of the water-structuring salt, K_3PO_4. This new system can be utilized to recycle hydrophilic ILs from aqueous solutions (Gutowski, *et al*., 2003). The viscosity and hydrophobicity of ILs also increases with the length of the alkyl chain ILs-based aqueous two phase systems are great candidates for the replacement of volatile solutions in typical liquid-liquid extraction due to their high extraction efficiency for different kinds of compounds, as listed in table 5.

Phenolics The distribution ratios of phenolic compounds are influenced significantly by the pH of the aqueous phase, nature of the IL and chemical structure of the phenols among other factors. Most of the phenolic compounds were extracted quantitatively from an aqueous solution into ILs at pH< p*K*a, when they were in the neutral form. Furthermore, picric acid was recovered into the ILs phase at high pH values (Khachatryan, *et al*., 2005). In a basic solution, phenols are ionized, and ion-exchange occurs during the separation. The H-bonding between anions and phenols is the main force for the extraction of phenols from water to ILs and those made of [Cl] combined with long chain imidazolium derivatives are a good choice. The higher extraction efficiency observed with ILs with $[BF_4]$ instead of $[PF_6]$ (Vidal, *et al*., 2004; Fan, *et al*., 2008) is due to the fact that $[BF_4]$ contains a more effective negative charge than $[PF_6]$ (Tsunekawa, *et al*., 2003) and is thus more hydrophilic and less viscous (Vidal, *et al*., 2004). The partition ratio of phenols between IL and water increases with the increasing length of the side chain in imidazolium, the effect of cations on the separation ratio between the two phases can be ascribed to hydrophobic interactions (Fan, *et al*., 2008). The extraction efficiency of phenols from water by a given IL is also affected by the structure of phenols and decreases in the order: 4-nonylphenol>4-octylphenol>phenol. Other factors that affect the extraction ratio are the proportion ILs to aqueous solution and the initial concentration of the solute in aqueous solution.

Alkaloids and heteroaromatics In the case of alkaloids, the separation mechanism varies according to their basic or neutral nature. For alkaloids such as caffeine and nicotine, the interactions between the cations and solutes are the main force because of their H-bonding acceptor property (Freire, *et al*., 2010). On the other hand, interactions between anions and solutes are the main forces for the partitioning of neutral alkaloids such as carbazole, because of their H-bonding donor property (ILs with [Cl] are effective) (Xie, *et al*., 2008). Another mechanism to consider is the salt-in effect of anions that is more effective in the

extraction of alkaloids from aqueous solution to ILs than salt-out ions (Freire, *et al*., 2010). For other heteroaromatic compounds, such as dibenzothiophene and aromatic carbohydrates, the interactions between cations and solutes are the main force for partition. Pyridinium and imidazolium ILs are more efficient than ammonium ILs, although ammonium ILs show high selectivity for aromatic hydrocarbons (Arce, *et al*., 2009). Addition of an alkyl group to the pyridinium ring increases the partition ratio with water (Holbrey, *et al*., 2008).

Organic acids The extraction ratio of organic acids is strongly affected by the pH of the aqueous phase and the chemical structure of the ILs. Similarly to phenolic compounds, organic acids can be extracted quantitatively from an aqueous solution at a pH below their p*K*a. [Bmim][PF_6] is a good solvent for organic acids because it is less viscous than [Omim][PF_6] and more hydrophobic than ILs with [BF_4]. No obvious effects due to temperature and ionic strength of the aqueous phase were reported.

Amino acids The pH value of the aqueous solution has a great effect on the characteristics of amino acids, as their charge state changes with increasing pH of the medium from cationsto zwitterions and anions successively. The higher extraction efficiency corresponds with the range in which the cationic form of the respective amino acids dominates. Arg and Lys can form cationic and dicationic species, why the pH range of efficient extraction of these amino acids is much broader. Crown ethers play an important role as a complexing reagent and H-bonding occurs between the ammonium center of cationic amino acid and the polyether. Unlike conventional organic solvents, it is not necessary to add counter-ions in the presence of a crown ether with [Bmim][PF_6] as a partitioning solvent from aqueous solution (Smirnova *et al*., 2004). The complexes of AAILs with copper enabled the separation of racemic amino acids, which was achieved through a chiral ligand-exchange mechanism (Tang *et al*., 2010).

Proteins For the extraction of proteins, pH, extraction time, and the volume of the ILs affect the extraction efficiency. The hydrophobic interactions, the electrostatic interaction and salting-out effects are important driving factors for the extraction of proteins from aqueous solution. Different types of ILs can be used for the extraction of proteins from aqueous solution, such as imidazolium based ILs with [Cl], [Br], [PF_6] anions.

Esters For the extraction of esters from organic solvents, larger distribution ratios were obtained with long chain cations. With the same cation, [PF_6] was more efficient than [BF_4] because of the hydrophobic property of [PF_6]. For example, [Omim][PF_6] proved to be a good choice for fatty acid esters such as omega-3 fatty acid methyl esters (Li *et al*., 2009).

Glycerol Deep eutectic solvents made of glycerol and choline chloride mixtures were used to extract glycerol from biofuel. A 1:1 ratio was better than 1:1.5, 1:2 due to the fact that a DES with less glycerol have a greater tendency

to attract glycerol molecules to form a DES with a higher ratio of glycerol (Hayyan *et al*., 2010).

Overall, except in case of glycerol, the pH value of the aqueous solution, the structures of the ILs and the addition of salts play an important role in the distribution of solutes between an IL and an aqueous solution.

Table 5. Application of ionic liquids (ILs) and deep eutectic solvents (DES) in the liquid-liquid extraction of different types of natural products.

extractants	ILs/DES	conclusions	Ref.
phenolic compounds			
endocrine-disrupting phenols	$[C_n mim][BF_4]$ (n=6,8) $[C_n mim][PF_6]$ (n=4,6,8)	①In the range of pH<7, most of the endocrine-disrupting phenols were extracted quantitatively from aqueous solution into the ILs; ②In the same acidic conditions, the extraction efficiency for a given phenol decreased in the order: [C8mim]>[C6mim]>[C4mim]; [BF4]>[PF6]; with a given IL extraction decreased in the order: 4-nonylphenol>4-octylphenol>phenol; ③The distribution ratio decreased slightly with as the phase volume of water/ILs increased, the best being 1:1; ④The distribution ratio of phenols increased with the increasing concentration of salts, such as $ZnSO_4$, Na_2SO_4, $Al_2(SO_4)_3$ and $NaClO_4$ in the aqueous phase; ⑤Compared with benzene and dichloromethane, the distribution ratio of the endocrine-disrupting phenols into $[Omim][BF_4]$ was about 496 times as high as that into dichloromethane under the same conditions; ⑥ There was no influence from the temperature.	Fan, *et al*., 2008
p-hydroxybenzoic acid	$[C_n mim][BF_4]$ $[C_n mim][PF_6]$ (n=6-10)	ILs with the $[BF_4]$ anion were more efficient in extracting *p*-hydroxybenzoic acid from an aqueous phase than $[PF_6]$.	Vidal, 2004
vanillin	$[C_4 mim]$ with anions([Cl],[dca],[Br],$[CH_3SO_4]$,[TfO],$[CH_3SO_3]$,$[CH_3CO_2]$), [Cl] with cations ($[C_n mim]$(n=2-10), $[OHC_2 mim]$, $[C_7H_7 mim]$)	① The extraction ability of ILs (with [Cl] as anions) followed the order: $[C_6 mim]$>$[C_4 mim]$> $[C_7H_7 mim]$ >$[C_7 mim]$>$[C_2 mim]$>$[OHC_2 mim]$>$[C_{10} mim]$; the extraction ability of ILs (with $[C_4 mim]$ as the cation followed the order: [Cl] > [dca] > [Br] > $[CH_3SO_4]$ > [TfO] > $[CH_3SO_3]$=$[CH_3CO_2]$, though the influence of cations was lower than that of anions; ②Temperature greatly affected the vanillin partition, and room temperature was the best for $[C_4 mim]$Cl; ③The partition coefficient of vanillin increased with the increase of the initial concentration of vanillin.	Cláudio, *et al*., 2010.
phenols	[Bmim] $[PF_6]$	①4-nitrophenol, 2,4-dinitrophenol, 2,6-dinitrophenol, 1-naphthol, 2-naphthol,4-chlorophenol, were extracted nearly quantitatively from aqueous solutions into the ILs at pH<p*K*a; ②Some phenols showed a high recovery ratio even at a pH of up to 12; ③The recovery	Khachatryan, *et al*.,

		of pyrocathechol and resorcinol is lower (20-58%).	2005
heteromatic compounds			
Neutral nitrogen compounds	[Bmim][Cl]	[Bmim][Cl] was found to be a very promising candidate for the extraction of neutral *N*-containing aromatic compounds from diesel with a high selectivity and up to 50% of indole and carbazole obtained in one step.	Xie, *et al*., 2008
Caffeine and nicotine	[Emim]with anions([$MeSO_4$] [$EtSO_4$] [Cl] [OAC] [TfO]) [Bmim]with anions([Me SO_3][Br][Cl][TfO][CF_3CO_2]) [$C_n$mim]Cl(n=2-12) [C_4C_1min][Cl] [C_7H_7mim][Cl] [OHC_2mim][Cl	①Nicotine showed higher partition coefficients compared to caffeine in all tested ILs because of its lower polarity ; ②The difference in partition coefficients between the cations (100%) was larger than the anions (50-60%); ③[C_4C_1min][Cl], [C_7H_7mim][Cl], [OHC_2mim][Cl] were the most efficient ILs because of multi- interactions between cation-solutes; ④[Cl],] [TfO], [CF_3CO_2] and [OAC] were effective anions because of the salting-in effect.	Freire, *et al*., 2010
Dibenzothophene	[Bpy] with anions([NTf_2][BF_4]) [Bmim] with anions ([BF_4][$C_8H_{17}SO_4$][TfO] [PF_6][SCN][OAC][Tf_2 N]) [BM_4Py] with s([BF_4][TfO] [SCN][TfO][Tf_2N]) [BM_3Py] with ([BF_4] [SCN][TfO][Tf_2N])	① The partition ratio of dibenzothophene to the ILs with the same anion increased in the order: [dimethylPy]>[MPy]>[Py]=[imidazolium]= [pyrrolidinium]; ②There was no significant difference in partition ratio between different anions.	Holbrey, *et al*., 2008
Aromatic hydrocarbons (benzene)	[Tf_2N] with cations like ([C_2mim], [C_2py][$N_{111}(C_2OH)$], [P_{66614}])	① No difference in efficiency was observed between the [C_2mim] and the [C_2py]; ② [$N_{111}(C_2OH)$] presented high selectivity but a low distribution ratio.	Arce , *et al*., 2009
organic acids			
ferulic acid (FA) and caffeic acid (CA)	[Bmim][PF_6] [Hmim][PF_6]	①Changes in temperature and type of inorganic salts had no obvious influence on the extraction ratio; ②The pH value had a big influence on the extraction efficiency, the appropriate pH value for FA was below 3.67, and for CA below 3.71; ③[Bmim][PF_6] showed higher extraction efficiency than [Hmim][PF_6]; ④The extraction efficiency of FA was higher than that of CA.	Yu, *et al*., 2007
3-indole butyric acid	[Bmim][PF_6] [Hmim][PF_6]/ [BF_4]	①Quantitative extraction was achieved with a pH below the p*K*a of IBA, ②With [Bmim][PF_6], higher extraction efficiency was achieved; ③No obvious effect from	Absalan, *et*

(IBA)	[Omim][PF_6]/ [BF_4]	temperature and ionic strength of aqueous phase was observed.	*al*., 2008
benzoic acid	[Bmim][PF_6]	With acetonitrile as a dispersing solvent, a high extraction ratio and a 96.6-99.3% recovery rate of benzoic acid from aqueous solution were obtained.	Zhang, *et al*., 2010
amino acids			
Trp, Gly, Ala, Leu	[Bmim][PF_6]	①Amino acids were extracted efficiently at low pH values of 1.5-4.0 (Lys and Arg at pH of 1.5-5.5); ②The most hydrophilic amino acid ,i.e., Gly was extracted as efficiently as the less hydrophilic amino acids (92-96%); ③Extraction of amino acids into ILs without crown ether is rather low, 5-10%, while quantitative extraction obtained with 0.05-0.1molL^{-1} crown ether was very high; ④No counter ions were needed.	Smirnova, *et al*., 2004
D/L-phe, racemic (D,L-Phe, D,L-Tyr, D,L-His)	Amino acid based ILs (AAILs) [$C_n$mim][L-Pro](n=4,6,8)	AAILs were efficient extraction solvents, showing enantioselectivity in amino acids extraction; more L-enantiomers of amino acids were extracted in the ILs phase than D-enantiomers, with a 50% enantiomeric excess value in a single-step extraction.	Tang, *et al*., 2010
proteins			
proteins from body fluids	[Bmim][Cl] with K_2HPO_4	①A distribution ratio of 10 was obtained with [Bmim][Cl]; ②Addition of an appropriate amount of K_2HPO_4 to the aqueous phase increased the distribution ratio ytwo-fold; ③No chemical (bonding) interaction was observed between ILs and protein and the electrostatic potential difference between the two phases and salting out effects facilitated the partition.	Du, *et al*., 2007
cytochrome C(Cyt-c)	[Bmim][PF_6]	①At pH 1, an extraction efficiency of 85% for Cyt-c from the aqueous solution was achieved; ②The high extraction yield was based on the exposure of hydrophobic groups of heme and a vacant position from the cleavage of the sixth coordinating bond of the ion at low pH value; ③ pH, extraction time, volume of the ILs affect extraction efficiency.	Cheng, *et al*, 2008
hemoglobin	[BTmsim][PF_6] [BBim][PF_6] [Bmim][PF_6]	① [btmsim][PF_6] showed high selectivity and efficiency in quantitative extraction of hemoglobin from an aqueous phase in the presence of other proteins at pH 7; ②The high selectivity and extraction ability were attributed to the interaction between the iron atom in the heme group of hemoglobin and the cation in ILs as the *penta*-coordinated ferrous atom provides a vacant binding site for the cationic [btmsim] moiety; ③A back extraction efficiency ca 80% for hemoglobin in ILs was achieved with sodium dodecyl sulfate solution as stripping reagent.	Cheng, *et al*., 2008

bovine serum albumin, trypsin, cytochrome C and γ-globulin	[Bmim]Br, [Hmim]Br, [Omim]Br	①It is noted that 75-100% of the protein could be extracted into the ILs phase in a single-step extraction; ②The extraction efficiency of protein increased with increasing temperature and the alkyl chain length of cation in ILs following the trend: [Omim]Br>[Hmim]Br>[Bmim]Br; ③The conformation of the protein was not affected after extraction into the ILs phase and 88-90% of the enzyme activity was maintained; ④ Hydrophobic interaction was the main driving force, although the electrostatic interaction and salting-out effects were also important factors.	Pei, *et al*., 2009
ester			
omega -3 polyunsaturated fatty acid methyl esters (PUFAM	[$C_n$mim] [BF_4] [$C_n$mim] [PF_6] (n=2,4,6,8)	①The extraction ratio of PUFAMEs increased significantly with an increase in the degree of unsaturation of PUFAMEs; ②The extraction ratio reached the optimum with [C_8mim][PF_6], which is about 5 times higher than [C_8mim][BF_4] and 2 times higher than [C_4mim][PF_6].	Li, *et al*., 2009
lovastatin and simvastatin	[C_6min][PF_6]	①The established ultrasound assisted ILs disperse extraction method from spiked aqueous solutions was applied to three water samples with recoveries in the range of 80.5-112.0%; ②The volume ratio of IL to aqueous solution played an important role in the extraction efficiency, 1:100 being the best; ③The extraction efficiencies reached an optimum witha pH value of the aqueous solution of 6.0.	Mao, *et al*., 2009

3.3. Extraction mechanism (Features of ILs that affect their extraction efficiency)

Considering information provided above, in this section some more general aspects of ILs as extraction solvents will be discussed. First, as in all dissolution processes, the interaction between solutes and solvent are the main driving force for extraction. Hydrogen bonding, misfit and Van der Waals interaction energy have been shown to effectively characterize the complex multiple interactions in the IL system. Thus the chemical structures of ILs (including the kind of anion, cation, and the alkyl chain length of the cation) have a significant influence on the extraction yield of analytes, owing to their distinct multiple interactions (Anderson *et al*., 2002). Second, the viscosity of ILs has to be considered as it has a great effect on the diffusion of solutes. Lastly, both the pH of the aqueous phase and the salting-out/in effect have to be taken into account since they could play an important role in liquid-liquid extraction.

Anion The extraction efficiency of most compounds is anion dependent. Solutes are strongly solvated, principally by forming H-bonds with the anions of the ILs (Hanke *et al*., 2002). COSMO-RS computation simulation revealed that the solubility of flavonoids in the same class of ILs was strongly anion-dependent (Guo *et al*., 2007). The above-mentioned experiments showed [Br], [BF_4], [Cl], [H_2PO_4], [SO_4], [DCA], [OAC] to be the most commonly used anions in the extraction of phenolic compounds, alkaloids, lignin and carbohydrates, all of which are hydrogen-bond donors. In the extraction of lignin, cellulose and starch from biomass, the H-bonding between solute and solvent molecules should be strong enough to break down inter- and intra-molecular H-bonds in the biomass and only ILs with anions with a strong H-bond accepting ability work. ILs with anions such as [Cl], [OAC], [alkylHPO_4] with a β value of around 1 are efficient solvents, whereas ILs with [Br], [BF_4] are inefficient. The same applies to DES formed between choline chloride and oxalic acid/citric acid, which were able to dissolve starch (Biswas *et al*., 2006). In the liquid-liquid extraction of neutral alkaloids and phenols from water, the H-bonding donor interactions between anions and solutes are the prevailing forces.

Cation The cation species also has an effect on the extraction efficiency. ILs which have cations with an electron-rich aromatic π-system produce stronger interactions with polarizable solute molecules, such as π–π and n–π interactions (Anderson *et al*., 2002; Crowhurst *et al*., 2003). The extraction yield of polyphenolic compounds was a little higher for [BPy]Cl than [Bmim]Cl, and lowest for $(CH_3)_4NCl$, because the *N*-butylpyridinium cation has a more aromatic character than the imidazolium based ILs, whereas the ammonium cation does not give π–π and n–π interactions (Du *et al*., 2009).

In the ILs aqueous two-phase systems, the extraction efficiency for heteroatomic compounds depends more on the nature of the cations than that of anions. ILs with the cations like imidazolium and pyridinium were effective in extracting heteroatomic compounds such as caffeine and nicotine from water. This was found to be driven by different factors including: 1) π-π interaction between the aromatic part of the solutes and the cations; 2) hydrogen-bonding interaction between solutes (the non-bonded electron pair in oxygen and nitrogen atoms) and acidic hydrogen atoms present in the cations of the ILs; and 3) dispersion-type interactions between alkyl groups of the solutes and the alkyl side chain of the cations. The influence of such factors are supported by the high extraction ability of [C_7H_7mim][Cl], [OHC_2mim][Cl], [C_4C_1mim][Cl] for caffeine and nicotine (Freire *et al*., 2010) and the increasing extraction capability of ILs with cations in the sequence[Py> [MePy]methylpyridinium > [dimethylPy] for dibenzothiophene (Holbrey *et al*., 2008). Furthermore, the aromaticity factor plays a more important role than the ring size; pyridinium and immidazolium based ILs showed the same extraction efficiency in the isolation of dibenzothiophene from dodecane (Holbrey *et al*., 2008).

The alkyl chain of the cation The melting point of ILs can be tuned by changing the length and symmetry of the alkyl chain of cations. Low symmetry

in substitution and a longer alkyl chain within a certain range leads to a low melting point. In the case of imidazolium cations, C_8 gives the lowest melting point (Zhang *et al*., 2008). Thus, ILs applied to the extraction of natural products have ([$C_n$mim], n = 2-8) cations, and and C_8 gives the lowest T_m (Zhang *et al*., 2009). The alkyl chain length of the cation affects the extraction yield of some natural products as its hydrophobicity increases with the increasing alkyl chain length. Apart from the hydrophobicity, viscosity also increases, so that while the longer chain length benefits the extraction of some middle to less polar compounds the corresponding increased viscosity limits the diffusion of compounds. ILs with a cation such as [Bmim] are most often used for mid-polar compounds and [Hmim] for non-polar compound (Du *et al*., 2009; Cao *et al*., 2009; Fan *et al*., 2009; Li *et al*., 2009).

The viscosity of ILs is strongly influenced by the cation and anion species. The longer the substituted chain of the cation, the more viscous the ILs. The high viscosity is one of the greatest obstacles for the application of ILs in natural products extraction. Two efficient ways are used to decrease their viscosity: increasing the temperature through heating or microwaves (Swatloski *et al*., 2002; Zhai *et al*., 2009) and dilution with water or organic solvents such as ethanol. To date, the most common solution has been the latter. The extraction ratio of compounds can be increased by 60-90% by adjusting the concentration of ILs (Cao *et al*., 2009; Du *et al*, 2007; 2009) showing that this factor is crucial since it balances not only the viscosity but also the extraction ability of the ILs solutions. The optimal concentration of ILs differs according to the type of ILs, the targeted compounds and the biomass (Lu *et al*., 2008; Zeng *et al*., 2010).

pH In liquid aqueous two- phase systems, the pH of the aqueous phase is an important factor since it can influence the existing form of ionic or ionizable compounds affecting thus, the extraction efficiency of organic acids, phenolic compounds, amino acids and proteins among others (Tzeng *et al*., 2008; Cheng *et al*., 2008). For example, ILs easily extracted organic acids or phenolic compounds in the neutral form (Yu *et al*., 2007; Fan *et al*., 2008) and amino acids as cations (Smirnova *et al*., 2004) from an aqueous phase.

Salting-out/in effect Another important factor affecting the extraction efficiency in liquid aqueous two-phase systems is the concentration of some inorganic ions, which can be explained by a salting-out effect. When salts are dissolved in water, they use up molecules of water for their solvation, reducing the concentration of free water, and increasing the relative concentration of solutes in the aqueous solution allowing them to be preferably extracted into the ILs phase. The stronger the hydration ability of a salt, the stronger its salting-out effect. The distribution ratio of phenols between ILs and aqueous phase increases as the concentration of salt present in the aqueous phase increases (Fan *et al*., 2008).

Concerning the influence of the ILs anion, there seems to be a similar salting-in/out behavior of the anions from ILs. Generally, salting-in inducing anions (low charge density ions) are more efficient at extracting solutes from a second liquid phase than salting-out (high charge density ions) inducing anion. For salting-in inducing anions the tendency to form hydration complexes is marginal and thus they tend to stabilize the solutes in solution by specific ion binding to the solute; On the other hand, salting-out inducing ions have a great tendency to form a hydration complex, and thus do not have interaction with solutes (Freire *et al*., 2009). With the same cation, ILs with [Cl], [Br], $[CH_3SO_4]$, $[CF_3SO_3]$ are more efficient than ILs with $[CH_3SO_3]$, $[CH_3CO_2]$ in extraction of vanillin (Cláudio *et al*., 2010), and ILs with [Cl], $[CF_3SO_3]$, $[CF_3CO_2]$ are more efficient than ILs with $[CH_3SO_3]$, $[CH_3SO_4]$ in the extraction of alkaloids (Freire *et al*., 2010).

The observations are important to achieve an improved understanding of the ILs solvation properties and reflect their versatility and their potential for tailoring the composition of IL for the efficient extraction of the desired natural compounds.

3.4. Combination of ILs with other technologies

3.4.1 Microwave-assisted extraction

ILs can efficiently absorb and transfer microwave energy (Hoffmann *et al*., 2003), and thus be employed to rapidly heat solvents and co-solvents in microwave-assisted extraction (MAE). MAE is rapid and effective compared to traditional extraction techniques (Zeng *et al*, 2010) and easy and cheap compared with other modern extraction techniques. Recently, microwave coupled with ILs aqueous solutions or pure ILs were evaluated for the extraction of some polyphenolic compounds (Du *et al*., 2007; 2009; Zeng *et al*., 2010), alkaloids (Lu *et al*., 2008; Ma *et al*., 2010) and essential oils (Zhai *et al*., 2009) from medicinal plants. Compared to conventional heat-reflux extraction (HRE), MAE had the advantage of remarkably higher efficiency (20–50% higher) and shorter extraction time (from 2 h to 90 s) (Lu *et al*., 2008).

The important parameters of the MAE procedure include the sample size, liquid-solid ratio, extraction time and temperature. The extraction rate of phenolic alkaloids with $[Hmim][BF_4]$ increased when the liquid-solid ratio was raised from 5:1 to 10:1 and decreases dramatically when the ratio further increases to 20:1. An extraction time of around 8-12 min was sufficient to obtain high extraction yields of polyphenolic compounds from 0.2-0.5 g of plant powder with 20-35 mL of a 1.5-2.0 mol/L [Bmim][Br] aqueous solution (Zeng *et al*., 2010). Another aspect to be considered is thermal stability. Phenolic compounds were found to be stable up to 100 ^{o}C during MAE (Liazid *et al*., 2007), but with ILs as the solvent the stability temperature decreased to 60-80 ^{o}C and compounds with a larger number of hydroxyl substituents were more readily degraded under the extraction conditions (Du *et al*., 2009).

MAE is highly efficient as an extraction method due to its unique mechanism. In MAE extraction, the direct interaction of the microwaves with the IL solution and free molecular water present in the cell, results in the rupture

of the cells and release of intracellular products into the solvent. The surface of the plant material was observed to be greatly destroyed and the structure of the cell walls ruptured after IL-MAE (Du *et al*., 2009; Zeng *et al*, 2010). Moreover, ILs improved the transfer of energy from the microwaves to the sample, which increased the speed of energy transfer and thus extraction efficiency (Eskilsson and Björklund, 2000).

3.4.2 Ultrasound-assisted ionic liquid extraction

IL-based ultrasound-assisted extraction (IL-UAE) methods have been developed for the effective extraction of alkaloids (Cao *et al*., 2009; Zhang *et al*., 2009) and phenolic compounds (Zhang *et al*., 2010) from plant material. The extraction time is one of the most important factors and optimal extraction efficiency can be achieved in 30-40 min. For example, the extraction efficiency of the optimized IL-UAE approach increased the yield of piperine by ca. 30-45% as compared with UAE but with a conventional solvent (ethanol) (Cao *et al*., 2009).

4 Analysis methods for extraction by ionic liquids

4.1 HPLC (high-performance liquid chromatography)

In general, the sample preparation for the HPLC analysis of an IL extract is similar to that required for conventional solvent extracts, involving its dilution with either water or the HPLC mobile phase (Fu *et al*., 2006) or alternately its injection as such (Fan *et al*., 2009), after filtration through a 0.45μm filter. A reversed-phase HPLC method using a C_{18} column and acetonitrile and 0.6% acetic acid aqueous solution as the mobile phase was applied to analyze the polyphenolic compounds in IL extracts of medicinal plants, with no interference from the ILs being observed (Du *et al*., 2007; 2009). The method showed a good repeatability, precision and accuracy, and no degradation of the target compounds. The presence of ILs did not affect usual chromatographic parameters such as peak resolution, peak shape, elution order and retention times in the separation of three phenolic alkaloids (Lu *et al*., 2008). Cao *et al*. (2009) came to the same conclusion when analyzing a non-diluted IL piperine extract with UPLC and a C_{18} column. In another case, HPLC-DAD-ELSDA with a C_{18} was used to determine the esterification of flavonoids in ILs (Lue *et al*., 2008). All these studies show that ILs can be used as extraction solvents for the analysis of natural products from a biological matrix, without the need to eliminate the ILs opening interesting novel opportunities for natural products extraction for quantitative analysis.

4.2 NMR (nuclear magnetic resonance spectroscopy)

^{13}C NMR spectroscopy was applied to analyze carbohydrates extracted with ILs to detect their content, conformation and interactions. The direct measurement of IL banana pulp extracts, gave well-resolved signals in the ^{13}C NMR spectra corresponding to different sugars in the anomeric carbon region

that could be used for quantitative analysis (Fort *et al*., 2006). Solid-state ^{13}C NMR spectroscopy was used to monitor the extraction of lignin from lignocellulose material (Tan *et al*., 2009). It is known that ^{13}C NMR spectroscopy is a very useful tool in the study of the conformational preferences of repetitive polysaccharides (Swalina *et al*., 2001) but a drawback for its application to ILs extracts is their high viscosity because it reduces molecular tumbling, resulting in low resolution and sensitivity. However, the spectrum recorded at 90 ^{o}C showed baseline resolution for most signals (Moulthrop *et al*., 2005). NMR relaxation measurements of the ILs ^{13}C and $^{35/37}Cl$ nuclei provided a better understanding of the mechanism of solvation of cellulose, the destruction of β-(1→4)-linked glucose oligomers (Moulthro *et al*., 2005) and the formation of H-bonds between the carbohydrate hydroxyl protons and the ILs chloride ions (Remsing *et al*., 2006; Xu *et al*., 2010). Two-dimensional heteronuclear overhauser enhancement (HOESY) spectrum of choline fluoride and urea showed intense cross-correlation between the fluoride ion and the primary amine protons of urea revealing the existence of hydrogen bonding in the liquid (Abbott *et al*., 2003). Thus, IL extracts can be analyzed by NMR spectroscopy, but the various signals of the protons in the IL constituent will, in fact, overlap with sample signals in certain spectral regions.

4.3 IR (infrared spectra)

FTIR spectra can provide useful information for identifying the presence of certain functional groups, and chemical bonds in a molecule, or interactions in a system. It has been applied in the rapid and nondestructive identification and quantification of medicinal plants, the analysis of interactions between ILs and proteins or DNA (Wang *et al*., 2007; Zhuo *et al*., 2007) and the identification of some secondary metabolites, including lignin (Tan *et al*., 2009) and polysaccharides (Fort *et al*., 2007). FT-IR is a useful tool to provide a characteristic fingerprint, reflectance peaks between 1800 and 700 cm^{-1} were selected as representative peaks for known carbohydrate compounds.

4.4 Others

Circular dichroism (CD) spectroscopy measures differences in the absorption of left- and right-handed circularly polarized light as a function of the wavelength. It occurs in structurally asymmetric molecules when a chiral (optically active) chromophore is in an asymmetric environment. CD spectroscopy is particularly good for demonstrating the conformational change of structure of proteins (Cheng *et al*., 2008) and DNA (Mamajanov *et al*., 2010).

Scanning electron microscope (SEM) allows the observation and characterization of heterogeneous organic and inorganic materials at a nano- to micrometer scale. The popularity of SEM stems from its capacity of obtaining three-dimensional-like images of the surfaces of a very wide range of materials. XL-30 SEM spectroscopy was applied to identify the microstructure of plant material after MAE using a [Bmim]Br solution as a solvent and SEM images shows that the structure of the cell walls was ruptured and the microstructure of leaves and tubers was greatly destroyed (Fig. 1) (Du *et al*., 2009).

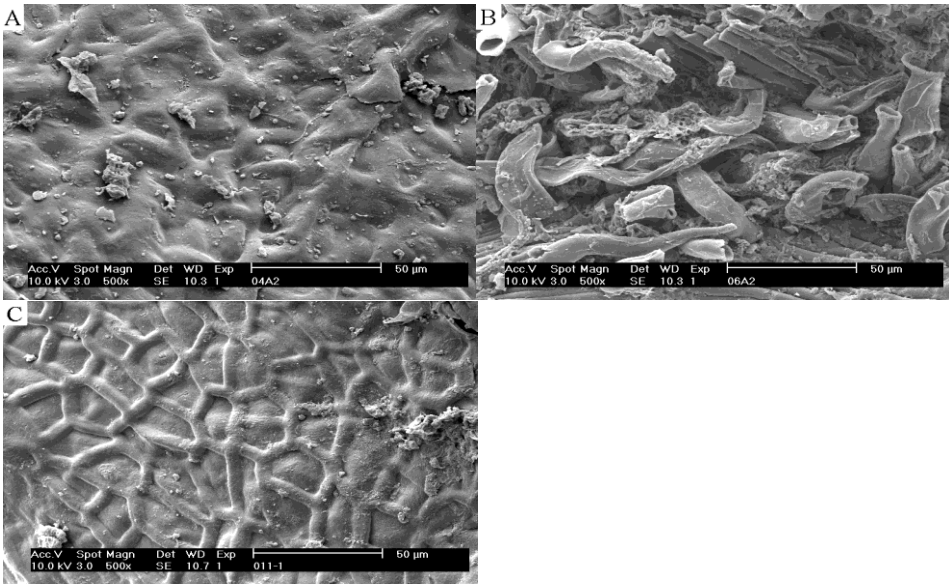


Fig. 1 Scanning electron micrographs of *Psidium guajava* leaves: (**A**) untreated *Psidium guajava* leaves; (**B**) after MAE for 10 min in [Bmim]Br and (**C**) heating extraction for 4 h. Image magnification is 500 for A, B and C (Du *et al*., 2009).

5 Recovery of compounds from ILs

The low volatility of ILs that on one hand makes them 'green' creates challenges for product separation and recovery. For volatile products, a back-distillation may be used to recover the product from ILs. In the case of a hydrophilic product in a hydrophobic IL, water may be used to remove the product from the ILs (Huddleston *et al*., 1998), but the biggest problem is to recover non volatile or thermally labile products from ILs. A number of methods have been used for the recovery of diverse compounds (carbohydrates, tannins, N-compounds, phenolic compounds) from diverse ILs (normal ILs, distillable ILs and DES).

5.1 Supercritical Carbon Dioxide

The low volatility of ILs that on one hand makes them "green" creates challenges for product separation and recovery. For volatile products, a back-distillation may be used to recover the product from ILs. In the case of a hydrophilic product in a hydrophobic IL, water may be used to remove the product from the ILs (Huddleston amd Rogers, 1998), but the biggest problem is to recover poorly volatile or thermally labile products from ILs.

Experiments using another type of "green" solvent, supercritical carbon dioxide (SC-CO_2), for product recovery from ILs solution have been reported. The volatile $scCO_2$ is insoluble in the non-volatile and polar ILs and they form two-phase systems. The principle of product recovery with these biphasic

systems is based on the solubility of the compounds in CO_2. During the process, high volatility and low polarity will favor the solubility of a solute in CO_2. Naphthalene was extracted from [Bmim][PF_6] using sc$CO_2$with recoveries of up to 94-96%, a near-quantitative recovery without detectable [Bmim][PF_6] in the extract (Blanchard *et al*., 1999). A variety of aromatic and aliphatic compounds were also quantitatively recovered from this IL with scCO_2without any IL contamination in the recovered product (Blanchard and Brennecke, 2001). In contrast, a mixture of CO_2 with conventional organic solvents results in a significant amount of the solvent in the CO_2-rich phase, due to their solubility. Diverse compounds such as *N*-acetyl-(*S*)-phenylalanine methyl ester and epoxides of olefins were extracted from ILs with scCO_2 by optimizing pressure, temperature and the type of IL, especially from enzyme-catalyzed reactions (Bortolini *et al*., 2003; Kroon *et al*., 2006). For example, 2-methylbutanoic acid that results from the asymmetric hydrogenation of tiglic acid in [Bmim][PF_6] was extracted with scCO_2. Moreover, the solution of the catalyst in [Bmim][PF_6] could be recycled without significant losses of both enantioselectivity and conversion (Brown *et al*., 2001).

The removal of a product from ILs by extraction with CO_2, however, only works for products that have a sufficiently high solubility in CO_2. For compounds that are insoluble in CO_2, crystallization from ILs with CO_2 as an anti-solvent has been explored. The addition of CO_2 lowers the solubility of the product in the ILs, thereby creating supersaturation (Kroon *et al*., 2006). With this method, removal of even solid, inorganic salts from [Hmim][Tf_2N] was achieved with CO_2 at 25 ^oC (Saurer *et al*., 2006). Strong hydrogen-bonding interactions between the ILs and the solutes make it more difficult to induce a separation. Further studies found that CO_2 acts primarily by disrupting the nonspecific interactions in the IL/organic mixtures (Mellein and Brennecke, 2007) and that the ability of CO_2 to act as an anti-solvent is highly dependent on the solubility of CO_2 in the IL/organic mixtures. Kroon *et al*. (2008) found that methyl-(*Z*)-*α*-acetamido cinnamate could be recovered from [Bmim][BF_4] by either shifting to a higher CO_2 concentration at a constant temperature (anti-solvent crystallization) or changing to lower temperatures at constant CO_2 concentration, showing the importance of exploring the effects of pressure, temperature and CO_2 concentration in each case.

The applicability of scCO_2 for extraction of solutes from an IL relies heavily on the phase behavior of the binary IL-CO_2 system. Solubility of CO_2 depends on the substituents on the cation and nature of the anion (Blanchard *et al*., 2001). Furthermore, experimental and molecular simulation studies found that the anion of the IL dominates the interaction with CO_2, with the cation playing a secondary role (Cadena *et al*., 2004). The solubility of CO_2 in the IL-rich phase was greater for IL with fluorinated anions, following the trend of [Bmim][PF_6] and [C_8mim][PF_6] > [C_8mim][BF_4] > [*N*-bupy][BF_4] > [Bmim][NO_3] > [Emim][$EtSO_4$] (Blanchard *et al*., 2001). The highest solubility of CO_2 is in ILs with anions containing fluoroalkyl groups, [TfO], [Tf_2N] while the least soluble in ILs with non-fluorinated anions, [NO_3] and [dca] (Aki *et al*., 2004). For the cations, CO_2 was more soluble in [Hmim][BF_4] than [Bmim][BF_4],

[Bmim][PF_6] than [Emim][PF_6] (Shariati *et al*., 2005). The solubility of CO_2 increases with the alkyl chain length at all pressures, with the increase being more apparent at higher pressure (Aki *et al*., 2004). In addition, the pressure and temperature also affect the solubility of CO_2 in ILs. The solubility of CO_2 in imidazolium-based ILs increases with increasing pressure and decreases with increasing temperature (Aki *et al*., 2004). Thus, by varying the substituents on the cation and nature of the anion, changing the pressure and temperature, one should be able to design ILs to achieve the desired phase behavior characteristics.

5.2 Anti-Solvents

In general, adding anti-solvent with agitation may result in the separation of solutes from ILs. Some examples use H_2O, ethanol, or acetone to isolate cellulose from [Bmim][Cl] or [Bmim][CH_3COO] (Swatioski *et al*., 2002; Xu *et al*., 2010), or H_2O, methanol, or acetone to isolate wool keratin [Bmim][Cl] (Xie *et al*., 2005), or mixtures of acetone-water (1:1) to isolate cellulose and lignins from [Emim][OAC] (Sun *et al*., 2009). Lignin was also separated by precipitation from [Emim][XS] by acidification to pH 2 at room temperature after which, the IL was recovered by neutralization, removal of water at reduced pressures and drying at 70 ^oC under high-vacuum conditions (Tan *et al*., 2009).

5.3 Re-crystallization

Re-crystallization by cooling or adding anti-solvent can be used to separate solution and target molecules. The addition of 1-butanol to [Gly][ChCl] with extra glycine and then subcooling to -20 ^oC, allowed the separation of choline chloride (Hayyan *et al*., 2010). Water was used as an anti-solvent (3:1 v/v H_2O to ILs) to partition and then recrystallize artemisinin from ILs; crystallization yielded 82% of the total extracted artemisinin and a 95% concentration of anhydrous artemisinin (Lapkin *et al*., 2006).

5.4 Back Extraction

Organic acids, *nitrogen-containing* compounds, phenolic compounds, and amino acids were removed from ILs by back extraction with aqueous solutions, making the necessary pH adjustments and the ILs were then quantitatively recovered after neutralization and evaporation of the solvent. For example, neutral *nitrogen-containing* compounds were recovered from [Bmim][Cl] by back extraction using water or methanol (Xie *et al*, 2008) and a 94-98% recovery of ferulic acid from [Bmim][PF_6] was achieved with a 0.02 M NaOH aqueous solution (Yu *et al*., 2007). In another case, 99% of phenols present were removed from [Bmim][PF_6] after back extraction with 0.1 mol L^{-1} NaOH aqueous solution (Fan *et al*., 2008) and amino acids were recovered from AAILs by adjusting the pH of the solution to 7.0 with HCl, thus neutralizing the amino group (Tang *et al*., 2010).

5.5 Chromatographic Techniques

In addition, some column chromatographic techniques have been developed for the separation and purification of compounds from ILs extracts. An anion-exchange resin has been successfully used in the isolation and purification of shikimic acid from [Bmim][Cl] extract with a 87% yield (Usuki *et al*., 2011). High speed counter current chromatography has been used in the separation and purification of three isoflavones from [C_8min][Br] extract with a purity above 95% (Sun et al., 2011). Non-porous membranes with a selective layer of hydrophilic or hydrophobic polymers ave been applied for the quantitative and selective recovery of solutes with different physicochemical properties from [Bmim][PF_6] (Schafer et al., 2001).

Thus, although ILs cannot be removed from extracts by evaporation, diverse methods have been employed succesfully to isolate compounds from ILs as described above. The solvents used in these processes are also green solvents such as SC-CO_2, water, or ethanol, ensuring the green extraction and isolation of natural products from live material with ILs. Furthermore, the ILs can also be reused several times decreasing the cost of the whole process.

6. Enzyme reactions in ILs and DES (factors affecting the enzyme activity in ILs and DES)

There have been many reports on enzyme reactions in ILs (table 6). Enzymes may exhibit enhanced activity, stability and selectivity in ILs, depending on the structure of ILs, properties, the concentration, water activity of the ILs, ILs coating effect and so on. Some enzyme reactions in DES have also been reported, as mentioned below.

6.1IL structure

The enzyme activity was found to be dependent on the type of anions, cations and their spatial configuration (Itoh *et al*., 2001). The enzymatic activity, stability and selectivity are greatly affected by the constituents of the ILs. [Bmim][BF_4] combined with acetone showed eight fold higher yields and three times faster reaction rates for the lipase-catalyzed (CaL B) synthesis reactions of ester of nucleoside drugs. However, the yield decreased drastically when the cationic part and anionic part were replaced, namely [Bmim][PF_6]and [Emim][BF_4] (Liu *et al*., 2006). The immobilized CaL B (*i*-CaL B) displayed enhanced activity when the alkyl chain of [$C_n$mim][BF_4] increased in length (n = 4-8) and no acylation reaction occurred in [C_4mim]Cl or [C_4mim]Br (Li *et al*., 2006). The mushroom tyrosinase treated with [Bmim][BF_4] retained a higher activity than that in [Bmim][PF_6] and [Bmim][$MeSO_4$](Yang *et al*., 2008). In addition, ILs with [PF_6] and [alkyl-SO_4] are also good solvents for some enzyme reactions. [Bmim][PF_6] and [Hmim][PF_6] were suitable reaction media for esterification of isoamyl acetate by CaL B (Fehér *et al*., 2008). The best synthetic activity was obtained when free CaL B was assayed in [CPMA][$MeSO_4$] (De Diego *et al*., 2009). [Emim][$MDEGSO_4$] was the most promising IL for laccase compared with [Emim][$EtSO_4$] and [Emim][$MeSO_3$] (Tavares *et al*., 2008).

Table 6. Examples of enzyme activities in ionic liquids (ILs) and deep eutectic solvents (DES).

enzymes	ILs/DES	reaction	conclusions	Ref.
lipase from *Candida Antarctica* (CaL)	[Bmim][PF_6]	acylation of L-carnitine with conjugated linoleic acid	the conversion in [Bmim][PF_6] was 2.13 and 1.56 times higher than that in acetonitrile and in solvent-free system, respectively.	Tian *et al*., 2010
	[$C_n$mim][dca], [$C_n$mim][Cl] (n=2,4,8) [$C_n$mim][PF_6] (n=4,6,8) [$C_n$mim][BF_4] (n=4,6) [$C_n$mim][Tf_2N] (n=2,4,6,8) [Bmim] with anions ([OSO_4], [$MDEGSO_4$] [NO_3][OAC])	synthesis of butyl butyrate by transesterification	1.CaLB exhibited greater stability in water-immiscible ILs than in water-miscible ILs. The highest activity was observed in [Omim][PF6]; 2.The activity of CaLB decreased following the trends: water-immiscible ILs(ILs with anions like [Tf_2N] and [PF_6]>hexane> water -miscible ILs (ILs with anions like [BF_4], [dca], [NO_3], [OAC], [OSO_4], [$MDEGSO_4$]), with the [Bmim] cation, the enzyme activity in [Tf_2N] is nearly 430 times higher than that in [OSO4]; 3.For ILs with the same anion, the synthetic activity was gradually enhanced by increasing alkyl length in cation: the enzyme activity in [Omim][PF_6] is nearly 6 times higher than that in [bmim][PF_6].	De Los Rios *et al*., 2007
	[Bmin][BF_4]/ [PF_6] [Emim][BF_4]	synthesis reactions of ester of nucleoside	Eight fold higher yields and three times faster reaction rates using a mixture solvent system composed of 90% acetone and 10% [Bmim][BF_4].	Liu *et al*., 2006
Immobilized Candida antarctica lipase B (i-CaLB)	[Emim][BF_4] [Bmim] with anions([HSO_4] [Cl] [Br] [NO_3] [BF_4])	enantioselective hydrolysis Of D,L-phenylglycine methyl ester	[Bmim][BF_4] is the most suitable IL for the reaction with the highest initial rate and enantioselectivity, while the reaction became much less active and enantioselective in the systems with [Bmim][HSO_4].	Lou *et al*., 2006
	Cation: $C_n$mim (n=4-8), Anion: BF_4, PF_6, Cl, Br	regioselective acylation of 1-*β*-D-arabinofuranosylcytosine	10% (v/v) [C_4mim][PF_6]-tetrahydrofuran gave the highest initial rate and substrate conversion.	Li *et al*., 2006
	[Bmim] with anions([PF_6][BF_4] [Cl]) [Hmim][PF_6] [Emim][tosy] [Mmim][$(Me)_2PO_4$]	esterfication of isoaml acetate	1. Both [Bmim][PF_6] and [Hmim][PF_6] are suitable reaction media because their hydrophobic properties and they can form two phase system with the substrates; 2.Water as a by-product of the esterification reaction can shift the equilibrium towards the direction of the hydrolysis; 3.The best results were obtained with 1 to 2% (w/w)	Fehér *et al*., 2008

			initial water content, while with 10% the reaction ratio approached to zero.	
	[Bmim][PF_6]	Esterification geraniol	1.The maximum initial reaction rate was found at aw=0.6; 2.The average value of reaction equilibrium constant was 12-20 fold lower than that in hexane.	Barahona *et al*., 2006
	27 ILs with different anions and cations	transesterification	1.Under microwave irridation, enhanced enzyme activities were observed with a layer of water; 2.The initial reaction rates bear no direct relationship with the viscosity and polarity of Ils, but have a loose correlationship with the hydrophobicity of ILs.	Zhao *et al*., 2009
lipases from (CaL A ,CaL B), *Thermomyces lanuginosus Rhizomucor miehei*	[Bmim][PF_6]/[BF_4],[Omim][PF_6]/[BF_4][Hmim][BF_4] [Bdmim][PF_6] [Bdmim][BF_4][Bmim][MS] [CPMA][MS]	transesterification	1.enzyme activities were clearly dependent on the nature of the ions, and the results improved as the alkyl chain length of the imidazolium cation increased; 2.The best synthetic activity was obtained when free CaL B were assayed in [CPMA][MS].	De Diego *et al*., 2009
lipases (CaL B, *i*-CaL B, CaL A and PcL)	ChCl-Acet, ChCl-malonic acid, ChCl-U, EAC:Acet, EAC-Gly, ChCl-EG, EAC-EG, ChCl-malonic acid	Transesterification of ethyl valerate with 1-butanol	1.In ChCl:Gly, all the lipases showed conversions comparable to that in toluene; 2. *i*-CaL B also showed conversions comparable to that in toluene for ChCl:EG, ChCl:U and EAC:Gly; 3.*i*-CaL B had the highest initial specific transesterification activity in EAC:Gly, which was twice as high as in [Bmin][Tf_2N] and 7 times higher than the activity in [Bmin][BF4]; 4. DES are also suitable as co-solvents for reaction in aqueous solution, increasing activity up to 20 fold.	Gorke *et al*., 2008
pseudomonas capaci lipase (PcL)	[*i*-C_4mim][PF_6] [C_4mim][PF_6] hexane	Transesterification reaction 2-phenylethanol with vinyl acetate	[*i*-C_4mim][PF_6] was the best among the three solvents. The initial reaction rate, the equilibrium conversion of 2-phenylethanol and the half-lifetime of the lipase in [*i*-C_4mim][PF_6] medium were about 1.5, 1.2 and 3-fold that obtained in [C_4mim][PF_6] medium, respectively.	Shan *et al*., 2008
lipase from *Candida rugosa* (CrL)	[Bmim][PF_6]	enantioselective esterification	the enantioselective esterification of (-)-menthol exceeded 53%.	Zhang 2008
	[Bmim]/[MMEP]with anions([PF_6] [NO_3][OAC]), [Bmim] with	transesterification of methyl methacrylate	1.The enzymatic activity was 1.5 times in [Bmin][PF_6] faster than that in hexane. However, no detectable activity was observed in all the "hydrophilic" ILs studied; 2.No lipase	Kaar *et al*., 2003

	anions ([PF_6][NO_3][OAC][TfO][CF_3CO_2][CH_3SO_3])		activity was observed in all ILs with [Bmin][PF_6] and more hydrophilic ILs; 3.Lipase exhibited greater stability in ILs than in organic solvents including hexane.	
CrL *Burkholderia cepacia* lipase (BcL)	[BDmim][cetyl-PEG10-sulfate] [Bmim][cetyl-PEG10-sulfate] [Bmim][BF_4] [BDmim][BF_4][BDmim][PF_6]	transesterification of 1-phenylethanol	A remarkable acceleration was accomplished by coating the lipase with IL in an *i*Pr_2O solvent system, reaching a 500-600 fold acceleration for some substrates.	Itoh *et al.*, 2006
i-BcL	[Bmim][PF_4] [Bmim][PF_6], 4-methyl-N-butylpyridinium [BF_4], and methyltrioctyl ammonium trifluoroacetate	butanolysis of triolein	1.Only methyltrioctylammonium trifluoroacetate was miscible with 1-butanol and triolein; 2.The addition of 80% methyltrioctylammonium trifluoroacetate to the reaction system gave the best result in terms of alcoholysis rate.	Miyawaki *et al*., 2008
Mucor javanicus lipase(MjL)	[Bmim][PF_6] [Emim][Tf_2N] [Bmim][BF_4] [Emim][BF_4]	the hydrolysis reaction	1.The activities of lipase pretreated with [Bmim][PF_6], [Emim][Tf_2N], [Bmim][BF4] and [Emim][BF_4] were 1.81, 1.66, 1.56 and 1.60 times higher than that of untreated lipase, respectively; 2.Activities of lipase in ILs were well maintained after 7 days of incubation in ILs at 60 ^oC, while untreated lipase in phosphate buffer was fully inactivated only after 12 h of incubation at the same temperature.	Dang *et al*., 2007
A feruloyl esterase (AnFaeA)	[Bmim][PF_6], [Omim][PF_6], [C_2OHmim][PF_6] [C_5O_2mim][PF_6]	the esterification of glycerol with sinapic acid	the esterase was able to catalyse both transesterification and esterification only in [C_2OHmim][PF_6] and [C_5O_2mim] [PF_6] with a high conversion yield around 72.5 ±2.1%.	Vafiadi *et al*., 2009
esterases from *Bacillus stearothermophilus*	[Bmim][Tf_2N] [Bmim][PF_6] [Bmim] [BF_4]	transesterification of 1-phenylethanol	1.Highest specific activity was obtained in n-hexane for both enzymes, while the specific activity was similar in organic solvents and in the ILs; 2.The enantioselectivity was independent of the solvent.	Persson, 2003
Protease (subtilisin)	[Emim][Tf]	esterification *N*-acetyl-*L*-phenyl alanine	The activity was improved 9-fold in [Emim][Tf] compared to acetonitrile and 3-fold to octane.	Noritomi *et al*., 2007

horseradish peroxidase (HRP)	[Bmim][BF_4]		1.No detectable activity exhibited in anhydrous [Bmim][BF4], while HRP was active in the presence of a small amount of water (4.53%, v/v); 2.The *i*-HRP was very sensitive and stable in H_2O-containing [Bmim][BF_4].	Wang *et al*., 2007
	water-in-hydrophobic [C_8mim][Tf_2N] (w/IL) microemulsions	oxidation of pyrogallol	1.The HRP-catalyzed oxidation of pyrogallol by hydrogen peroxide in water/IL microemulsions is much more effective than in general water/isooctane microemulsion; 2.HRP retained almost 70% of its initial activity after incubation at 28 ^oC for 30 h.	Monir uzzaman *et al*., 2008
Chloroperoxidase from *Caldariomyces fumago*	citrate buffer/ILs mixtures,	oxidation of 1,2-dihydronaphthalene	The enzyme activity is retained for 24 h, but it falls to 3 h using non-ionic organic solvents such as *t*-BuOH or acetone.	Sanfilippo *et al*., 2004
mushroom tyrosinase	[Bmim][PF_6], [Bmim][BF_4] [Bmim][$MeSO_4$]	Oxidation of 4methylcatechol to 4-methyl-*O*- quinone.	1.The three ILs were able to trigger the enzyme activity; 2.The enzyme treated with [Bmim][BF4] retained a higher activity than that in the other two; 3.The enzyme could be stabilized by addition of $KMeSO_4$ and $NaBF_4$.	Yang *et al*., 2008
laccase (DeniLite base)	[Emim][$MDEGSO_4$] [Emim][$EtSO_4$] [Emim][$MeSO_3$]		1.[Emim][$MDEGSO_4$] was the most promising IL for laccase with an activity loss of about 10% after 7 days of incubation; 2.The enzyme maintained activity at pH 9.0 for all tested ILs.	Tavares *et al*., 2008
Alcohol dehydrogenase glucose dehydrogenase	[BMP][Tf_2N] a water immiscible IL	ketone reductions	1.Employing 10% (v/v) [BMP][Tf_2N], facilitated conversion of 50 gL^{-1} ketone to the chiral alcohol in less than 24 h; 2.The initial rate of reaction was improved more than 4 times with 10% (v/v) [BMP][Tf_2N] compared with organic solvents and aqueous buffer.	Hussain *et al*., 2008
cellulase	[Emim] [Me_2PO_4]	enzymatic saccharification	1.When the volume of IL to water was greater than 3: 2(v/ v), little cellulase activity was observed; 2.An IL to water ratio of 1: 4 resulted in over 70% of the starting amount of cellulose being converted to glucose and cellobiose.	Kamiya *et al*., 2008

The cations and, particularly, the anions of ILs have a significant effect on the selectivity of enzyme reaction except for reaction activity. [Bmim][BF_4] gave the highest initial rate and enantioselectivity among various ILs examined for the hydrolysis of D,L-phenylglycine methyl ester, while [Bmim][HSO_4] makes the reaction much less active and enantioselective (Lou *et al*., 2006). In [Bmim][BF_4], best selectivity towards the formation of the monoester (74% at 1 h) over the diester (0% at 1 h) was obtained (Yang *et al*., 2008). Nevertheless,

in [Bmim][PF_6] a higher yield was obtained, though no selectivity was obtained with any substrate.

The spatial configuration of ILs is also considered a key factor affecting the behavior of the enzyme in ILs. The [*i*-C_4mim][PF_6] has good biocompatibility, showing superiority in the initial reaction rate, the equilibrium conversion and the half-lifetime of the lipase over [*i*-C_4mim][PF_6] and hexane (Shan *et al*., 2008). So [*i*-C_4mim][PF_6] can be used as green solvent in various biocatalytic reactions to improve the activity and stability of enzyme.

6.2 Properties of ILs

The effect of ILs on the enzyme can be largely attributed to their interaction with the enzyme, substrate and water, depending on their hydrophobicity, H-bonding basicity, ion's kosmotropoc and nucleophilicity (Yang *et al*., 2008). Kosmotropic is the ability of solutes to increase the stability of intermolecular forces in water-water interactions.Kosmotropic ions contribute to the stability and structure of an enzyme and tend to be small or having high charge density, e.g. SO_4^{2-}, Mg^{2+}. However, none of these properties are solely responsible for the enzyme functions in ILs and multiple factors have to be considered.

Hydrophobicity was considered a key parameter for analyzing the behavior of enzymes in ILs. In general, enzymes are more stable and exhibit higher activity in hydrophobic ILs than hydrophilic ILs. The initial reaction rates of lipase B have a loose correlation with hydrophobicity value of ILs (Zhao *et al*., 2009). The hydrophobic property is anion dependent and ILs with anions like [PF_6], [Tf_2N], [BF_4] are hydrophobic. The enzymatic activity of free lipase (Kaar *et al*., 2003) and lipase B (De Los Rios *et al.*, 2007) are higher in [Bmim][PF_6] or [Omim][PF_6] than in hexane, while no detectable or lower activity was observed in all the "hydrophilic" ILs studied. On the other hand, for the ILs based on the same anion, the hydrophobicity increased with increasing length of the alkyl group on the cation (Ropel *et al*. 2005). With [PF_6] or [dca] as anions, the activity of lipase B increases in the trend: [Bmim] > [Hmim] >[Omim], which can be attributed to the gradually enhanced hydrophobicity (De Los Rios *et al* 2007). The initial lipase activity in [Amim110][dca] is the same as in [Emim][Tf_2N], [BuPy][Tf_2N] and more than 7 times higher than that in [Bmim][dca]. In addition, the lipase has activity even in [Amim110][Cl]. These can be attributed to the hydrophobicity of [Amim110] cation (Zhao *et al*., 2009). However, there are some exceptions. With [PF_6] as the anion, the enzyme is active in ILs with cations like [C_2OHmim] and [C_5O_2mim] and inactive in [Bmim] and [Omim] based ILs, which can be correlated with the amphiphilic properties caused by the hydrophobic anions [PF_6] and hydrophilic cations [C_2OHmim] and [C_5O_2mim] (Vafiadi *et al*., 2009). Moreover, in DES with polarity equal to methanol to glycerol, lipases also show conversions comparable to that in toluene (Gorke *et al*., 2008). In all, not only the hydrophobic ILs are suitable solvents for enzyme activity, some

hydrophilic ILs are also good solvents depending on the type of enzyme and solvents.

H-bonding basicity (the H-bond acceptor strength) could have a considerable impact on the enzyme stabilization in some ILs. The strength of anion coordination is dependent on and consistent with the H-bonding basicity of the anions (β), listed in an increasing order (Henderson, 2007), [Tf_2N] < [PF_6], [BF_4] < [Br] < [Cl]. This order represents the strength of interactions between the anions and the charged surface of macromoleculars. [Bmim][BF_4], [Bmim][PF_6] and [Bmim][Tf_2N] have similar H-bonding basicities, which are much lower than that of [Bmim][Cl] (Anderson *et al*., 2002). The enzyme is less active in ILs with [Cl] than [dca], which can be attributed to the stronger H-bond accepting ability of [Cl] than [dca] (Zhao *et al*., 2009).

6.3 Concentration of ILs

The concentration of ILs or the water content in ILs is an important factor affecting the efficiency and selectivity of enzyme biocatalysis. The enzyme activity of *i*-CaL B in hydrolysis of D,L-phenylglycine methyl was strongly dependent on [Bmim][BF_4] content in the co-solvent system and the favorable content of the IL was 20% (v/v) (Lou *et al*., 2006). The volume ratio of methyltrioctylammonium trifluoroacetate (MTOATFA) in the solution with 1-butanol had an effect on the production rate of lipase-catalyzed *Burkholderia cepacia* lipase (BcL) butanolysis of triolein: a small amount of IL seemed inhibitory, a large amount of IL accelerated the reaction and the addition of 80% MTOATFA gave the best result in spite of the reduction in substrate concentration (Miyawaki and Tatsuno, 2008). Increasing the IL content in aqueous solution results in a decrease of the laccase activity and at high IL content (75%, v/v) the enzyme precipitates (Tavares *et al*., 2008). For horseradish peroxidase (HRP), no detectable activity was found in anhydrous [Bmim][BF_4], but in the presence of a small amount of water (4.53%, v/v) it was active (Wang *et al*., 2007). The water content in [Emim][Tf] affected the activity of subtilisin in the esterification of *N*-acetyl-(L)-phenylalanine and the initial rate with 0.5% water is enhanced 2.4-fold compared to that with 0.2% water (Noritomi *et al*., 2007). For cellulase, decreasing the volume ratio markedly enhanced enzymatic activity: an IL to water ratio of 1: 4 (v/ v) resulted in over 70% of the starting amount of cellulose being converted to glucose and cellobiose (Kamiya *et al*., 2008).

10% (v/v) [Bmim][PF_6]-tetrahydrofuran gave the highest initial rate and substrate conversion in the regioselective acylation of 1-beta-D-arabinofuranosylcytosine (ara-C) catalyzed by *i*-CaL B (Li *et al*., 2006). 10% (v/v) [BMP][Tf_2N] facilitated stereoselective conversion of ketone to the chiral alcohol with alcohol dehydrogenase and improved the initial rate of the reaction more than four times compared to no co-solvent (Hussain *et al*., 2008). Gervaise *et al*. (2009) found that the regioselectivity of lipase from *Candida cylindracea* (LCC) can be influenced by the proportion of [Bmim][PF_6]-phosphase buffer. The influence on the regioselectivity of the enzyme reaction can be explained

by the ILs-cosolvent mixture having an impact on the surface of enzyme and consequently leading a conformational change.

6.4 Water content

Water content (α_w) is known to play an important role in affecting enzyme activity in non-aqueous environments and the thermodynamic water activity is an important variable affecting the activity of enzymes in non-aqueous solvents. The maximum initial reaction ratio was observed at α_w=0.6 in the conversion of geraniol with CaL B (Barahona *et al*., 2005). In the regioselective acylation of 1-beta-D-arabinofuranosylcytosine, catalyzed by *i*-CaL B in [C_4mim][PF_6], at α_w=0.07, the initial rate, substrate conversion and the regioselectivity were 94.0 mM h^{-1}, 98.5% and 99%, respectively. However, when α_w = 0.85, the reaction rate was near to zero (Li *et al*., 2006).

6.5 ILs-coating effect

The activity and stability of *Mucor javanicus* lipase (MjL) pretreated with ILs (such as [Bmim][PF_6], [Emim][Tf_2N], [Bmim][BF_4] and [Emim][BF_4]) were higher than those of untreated lipase for the hydrolysis reaction in an aqueous medium; activities of lipase in ILs were well maintained even after 7 days of incubation, while untreated lipase in phosphate buffer was fully inactivated already after 12 h of incubation at the same temperature (Dang *et al*., 2007). A remarkable acceleration was accomplished by the lipase coated with IL in transesterification of 1-phenylethanol catalyzed by the *Burkholderia cepacia* lipase (BcL) (Itoh *et al*., 2006). Lipase coated by [PPmim][PF_6] (PPmim 1-(3´-phenylpropyl)-3-methyllimidazolium) showed higher enantioselectivity than that of commercial lipase in toluene (Lee and Kim, 2002). With ILs as the coating materials, the activity, stability and enantioselectivity might be enhanced due to a change in the structure of the enzyme coated with ILs.

In addition, there are also some factors affecting the enzyme activity in ILs, such as pH (Ren *et al*., 2008), temperature (Zhang *et al*., 2008), microwaves (Zhao *et al*., 2009), which should be taken into consideration when optimizing the conditions for enzyme reaction in ILs. Enzymes in ILs maintain their activity and also their structure, over a much longer period than in organic solvents and often even at a much higher temperature. Van Rantwijk and Sheldon (2007) suggested that the underlying cause of this stabilizing effect is the high viscosity of ionic liquids, which slows the migration of protein domains from the active conformation into the inactive one.

6.6 Deep eutectic solvents

There are a few applications of DES in the extraction of natural products. This is probably due to their high viscosity and most are in nearly solid state at room temperature. However, DES made of glycerol and choline have relative low viscosity and are good solvents for enzyme reaction. Hydrolases show good catalytic activity in DES including choline chloride-acetamide, choline

chloride-glycerol, choline chloride-urea. In those DES, lyophilized *Candida antarctica* lipase B shows higher activity than that in the conventional solvent, toluene. Particularly, in choline chloride-glycerol, the conversion is much higher than in toluene (22% *vs* 5%) with *lyophilized Burkholderia cepacia* lipase. Furthermore, the enzyme activity of lyophilized *Candida antarctica* lipase B did not decrease in choline chloride-urea at 60 ^{o}C over 90 min (Gorke *et al*., 2008). The high activity and stability of hydrolases in DES show the promise of DES as solvents in biotransformation. The enzymatic transesterification of Miglyol oil 812 catalyzed by Novozym 435, a commercial immobilized *Candida antarctica* lipase B, showed high reaction rates in choline acetate:glycerol (1:1.5) at 50 ^{o}C. The high conversion rate of up to 97% ensures the further exploration of DES as potential solvents in enzyme reactions (Zhao *et al*., 2011).

7. Conclusions and Perspective

ILs are promising solvents for natural products research basically due to their high extraction efficiency of a wide range of metabolites and their low environmental impact. They are compatible with HPLC analytical procedures and the compounds can be recovered from the ILs. Green ILs-AAILs- have already been used in the chiral liquid-liquid extraction of amino acids displaying high enantioselectivity. All these results show their great potential for further applications in natural products-related areas such as food additives and pharmaceuticals or cosmetics.

ILs, with their special properties, constitute a new and green type of solvents. They are of great importance for applications in all areas where general organic solvents are used. For natural products, they offer a perspective of novel extraction processes, with high efficiency and yielding a greater variety of compounds given their high dissolving power. This feature allows ILs to be considered as a new kind of solvent capable of extracting all metabolites for total metabolomics, for example. Most ILs that have been used in the extraction of natural products, however, were imidazolium-based, and only a few of them were suitable for certain extraction applications. The application of other types of ILs and DES in natural products extraction should be further investigated, especially regarding their physical properties.

All natural products are soluble in ILs, although, as mentioned before, when used to extract compounds from plant material, high viscosity may be a problem. There are a number of reports of the efficient extraction of certain types of natural products using aqueous ILs solutions combined with microwaves. Some thermally unstable natural products, such as flavonoids, however, should be extracted at lower temperatures. The combination of ILs/supercritical CO_2 might also be a good choice for the extraction of natural products especially for thermally unstable compounds.

ILs might be applied to extract natural products without polluting the environment. Obviously, for certain applications of natural ILs, the extract maybe used as such, e.g. as food additives and in pharmaceutical or cosmetic

products. How to isolate compounds from ILs, however, is still an important challenge. Some recovery methods with supercritical CO_2 have been established for simple organic model compounds as a means of exploring the recovery mechanism. It may also be useful to develop a liquid/solid separation using different column materials to separate target solutes and ILs. Further studies are needed to optimize the recovery of natural products from ILs.

Finally, considering all the facts mentioned above, our group has recently postulated that ILs and DES also occur in nature (Choi *et al*., 2011). The existence of Natural Deep Eutectic Solvents (NADES) would explain the biosynthesis of the water insoluble macromolecules cellulose, starch, lignin, the biosynthesis of small water insoluble molecules, drought and cold resistance of all kinds of organisms, the germination of an almost dry seed, etc. Where could they occur? In our view, they 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. Once more, 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 natural ILs and DES we are at the beginning of something very fundamental which on the one hand may explain many basic biological cellular processes and on the other hand will generate many applications in the field of extractions and enzymatic reactions. For example, most biosynthetic enzymes have been characterized in an aqueous environment. The finding that in certain ILs enzymes are more active than in the classical solvents shows that in nature the biosynthetic pathways also may be controlled by e.g. water contents and water cativity, the presence of NADES. The green chemistry has opened a realm of pandora.

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Chapter 3

Natural Deep Eutectic Solvents as new potential media for green technology

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Abstract

Developing new green solvents is one of the key subjects in Green Chemistry. Ionic liquids (ILs) and deep eutectic solvents (DES), thus, have got much attention as replacement for current toxic organic solvents and are now applied in many chemical processes such as extraction and synthesis. However, current ILs and DES still have limitations for applications in commercial chemical industry due to toxicity for humans and environment, as well as high cost of ILs and the solid state of most DES at room temperature. Recently we discovered that many plant abundant primary metabolites changed their state from solid to liquid when mixed in a proper ratio. This finding made us to hypothesize that natural deep eutectic solvents (NADES) play a role as alternative media to water in living organisms and therefore tested a wide range of natural products, which resulted in a discovery of over 100 NADES from natural ingredients. In order to characterize the deep eutectic solvents the interaction between the molecules was investigated by nuclear magnetic resonance spectroscopy. All the tested NADES show clear hydrogen bonding between the components. As a next step physical properties of NADES such as water activity, density, viscosity, polarity and thermal properties were measured as well as the effect of water on the physical properties. In the last stage the novel NADES were applied to the solubilization of a wide range of biomolecules such as non-water soluble bioactive natural products, gluten, starch, and DNA. In most cases the solubility of the biomolecules evaluated in this study was much higher than in water. Based on the results these novel NADES are potential green solvents at room temperature in diverse fields of chemistry.

Key words: Natural deep eutectic solvents; ionic liquids; physicochemical properties; green technology; solubility

1. Introduction

Currently green technology is one of the key issues in chemistry because it aims to preserve the environment and to reduce the possible negative effect on human health. The green technology reduce the use of hazardous media by offering new environmentally acceptable solubilization techniques by controlling physical properties of media such as temperature and pressure, and developing new green solvents. In the further development of green technology, new green solvents may be the most important goals. In this context, ionic liquids and deep eutectic solvents have been developed to replace current harsh organic solvents.

It is a well-known phenomenon that pure solid chemicals can become liquid by mixing in certain ratios as in the case of ionic liquids and deep eutectic solvents. Ionic liquids (ILs) are a class of organic salts with a low melting point. Recently, with the aim of developing environmentally friendly solvents, ILs have received increasing attention because they have a negligible vapor pressure and can be tailored concerning polarity and selectivity for different applications such as chemical or enzymatic reactions (Welton, 1999; Visser et al., 2000). Another type of solvent with similar physical properties and phase behavior to ILs are deep eutectic solvents (DES) (Abedin and Endres, 2007). These solvents are mixtures of compounds that have a much lower melting point than that of any of its individual components, mainly due to the generation of intermolecular hydrogen bonds. The creating DES was demonstrated for mixtures of quaternary ammonium salts (Abbott *et al*., 2003) with a range of amides and carboxylic acids (Abbott *et al*., 2004), and later extended to choline chloride with alcohols (Gorke *et al*., 2008), and urea with sugars or organic acids (Imperato *et al*., 2005; Gore *et al*., 2011). Some features of these DES make that they have an advantage over ILs because they are easier to prepare with high purity at low cost. Higher melting points of many DES, however, can hamper their application as a green solvent at room temperature. Compared to the broad applications of ILs (Tang *et al*., 2012; Park *et al*., 2003; Han and Armstrong, 2007; Liu *et al*., 2009), the application of DES has been so far limited to organic reactions (Imperato *et al*., 2005; Gore *et al*., 2011; Ilgen and Konig, 2009) organic extractions (Abbott *et al*., 2009), electrochemistry (Nkuku and Lesuer, 2007; Figueiredo *et al*., 2009; Jhong *et al*., 2009), and enzyme reactions carried out at 60 ^{o}C (Gorke *et al*., 2008). Moreover, the synthetic ILs suffer from high toxicity of some of the ingredients (Docherty and Kulpa, 2005; Zhao *et al*., 2007), which is hampering their use in pharmaceutical and food related products.

In order to increase the number of candidates for ILs and DES and extend their applications, apart from synthetic compounds, attention has directed towards natural products such as organic acids (Abbott *et al*., 2004; Gore *et al*., 2011; Fukaya *et al*., 2007) , amino acids (Fukumoto *et al*., 2005), sugars (Poletti *et al*., 2007; Imperato *et al*., 2005), choline (Abbott et al., 2003; Gorke *et al*., 2008), or urea (Abbott et al., 2003; Imperato *et al*., 2005; Gore *et al*., 2011).

Natural products are indeed a plentiful and ideal source of ILs and DES due to their enormous chemical diversity, biodegradable properties and pharmaceutically acceptable toxicity profile. There is, however, an even more interesting aspect. We recently postulated that in living organisms there is an alternative medium to water and lipids, because if they were the only two media it would be difficult to explain a great number of biological processes that occur in all organisms, such as biosynthesis of poorly water soluble metabolites and macromolecules in the aqueous environment of cells, also survival of organisms in extreme drought (e.g. cacti, resurrection plants, lichen, prokaryotes), and/or cold conditions (e.g. seeds, prokaryotes). The occurrence of ILs and DES in living organisms can explain many of these biological phenomena (Choi *et al*., 2011). This hypothesis was based on the observation that many potential ingredients of ILs and DES are always observed in large and about similar amounts in NMR-based metabolomics of all type of cells and organisms.

To prove our recently published hypothesis (Choi *et al*., 2011) that ILs and DES might play an important role as a liquid phase for solubilizing, storing, and transporting non-water soluble metabolites in living cells and organisms, we tested different mixtures of various abundant cellular constituents (primary metabolites) to make liquid, measure their important physicochemical properties, and test their solubilization ability for poorly water soluble metabolites and also macromolecules. Furthermore, the existence of NADES in plants was also explored.

2. Material and Methods

2.1 NADES preparation

Two methods were used for preparing natural deep eutectic solvents (NADES): a vacuum evaporating and a heating method. Evaporating method: components were dissolved in water and evaporated at 50 ^{o}C with a rotatory evaporator. The liquid obtained was put in a desiccator with silica gel till they reached a constant weight. Heating method: this method was employed to obtain NADES with a known amount of water. The two-component mixture with calculated amounts of water was placed in a capped bottle with a stirring bar and heated in a water bath below 50 ^{o}C with agitation till a clear liquid was formed (about 30-90 min). The viscous liquids were tested on a 1H NMR spectrometer at 40 ^{o}C.

2.2 NMR Spectroscopy

NMR experiments: ^{1}H NMR spectra, 2D NOESY and HOESY spectra were recorded at 25 and 40 ^{o}C on a Bruker 500 MHz DMX NMR spectrometer (500.13 MHz proton frequency) equipped with a TCI cryoprobe and Z-gradient system. For 1D-^{1}H NMR spectra, a total of 32768 data points were recorded covering a spectral window of 9615 Hz; 128 scans of a standard one-pulse sequence with 90^{o} flip angle for excitation and presaturation during 2.0 s

relaxation delay. An exponential window function with a line-broadening factor of 0.3 Hz was applied prior to Fourier transformation. The resulting spectra were manually phased and baseline corrected. ^{1}H-^{1}H NOESY spectra were acquired with presaturation (B_1 = 50Hz) during a relaxation delay of 1.5 s. A data matrix of 1024 x 2048 points covering 7739.4 x 7739.4 Hz was recorded with 16 scans for each increment. Data were zero filled to 2048 x 2048 points prior to States-TPPI type 2D Fourier transformation and a sine bell-shaped window function was applied in both dimensions. Mixing time was 100 msec. 2D NOESY spectra were recorded at 25 ^{o}C on a Bruker 400 MHz HR-MAS NMR spectrometer at frequence of 3000 Hz without buffer and D_2O.

2.3 Physicochemical properties tests

Thermogravimetric analysis (TGA) was performed using PerkinElmer TGA 7, heating from room temperature to 100 ^{o}C, kept at 100 ^{o}C for 1 h, and then up to 300 ^{o}C at a rate of 10 ^{o}C min^{-1} in air. Differential scanning calorimetry (DSC) curve was recorded using a PerkinElmer Diamond DSC, from -120 ^{o}C to 50 ^{o}C at a rate of 10^{o}Cmin^{-1} with heat down and heat up process in nitrogen. Density tests were performed using a density meter (DMA 5000) at 40 ^{o}C. Viscosity test was performed using a viscometer 16983, type U-tube reverse (P.M. Tamson B.V. Zoetermeer, The Netherlands) in a thermostatic bath TVB 445 (Labovisco B.V., The Netherlands) at 40 ^{o}C. Water activity test was performed in a water activity measurement equipment at 40 ^{o}C. Polarity testing was done with Nile red (NR) as a solvatochromatic probe. The λ_{max} was determined and used in the formula E_{NR}(kcal/mol) $=hc\ N_A/\lambda_{max}=28591/\lambda_{max}$ to obtain E_{NR} (Ogihara *et al*., 2004).

2.4 Solubility tests

Solubility tests were carried out by saturating NADES with an excess of the tested compound in a bottle with a cap, stirring at 40 ^{o}C for 2 h and leaving to rest for 3 h for precipitation (centrifuging 20 min for DNA and gluten after dissolving for 2h). Triplicate samples of the resulting solution were diluted with water. The diluted solutions were analyzed with HPLC-UV at wavelength of 360 nm for rutin, 370 nm for quercetin, 272 nm for cinnamic acid, 517 nm for carthamin, 472 nm for 1,8-dihydroxyanthaquinone, and quantified with a UV/Vis spectrophotometer at 217 nm for ginkgolide B, 228 nm for taxol, 260 nm for DNA, and 595 nm for gluten with Bradford method. Solubility of starch was performed as follows: a known amount of starch was placed in a glass vial with a cap and 5 mL of NADES were added. The vial was vortexed, then loosely capped, placed in a microwave oven and repeatedly heated with 5-10 s pulses at full power till the liquid boiled. Between pulses, the vial was removed, and 1/10 of the original amount of the sample was added, vortexed and replaced in the oven if fully soluble. If not soluble, the sample amount was reduced and the above steps were repeated till a cloudy solution became clear when boiled (Swatloski *et al*., 2002).

3. Results and Discussion

Previously we discovered that many plant primary metabolites in solid state became liquid when they mixed in a certain condition (Choi *et al*., 2011). We hypothesized that these liquids may play a role as alternative media to water in living organisms. To prove our recently published hypothesis that ILs and DES might play an important role as a liquid phase for solubilizing, storing, and transporting non-water soluble metabolites in living cells and organisms, we tested different mixtures of various abundant cellular constituents (primary metabolites) such as sugars, sugar alcohols, amino acids, organic acids, and choline derivatives. Indeed many combinations of these compounds were found to be liquids. We introduced the term Natural Deep Eutectic Solvents (NADES) for these liquids. The exploration of different combinations of these common metabolites abundantly present in all types of cells and organisms provided over 100 combinations of NADES (Table 1). Choline chloride, for example, in combination with any kind of primary metabolite, can make liquids. One may distinguish five main 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. Surprisingly, different kinds of sugars or organic acids mixtures can also form liquids, such as fructose-glucose-sucrose (Choi *et al*., 2011) and malic acid-citric acid. Other combinations of more than two components can also lead to clear liquids, such as glucose-sorbitol-malic acid or choline chloride-proline-malic acid. These multi-component mixtures might be closer to the NADES found in plants since plants have, naturally, a pool of all these metabolites.

Table 1. Different combinations of natural ionic liquids or deep eutectic solvents from natural products made through vacuum evaporating method.

components			**mole ratio**
component 1	**component 2**	**component 3**	
choline chloride	lactic acid		1:1
choline chloride	malonic acid		1:1[a]
choline chloride	maleic acid		1:1, 2:1[a],
choline chloride	DL-malic acid		1:1, 1.5:1,
choline chloride	citric acid		1:1, 2:1,
choline chloride	aconitic acid		1:1
choline chloride	L-(+)-tartaric acid		2:1
choline chloride	glycol		1:1,1:2
choline chloride	1,2-propanediol		1:1, 1:1.5, 1:2, 1:3
choline chloride	1,2-propanediol		2:1 [a]
choline chloride	glycerol		1:1, 3:2
choline chloride	*meso*-erythritol		2:1 [a]
choline chloride	xylitol		5:2
choline chloride	ribitol (adonitol)		5:2

choline chloride	D-sorbitol		3:1, 5:2
choline chloride	D-xylose		2:1, 3:1
choline chloride	A-L-rhamnose		2:1
choline chloride	D-(+)-glucose		1:1, 2:1[a]
choline chloride	D-(+)-glucose		5:2
choline chloride	D-(-)-fructose		1:1, 1:1.5, 1:2[a]
choline chloride	D-(-)-fructose		5:2
choline chloride	sorbose		5:2, 1:1
choline chloride	D-mannose		5:2
choline chloride	D-(+)-galactose		5:2
choline chloride	sucrose		4:1, 1:1
choline chloride	D-(+)-trehalose		4:1
choline chloride	maltose		4:1
choline chloride	raffinose		11:2
choline chloride	proline	DL-malic acid	1:1:1[a]
choline chloride	xylitol	DL-malic acid	1:1:1
choline bitartrate	D-(+)-glucose		1:1
betaine	D-(+)-glucose		5:2 [a]
betaine	sucrose		4:1, 1:1[a]
betaine	sucrose		2:1
betaine	D-(+)-trehalose		4:1
betaine	D-sorbitol		3:1[a]
betaine	DL-malic acid		1:1
betaine	L-(+)-tartaric acid		2:1
betaine	D-mannose		5:2
betaine	inositol	raffinose	9:1:1[a]
betaine	sucrose	proline	1:1:1
betaine	sucrose	proline	5:2:2
betaine	D-(+)-glucose	proline	1:1:1
betaine	DL-malic acid	D-(+)-glucose	1:1:1
betaine	DL-malic acid	proline	1:1:1
betaine	DL-malic acid	inositol	1:1:1[a]
betaine	oxalic acid	D-(+)-glucose	1:1:1
betaine	citric acid		1:1
lactic acid	D-(+)-glucose		5:1
lactic acid	ß-alanine		1:1
DL-malic acid	D-xylose		1:1[a]
DL-malic acid	D-(+)-glucose		1:1, 1:2[a]
DL-malic acid	sucrose		1:1
DL-malic acid	D-(-)-fructose		1:1[a]
DL-malic acid	D-mannose		1:1

DL-malic acid	sucrose		1:1, 2:1
DL-malic acid	maltose		2:1[a]
DL-malic acid	D-(+)-trehalose		2:1[a]
DL-malic acid	lactose		2:1, 1:1
DL-malic acid	raffinose		3:1[a]
DL-malic acid	xylitol		1:1[a]
DL-malic acid	adonitol		1:1[a]
DL-malic acid	D-sorbitol		1:1
DL-malic acid	D-(+)-glucose	D-(-)-fructose	1:1:1
DL-malic acid	D-(+)-glucose	glycerol	1:1:1
DL-malic acid	sucrose	glycerol	1:1:2
DL-malic acid	L-proline	choline chloride	1:1:1
citric acid	D-xylose		1:1[a]
citric acid	D-(+)-glucose		2:1[a]
citric acid	D-(-)-fructose		1:1
citric acid	sorbose		1:1[a]
citric acid	D-mannose		1:1
citric acid	D-(+)-galactose		1:1[a]
citric acid	sucrose		1:1
citric acid	maltose		2:1
citric acid	D-(+)-trehalose		2:1[a]
citric acid	raffinose		3:1
citric acid	D-sorbitol		1:1
citric acid	ribitol		1:1
citric acid	xylitol		1:1
citric acid	adonitol		1:1
citric acid	L-proline		1:1, 1:2, 1:3
citric acid	DL-malic acid		1:1[a]
phytic acid sodium	betaine		1:6
phytic acid sodium	DL-malic acid		1:6
phytic acid sodium	glycerol		1:6
phytic acid sodium	L-proline		1:6
phytic acid sodium	D-(+)-glucose		1:6
phytic acid sodium	choline chloride		1:3
D/L-proline	sucrose		2:1, 3:1
D/L-proline	sucrose		4:1,1:1[a]
D/L-proline	D-sorbitol		1:1
D/L-proline	D-(+)-glucose		1:1, 5:3

D/L-proline	lactic acid		1:1
D/L-proline	DL-malic acid		1:1
D/L-proline	citric acid		1:1, 2:1
D/L-proline	malonic acid		1:1[a]
D-proline	D-(+)-glucose		5:3
L-proline	D-(+)-glucose		5:3
L-serine	DL-malic acid		3:2, 1:1
L-serine	D-(+)-glucose		5:4[a]
L-glutamic salt	sucrose		2:1
L-glutamic salt	D-(+)-glucose		1:1
D-(+)-glucose	DL-malic acid		1:1[a]
D-(+)-glucose	citric acid		1:1
D-(+)-glucose	L-(+)-tartaric acid		1:1
D-(+)-glucose	D-(-)-fructose	sucrose	1:1:1[a]
D-(-)-fructose	sucrose		1:1
ß-alanine	DL-malic acid		3:2, 1:1
ß-alanine	citric acid		1:1

[a] Not stable; solid precipitate within 7 days.

3.1 Discovery of NADES in nature

Similar combinations are also observed in plant secretions, and plants in drought, or cold conditions. In fact, the ingredients for natural ILs and DES are abundant in organisms, which leads to our hypothesis that the natural ILs and DES play important physiological roles as a third medium polar liquid in living cells and organisms (Choi *et al*., 2011). For example, the NMR spectrum of nectar of flowers, a liquid, shows that it is composed mainly of sugars which are individually in their solid form at room temperature; but the composition of the mixture of sugars in this secretion is a liquid similar to our proposed composition, a fructose-glucose-sucrose NADES (Choi *et al*., 2011), and the same applies for the components found in the honey, which is composed of glucose and fructose (Fig. 1a). Components of NADES were also observed in desert plants of the *Selaginela* species, like Mexican moss, but also in microorganisms, lichen, and various other organisms that can survive longer periods without water, such as barley seeds with a high amount of sucrose and choline in episperm during dormancy (Fig. 1b). Various investigations showed that the level of primary metabolites increases in the case of water shortage even for normal plants, such as *Arabidopsis* which shows increased levels of sugars (sucrose), amino acids (proline, alanine, arginine), organic acids (succinic acid, fumaric acid, malic acid) and amines (choline) in water depleted conditions as compared to its normal growing conditions. Also cold resistance might be related to NADES, and in fact the commonly used cryoprotectants for plants, like sugars, sugar alcohols and proline, are all ingredients of NADES. Furthermore, the NADES can also explain the biosynthesis and storage of poorly water soluble compounds since NADES show high solubilizing capacity

for those compounds, as shown in the below solubility results. In particular, NADES may be involved in solubilizing e.g. water insoluble flavonoids in flowers at very high level. NADES in our view function as an alternative liquid phase to water in nature to protect organisms from drought, cold, and to enable the biosynthesis and storage of poorly water-soluble molecules, including high molecular weight molecules.

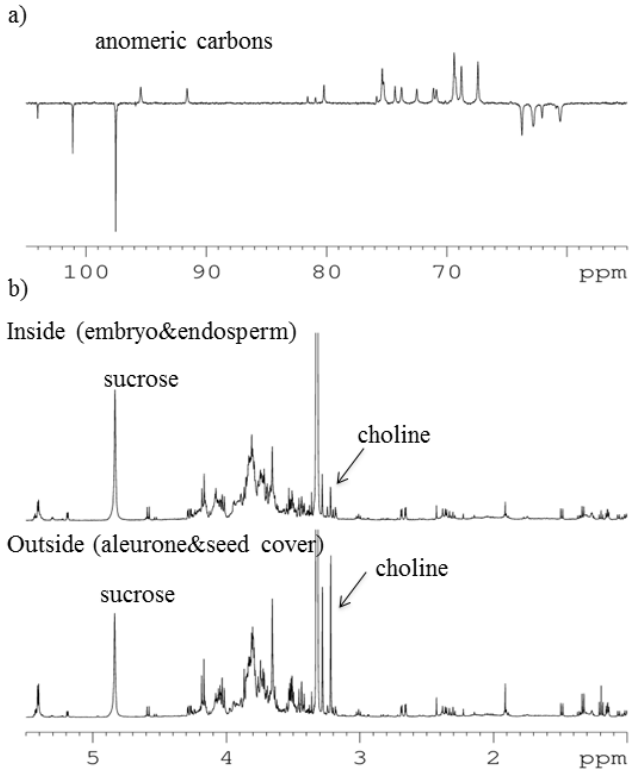
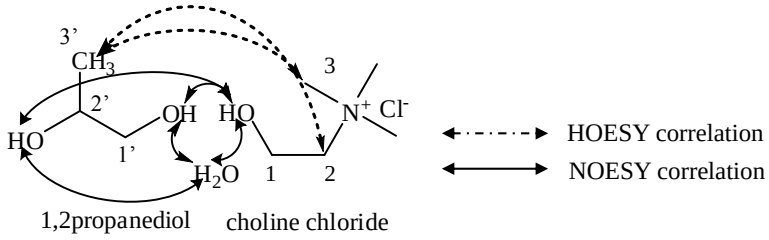


Fig. 1. ^{13}C NMR spectrum of a) honey and ^{1}HNMR spectra of b) the inside and outside parts of barley seeds.

3.2 Structures of NADES

Nuclear magnetic resonance (NMR) spectroscopy was applied to NADES in search for molecular interactions involved in this phenomenon. To start with, the existence of hydrogen bonds in these NADES was observed. Abbott *et al*. (2003) observed a cross-correlation between the fluoride ion from choline fluoride and protons from urea using heteronuclear Overhauser spectroscopy (HOESY). Also, Mele *et al*. (2003) observed a direct intermolecular and intramolecular interaction between 1-*n*-butyl-3-methylimidazolium tetrafluoroborate molecules through ^{1}H-^{1}H-nuclear overhauser spectroscopy (NOESY). In our study, for example, the HOESY spectrum of 1,2-propanediol-choline chloride-H_2O (PCH) revealed a signal corresponding to the proton on the methyl group of 1,2-propanediol interacting with both the methyl carbon

and methylene (connected to nitrogen) carbon of choline chloride. This implies that the protons of the hydroxyl group of 1,2-propanediol may form a hydrogen bond with choline chloride (Fig. 2a). The NOESY spectrum of PCH shows strong interaction between the protons on the hydroxyl groups from choline chloride, 1,2-propanediol, and water (Fig. 2b), implying that hydrogen bonds are formed between these hydroxyl groups. This example suggests that water might also participate in the supermolecular structure of NADES. A similar interaction was observed in other combinations, such as proline-malic acid-water (PMH) (Fig. 3).



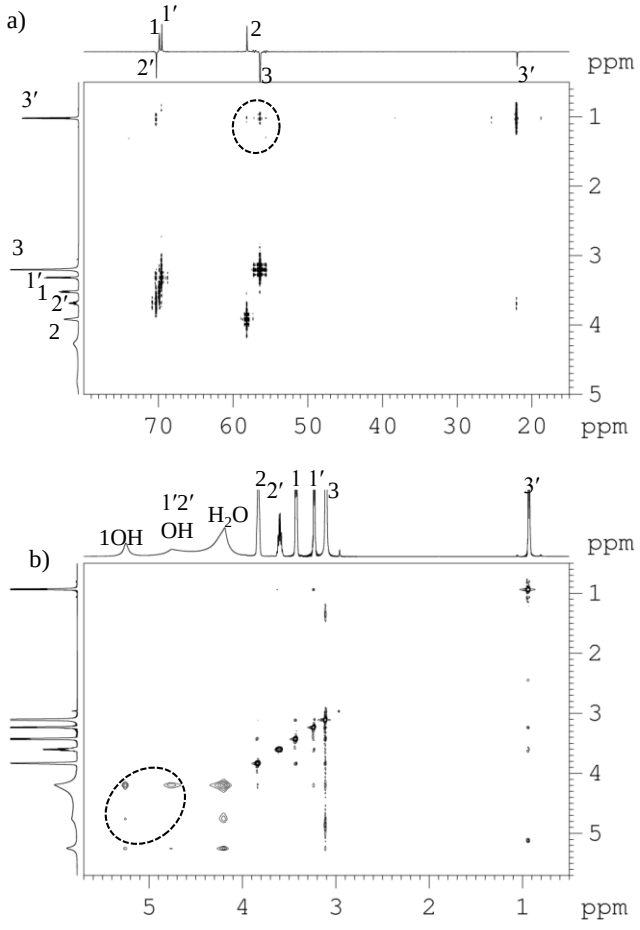


Fig. 2. 2D NMR spectra of 1,2-propanediol-choline chloride-water (1:1:1, molar ratio). a) Heteronuclear overhauser spectroscopy (HOESY); b) ^{1}H-^{1}H-Nuclear Overhauser enhancement spectroscopy (NOESY).

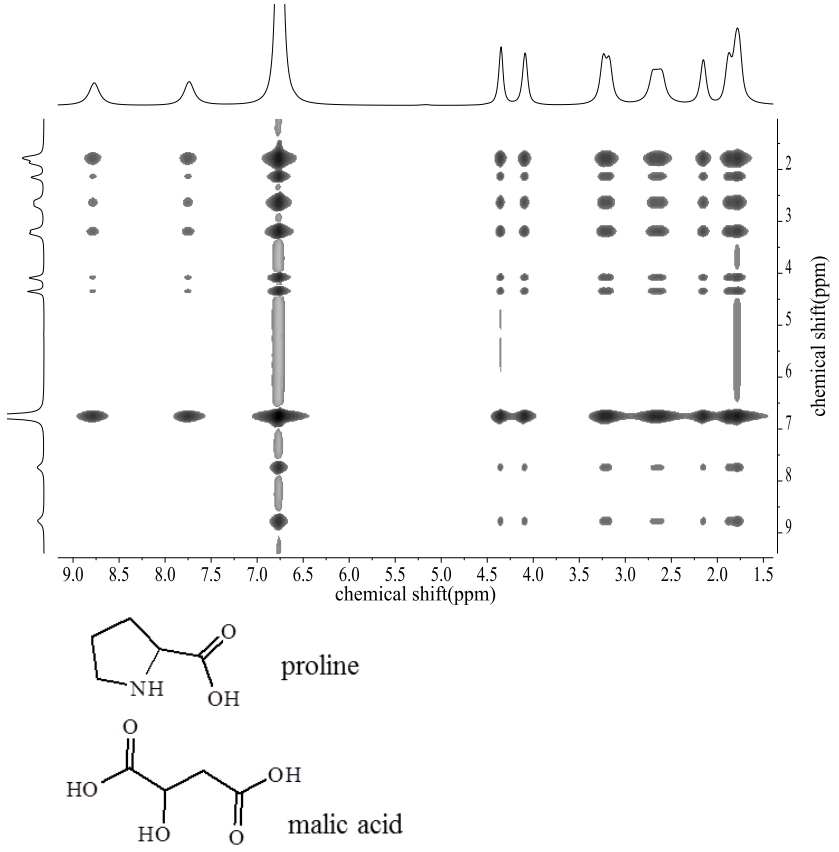


Fig. 3. ^{1}H-^{1}H-Nuclear Overhauser enhancement spectroscopy spectrum (NOESY) of proline-malic acid-H_2O.

Different ratios of the components of NADES, may affect stability of NADES in terms of the mixture remaining in the liquid phase for prolonged periods. To test this, the stability of mixtures prepared with different molar ratios of compounds was evaluated. In the case of sugars-choline chloride mixture, for example, glucose: choline chloride, a ratio of 2:5 moles is stable, but with 2:1, 1:1 or 1:4 mole ratio, a clear liquid can be prepared by mixing, but solid (crystalline) precipitate will gradually appear. Similar cases are listed in Table 1. These observations lead to our conclusion that one chloride ion from choline chloride can form two hydrogen bonds with two hydroxyl groups from sugars, thus behaving similarly as in a mixture of a choline chloride and a carboxylic acid (Abbott *et al*., 2004). An 1:1 molar ratio is suitable for most other combinations. Sugar/sugar alcohol-organic acid/amino acid, amino acid-organic acid, all components are both hydrogen-bond donors and acceptors,

which is thought to be the basis for the complexation of the solids yielding liquids with a supermolecular structure. In fact, NADES are like liquid crystals in which all molecules are arranged through hydrogen bonding and other physical intermolecular binding forces.

Table 2. Physical properties of natural ionic liquids or deep eutectic solvents with water and methanol as references.

name	composition (mole ratio)	water (wt %)	water activity (40 °C)	density(40 °C) g/cm^3	viscosity (40 °C) mm^2/s	T_d[a] / °C	T_g[b] / °C	E_{NR}[c] (kca/mol)
MCH	malic acid:choline chloride:water(1:1:2)	11.6%	0.195	1.2303	445.9	201	-71.32	44.81
GlyCH	glycerol:choline chloride:water(2:1:1)	5.26%	0.126	1.1742	51.3	187	-101.6	49.55
MAH	malic acid:*β*-alanine:water (1:1:3)	19.5%	0.573	1.352	174.6	164	-70.88	48.05
PMH	proline:malic acid: water (1:1:3)	17.8%	0.591	1.3184	251	156	-61.29	48.3
FCH	fructose: choline chloride: water (2:5:5)	7.84%	0.151	1.2078	280.8	160	-84.58	49.81
XCH	xylose: choline chloride: water (1:2:2)	7.74%	0.141	1.2095	308.3	178	-81.8	49.81
SCH	sucrose: choline chloride: water (1:4:4)	7.40%	0.182	1.2269	581	>200	-82.96	49.72
FGSH	fructose:glucose:sucrose : water (1:1:1:11)	22.0%	0.662	1.3657	720	138	-50.77	48.21
GCH	glucose: choline chloride: water (2:5:5)	7.84%	0.162	1.2069	397.4	170	-83.86	49.72
PCH	1,2-propanediol: choline chloride: water (1:1:1)	7.70%	0.242	1.0833	33	162	-109.55	50.07
LGH	lactic acid:glucose: water (5:1:3)	7.89%	0.496	1.2497	37	135	-77.06	44.81
SoCH	sorbitol: choline chloride: water (2:5:6)	9.23%	0.12	1.1854	138.4	>200	-89.62	49.98
XoCH	xylitol:choline chloride: water (1:2:3)	11.2%	0.116	1.17841	86.1	>200	-93.33	49.72
H_2O	water	100%	1	0.992	≈1	-	-	48.21
MeOH	methanol	-	-	0.791	-	-	-	51.89

[a] decomposition temperature; [b] glass transition temperature; [c] $E_{NR}= hcN_A /\lambda_{max}=28591/\lambda_{max}$.

Another feature to evaluate was the influence of the structure of the compounds on the formation and stability of NADES. To study this, different compounds with similar structures were tested (Table 1). The number of hydrogen bond donor or acceptor groups, the spatial structure of those groups and the position of the bonds appeared to significantly influence the formation and stability of NADES. For example, in the case of organic acids, succinic acid does not form a liquid with choline salts, whereas malic acid, citric acid, and tartaric acid do. Considering the structure of these acids, it is possible to conclude that the presence of extra hydroxyl or carboxyl groups, allows more hydrogen bonds to be formed, thus increasing the stability of the liquids. The same applies for the combinations of organic acids and sugars, those that have more carboxylic groups, such as citric acid, can form stable liquids with more kinds of sugars than those with less carboxylic groups as is the case of malic

acid. Not only the number of hydroxyl groups but also their spatial structure has a great influence on the formation and stability of hydrogen bonds. The liquid formed with galactose and choline chloride is not stable and precipitates while the combination of choline chloride with glucose is a stable liquid. A similar phenomenon was also observed with other sugars and sugar alcohols, like sorbitol *vs* mannitol, sorbitol cam form stable liquid while mannitol fails. In the case of disaccharides, trehalose can form a liquid with choline salts or organic acids, while cellobiose, which has a different glycosidic bond to trehalose, does not form a liquid.

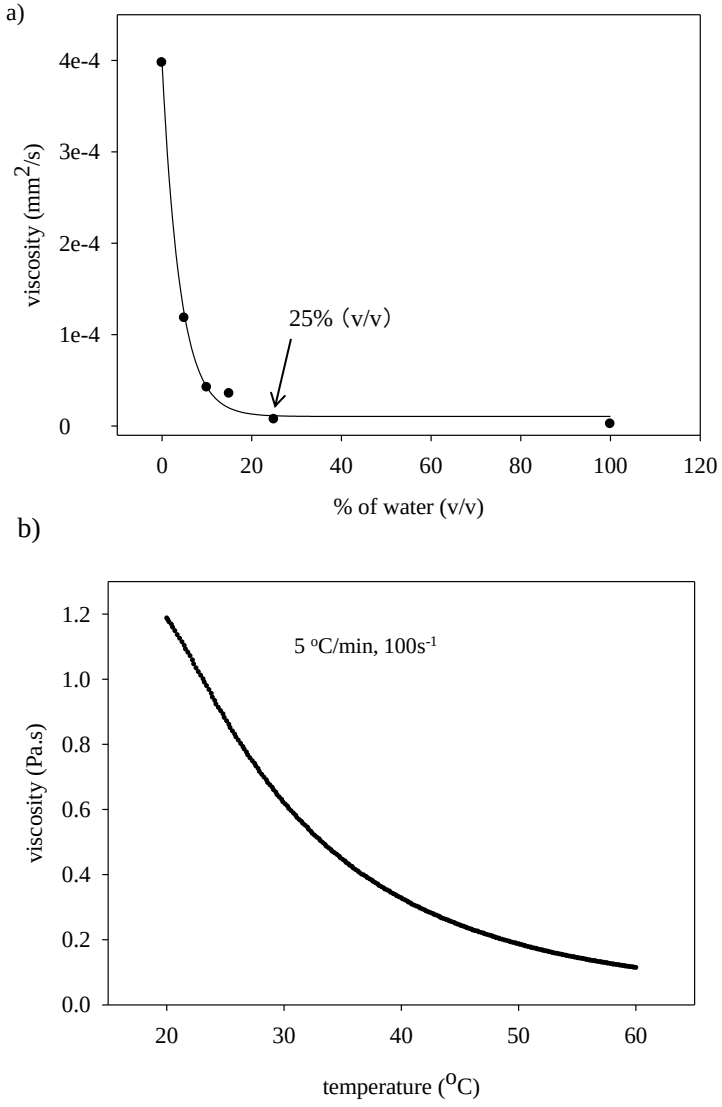


Fig. 4. Relationship between a) viscosity and added water percentage (v/v), b) viscosity and temperature of glucose-choline chloride-water (2:5:5, molar ratio).

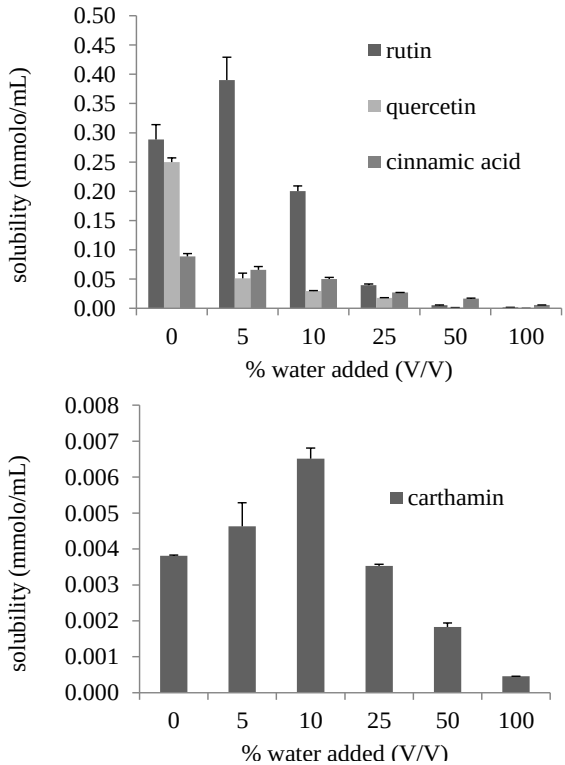


Fig. 5. Solubility of rutin, quercetin, cinnamic acid and carthamin in glucose-choline chloride-water (2:5:5, molar ratio) diluted with different percentage of water. The data is expressed in mean ± SD (n=3).

One of the interesting applications of ILs and DES is their potential use as solvents. The physical properties (Widegren *et al*., 2005; Koddermann *et al*., 2006) and solubilizing capacity (Najdanovic-Visak *et al*., 2003; 2005) of ILs, can be modified by the addition of small quantities of water. In the case of DES, previous studies have reported that most of them were not liquid at room temperature (Abbott *et al*., 2003; 2004; Imperato *et al*., 2005) and consequently there is a limitation for their application as extraction or reaction media at room temperature. In this study we found that small amounts of water (around 5-10% of water, e.g. 2:5:5 in molar combinations in case of GCH, Table 2) resulted in a liquid at room temperature and even at lower temperature. This fits in with our hypothesis of the role of NADES in nature as regards to desiccation of various organisms in which NADES are formed after the evaporation of water. However, extened dilution of DES with water will result in the loss of existing hydrogen bonds, and consequently, the disappearance of the special structure of DES (Gutiérrez, *et al*., 2009). Adding a small amount of water to a NADES has other effects such as reducing the preparation time and temperature, and decreasing their viscosity (Fig. 4a). The molar ratio of water that is compatible

with the stability of liquid NADES at room temperature is listed in Table 2. The water activity values of most DES are close to 0.2, much lower than the mole percentage of water in each DES, indicating that the water in NADES is difficult to evaporate as it is in the form of bonded water. Most importantly, the physical properties of NADES, e.g. solubilizing capacity, can be tuned by varying the water content, leading in some cases, to a higher solubility of plant secondary metabolites such as rutin, carthamin and cinnamic acid (Fig. 5)

3.3 Preparation of NADES

Two methods have been described for the preparation of DES: freeze-drying (Gutiérrez *et al*., 2009) and heating a mixture of the solids to around 80 ^{o}C (Abbott *et al*., 2003). The NADES evaluated in this study can be obtained by heating with stirring at 50 ^{o}C in 0.5-2h when adding a small amount of water. This method is not only cheaper but also safer considering that the components are usually thermally unstable, as is the case of sugars or amino acids. Both vacuum evaporating and heating methods tested in this paper show the same chemical profile for the liquid obtained as shown by the ^{1}H NMR spectra.

3.4 Physicochemical properties of NADES

The thermal behavior of NADES was studied using thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) (Table 2). The weight loss at 100 ^{o}C indicates that the water content in glucose-choline chloride-water (GCH) is 7.8%, which is consistent with the amount of water added. All the NADES were heated to 100 ^{o}C for 1 h, without any evident decomposition. The NADES made of sugars have a low decomposition temperature (T_d) of approximately 135 ^{o}C but others have a T_d that is even above 200 ^{o}C. All NADES evaluated by DSC revealed that they have glass transition points (T_g) below -50 ^{o}C but without a melting point, which confirms that those NADES are supermolecular complexes, with a stable liquid status over a wide temperature range. The liquid state of NADES at low temperature supports our hypothesis that NADES play an important role in plant for cold resistance. Also, it implies that NADES can be used as solvents in a range between at least -20-100 ^{o}C.

Important physical properties including density, viscosity and polarity were examined (Table 2). The densities of all tested NADES proved to be higher than that of water. Viscosity is one of the most important characteristics and also one of the largest obstacles for the application of ILs and DES. The viscosity of NADES is affected by water percentage and temperature. In the case of GCH with different water percentages (v/v) (Fig. 4a), its viscosity decreased by 1/3 when diluted with 5% water, and decreased to 1/10 of the original value with the addition of 10% water. The viscosity of GCH decreased by 2/3 when the temperature increased from 20 to 40 ^{o}C (Fig. 4b). Polarity is another important property of NADES, since it affects theirs solubilizing capacity. Organic acid-based NADES are the most polar (44.81 kcal mol^{-1}), followed by amino acids and pure sugar based NADES with a polarity similar to water (48.21 kcal mol^{-}

[1]). Both sugar and polyalcohol based NADES are the least polar, with a polarity close to that of MeOH (51.89 kcal mol^{-1}). In addition, polarity of NADES may be affected by the addition of water. The evaluation of the polarity of PCH and lactic acid-glucose-water (LGH) with different ratios of water (Table 3) showed an obvious change of polarity with the addition of 50% (v/v) water indicating that this amount of water provoked a dramatic change in the structure of PCH and LGH, very likely due to the rupture of hydrogen bonding between the two components. This is in agreement with the results of previously reported dilution experiments of DES made of glycerol-choline chloride and urea-choline chloride (Gutiérrez *et al*., 2009; 2010).

Table 3. Polarity of 1, 2 propanediol- choline chloride-water (PCH) and lactic acid-glucose- water (LGH) diluted with different ratios of water.

% of added water (v/v)	E_{NR}(kca/mol)	
	PCH	LGH
0%	50.07	44.81
5%	50.16	44.81
10%	49.90	44.81
15%	49.64	44.81
25%	49.13	44.88
50%	48.38	47.97
75%	48.38	48.13

3.5 Solubilizing ability of NADES

Considering the different polarities of the NADES, it is possible that they act as an alternative media to water in organisms, dissolving non water-soluble metabolites or macromolecules. In particular, many plant secondary metabolites are not soluble in water at all, but are synthesized, stored, and transported in plants. A model experiment was carried out to explore the solubilizing capacity of NADES. For this, the solubility of the non-water soluble or poorly water soluble natural products rutin, quercetin, cinnamic acid, carthamin, taxol, ginkgolide B, and 1,8-dihydroxyanthraquinone in LGH, GCH, PCH, and xylitol-choline chloride-water (XoCH) was measured and compared with their solubility in water (Table 4). The results indicate that the solubility of most tested compounds was highest in PCH, which is reasonable, considering that PCH is the least polar. It is noteworthy that the solubility of these compounds increased in NADES by 18 to 460,000 times compared to water. As a further step, the influence of the water content on the solubilizing capacity of GCH was investigated (Fig. 5). In this case, addition of a small amount of water in GCH can increase its solubilizing capacity and the optimum water content depended on the compound. For example, rutin showed the highest solubility in GCH with 5% (v/v) water and carthamin was best soluble in GCH with 10% (v/v)

water. These results show that water is an important factor in optimizing natural NADES. The solubility of quercetin and carthamin was higher in XoCH than in PCH. Taxol and 1,8-dihydroxyanthraquinone have the highest solubility in LGH, although LGH is the most polar of the tested NADES. In order to gain more insight into the solubilizing mechanism of NADES, high-resolution magic angle spinning (HR-MAS) NMR was selected to analyze a saturated solution of quercetin in XoCH. Obvious cross peaks between quercetin and XoCH were observed in the HR-MAS-NOESY spectrum (Fig. 6) indicating that the interaction forces, might result from hydrogen bonding between NADES molecules and solutes, providing an explanation of the high solubilizing capacity of NADES. In addition, the solubility of compounds in NADES affected a lot by temperature. Increasing temperature from 40 to 50 ^{o}C, the solubility of quercetin increased to 2.3 times higher in GCH and 1.65 times higher in PCH (Table 5).

Table 4. Solubility of small molecules (m mole) including rutin, quercetin, cinnamic acid, carthamin, 1,8-dihydroxy-anthraquinone, taxol, ginkgolide B and macromolecules (g/mole$_{solvent}$) including gluten, DNA and starch in different natural deep eutectic solvents or ionic liquids (n=3).

	compounds	**H_2O**	**PCH**[d]	**GCH**[d]	**LGH**[d]	**XoCH**
small molecules	rutin[a]	0.01	107.0	120.6	20.36	114.15
	quercetin[a]	0.000	117.6	106.1	2.57	166.95
	cinnamic acid[a]	0.13	128.4	40.54	124.6	44.29
	carthamin[a]	0.02	4.21	2.77	0.41	6.77
	1,8-dihydroxy	0.00	0.12	0.26	0.41	0.14
	taxol[a]	0.000	2.95	0.46	3.45	0.13
	ginkgolide B[a]	0.01	38.34	6.51	2.49	11.62
macromolecules	gluten[a]	0.03	0.06	0.10	2.64	0.23
	DNA[a]	4.56	0.92	1.20	157.0	1.81
	starch[b]	- [c]	2.47	7.55	1.67	-[c]

[a] Detection temperature at 10 ^{o}C; [b] Detection temperature at 100 ^{o}C; [c] Not detected; [d] 1,2-propanediol-choline chloride-water (PCH), glucose-choline chloride-water (GCH), lactic acid-glucose-water (LGH) and xylitol-choline chloride-water (XoCH).

Other usual components of cells are macromolecules such as DNA, proteins, and starch. The solubility of such compounds was also tested in several NADES (Table 4). Starch was found to be more soluble in GCH than PCH, yielding solutions that remained clear at room temperature. On the other hand, gluten and DNA were best soluble in LGH, which is in agreement with their polarity, LGH being the most polar. The solubility in LGH increased 34 times for DNA and 101 times for gluten as compared to that in water. DES made of choline

chloride and glycerol have been reported to be good solvents for DNA, allowing it to keep its native folded structure (Mamajanov *et al*., 2010). This is in agreement with the solubility of DNA in NADES that we observed, a fact that supports the possible importance of NADES in cells.

Table 5. Solubility (m mole) of rutin and quercetin in 1, 2 propanediol- choline chloride-water (PCH) and glucose-choline chloride- water (GCH) at different temperature (*n*=3).

	rutin		**quercetin**	
temperature(^{o}C)	40	50	40	50
GCH	120.61	163.04	106.18	244.76
PCH	107.09	131.39	117.60	194.42

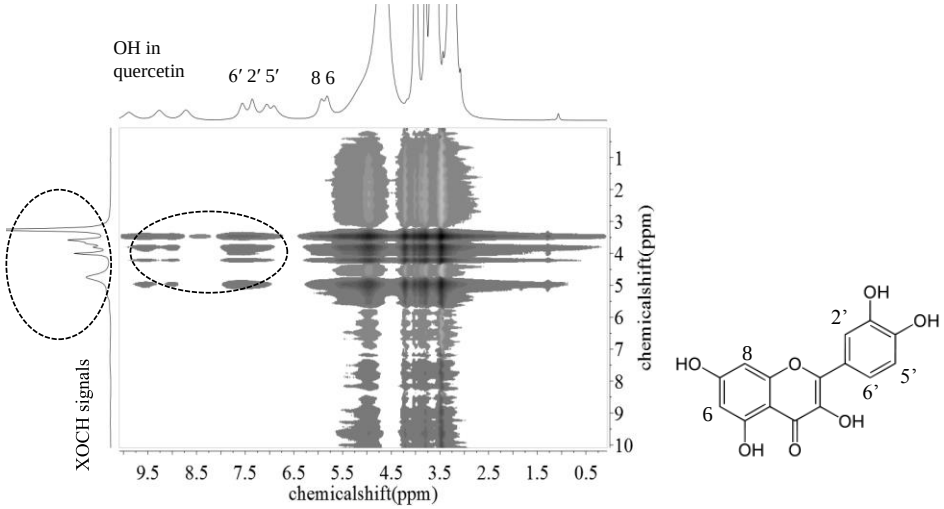


Fig. 6. ^{1}H-^{1}H-Nuclear Overhauser enhancement spectroscopy of correlation between solute (quercetin) and xylitol-choline chloride-water (XoCH) (1:2:3, molar ratio) by high-resolution magic angle spinning (HR-MAS) NMR.

4. Conclusions

This investigation demonstrates that mixtures of many abundant primary metabolites from all kinds of organisms can form natural deep eutectic solvents (NADES) when mixed in adequate ratios. Their NOESY spectra show that they have a supermolecular structure mainly due to hydrogen bonds between the molecules. Also, water can be part of such NADES, and is then strongly bound. Despite high viscosity, the NADES are still liquid at room temperature and even at low temperature. Their viscosity decreases significantly with the addition of

small amounts of water, but preserving their characteristics. In addition, they cover a wide range of polarities, from more polar than water to the same as methanol. The NADES proved to be excellent solvents for a wide range of metabolites 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. Their high solubilizing capacity is related to their supermolecular structure and broad polarity range. The existence of NADES in plants and their properties indicate that NADES might be involved in the biosynthesis and storage of various non-water soluble metabolites in cells and imply the role of NADES in protecting organisms from extreme conditions. This implies that biosynthesis of poor water-soluble compounds occurs in a NADES in which both substrates and enzymes are dissolved. Consequently, the enzymatic reaction does not occur in water. Thus characteristics of the enzyme might be quite different, e.g. measuring enzyme kinetics with poorly soluble substrates may give erroneous results. Finally, the nontoxic and environmentally friendly NADES makes them fit for numerous various applications in e.g. food, cosmetic, agrochemical and pharmaceutical industry as new Green Technology media.

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Chapter 4

The effect of water content on the characteristics of Natural Deep Eutectic Solvents

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Abstract

Recently, binary systems made of ionic liquids and co-solvents have been explored in order to expand the applications of ionic liquids. Mixing ionic liquids with organic solvents or water is an efficient and controllable way to design functional ionic liquids through adjusting the physicochemical properties. In this study, binary systems of natural deep eutectic solvents (NADES) and water, the most abundant liquid on earth, were studied in terms of their structures, physicochemical properties, and solubilization capacity. FT-IR spectra demonstrated H-bonding interactions between the two components of NADES. NMR spectra showed that dilution of the NADES causes a gradual weakening of the H-bonding interactions between the two components and that disappears completely at around 50% (v/v) water dilution. A small amount of water (e.g. 5% in weight) can reduce the viscosity of NADES to the range of water. Conductivity of NADES can be increased by up to 100 times with water dilution for some NADES. Water activity and density of NADES have a quantitative relationship with the water content in NADES. The solubility of quercetin decreases significantly in PCH with water dilution, while the solubility of carthamin reaches the maximal value with 5% water (v/v) in PCH. This study provides the basis for modulating NADES for applications in pharmaceuticals, cosmetics, enzyme reactions, and food processing.

Key words: Natural deep eutectic solvents; water content; Hydrogen bond interactions; physicochemical properties; solubility.

1. Introduction

A new type of green solvents, natural deep eutectic solvents (NADES) have been proposed by our group (Choi *et al.*, 2011; Dai *et al.*, 2013) to extend the range of ionic liquids (ILs) and deep eutectic solvents (DES) and explore their applications in health related fields. Ionic liquids are a class of organic salts with a low melting point (Welton, 1999). Deep eutectic solvents are mixtures of compounds that have a much lower melting point than any of its individual components (Abbott *et al.*, 2004). Natural deep eutectic solvents are composed of natural primary metabolites from organisms such as sugars, sugar alcohols, organic acids, amino acids, and amines, and additionally often contain water in certain molar ratios. They are characterized by extensive intermolecular interactions (Choi *et al.*, 2011; Dai *et al*., 2013). They can be approximately classified into five main groups: ionic liquids formed by 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. Natural deep eutectic solvents possess excellent properties as solvents such as negligible volatility except for the water contained, liquid state even below 0 ^{o}C, a broad range of polarity and high solubilization strength for a wide range of compounds, indcluding especially for poorly water-soluble compounds (Dai *et al.*, 2013). As solvents, from an environment and economic perspective, NADES also offer many striking advantages including biodegradability, sustainability, low costs, simple preparation, and low toxicity. All these properties make them of interest for applications in health related areas such as food, pharmaceuticals and cosmetics. They have already been used to dissolve DNA (Mamajanov *et al.*, 2010; Dai *et al*., 2013), as media for enzyme reactions (Zhao *et al.*, 2011; Choi *et al*., 2011), and biotransformations (Gutiérrez *et al.*, 2010).

These solvents, however, share some of the limitations observed in synthetic ILs and DES. Their high viscosity (typically 200-500 mm^2/s at 40 ^{o}C) (Dai *et al*., 2013) is the most obvious issue, which leads to some practical problems. These include time consuming solvent transfer operations and slow mass transfer in dissolutions and extractions. In ILs, this is generally overcome by applying external physical forces, such as microwave (Swatloski *et al*., 2002) and stirring at high temperature (Abe *et al*., 2010), thus accelerating the dissolving process. Another way to get around this problem is diluting the ILs with water, and the viscosity of diluted NADES or synthetic ILs can be decreased even down to the range of water (Jacquemin *et al.*, 2006; Dai *et al*, 2013). Another important issue to be solved is to design appropriate NADES for certain applications. Mixing ILs with organic solvents or water is an efficient way to engineer functional solvents (Kohno and Ohno, 2012). Therefore, dilution with water might be a solution to solve these practical problems and realize or extend the applications of NADES in health related areas.

There is an even more interesting aspect to understand the effect of water on NADES. Water is the most abundant liquid on earth and it plays an important role in biological systems. NADES could play a major role as a third type of solvent in cells and living organisms, besides water and lipids (Choi *et al*., 2011; Dai *et al*., 2013). From biological viewpoint, given the coexistence of the two liquids (NADES and water) in organisms it is of great importance to learn more about how water affects NADES.

Different aspects of the effect of water or other organic solvents on ILs have been studied, including structures, physical properties and applications aspects by different groups. In general, the structures and corresponding physicochemical properties of ILs will change with dilution. With the addition of water, the interaction of the two components of ILs is weakened and new hydrogen bonds are formed between the ions and water, as discovered by IR and NMR spectroscopy (Zhang *et al.*, 2010; Mele *et al.*, 2003; Hayes *et al.*, 2012). Certain amounts of water in ILs decrease their viscosity and density (Seddon *et al.*, 2000; Jacquemin *et al.*, 2006), accelerate the diffusion (Schröder *et al.*, 2000), and change the polarity (Fletcher and Pandey, 2003) and conductivity (Widegren *et al.*, 2005) of ILs. ILs diluted with a large amount of water or ethanol or methanol have been used in the extraction of diverse natural products (Du *et al.*, 2007; Cao *et al.*, 2009; Bica *et al*., 2011; Usuki *et al.*, 2011) and enzyme reactions (Barahona *et al.*, 2006). The extraction yield of certain compounds can be increased by 60-90% through adjusting its water content and the optimal concentrations of ILs for extraction are in the range of 0.5-2 M (Cao *et al.*, 2009; Du *et al.*, 2007; 2009; Dai *et al*., chapter 1). However, the applications of synthetic ILs in the health-related areas are limited because of the high toxicity of some ingredients, their irritation properties, and high costs of synthesis of the components (Docherty *et al*., 2005; Quijano *et al*., 2011). Therefore, in view of their potential applications and the fact that their intermolecular interactions might differ from those in ILs and DES, it is necessary to investigate their behavior when diluted with water.

Thus we explored NADES and water mixtures in terms of supermolecular structures, physicochemical properties, and solubilizing capacity for non-water soluble compounds (quercetin) and a medium polar compound (carthamin) to demonstrate the role of water dilution in developing tailor-made NADES for specific applications. This study provides the basis for deeper understanding the chemical structures and physical properties, and lays the basis for design an adaptable NADES for specific applications in health-related areas.

2. Material and methods

2.1 Chemicals and material

Water was deionized water. Malic acid, lactic acid, proline, glucose, sucrose, 1,2-propanediol, and choline chloride were purchased from Sigma (St. Louis,

MO, USA). Deuterium oxide was obtained from CortecNet (Voisins-Le-Bretonneux, France.) Carthamin and quercetin were previously isolated in our laboratory.

2.2 Solvent preparation

NADES including malic acid-choline chloride (MC); proline-malic acid (PM); and glucose-choline chloride (GC) were prepared by heating with stirring at 70 ^{o}C until a clear liquid was formed. Other NADES including malic acid-choline chloride-water(molar ratio, 1:1:2; MCH), proline-malic acid-water (1:1:3; PMH), glucose-choline chloride-water (2:5:5; GHC), 1,2-propanediol-choline chloride-water (1:1:1, PCH), lactic acid-glucose-water (5:1:3, LGH); sucrose-choline chloride-water (1:4:4, SuCH) and other NADES listed in table 1 were prepared with the following method: the mixture of two components with certain amount of water was placed in a bottle with a cap and heated in a water bath at 50 ^{o}C with agitation till a clear liquid was formed (Dai *et al.*, 2013). 1,2-propanediol-choline chloride-water (1:1:1, PCH) were diluted with different percentage (v/v) of deuterium oxide one day before the ^{1}H NMR measurement.

2.3 Solubility test

Solubility tests were carried out by saturating NADES with an excess of the tested compound at 40 ^{o}C, as reported in our former study (Dai *et al.*, 2013). The resulting solution was analyzed with HPLC-UV at a wavelength of 370 nm for quercetin and 517 nm for carthamin. The solubility test was repeated three times.

2.4 Apparatus and analysis

FT-IR spectra over the range from 4000 to 300 cm^{-1} were collected at room temperature (25 ^{o}C) using a Bruker FT-IR spectrometer. The ^{1}H NMR spectra of samples were recorded at 40 ^{o}C on a 500 MHz Bruker DMX-500 spectrometer (Bruker, Karlsruhe, Germany) with the parameters previously reported (Dai *et al.*, 2013). Water activity test was performed in a Labmaster water activity equipment (Novasina, Switzerland) at 40 ^{o}C. Density tests were performed using a density meter (DMA 5000) at 40 ^{o}C. Conductivity test was recorded at ambient temperature (27 ℃) on a 756 KF coulometer (Metrohm) equipped with 728 Stirrer and 756 KF Coulometer keyboard). Polarity testing was done with Nile red (NR) as a solvatochromatic probe. The λ_{max} was determined with a UV/Vis spectrophotometer and calculated in the following formula to obtain E_{NR} (Ogihara, *et al*., 2004):

E_{NR} (kcal/mol) $= hc\, N_A/\lambda_{max} = 28591/\lambda_{max.}$

3. Results and discussion

3.1The structure of NADES with and without water

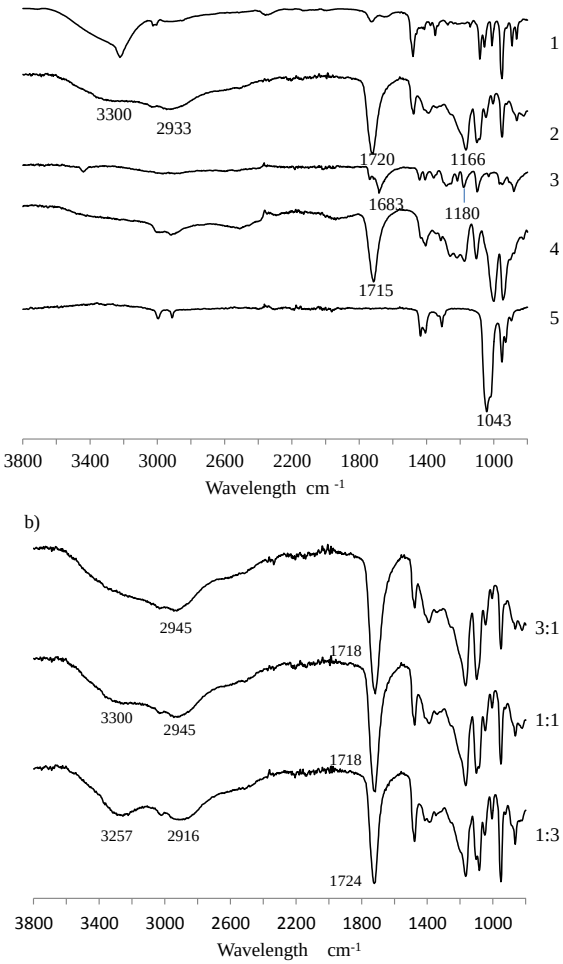


Fig. 1. FT-IR **a**) of **1**) choline chloride, **2**) malic acid-choline chloride (1:1, molar ratio; MC), **3**) malic acid solid, **4**) malic acid in DMSO and **5**) DMSO and **b**) malic acid-choline chloride with increasing amount of choline chloride (the molar ratio of malic acid to choline chloride 3:1, 1:1; 1:3) at room temperature.

3.1.1 The structure of NADES. Previous studies using NMR spectroscopy showed that there are cross interactions, and particularly hydrogen bonds, between the two components of NADES (Abbott *et al.*, 2004, Dai *et al.*, 2013). The structure of NADES was further investigated using IR to explore the type of interactions, the atoms involved in the interactions and even the possible ratios of the two components. Various categories of changes may occur on the establishment of an hydrogen bond, which can be characterized by the following structural and spectroscopic features (Reichardt, 2003): 1) The distance between the neighboring atoms involved in the hydrogen bonds are

considerably smaller than the sum of their van der Waals radii, resulting in correlation signals in nuclear Overhauser effect spectroscopy (NOESY) or Heteronuclear Overhauser Effect Spectroscopy (HOESY) spectra; 2) The electron density at H-atoms involved in H-bonds is reduced, resulting in downfield shifts of their ^{1}H NMR signals; 3) The bond length and corresponding vibrations of H-bonds are changed, which can be detected in IR. The vibrations involve the H-bond donor, such as the bending bond $\delta_{C\text{-}O\text{-}H}$ of a C-O-H group. Vibration changes also occur in the vicinity of the H-bonds, such as the stretching bond $\nu_{C\text{-}OH}$, as well as in the H-bond acceptor, such as the $\nu_{C=O}$ in carboxyl or carbonyl groups (Marécchal, 2007).

The IR spectrum of malic acid-choline chloride (Fig. 1a) shows broad moderate bands centered around 3300 and 2933 cm^{-1}, the stretching vibration of hydrogen-bonded hydroxyl group $\nu_{O\text{-}H}$ in malic acid and choline chloride and carboxyl group in malic acid, which means the formation of hydrogen bonds between malic acid and choline chloride. The strong band at 1683 cm^{-1} is ascribed to the stretching vibration of carboxylic acid $\nu_{C=O}$ dimers. The band of $\nu_{C=O}$ is shifted upwards to 1720 cm^{-1} in malic acid-choline chloride, which is attributed to new H-bonds formed in the malic acid-choline chloride complex with malic acid as H-bond donor. Concerning the strength of the H-bond, -COOH is more likely to form an H-bond with Cl^{-} than with the O in choline chloride (Barańska *et al*., 2003). So, a hydrogen bond (–COOH---Cl^{-}) is formed between malic acid and choline chloride. On forming the malic acid–choline chloride complex, the deformation vibration absorption band of δ_{CH2} increased in intensity and is shifted from1180 cm^{-1} down to 1166 cm^{-1}, suggesting that malic acid has a different configuration in the malic acid–choline chloride eutectic mixture than in solid state (Barańska *et al*., 2003).

In addition, the type and position of hydrogen bonds were confirmed in the following two experiments. In malic acid DMSO solution, the strong band of the carboxyl group in malic acid $\nu_{C=O}$ shifted from 1683 cm^{-1}upwards to 1715 cm^{-1} in a malic DMSO solution (Fig. 1a), which is attributed to new H-bonds formation in the malic acid-DMSO complex with malic acid as H-bond donor (S=O---HO-C=O). It is the similar to the malic acid–choline chloride complex. The hydrogen bonds were confirmed by the downwards shift of the stretching vibration band $\nu_{S=O}$ from 1042 cm^{-1} to 1000 cm^{-1}. The IR spectra of malic acid-choline chloride mixtures with different ratios (Fig. 1b) confirm that there are at least two kinds of H-bonds between malic acid and choline chloride. The formation of an H-bond affects two characteristic absorptions of the carboxylic acid, namely the stretching vibrations $\nu_{C=O}$ around 1720 cm^{-1} and $\nu_{H\text{-}O}$ from COO-H around 3000 cm^{-1}. With the increasing amount of choline chloride, the upward shift of $\nu_{C=O}$ from 1718 cm^{-1} to 1724 cm^{-1} and downward shift of $\nu_{H\text{-}O}$ from 2945 cm^{-1} to 2916 cm^{-1} indicates more H-bonds (e.g. –COOH---Cl^{-}) are formed between malic acid and choline chloride. The characteristic vibration band of $\nu_{O\text{-}H}$ around 3300 cm^{-1} increased in intensity and also shifted downward

to 3257 cm^{-1} with the increasing amount of choline chloride, suggesting that hydroxyl groups in the mixture also form H-bonds.

Similar hydrogen bonds are also detected in the 1,2-propanediol-choline chloride mixture. The IR spectrum of 1,2-propanediol and choline chloride also indicates the existence of H-bonds (CH-OH---Cl^-) between 1,2-propanediol and choline chloride (Fig. 2). On forming a eutectic mixture, the $\nu_{C\text{-}OH}$ of 1,2-propanediol shifted form 1038cm^{-1} to 1044 cm^{-1} indicating the H-bond formation between the hydroxyl groups of 1,2-propanediol and choline chloride with 1,2-propanediol as H-bonding donor. Concerning the strength of the H-bonding, C-OH is more likely to form H-bonding (CO-H---Cl^-) with Cl^- than O in choline chloride. In addition, the formation of a H- bond with CH_2-OH in choline chloride as H-bond donor was revealed by the shift of the vibration band of $\nu_{C\text{-}O}$ in CH_2-OH of choline chloride from 1012 cm^{-1} in the solid to 1006 cm^{-1}in the 1,2-propanediol-choline chloride mixture. All this structural information is in agreement with our reported 2D NMR results (Dai *et al*., 2013). Clearly, there is an extensive hydrogen-bonding network between the components of NADES; thus NADES are supermolecules.

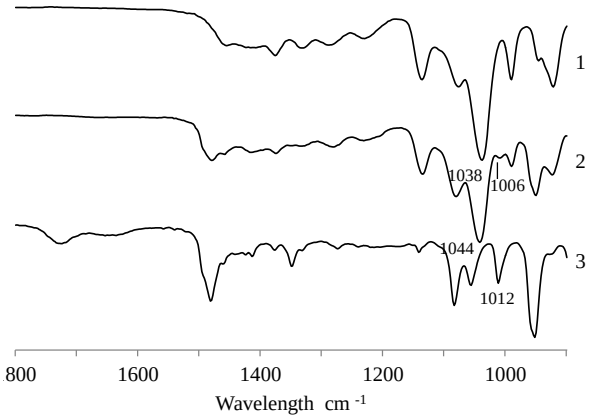


Fig. 2. FT-IR of **1**) choline chloride, **2**) 1,2propanediol:choline chloride (molar ratio 1:1) and **3**) choline chloride.

The NOESY spectrum of PMH showed strong interactions between malic acid and proline (Dai *et al.*, 2013). Infrared spectra of the proline-malic mixture, solid malic acid, and solid choline chloride (Fig. 3a) may provide more detailed information on the supermolecular structure of PMH. Proline exists in the ionic form in solid state, as indicated by the stretching bond $\nu_{C=O}$ at 1615 cm^{-1}. A strong band at 1683 cm^{-1} indicates that malic acid exists in the dimeric form in solid state. In the malic acid-proline mixture, new H-bonds form between the two components, as shown by the broad band at 3062 cm^{-1} of ν_{OH} in COOH and the upward shift of the $\nu_{C=O}$ to 1717 cm^{-1} in malic acid. The stretching bands of hydroxyl groups shifted downwards to ca. 2450 cm^{-1} and 1920 cm^{-1} which is

indicative of the appearance of a H-bond interaction (O=C-O-H---N) between malic acid and proline in the liquid crystal (Barańska *et al*., 2003; Xu *et al*., 2005). On forming the malic acid–proline complex, in malic acid the deformation vibration absorption band of δ_{CH2} increased in intensity and shifted from1180 cm^{-1} down to 1170 cm^{-1}, and in proline the strong in-plane bending vibration of the carboxyl group δ_{OH} shifted from 1374 cm^{-1} to 1397 cm^{-1} and the strong broad band of amine δ_{NH} shifted from 1557 cm^{-1} to 1660 cm^{-1}, suggesting that malic acid and proline have a different configuration in the malic acid–proline eutectic mixture than in their solid state (Barańska *et al*., 2003; Sheena Mary *et al*., 2009). The IR spectra of malic acid-proline mixed in different molar ratios (1:3; 1:1; 3:1) (Fig. 3b) show that the band of $v_{C=O}$ at 1708 cm^{-1} shifts up to 1716 cm^{-1} and v_{OH} in carboxyl group shifts from 2972 cm^{-1} to 2980 cm^{-1}, indicating that more H-bonds between proline and malic acid are formed when the ratio of proline–malic acid increases from 1:3 to 1:1.When the amount of proline–malic acid increases to 3:1, there is no shift of the v_{CO} and v_{OH} in carboxyl group. This phenomenon suggests that 1:1 is a proper ratio for malic acid and proline to form a eutectic mixture.

3.1.2 The structure of NADES with water dilution. To further explore the supermolecular structure of NADES after dilution with water, the NADES made mixture of 1,2- propanediol and choline chloride (PCH) was diluted with 0% to 75% (v/v) deuterium oxide and investigated with NMR as an example. FT-IR and the NOESY spectra confirm the existence of hydrogen bonds between the hydroxyl groups of 1,2-propanediol and chloride ion of choline chloride and between all the hydroxyl groups from molecules of 1,2-propanediol, choline and water (Dai *et al.*, 2013). The ^1H NMR spectra of 1,2-propanediol-choline chloride with increasing amount of D_2O (Fig. 4, Table 1) show a continuous downfield shift of all signals from the two components except the peaks of methyl and methylene groups vicinal to the nitrogen atom, suggesting hydration of the two organic components of the mixture PCH. Also, the relative number of detected protons on the hydroxyl groups (relative to the protons on the methyl group) decreased during dilution (Table 1). The decreasing number of detected hydroxyl groups implies the rupture of the hydrogen bond between 1,2-propanediol and choline chloride. When the dilution increased from 25% to 50%, all the signals of the hydroxyl groups disappeared and there was no further shift of the water signal with a further dilution to 75% D_2O. This indicates the complete rupture of the hydrogen bond between 1,2-propanediol and choline chloride with 50% D_2O. The above results show that the supermolecular complex interactions of PCH are perserveed with less than 50% water dilution, but further dilution produce a solution of the free forms of the individual components in water (Gutiérrez *et al.*, 2009). (The further downfield shift of peaks from all methylene or methyne connected to hydroxyl group is due to the effect of concentration as the diluted increased from 50% to 75% (Kim *et al*., 2006)).

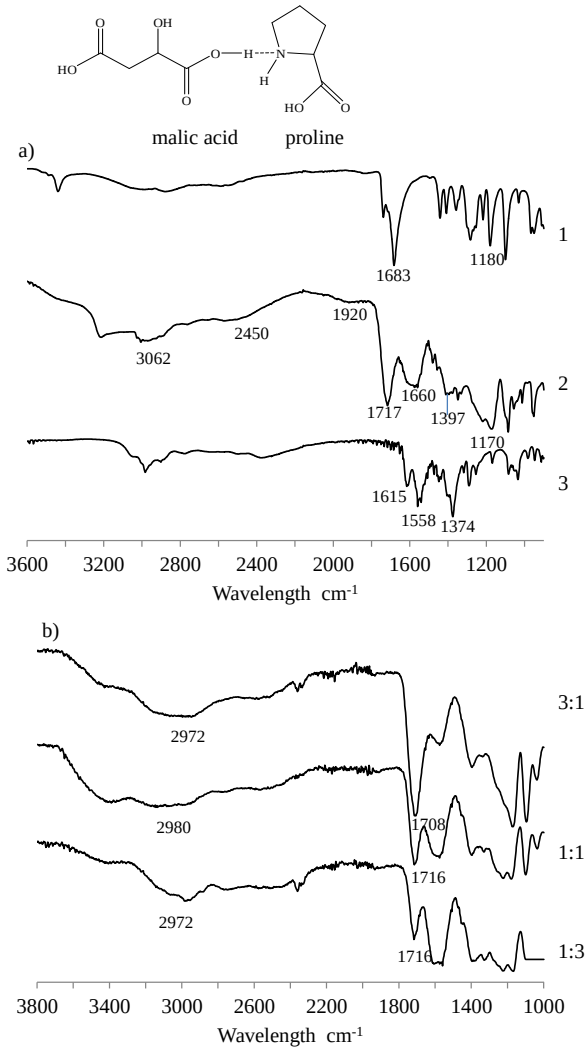


Fig. 3. FT-IR **a**) of **1**) malic acid, **2**) malic acid-proline (1:1, molar ratio; PM) and **3**) proline solid at room temperature and **b**) malic acid:proline (PM) with different molar ratio (3:1; 1:1; 1:3).

It was reported that with the addition of water, the interaction of the two components of ILs is weakened (López-Pastor *et al.*, 2006; Kohno and Ohno, 2012). With increasing water concentration, association of water molecules with the anions is observed. With further dilution, the association with water molecules increases and ends with hydrated ions. In agreement with ILs, dilution of NADES with a large amount of water will lead to the hydration of each component, resulting in a solution of two free components.

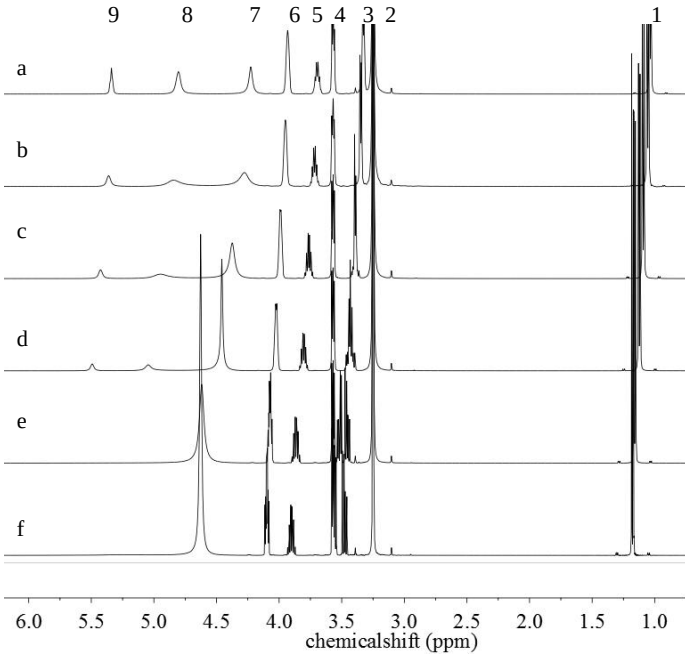


Fig. 4. ^{1}H NMR recorded at 40 ^{o}C of 1,2-propanediol-choline chloride-H_2O (1:1:1) diluted using deuterium oxide from 0% (**a**), 5% (**b**), 15% (**c**), 25% (**d**), 50% (**e**), 75% (**f**) D_2O (v/v) with details of downfield shifts of HDO (peak 7), choline (peaks 2, 4, 6), 1,2 propanediol (peaks 1, 3, 5) and hydroxyl groups from 1,2-propanediol (peaks 8) and choline (peaks 9).

Table 1. Chemical shift and some integrated areas of peaks in ^{1}H NMR spectroscopy of 1,2-propanediol- choline chloride-H_2O diluted with different percentage of deuterium oxide at 40 ^{o}C.

PCH% (V)	**1,2-propanediol** $HOCH_2$-(OH)CH-CH_3				**choline chloride** $(CH_3)_3NCH_2CH_2OH$				**HDO**
	CH_3	$HOCH_2$	(OH) CH	-OH	NCH_3	NCH_2	CH_2 OH	CH_2OH	HDO
	6	4	5	8	3	2	1	9	7
100%	1,04[a]	3,69	3,33	4.8(1.24H[b])	3,25	3,57	3,93	5,34(0,6H)	4,23(1,29H)
95%	1,05	3,72	3,35	4.84(0.98H)	3,25	3,57	3,95	5,36(0,47H)	4,28(1,56H)
85%	1,09	3,76	3,39	4.94(0.77H)	3,25	3,57	3,99	5,43(0,38H)	4,37(2,55H)
75%	1,12	3,8	3,43	5.04(0.55H)	3,25	3,57	4,02	5,49(0,27H)	4,46(2.55H)
50%	1,16	3,87	3,49		3,25	3,57	4,07		4,62(6,16H)
25%	1,18	3,90	3,52		3,25	3,56	4,1		4,63(11,64H)

[a] the chemical shift of the proton underlined; [b] the relative integrated area of the proton in hydroxyl groups (relative to the protons on the methyl group).

3.2 Properties of NADES diluted with water

3.2.1 Viscosity. The viscosity of NADES is greatly affected by the water content. For example, the viscosity of GCH decreases to 1/10 of the original value (397.4 mm^2/s) with the addition of 10% water (Dai *et al.*, 2013). Even for NADES with the lowest viscosity, PCH, the viscosity decreases to 1/15 of the original value (33 mm^2/s) when diluted with the same volume of water following a function $y=0.0008x^{-1.524}$ (r=0.99) (Fig. 5a). The high viscosity of NADES is often attributed to the presence of extensive hydrogen bonding network between the components (Zhang *et al.*, 2012), which is in agreement with our IR results mentioned above. In fact one may consider the NADES to be liquid crystals. Through dilution with water, the interactions between the components of NADES become weaker, as mentioned before. The change in structure leads to a large decrease in viscosity of NADES. After adding 25% water to GCH and PCH, their viscosity decreased to 7.2 mm^2/s and 6.10 mm^2/s, respectively, which is in the range of the viscosity of water (0.66 mm^2/s), whereas they still possess the supermolecular characteristic.

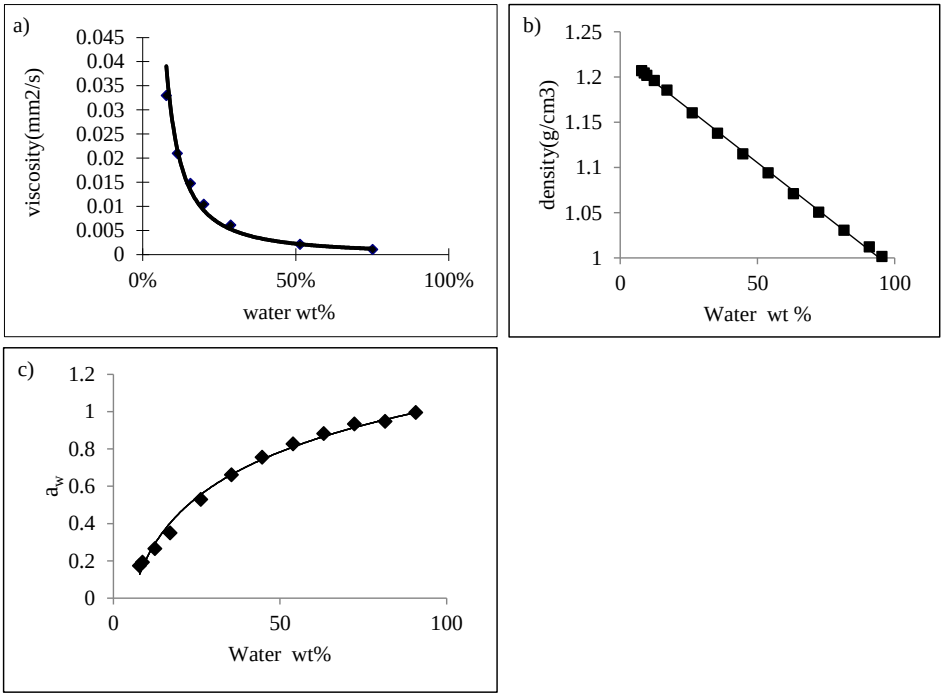


Fig. 5. The relationship curves a) between the viscosity and water percentage in weight (w), b) between the density (g/cm^3) and water percentage and c) between water activity and water percentage in glucose-choline (1:2.5).

3.2.2 Conductivity. The conductivity of 16 typical NADES and four kinds of diluted NADES with water was investigated at room temperature. Big differences in conductivity were observed for the NADES, as shown in Table 2.

Polyalcohol-choline has the highest conductivity value (GlyCH, 13.75 mS/cm) while sugar-sugar (FGSH, 0.001 mS/cm) has the lowest, being similar to water (0.002 mS/cm) and methanol (0.004 mS/cm). In this perspective, the NADES made of sugars can be considered as neutral NADES. The conductivity of NADES decreased in the following sequence: base-polyalcohol > base-organic acid ≈ base-sugar > organic acid-non-polar amino acid > organic acid-sugar> sugar-sugar.

Table 2. The conductivity (mS/cm) of natural deep eutectic solvents at room temperature with water and methanol as reference (at 27 ℃).

compositions	**NADES**	**conductivity**
fructose:glucose:sucrose: water (1:1:1:11)	FGSH	0.0012
lactic acid:glucose: water (5:1:3)	LGH	0.114
malic acid:*β*-alanine:water (1:1:3)	MAH	0.551
proline:malic acid: water (1:1:3)	PMH	1.06
fructose: choline chloride: water (2:5:5)	FCH	3.1
xylose: choline chloride: water (1:2:2)	XCH	3.15
sucrose: choline chloride: water (1:4:4)	SuCH	3.39
malic acid: choline chloride: water(1:1:2)	MCH	5.91
lactic acid: choline chloride: water (1:1)	LC	6.76
sorbitol: choline chloride: water (2:5:6)	SoCH	6.77
glucose: choline chloride: water (2:5:5)	GCH	6.81
1,2-propanediol: choline chloride: water (1:1:1)	PCH	12.09
xylitol:choline chloride: water (1:2:3)	XoCH	13.55
glycerol:choline chloride:water(2:1:1)	GlyCH	13.78
Water	water	0.00208
Methanol	methanol	0.00424

NADES with high and low conductivity values are selected to detect the effect of water content on the conductivity of NADES. The conductivity is first increased with increasing water content, and then decreased after reaching a peak value of around 10-100 times higher than that of pure NADES (Fig. 6). SuCH, GCH, PCH have the highest conductivity with 60% added water and LGH has the highest one with 80% added water. A similar phenomenon was also reported with ILs diluted with different percentage of water, in which the conductivity of ILs increased more than 10 times (Liu *et al.*, 2008). So, the conductivity of NADES can be tailored through changing the water content.

These results show that the conductivity of NADES is correlated with their composition and viscosity, similar as for ILs (Every *et al.*, 2004; Widegren *et*

al., 2005; Chiappe and Pieraccini *et al.*, 2005). Comparing NADES with one same component, GCH (6.81 mS cm^{-1}) has much higher conductivity than LGH (0.11 mS cm^{-1}), and MCH (5.91 mS cm^{-1}) higher than PMH (1.06 mS cm^{-1}), which may be due to the ionic form of choline chloride and partial ionization of lactic acid (p*k*a 3.86) and proline (p*k*a 1.99).Thus, it may be concluded that NADES with choline chloride have a high conductivity while NADES with organic acid have low values. The second important factor is viscosity. The second important affecting factor for the conductivity of NADES is their viscosity. NADES made of polyalcohol-base such as GlyCH are highly conductive, and this might be correlated to their low viscosity (51 mm^2/s), around 1/10 of that of sugar-choline (Dai *et al.*, 2013). Most NADES with a low conductivity (lower than 10) are highly viscous, especially NADES containing a sugar or amino acid (300-600 mm^2/s). Through dilution with water, the interactions between the components become weaker leading to a decrease of the viscosity of the NADES and as a result the conductivity increases by 10-100 times. No relationship between the conductivity and polarity of NADES is observed.

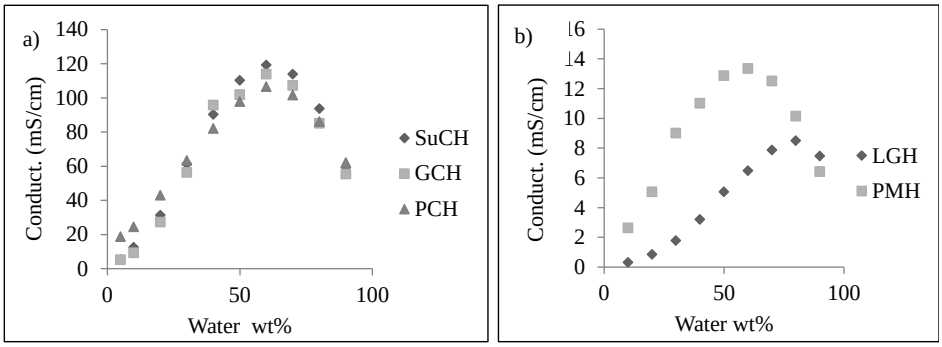


Fig. 6. Conductivity data of diluted NADES at ambient temperature (27 ℃) of **a**) sucrose: choline chloride: water (1:4:4, SuCH), glucose: choline chloride: water (2:5:5, GCH), 1,2-propanediol-choline chloride (1:1:1, PCH), and of **b**) lactic acid-glucose (5:1:3, LGH).

3.2.3 Polarity, density and water activity. Other physical properties, such as polarity, density and water activity, are also affected by the water content, like in ILs (Seddon *et al.*, 2000). Previous experiments showed that the water content has a great effect on the polarity of NADES (Dai *et al.*, 2013). In those experiment LGH with the highest (44.81 kcal/mol) and PCH with lowest polarity (50.07 kcal/mol) were selected to test the effect of water content on the polarity of NADES (Table 3, chapter 2). Notably, the polarity value of LGH remained the same when the water content increased from 0 to 25%, but increased considerably with 50% water dilution and finally reached a similar polarity value as water (48.2 kcal/mol) at 75% dilution. Conversely, the polarity

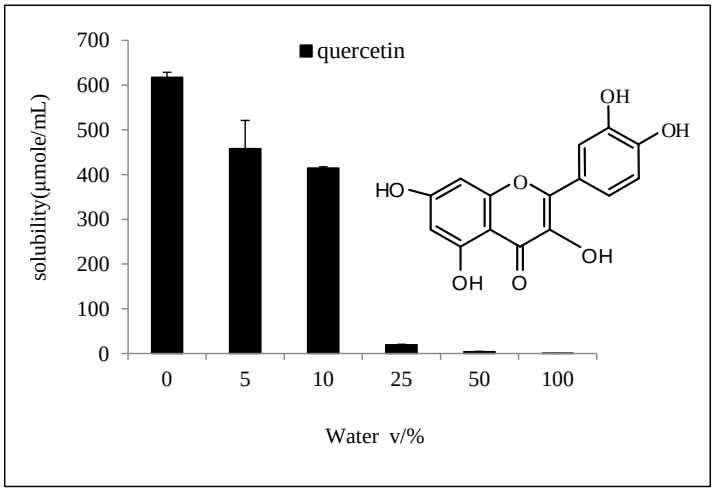
value of PCH decreased with the addition of water because the polarity value of PCH is bigger than water (Dai *et al.*, 2013). With 10% percent, there is no obvious change of the polarity of PCH, but it decreased with the addition of 15-50% water and finally stabilizes at 75% dilution. So water dilution results eventually in a polarity similar to water. The density of GCH decreased linearly with the addition of water which can be expressed in a function y=-0.0024x+1.224, r=0.99, as shown in Fig. 5b. A similar linear relationship was also observed in SuCH (y=-0.0025x+1.2418, r=0.99) and PCH (y=-0.0009x+1.0873, r=0.99), which is consistent with the findings for synthetic ILs (Seddon *et al.*, 2000). The last property investigated in diluted NADES is the water activity. The water activity of NADES increased gradually with the addition of water, which is quantitatively expressed in a function y = 0.354ln(x) - 0.6008 for GCH (Fig. 5c). Above a 50% dilution of GCH with water, there is a linear relationship (y=0.004x+ 0.6016, r=0.99) between the water content and water activity, which may be correlated with above mentioned effect of water content on the supermolecular structure of NADES.

3.3 Applications of NADES diluted with water

The possibility of adjusting NADES properties quantitatively by adding water allows them to be adapted to different applications. An important application of NADES is their high solubilizing capacity. The solubility of quercetin (a widely spread flavonoid in plants) and carthamin was investigated in PCH with different water contents. The solubility of quercetin in PCH is 30,000 times higher than in water. However, its solubility changes with an increasing water content in PCH, decreasing in PCH with 5 v% water, keeping similar value with 10% water but decreasing drastically upon dilution with 25% water (Fig. 7). PCH is the NADES with the lowest polarity (50.0 kcal/mol, similar as methanol) of all our tested NADES (Dai *et al.*, 2013). The dilution of PCH leads to an increase of its polarity and consequently to a decrease in the solubility of nonpolar compounds. Notably, the case is different for carthamin (a natural dye and antioxidant), which is most soluble in PCH with 5% water, probably due to its medium polarity. Most interestingly, both the solubility of both quercetin and carthamin decrease strongly when the water content increases from 25% to 50% in PCH, reaching nearly the same values as in water. These phenomena are coherent with the above-mentioned effect of water dilution on the structure of PCH.

The physicochemical characteristics of NADES with different water contents are useful to predict the solubilization capacity of NADES. Our previous study showed that quercetin has the highest solubility in pure GCH, rutin with 5% (v/v) water in GCH and carthamin with 10% (v/v) water in GCH (Dai *et al.*, 2013). Firstly, this is due to the different polarity of the compounds and more water dilution is needed for more polar compounds to reach the highest solubility in NADES. The conclusion can be drawn that nonpolar compounds have the highest solubility in pure NADES and 5-10% water

dilution can increase the solubility of medium polar compounds in NADES. Secondly, the optimal water content is correlated with the viscosity of NADES instead of polarity due to the supermoelcular structure of NADES. For example, carthamin has the highest solubility in GCH with 10% (v/v) water and in PCH with 5% (v/v) water. The viscosity of GCH (397 mm^2/s) is much higher than PCH (33 mm^2/s). The polarity of GCH is more close to water while PCH is more close to methanol (Dai *et al*., 2013). However, to reach the highest solubility, more water is needed in GCH than in PCH and so this correlates with the high viscosity of GCH. This points to a strong liquid crystal like structure in GCH with high viscosity where there is no space for dissolving various solutes and more water is needed to loosen the H-bonded structure to dissolve solutes.



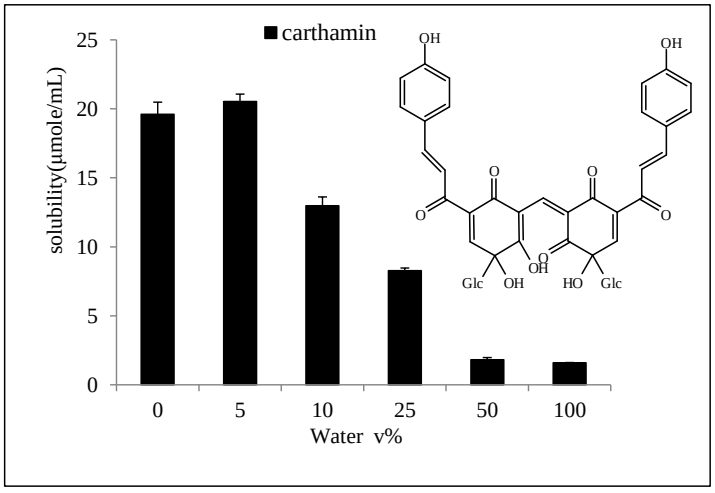


Fig. 7. The solubility of quercetin and carthamin in 1,2-propanediol-choline chloride-water (1:1:1, molar ratio) diluted with a different percentage of water. The data is expressed in mean ± SD (n=3).

The second important application of diluted NADES is for enzymatic reactions. For example, the water activity of ILs is an important factor affecting the microenvironment of enzymes and thus their activity. Enzyme activity is often low at both high and low water activity, thus requiring optimization of the conditions. We observed that laccase was inactive in malic acid-choline chloride-water (MCH) but was activated after dilution with 50% water. Similar phenomena were reported, for lipase with a maximum of the initial reaction rate at a_w=0.6 in [bmim][PF_6] (Barahona *et al.*, 2006) and for horseradish peroxidase being activated in the presence of a small amount of water (4.53%, v/v) in [Bmim][BF_4] (Wang *et al.*, 2007). This aspect is of interest in connection with the functioning of enzymes in cells, apparently reaction rates can be changed by altering the water environment of the enzyme. It may also explain the germination of seeds and the survival of resurrection plants, which store enzymes in NADES and the enzymes can be activated after absorbing water.

4. Conclusion

NADES are supermolecules with hydrogen-bonding interactions between the components, which will be weakened upon water dilution and even disappears when the water content is around 50 v%. The physicochemical properties can be tailored in a controllable way when diluted with water. With increasing water content, viscosity decreases considerably down to the level of water at around 25% water level; Conductivity of NADES covers a broad range and increases with water dilution reaching a peak value with 60-80% water of around 10-100 times that of pure NADES, which is correlated with high viscosity of NADES; Density decreases with water dilution in a linear way; water dilution results in a polarity value similar to water; water activity increases with water dilution.

The changed properties of diluted NADES provide a basis to develop tailor-made for various applications. The water content in NADES significantly affects the solubility of compounds in NADES and enzymetic reactions, revealing their enormous potential for diverse uses in health related areas.

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Chapter 5

Natural Deep Eutectic Solvents providing enhanced stability of natural colourants from safflower (*Carthamus tinctorius*)

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Abstract

As promising new solvents for food, cosmetic and pharmaceutical industry, natural deep eutectic solvents (NADES) show a good solubilizing capacity for compounds with diverse polarities. In addition to the solubility data, the stability of compounds in NADES should be investigated for further applications. As a model, the stability of carthamin, an unstable pigment, was evaluated in some typical NADES. The different factors affecting the stability of compounds are heat, light, and storage time. In all tested conditions, carthamin was more stable in sugar-based NADES than in water or 40% ethanol. Moreover, carthamin together with hydroxysafflor yellow A and cartormin, the main active compounds and major pigments in safflower, exhibited improved stability in sugar-based NADES extracts at ambient conditions in daylight. Notably, NADES extracts with low water content and high viscosity exhibited even better stability. The strong stabilization capacity of some NADES was found to be due to the formation of hydrogen bonding interactions between solutes and NADES molecules, which is related with the high viscosity of NADES. These results show the stabilizing ability of NADES for phenolic compounds and holds great promise for their applications in food, cosmetic and pharmaceutical industry.

Key words: natural deep eutectic solvents; stabilizing capacity; carthamin; safflower; water content; hydrogen bonding.

1. Introduction

To extend the range of green solvents, we proposed natural ionic liquids and deep eutectic solvents (NADES) for applications in health-related areas such as food, pharmaceuticals and cosmetics (Choi *et al*., 2011; Dai *et al*., 2013). NADES are liquid supermolecules made of natural primary metabolites bound together by inter-molecular interactions, particularly hydrogen bonding. They have several advantages over synthetic ionic liquids, e.g. their low cost, biodegradability, non-toxicity, sustainability, and simple preparation methods. Moreover, they show very good physicochemical properties as solvents: negligible volatility, liquid state even below 0 ^{o}C, adjustable viscosity, wide polarity range, and high solubilization strength for a wide variety of compounds (Dai *et al*., 2013). All those properties imply their potential for various types of applications in health related areas. Until now, they have been used in metabolite extraction (Usuki *et al*., 2011), enzyme stability and enzymatic reactions (Kragl *et al*., 2002; Kaar *et al*., 2003; Gorke *et al*., 2008). Few studies have been done to evaluate the stabilization ability of NADES for natural products. Undoubtedly, it is essential to determine the stability of compounds in NADES in order to evaluate its applicability to all kinds of natural products extraction and processing.

Natural deep eutectic solvents (NADES) have a great potential as stabilizing media for e metabolites due to their unique physicochemical properties. In our view, NADES occur in all organisms and cells, where NADES exist around the membranes and are involved in the biosynthesis, solubilization and storage of various poorly water-soluble metabolites and unstable compounds in cells (Choi *et al*., 2011; Dai *et al*., 2013). This hypothesis does give a number of ideas for applications of NADES. For example, carthamin is stable in the plant which implies that in safflower NADES may stabilize carthamin as well as other pigments.

Safflower (Flos carthami), the corolla from *Carthamus tinctorius* L. (Asteraceae) is used as a natural dye, food additive, and cosmetic. Also, it is widely used as traditional medicine for cardiovascular diseases (Huang *et al*., 1987; Shi and Liu, 2006; Zhou *et al*., 2008). Antioxidant and neuroprotective properties have also been reported for safflower extracts (Hiramatsu *et al*., 2009; Lee *et al*., 2009). The dried petals of safflower contain yellow and red pigments. Safflower yellow pigment is one of the few water-soluble yellow pigments found in nature. The major components of safflower yellow pigments are hydroxysafflor yellow A (HSYA), safflor yellow B and some other minor components, such as cartormin (Kazuma *et al*., 2000; Yin and He 2000). HSYA was reported to have an antithrombotic and neuroprotective effect (Zhu *et al*., 2003; Wei *et al*., 2005). Carthamin is the major red pigment in safflower and it has antioxidant activity (Takahashi *et al*., 1982; Wang and Zheng 2006).

Thebasic structure of those pigments is a *C*-glucosyl quinochalcone (Jin *et al*., 2008; Kazuma *et al*., 2000).

However, carthamin is very unstable in an aqueous solution. It is usually extracted with an alkaline solution, but the red colour fades progressively to reddish orange, orange-yellow, yellow and light yellow (Saito *et al*., 1992; Wang and Zheng, 2006). Carthamin has been reported to be more stable in alkaline than in acid or neutral conditions (Fatahi *et al*., 2009). But it degrades fast when heating, having a half-live in alkaline conditions of 12.5 hours at 25 ^{o}C and 0.75 hours at 60 ^{o}C (Kim and Paik, 1997). The presence of NADES in the plant may, to some extent, solve the instability of carthamin in the plant cells.

NADES have totally different characteristics if compared to conventional solvents. The major components of NADES are natural primary metabolites, e.g. sugars, sugar alcohols, organic acids, amino acids and amines, which have several hydroxyl groups, carboxyl groups, or amino groups (Choi *et al*., 2011; Dai *et al*., 2013.) Hydrogen-bonding interactions appear between molecules with these groups, leading to highly structured viscous liquids. Those liquids can in turn, form hydrogen bonds with solutes (Dai *et al*., 2013) increasing their solubility, e.g. phenolic compounds. NADES are like liquid crystals in which all molecules are arranged in a matrix with optimum interactions via inter- and intra- molecular H-bonding of the constituents. Studies are needed to explore the full potential of NADES as solvents for food, medicine and cosmetics.

This chapter describes the stabilizing ability of some typical NADES for carthamin. The stabilizing mechanism was investigated, and NADES with high stabilization ability for unstable phenolic compounds like carthamin were developed. As an important factor, the effect of the water content in NADES on the stability of carthamin was investigated.

2. Materials and methods

2.1. Chemicals, material and reagents

Carthamin was isolated from safflower bought from Xinjiang province in China. The plant material was identified by Dr. Young Hae Choi, and a voucher specimen (NPL-safflower-0913) was deposited in the Natural Products Laboratory, Institute of Biology, Leiden University. The dry plant material was ground into a powder in a blender with liquid nitrogen. Ethanol of analytical grade and acetonitrile of HPLC grade were purchased from Biosolve BV (Valkenswaard, The Netherlands). Water was of deionized water quality. Malic acid, lactic acid, proline, sucrose, glucose, xylitol and choline chloride were purchased from Sigma (St. Louis, MO, USA).

2.2. Solvent and sample preparation

All NADES including glucose-choline chloride (GCH); sucrose-choline chloride (SuCH); proline-malic acid (PMH); lactic acid-glucose (LGH); xylitol-choline chloride (XoCH) were prepared using mild heating combined with stirring at 50 ^{o}C (Dai *et al*., 2013). The 40% EtOH, 90% GCH, 75%, 50% and 25% PMH and SuCH were prepared diluting a certain volume of NADES with deionized water.

Carthamin solutions were prepared by dissolving carthamin in each solvent (water, 40% EtOH, NADES) with agitation for 30 min at room temperature. The samples were transferred into an Eppendorf tube, centrifuged at 1300 rpm for 20 min and then the supernatant was used for further tests. Extraction was performed in sealed bottles with 50 mg plant material and 3 mL NADES or 40% ethanol, heating and stirring at 40 ^{o}C for 30 min. The sample was transferred into an Eppendorf tube, centrifuged at 1300 rpm for 20 min and then the supernatant was filtered through a 0.45 μm cellulose membrane. The resulting solutions were used to test the effect of storage at ambient room conditions with daylight on the stability of dissolved phenolic compounds.

2.3. Stability tests

The effects of heating, light, ambient conditions in daylight, storage time, and water content in NADES on the stability of carthamin and safflower extract were investigated with the methods described bellow.

For thermal stability, carthamin solutions were put in glass vials with screw-caps and placed in a preheated water bath at 80 ^{o}C, 60 ^{o}C and 40 ^{o}C. Three tubes of each group were removed from the water bath after 10, 20, 40, 60, 80, 100, 120 min and rapidly cooled to room temperature.

The effect of light from artificial light was determined with carthamin solutions at room temperature. Tubes with the test solutions were placed one meter below a lamp (TL 40 W, Philips) or covered with aluminum foil, and three tubes of each group were taken at day 0, 3, 7, and 15 for UV spectroscopy.

The effect of storage stability was investigated at -20 ^{o}C and 4 ^{o}C in the dark with carthamin solution and three tubes of each group were tested at day 0, 3, 7, 15, 30 and60.

The effect of ambient conditions in daylight was studied with carthamin solutions and safflower extract solutions. Each solution was exposed to room conditions in the daylight of late spring in The Netherlands and three samples of each group were removed at day 0, 3, 7, 15 for HPLC analysis.

The effect of water content in NADES on the stability of carthamin was investigated with two NADES (PMH and SuCH) at 4 ^{o}C and -20 ^{o}C in the dark. PMH and SuCH were used with 0%, 25%, 50%, and 75% (v/v) water. All stability tests were done by triplicate.

2.4 Apparatus and analysis

UV-Vis spectrophotometer (Shimadzu, Japan) was used for the stability test of carthamin using a wavelength of 520 nm. Extracts were analyzed with a

Agilent 1200 HPLC-DAD on a Phenomenex Luna C18(2) (4.6 μm x 250 mm, 5 μm) column. The mobile phase consisted of 0.5% H_3PO_4 (A) and acetonitrile (B) in a linear gradient program as follows: 5%-11% B (0-10 min), 11%-14% B (10-16 min), 14% B (16-23 min), 14%-20% B (23-30 min), 20%-35% B (30-70 min), 35%-60% B (70-80 min) at the flow rate of 1.0 mL/min (Wang *et al*., 2008). The injection volume was 10 μL. Chromatograms were recorded at 520 nm, 403 nm, and 280 nm. FT-IR spectra over the range from 4000 to 300 cm^{-1} were registered at room temperature (25 ^oC) using a Bruker FT-IR spectrometer. The pH of the diluted NADES with 90% (v/v) deionized water was tested with pH indicator paper (Merck, Darmstadt, Germany).

2.5 Data analysis

Calculation of kinetic parameters of carthamin degradation at high temperature: the first-order reaction rate constants (k) and half-lives ($t_{1/2}$), for degradation of 50% of carthamin, were calculated by the following equations (Kırca *et al*., 2007):

$$\ln(C/C_0) = -k\,t$$

$$t_{1/2} = -\ln(0.5)/k$$

where $C/C_{0=}\,A/A_{0,}\,C_0$ and A_0 is the initial concentration and absorption of diluted carthamin, and C and A is the concentration and absorption value of diluted carthamin after heating time (t) at a given temperature, respectively.

3. Results and discussion

To test the effect of viscosity, polarity, and composition of NADES (acidic or basic) on their stabilization ability, the following five typical NADES were selected: PMH, LGH, GCH, SuCH and XoCH (Table 1). Physical properties of NADES with diverse compositions show that all the NADES are different in composition (acidic or basic), viscosity and polarity (Dai *et al*., 2013). LGH and PMH are acidic with a pH=3 when diluted with 90% (v/v) of water, and the other NADES have pH values from 6 to 7 after dilution (table 1). Regarding their viscosity, PMH, SuCH and GCH are the most viscous, followed by XoCH, while LGH has the lowest viscosity. LGH is the most polar of the studied NADES, whereas PMH is similar to water, and sugar/sugar alcohol-choline is slightly less polar than water. It was reported that 40% ethanol has the highest extraction ability for the pigments from safflower (Zhang *et al*., 2009) while water is a common general extraction solvent for the safflower yellow pigment (Wang and Zhang, 2007; Zhu and Pan, 2007). Thus, the stabilization ability of NADES was investigated using both water and 40% ethanol as references.

3.1 Stability of carthamin during heating

Table 1. Some physical properties data of the selected natural deep eutectic solvents (NADES) and a summary of the stability of typical phenolic compounds (carthamin (1), hydroxysafflor yellow A (2), and cartormin (3)) in NADES with water and 40% ethanol as references.

	physical data			**conditions**[c]						
solvents	**compositions (molar ratio)**	**viscosity**[a] **(40 °C)**	**pH**[b]	**heating (40-80 °C)**	**light from lamp (~25 °C)**	**storage time**		**sunlight at ambient conditions (~25 °C)**		
						4 °C	**-20 °C**			
				1				**1**	**2**	**3**
LGH	lactic acid:glucose (5:1)	37	3		-	+	++	-	-	-
PMH	proline:malic acid (1:1)	251	3		-	-	++	-	-	-
GCH	glucose:choline chloride (2:5)	397	6-7		++	++	++	++	++	++
SuCH	sucrose:choline chloride (1:4)	581	6-7		+	++	++	+	++	++
XoCH	xylitol: choline chloride (1:4)	86	6-7	+						
water		1	7	-	-	-	-	-	+	+
EtOH (40%)	40 v% ethanol in water				-	-	-	-	+	+

[a] the viscosity and polarity data are for the pure NADES from previous report (Dai, *et al*., 2013).
[b] the pH value were detected with 90% (v/v) water dilution of NADES.
[c] "-" not stable; "+" more stable than "-" ; "++" more stable than"+"

The degradation rate of carthamin in aqueous solution was reported to increase at high temperatures by Fatahi *et al*. (2009). This was also observed in our experiments, in which the degradation rate of carthamin increased with increasing temperature (Fig. 1). However, carthamin was much more stable in XoCH than in water. At all tested temperatures, the degradation rate of carthamin in XoCH was much slower than in water. At 60 and 40 °C, first-order kinetics were observed for the degradation of carthamin in both XoCH and water, which is in agreement with a previous report on the degradation of carthamin in aqueous solution (Kim and Paik, 1997). At 60 °C, the half-live ($t_{1/2}$) of carthamin in XoCH was more than twice that in water (Table 2). Furthermore, if compared to the $t_{1/2}$ of carthamin at 60 °C, the $t_{1/2}$ in XoCH is 5 times higher and 8-time higher than that in XoCH and water at 40 °C, respectively. Therefore, compared with water, XoCH has a clear protective effect on carthamin against thermodegradation.

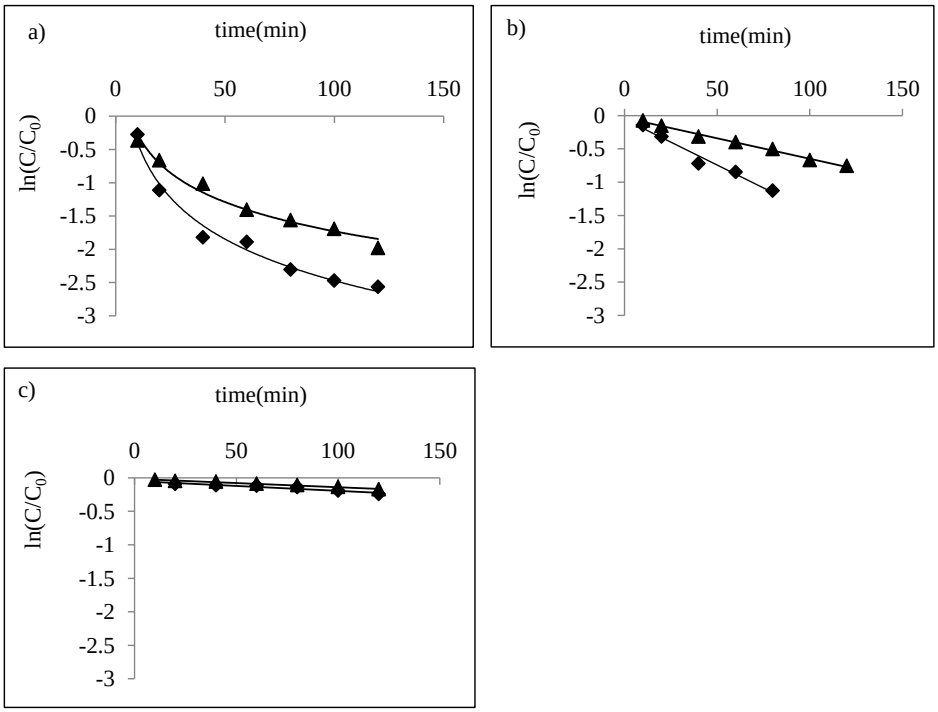

Fig.1. Stability of carthamin in xylitol-choline chloride (▲) and water (♦) at high temperature (**a**) 80 °C, **b**) 60 °C and **c**) 40 °C) (n=3). C_0 is the initial concentration of diluted carthamin and C is the concentration value of diluted carthamin after heating time (t) at a given temperature.

3.2 Stability of carthamin in light

The stability of carthamin in light was investigated at room temperature exposing a solution to 24 hours light under a daylight lamp over a 15-day period.

The reference was a similar solution that was kept in the dark. The effect of light on the stability of carthamin differed according to the solvents (Fig. 2a). The degradation curve of carthamin in light and in the dark overlapped in three of the solvents, GCH, SuCH and water, implying that light has no obvious effect on the stability of carthamin in those three solvents at room temperature for 15 days (Fig. 2b). With light, carthamin degraded faster than in the dark when dissolved in LGH, and 40% ethanol, but especially more in PMH (Fig. 2c), suggesting that light accelerates the degradation of carthamin in these solvents at room temperature.

Table 2. Degradation kinetics parameters of carthamin in water and xylitol-choline chloride (XoCH) at high temperature including reaction rate constants (k) and half-lives ($t_{1/2}$), and the degradation functions (n=3).

temperature	solvent	k	R^2	$t_{1/2}$	function
80 °C	XoCH	0.0482	0.9757		y = -0,6345Ln(x) + 1,1927
	H_2O	0.9012	0.9817		y = -0,9012Ln(x) + 1,6792
60 °C	XoCH	0.0061	0.992	113.6	y = -0,0061x - 0,0348
	H_2O	0.0138	0.9723	50.2	y = -0,0138x - 0,0546
40 °C	XoCH	0.0012	0.9841	577.6	y = -0,0012x - 0,0155
	H_2O	0.0017	0.9532	407.7	y = -0,0017x - 0,756

As regards different solvents, comparing the degradation curve of light-exposed solutions of carthamin in 90% GCH and 75% SuCH, the stability was very much higher than 40% ethanol and water solution. Thus, SuCH and GCH are good solvents for carthamin that exert a higher stabilization effect on solutes than conventional solvents and show no obvious effect from light.

3.3 Stability of carthamin according to storage time

At -20 °C, carthamin was stable in all tested solvents over a 7- day period. After 15 days, it was still stable in SuCH, and less than 10% degradation occurred in other NADES (PMH, 90% GCH, LGH), while substantial degradation was observed in water and 40% ethanol (Fig. 3a). At 4 °C, carthamin remained stable in SuCH over one month, while it showed degradation in other solvents including NADES. The stability of carthamin solutions decreases in the following sequence: SuCH> GCH> LGH> PMH=40% EtOH> water (Fig. 3b). In particular, at 4 °C the degradation of carthamin was below 5% in the NADES in the first 3 days, while it was near 15% in ethanol and 38% in water. Comparing -20 °C and 4 °C, the degradation of carthamin was increased from 35% to 61% in ethanol and from 16% to 26% in GCH after 30 days. Thus, NADES also exert a stabilizing effect on carthamin during storage. Therefore, carthamin can be preserved in SuCH at 4 °C for at least one month.

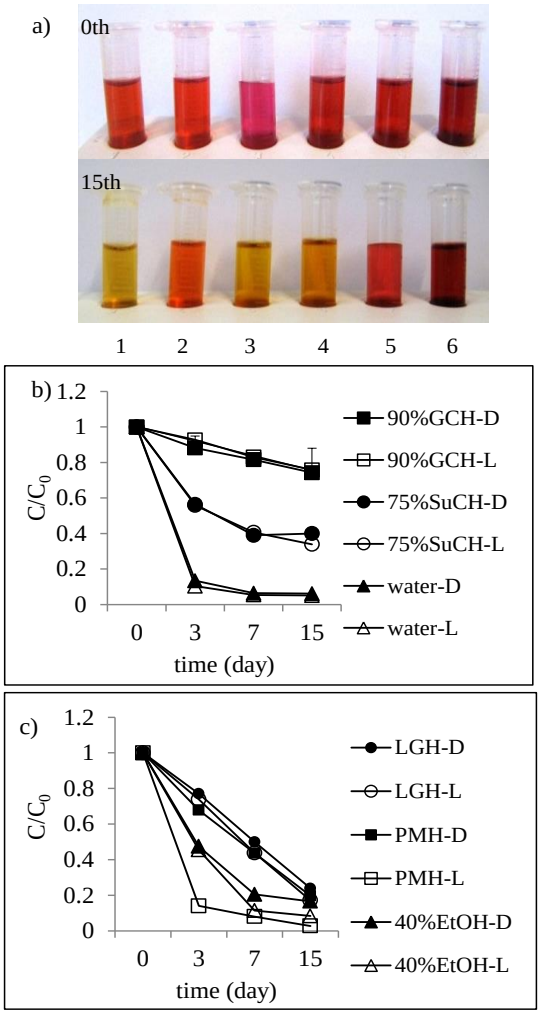

Fig. 2. Stability of carthamin in light from lamp at ambient temperature **a**) Pictures of carthamin solution before and after 15 days' 24-hour light exposure (**1**, water; **2**, 40% EtOH; **3**, lactic acid-glucose (LGH); **4**, proline-malic acid (PMH); **5**, sucrose-choline chloride (SuCH); **6**, 90% (v/v) glucose-choline chloride (GCH)) and degradation curve of carthamin in two groups of solvents with daylight (L) and in the dark (D) (group **b**: 90% GCH, 75%SuCH, and water; group **c**: LGH, PMH, and 40% (v/v) ethanol) (*n*=3).

Carthamin is more stable in SuCH and GCH than in PMH and LGH in all forementioned conditions (table 1). The big difference among those NADES is their viscosity; SuCH and GCH have a much higher viscosity than PMH and LGH. Thus, the stabilization ability of NADES may have a relationship with their viscosity. Moreover, the viscosity of NADES is affected by temperature

inversely, so that the viscosity of NADES decreases with increased temperature, which may partly explain why the stability of carthamin decreases with increased temperatures. For instance, in the case of PMH and LGH, the stability of carthamin decreased significantly when the temperature increased from -20 ^{o}C to 4 ^{o}C. The high viscosity at low temperature decreases the movement of molecules, allows stable molecular interactions between solvents and carthamin (section 3.6), and therefore possibly reduces the degradation of carthamin.

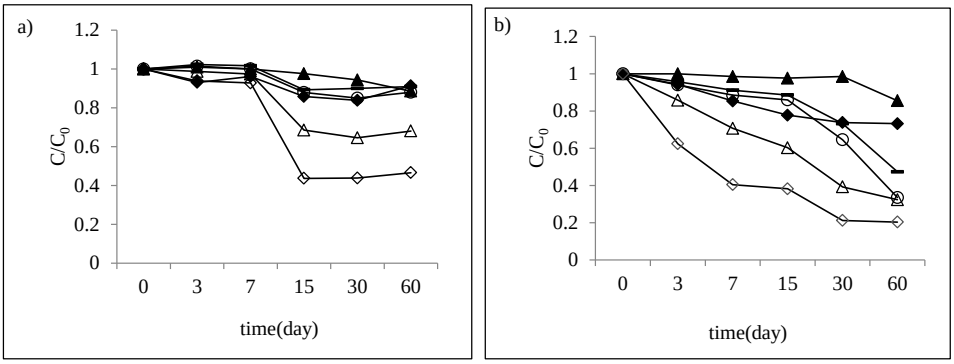

Fig. 3. Stability of carthamin standard in different natural deep eutectic solvents in two months at **a**) -20 ℃ and **b**) 4 ℃ (SuCH (▲); 90% GCH (♦); LGH (-); PMH (○);40% EtOH (Δ); water (◊))(*n*=3).

3.4 Stability of carthamin and safflower extraction at ambient conditions in sunlight

The red colored safflower was selected to explore the stabilizing ability of NADES for aromatic pigments with different polarities at ambient conditions in sunlight. The retention time in HPLC profile corresponded well with their polarity. There are numerous peaks in the UV-trace, including those visible at different wavelengths (280 nm, 520 nm and 403 nm) (Fig. 4). Because of the complexity derived from overlapping and minor compounds, three typical peaks with different retention times (Tr) were selected to evaluate the stabilizing ability of NADES for phenolic compounds in safflower. Three representative peaks included: hydroxysafflor yellow A (HSYA) (Tr=21.9 min), cartormin (Tr=40.0 min) and carthamin (Tr=72.9 min). The chosen compounds represent a whole range of polarity and include major and important active metabolites in the safflower extract, which should reflect the stabilization ability of NADES for phenolic compounds in terms of polarity. The stability of the safflower extract and carthamin standard in NADES were investigated at ambient conditions in daylight over a 15-day period.

The results show that the degradation of the carthamin standard was very different in each solvent (Fig. 5). The stability of carthamin decreased in the following sequence, 90% GCH>75% SuCH=LGH>40% EtOH>PMH=water (Fig. 5a). The carthamin in the safflower extract solution showed a similar stability profile to the carthamin standard after 15 days (Fig. 5b). The

degradation of carthamin was found to be 25% in 90% GCH three days after treatment, and 60% in water and 40% ethanol. Thus, the stability of carthamin is markedly improved in 90% GCH ascompared with that in water and EtOH (40%).

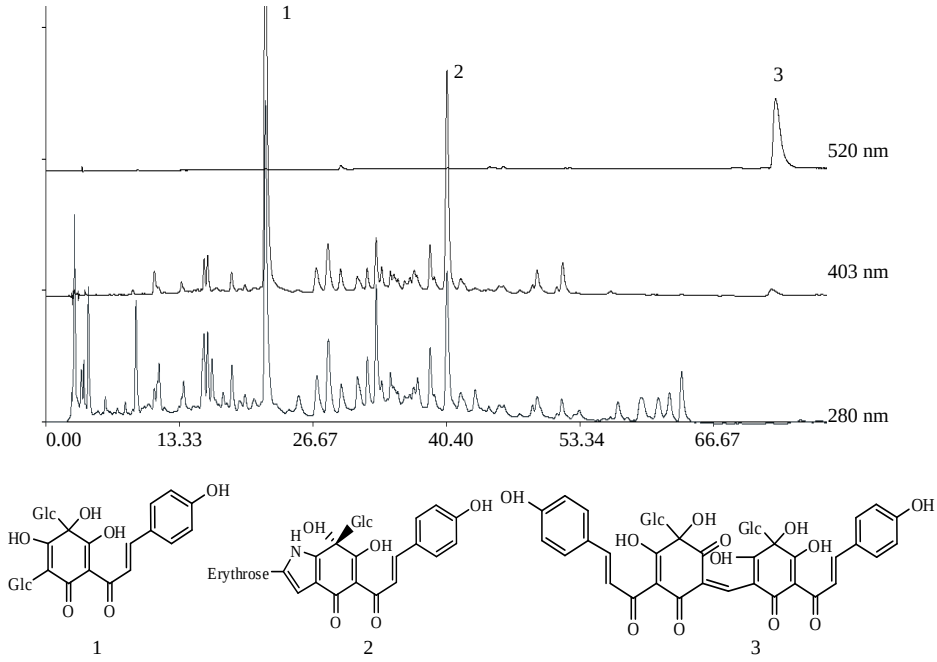

Fig. 4. HPLC chromatograms of safflower extract at 280, 403, and 520 nm (including **1**, hydroxysafflor yellow A; **2**, cartormin and **3**, carthamin).

The behaviour of HSYA and cartormin is very different to carthamin. HSYA was stable in 90% GCH and 75% SuCH, and showed a 5% degradation in water and 40% ethanol in 15 days. However, it degraded rapidly in LGH and 75% PMH (Fig. 5c). Cartormin was also stable in 90% GCH and 75% SuCH, exhibited around 10% in water and 40% ethanol in 15 days, and also degraded dramatically in LGH and 75% PMH (Fig. 5d). Light has been reported to affect the stability of a safflor yellow extract (main components including HSYA and cartormin) in buffer solution (Fatahi *et al*., 2009); the main components of safflor yellow, HSYA and cartormin, however, are more stable in 90% GCH and 75% SuCH than in water and 40% ethanol in our studies. This indicates that 90% GCH and 75% SuCH are far better solvents for storage of phenolic compounds in safflor yellow at ambient than water and 40% ethanol. Ultimately, SuCH and GCH are promising as solvents and color protectors for safflower yellow extracts when applied in the food or pharmaceutical industry.

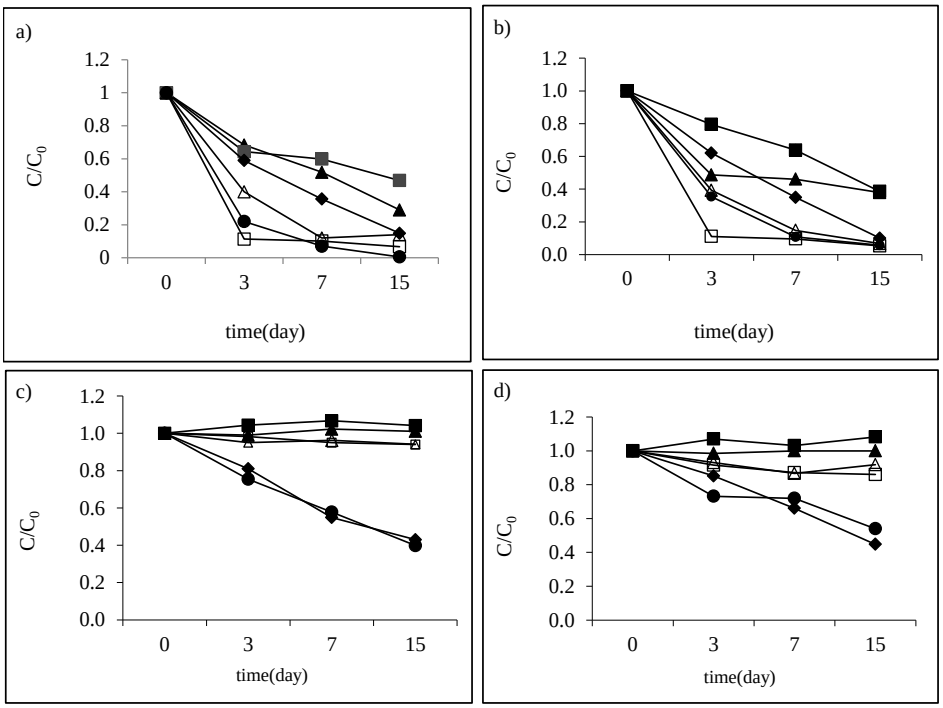

Fig. 5. Stability of three typical phenolic compounds from the safflower extract in different natural deep eutectic solvents at ambient conditions with sunlight in 15 days, compared with in water and 40% ethanol (**a**) carthamin standard, **b**) carthamin in extract, **c**) hydroxysafflor yellow A in extract, and **d**) cartormin in extract). (■: 90% GCH; ▲: 75% SuCH; ♦: LGH; ●:75% PMH; Δ: 40% ethanol; □: water) (*n*=3).

Our results revealed that carthamin (red pigment), HSYA and cartormin (major components of safflor yellow) are more stable in GCH and SuCH than in PMH, LGH, water or 40% ethanol. Sugars (xylose, glucose, sucrose, fructose, lactose) were reported to protect the color of safflower yellow B (a major component of safflor yellow) at high temperature (Saito and Murata, 1994). Therefore, in the first place, it could be possible that the sugar component in NADES may play an important role in stabilizing the safflower extract in ambient conditions, probably due to hydrogen bonding with solutes. Secondly, pH is reportedly an important factor for the degradation of safflower extracts (Saito and Mori, 1994). Safflor yellow is more stable in acidic (pH 2-6) than basic solutions (Yoon *et al*., 2003; Zhu and Pan, 2007; Fatahi *et al*., 2009). LGH and PMH contain acidic ingredients and they are thus more acidic than SuCH and GCH when diluted with 90% (v/v) water. However, HSYA and cartormin are much more stable in GCH and SuCH than in LGH and PMH. Thus, there is no relationship between the stability of HSYA and cartormin and the presence of acids in NADES. Carthamin, on the other hand, is more stable in GCH and SuCH than in PMH and LGH, which is in agreement with the report that carthamin is more stable in basic than acidic aqueous solution (Kim

and Paik, 1997; Fatahi *et al*., 2009). Lastly, the direct relationship between the stabilizing ability of NADES and their high viscosity is confirmed. The viscosity of NADES is greatly affected by the water content (Dai *et al*., 2013) so that the water content may affect the stabilizing ability of NADES, as demonstrated below (section 3.5). All things considered, safflower extracts are more stable in high viscous non-acid NADES.

3.5 Stability of carthamin in NADES with different water contents

The effect of the water content (in the form of water percentage) on the stability of carthamin was investigated in two NADES (SuCH and PMH) at 4 ^{o}C and -20 ^{o}C. Both are highly viscous (Dai *et al*., 2013), but have a different compositionand acidic properties after dilution. Their viscosity is affected by the water percentage and temperature (Dai *et al*., 2013). As discussed previously the viscosity of NADES affects their solubilization ability according to their dilution with water (Dai *et al*., 2013) and may also affect their stabilization ability. Therefore, it is necessary to evaluate the effect of the water content on the stability of compounds in NADES for their application in dissolving and storage of compounds.

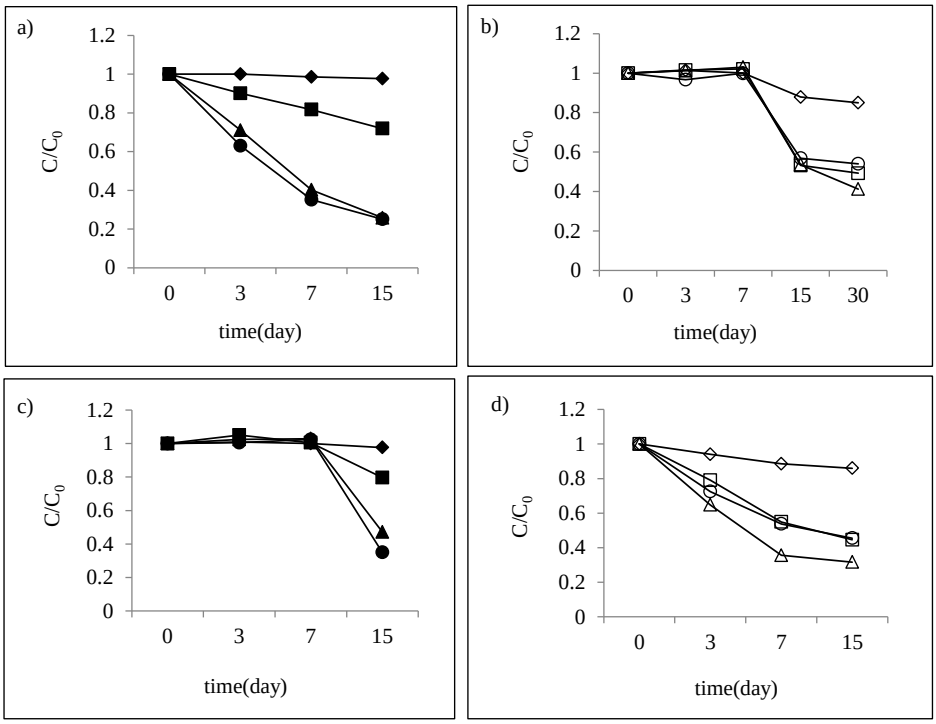

Fig. 6. Stability of carthamin standard in sucrose- choline chloride (SuCH) and proline- malic acid (PMH) with different percentage of water in the dark at at 4 ^{o}C (**a**, **b**) and a-20 ℃ **(c**, **d**). (♦: 100% SuCH; ■: 75% SuCH; ●: 50% SuCH; ▲: 25% SuCH; ◊: 100% PMH; □: 75% PMH; ○:50% PMH; Δ: 25% PMH) (*n*=3).

The results showed that the water content of NADES plays an important role on the stability of carthamin. At 4 ^{o}C, carthamin was stable in SuCH, but less stable in water-diluted SuCH, with a decreasing stability from SuCH: H_2O (3:1), to SuCH: H_2O (1:1), and SuCH: H_2O (1:3) (Fig. 6a). In PMH, the stability of carthamin decreased in the following sequence, PMH > PMH: H_2O (3:1) = PMH:H_2O (1:1) > PMH:H_2O (1:3) (Fig. 6b). After 15 days, the level of carthamin in pure SuCH was still the same, but decreased a 25% in SuCH:H_2O (3:1), and a 75% in both SuCH:H_2O (1:1) and SuCH:H_2O (1:3). Therefore, the stabilization ability of NADES increases with increasing viscosity (low water content). At -20 ^{o}C, the effect of the water content in NADES on the stability of carthamin was lower and became visible after 7 days storage (Fig. 6c, d). Thus, lower water content in highly viscous NADES at low temperature is good for the stability of carthamin.

3.6 Mechanism of the stabilizing ability of NADES for phenolic compounds

FT-IR was recorded for a typical phenolic compound, quercetin, dissolved in GCH (Fig. 7). The spectra show different absorption bands of the C-OH and aromatic ring as well as the carbonyl group from quercetin in solid state and dissolved in GCH. The stretching vibration absorption band of the C-OH of quercetin shifted from 1168 cm^{-1} to 1164 cm^{-1}, indicating that the hydroxyl groups in quercetin donate protons to form hydrogen bonds with solvent molecules. The deformation vibration absorption bands of the C-OH in quercetin shifted from 1355 cm^{-1} to 1369 cm^{-1} and from 1210 cm^{-1} to 1200 cm^{-1}, which confirms the formation of new hydrogen bonds between quercetin and GCH and also implies that quercetin has a different conformation in GCH than in solid state. The shift of the characteristic band of the aromatic ring of quercetin from 1615 and 1600 confirmed the structure deformation of quercetin in GCH (Heneczkowski *et al.*, 2001). In addition, the downward shift of C=O from 1669 cm^{-1} to 1654 cm^{-1} indicates H-bonding (C=O---HO) between the carbonyl group of quercetin and solvent hydroxyl groups (glucose and choline). All the above spectral characters of quercetin reveal the existence of multi H-bond interactions between quercetin and NADES, which is in agreement with the interaction signals in the NOESY spectrum of quercetin in XoCH (Dai *et al*., 2013).

The H-bond interactions between solute and molecules of NADES provide an explanation for the high stabilizing ability of the sugar-based NADES such as SuCH. It was reported that the water extract of safflower is stable in acid conditions (pH 2-6), in which most phenolic compounds are in the neutral form. Further studies showed that sucrose, glucose, starch and the common ions Ca^{2+}, Mg^{2+} and Zn^{2+},can improve the stability of the extract (Zhu and Pan, 2007). All these examples confirm the conclusion that the formation of hydrogen bonds or chelates can stabilize the structures of the phenolic compounds.

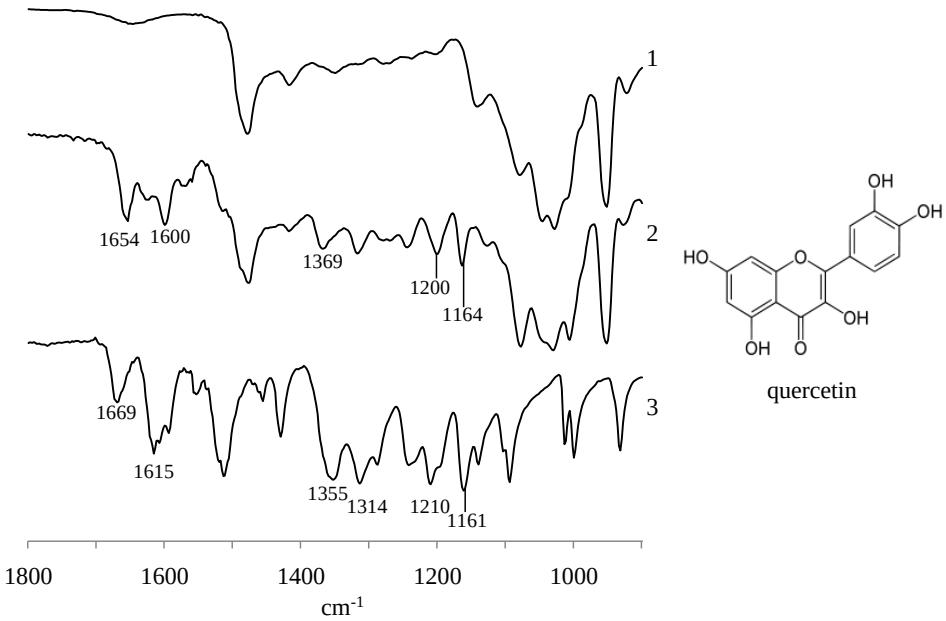

Fig. 7. FT-IR spectra of **1**) glucose-choline chloride **2**) quercetin in glucose-choline chloride; and **3**) quercetin.

All things considered, we hypothesize that the NADES solution of the safflower extract are, in fact, liquid crystals in which the dye molecules are fixed in the crystals. Degradation by oxidation only thus occurs on the surface of the liquid crystals. Melting the crystals by increasing the temperature or adding water allows the phenolics to diffuse freely through the system and thus increased oxidation will occur.

The water extract of safflower is characterized by a high amount of glucose, fructose and choline, as shown in the ^{1}H NMR spectrum of the water extract of safflower. The red and yellow pigments in safflower are accumulated in flowers exposed to the sunlight. However, pure carthamin is unstable in water solution. These findings provide indirect evidence, therefore, to support our hypothesis that NADES in plants are involved in the storage of various non-water soluble metabolites or unstable compounds in cells.

4. Conclusions

In this study, some typical NADES were demonstrated to be better solvents for the stabilization of phenolic compounds than general solvents. The NADES improve the stability of carthamin under various conditions such as high temperature, light and storage time, if compared to water and 40% ethanol. Furthermore, the higher color stability of carthamin as well as of the other two

phenolic compounds in the safflower extract was also observed in NADES when exposed to sunlight at ambient conditions.

The high stabilizing ability of NADES seems to be correlated with the strong hydrogen bonding interactions between solutes and solvent molecules. High viscosity of sugar-based NADES with low water contents allows stable molecular interactions and plays an important role in the stabilizing effect of NADES for phenolic compounds. NADES are like liquid crystals in which the dye molecules are fixed in the crystals.

The high stability of the three typical phenolic compounds from safflower in sugar-based NADES throws new light on the stabilizing ability of NADES for phenolic compounds. Further studies on the stabilization effect in other sugar-based NADES and NADES made of organic acids may not only provide further understanding of the principle of the stabilizing ability of NADES, but also lead to novel applications of NADES in the food and pharmaceuticals industry.

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Chapter 6

Natural Deep Eutectic Solvents facilitating the extraction and storage of anthocyanins

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Abstract

Natural deep eutectic solvents (NADES) have a high solubilizing capacity, which is promising for their application in the extraction of active compounds from herbal medicines. Natural deep eutectic solvents are composed of neutral, acidic or basic compounds that form liquids of high viscosity when mixed in certain molar ratios. The viscosity may affect the extraction properties and stabilizing ability of NADES as well as the chromatographic properties of the extracts obtained. To address these problems, as a model, extraction methods with NADES were investigated in combination with HPLC-UV based metabolomics for the analysis of anthocyanins in flower petals of *Catharanthus roseus*. Stability tests of the anthocyanin extracts were also done. Multivariate data analysis indicates that NADES made of lactic acid-glucose (LGH), and 1,2-propanediol-choline chloride (PCH) present similar extractability of anthocyanins as acidified methanol. Furthermore, LGH exhibits higher stabilizing ability for cyanidin than acidified ethanol. Cyanidin was stable for 7 days at 4 ℃ and 3 month at -20 ℃ in LGH in the dark, which facilitates their extraction and analysis process. Compared with conventional organic solvents, NADES provide a greener and more stable approach for the extraction and storage of anthocyanins, implying that they have the potential to replace conventional organic solvents in health related areas such as food, pharmaceuticals, and cosmetics.

Key words: Natural deep eutectic solvent; anthocyanins; extractability; stabilizing ability; chromatographic behavior; *Catharanthus roseus*.

1. Introduction

A new type of green solvents, the natural deep eutectic solvents (NADES) were proposed in our group (Choi *et al*., 2011; Dai *et al*., 2013). NADES are liquid supermolecules made of natural primary metabolites (e.g. sugars, sugar alcohols, organic acids, amino acids, and amines) held together by intermolecular interactions, particularly H-bonding. NADES present many advantages for extraction (Dai *et al*., 2013), e.g., negligible volatility, adjustable viscosity, and high solubilization strength. For example, some NADES show a very high solubilization ability for rutin, as much as 12,000 times higher than water (Choi *et al*., 2011; Dai *et al*., 2013). From an environmental and economic perspective, NADES also present great advantages including their biodegradability, sustainability, low cost, and simple preparation. All those properties indicate their great promise as good extraction solvents for natural products and their potential applications in health related areas such as food, pharmaceuticals and cosmetics.

The structure of NADES and relevant physicochemical characteristics are to be considered. The major components of NADES have several functional groups such as hydroxyls, carboxyl or amino groups (Choi *et al*., 2011; Dai *et al*., 2013). Those groups can interact forming H-bonds between the components, leading to highly structured viscous liquids, which account for their special physical properties and different solvent behavior compared to conventional solvents. Those liquids can also form hydrogen bonds with solutes, thus greatly increasing the solubility of compounds in NADES, e.g. phenolic compounds. NADES can be divided into five groups according to the nature of their components: ionic liquids with an acid and a base, NADES with neutral compounds, sugar-based NADES with an acid, sugar-based NADES with a base and sugar-based NADES with an amino acid (Dai *et al*., 2013). The different compositions of NADES result in their broad range of physical properties, probably leading to different behavior in applications such as extraction, analysis and storage of natural products. Therefore, further studies are required to develop diverse applications of NADES, such as the extraction of herbal medicines.

Anthocyanins are a widespread group of natural water soluble phenolic compounds that occur in plants. They exist mainly in flowers, fruits, and vegetables, being responsible for a great part of the orange, red, purple, and blue colors (Castañeda-Ovando *et al*., 2009). Since the 1990s, there is a renewed interest in anthocyanins because of their possible health benefits as antioxidant and anti-inflammatory agents, especially for the prevention of diseases such as cardiovascular diseases and cancer (Wang *et al*., 1999; Noda *et al*., 2002; Hou, 2003; Kong *et al*., 2003; Albrecht *et al*., 2004). Plant extracts containing anthocyanins, such as pomegranate extract, have also been developed as "functional foods" and "herbal/ nutritional supplements" in developed countries (Ismail *et al*., 2012). In plants, anthocyanins are almost always found in the form of glycosides with different sugar substituents, and different aliphatic or aromatic carboxylic acids bonded to sugar units (Kong *et al*., 2003). There are six major

aglycones (anthocyanidins): delphinidin, cyanidin, pelargonidin, petunidin and malvidin, differing only in the number and position of hydroxyl or methoxyl substitutions in the B-ring.

The general solvents for the extraction of anthocyanins are typically mixtures of water with ethanol, methanol, or acetone (Kähkönen *et al*., 2001; Kalia *et al*., 2008). The instability of anthocyanins causes many inconveniences for their extraction, preparation, analysis process and storage (Melgarejo *et al*., 2011; Oidtmann *et al*., 2012). The anthocyanin extracts or isolated anthocyanins are highly instable and very susceptible to degradation due to temperature, light andsolvent among other factors (Giusti and Wrolstad, 2003; Castañeda-Ovando *et al*., 2009). Thismake the protocols for sample preparation and analysis of anthocyanins complex, and the whole process can often be very time-consuming. (Mazza, 2004; Dai *et al*., 2009; Navas *et al*., 2012). In general, it is recommended that the extraction of anthocyanins be performed in the dark at a low temperature (Awika *et al*., 2005; Piovan *et al*., 1998). Therefore, it is very useful to develop new green solvents that combine high extractability and stabilization ability for anthocyanins.

In this study, the extractability and storage stabilization properties of some typical NADES for anthocyanins were explored. A simple extraction method for anthocyanins with NADES combined with HPLC/UV-based metabolomics for anthocyanins was designed. As a model, *Catharanthus roseus* petals of purple and orange color were selected because they contain different kinds of anthocyanins. The stability of cyanidin in NADES was investigated considering the effects of temperature, light and storage time. The physical properties of NADES which may affect the extraction, analysis, and storage process are discussed, laying the basis for their further applications.

2. Materials and methods

2.1 Chemicals and materials

Two different flower-colored *Catharanthus roseus* varieties were purchased from Intratuin, Pijnacker (Postbus 1016, 1700BA, Heerhugowaard, The Netherlands), belonging to the Pacific series i.e. Pacifica XP Apricot (orange color), and Pacifica Orchid Halo (purple, color) were used in this study. Flower petals were collected, ground to powder with liquid nitrogen and freeze dried in September 2009 during the blooming season of *C. roseus*. Methanol and ethanol of analytical grade and methanol of HPLC grade were purchased from Biosolve BV (Valkenswaard, The Netherlands). Water was of deionized water quality. Malic acid, lactic acid, proline, glucose, fructose, sucrose, 1.2-propanediol, and choline chloride from Sigma (St. Louis, MO, USA) and cyanidin standard (Carl Roth, Karlsruhe) were used.

2.2 Natural deep eutectic solvents and solution preparation

All NADES including 1,2-propanediol-choline chloride (PCH); lactic acid-glucose (LGH); proline-malic acid (PMH); malic acid-choline chloride (MCH); glucose-choline chloride (GCH); glucose-fructose-sucrose (FGSH) were prepared (Dai, *et al*., 2013). Proline aqueous solution (65 mg/ml) and malic acid aqueous solution (50 mg/ml) were prepared with deionized water.

2.3 Extraction methods optimization

*Heating and stirring***.** Plant material (50 mg) with 1.5 mL NADES or methanol with 3% formic acid was stirred at 40 ^{o}C for 30 min in a sealed glass bottle. The sample was transferred into an eppendorf tube, centrifuged at 1300 rpm for 20 min and then filtered through a 0.45 μm cellulose acetate filter and diluted to double the volume with 3% aqueous formic acid. All extractions were performed in triplicate.

Ultrasound-assisted extraction (UAE). Plant material (50 mg) was extracted with 1.5 mL solvent in an ultrasonicator bath at room temperature for 30 min. Ultrasound-assisted extraction with heating (UEH) was carried out in an ultrasonicator bath at 40 ^{o}C for 30 min. The extract was centrifuged, filtered, and diluted as described above.

2.4 Stability test

Solutions of cyanidin reference standard in LGH (0.1 mg/mL) and ethanol with 3% formic acid (0.1 mg/mL) were used in the following stability tests. For thermal stability, cyanidin solutions in tubes with screw-caps were placed in a preheated water bath at 80 ^{o}C, 60 ^{o}C and 40 ^{o}C, respectively. Three tubes of each group were removed from the water bath at 10, 20, 40, 60, 80, 100, 120 min and rapidly cooled to room temperature. The stability of cyanidin solutions stored for three months at -20, 4 and 25 ^{o}C in the dark was evaluated by analyzing three tubes of each group at day 1, 3, 7, 15, 30, 60, 90. For the effect of light, cyanidin solutions were exposed to sunlight or kept in the dark in ambient conditions and three tubes of each group were removed at day 1, 3, 7, 15. All experiments were done by triplicate.

2.5 Apparatus and analysis

HPLC analysis was carried out on a Agilent chromatographic system with Phenomenex Luna C18(2) column (4.6 μm x 250 mm, 5 μm) at 35 degree. The mobile phase consisted of water with 3% formic acid (A) and methanol with 3% formic acid (B) in a linear gradient program as follows: 20-25% B (0-30 min), 25-45% B (30-35 min), 45-80% B (35-40 min) at a flow rate of 1.0 mL/min. Chromatograms were record at 520 nm. The injection volume was 10 μL. A UV-Vis spectrophotometer (Shimadzu, Japan) was used for measuring the stability test at a wavelength of 520 nm.

2.6 Data analysis

Calculation of kinetic parameters of cyanidin degradation at high temperature: the first-order reaction rate constants (k) and half-lives ($t_{1/2}$) were calculated by the following equation (Kırca *et al*., 2007):

$$\ln(C/C_0)=-kt$$

$t_{1/2}$=-In(0.5)/k

where $C/C_{0=}$ $A/A_{0,}$ C_0 and A_0 is the initial concentration and absorption of diluted carthamin, and C and A is the concentration and absorption value of diluted carthamin after heating time (t) at a given temperature, respectively.

The integrated areas of peaks of anthocyanins in HPLC-UV chromatograms from three replicates were analyzed with the SIMCA-P software (V. 12, Umetrics, Umeå, Sweden) for PCA analysis using the Pareto scaling method. Analysis of variance (ANOVA) was performed in SPSS software (version 14.0, Chicago, IL, USA) using the integration areas of the peaks in the HPLC chromatograms at 520 nm with P values ≤ 0.05 considered as significant.

3 Results and discussion

3.1 Comparing the extraction of anthocyanins with NADES and methanol

Catharanthus roseus has been wellstudied in our lab for many years (Mustafa *et al*., 2007). They have petals with more than 5 different colors, e.g. pink, light pink, purple, red, and orange. Higher amounts of hirsutidin 3-*O-(6-O-p-coumaroyl)* glucose and petunidin 3-*O-(6-O-p-coumaroyl)* glucose, an intermediate level of malvidin 3-*O-(6-O-p-coumaroyl)* glucose, and a low level of petunidin 3-*O*-glucose and trace amounts of some others were observed in *C. roseus* (Piovan *et al*., 1998). The anthocyanin profiles in *C. roseus* are different for the 5 different flower colors and those petals of the purple and orange flowers exhibit more peaks (6-9) at the wavelength of 520 nm than the other colors, as shown in a previous study (Fig. *S*1, unpublished). The purple- and orange-colored petals of *C. roseus* were selected to investigate the extractability of NADES for anthocyanins.

The HPLC fingerprints at 520 nm show the same qualitative chemical profiles of anthocyanins in some tested NADES and methanol with 3% formic acid extracts. Nine peaks were observed for the purple petal extracts (Fig. 2a) and 6 for the orange petals (Fig. 2b). To quantify the different extractability of NADES and methanol for anthocyanins, multivariate data analysis (PCA) was applied with the peak areas of all peaks at 520 nm as variables. PCA is one of the most widely used multivariate data analysis methods to reduce the dimensionality of a multivariate dataset. The PCA gives a score plot of principle components, which can be used to identify the separation and similarities among all the analyzed samples. The variables responsible for the separation or grouping among samples are plotted in a loading plot and variables with a high value far from the center are most important. The score plot of PCA (Fig. 3a) with the first two components (R^2 =0.94 and Q^2 =0.67) shows a separation of the extracts from purple petals into 2 groups, separated by PC1. The LGH, PCH, 75% FGSH and acidified methanol extracts are in the positive area of PC1 (group I) and PMH, MCH, GCH are in the negative part of PC1 (group II). The score plot implies that LGH and PCH have similar extraction characteristics to acidified methanol for anthocyanins from the purple flowers of *C. roseus*. The loadings plot (Fig. 3b) shows that all anthocyanins peaks are located in the positive area of PC1 (group I), indicating that solvents in group I

(PGH, LGH, 75% FGSH and acidified methanol) are better solvents than those in group II (PMH, MCH and GCH). Methanol with 3% formic acid was selected as a reference because of its high extraction yield of anthocyanins (Metivier *et al*., 1980; Awika *et al*., 2005). Both LGH and PCH are suitable extraction solvents for anthocyanins, being as efficient as acidified methanol.

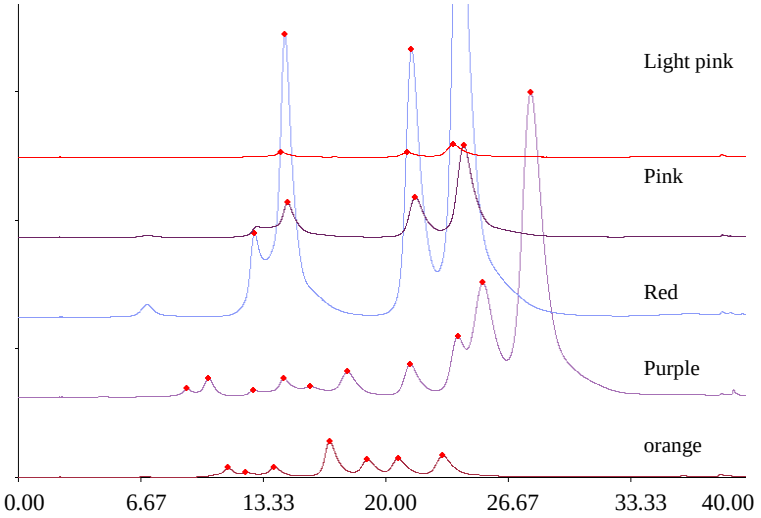

Fig. 1. HPLC-UV chromatograms at 520 nm of anthocyanins from *Catharanthus roseus* with different flower colors extracted with methanol 3% formic acid.

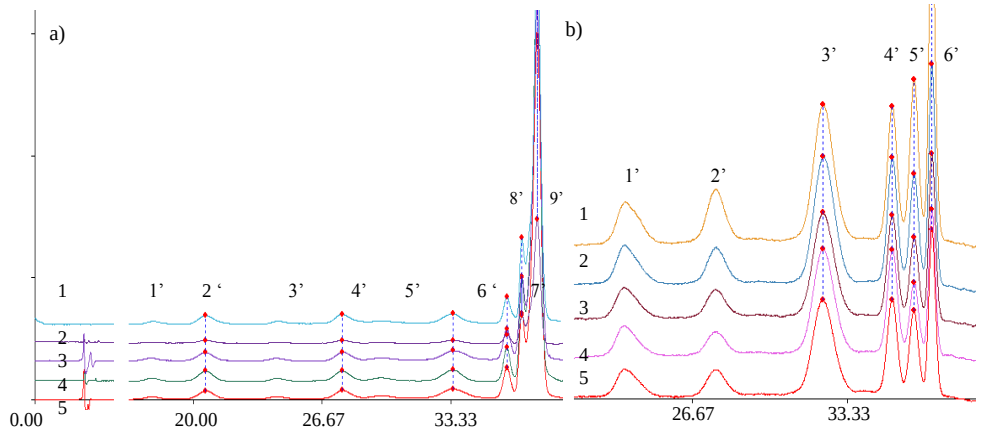

Fig. 2. HPLC-UV chromatograms of anthocyanins from *Catharanthus roseus* with **a**) purple and **b**) orange flower petals extracted with different solvents at 520 nm. (**1**) methanol; **2**) lactic acid-glucose(LGH); **3**) malic acid-choline chloride(MCH); **4**) prolinel-malic acid(PMH); **5**) glucose- choline chloride(GCH)).

For the anthocyanins in the orange-colored petals of *C. roseus*, a similar separation model was observed. The score plot of PCA with the first two components (R^2 =0.95 and Q^2 =0.82) shows that all the extracts were separated into two groups by PC1 (Fig. 3c), with acidified methanol, LGH, PCH in the positive

area of PC1 (group I) and PMH, MCH, and GCH in the negative area of PC1 (group II). The loading plot (Fig. 3d) shows that all peaks of anthocyanins are located in the positive area of PC1, indicating that solvents in group I give higher extraction yields than solvents in group II.

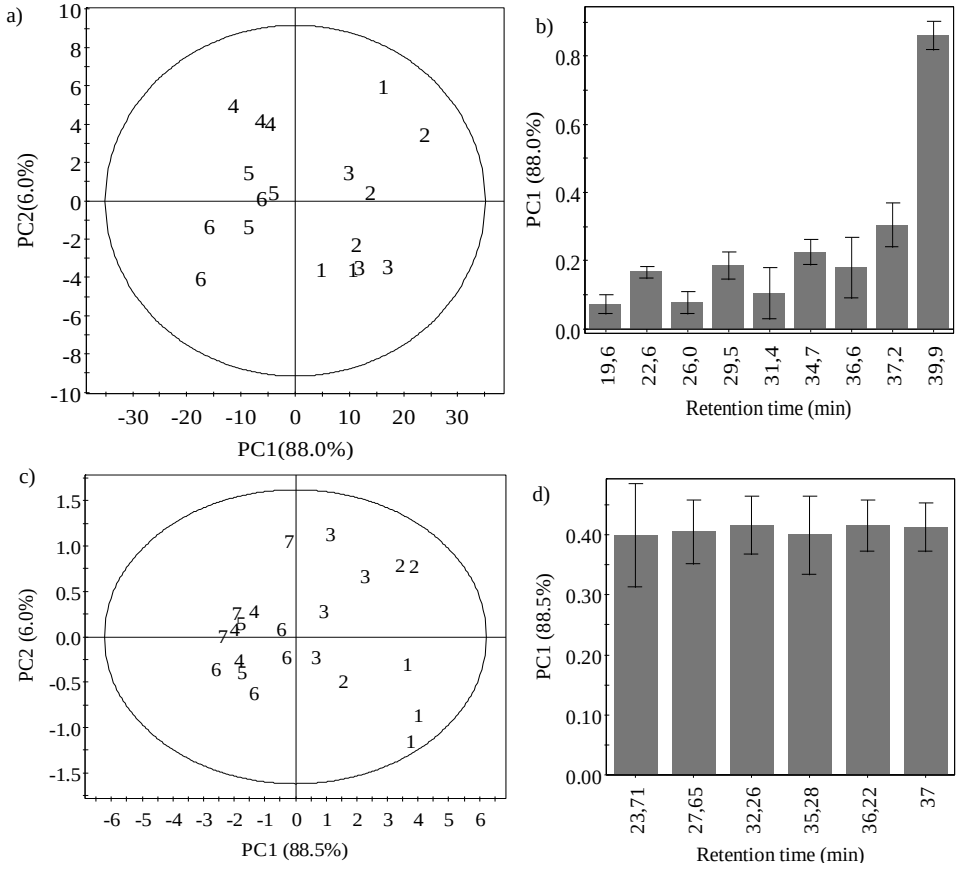

Fig. 3. Score plots (**a**, **c**) and loadings plots (**b**, **d**) of principal component analysis of extracts of *Catharanthus roseus* flowers with purple (a,b) and orange color (c,d) by different solvents (methanol 3% formic acid (**1**); 1,2-propanediol-choline chloride-water (**2**); lactic acid-glucose-water (**3**); proline-malic acid (**4**); malic acid- choline chloride (**5**); glucose-choline chloride (**6**); 75% glucose-fructose-sucrose (7).

A further ANOVA test on the average peak area of anthocyanins in HPLC-UV chromatogram confirms that LGH and PCH have the same extraction yields of both colored anthocyanins as acidified methanol, which is more efficient that the other tested NADES. ANOVA shows that the area of the peak (retention timeat 39.9 min) is significantly higher in LGH, PCH, and acidified methanol than in the others (Fig. 4), which confirms the results from PCA that LGH, PCH, 75% FGSH

possess similar solvent strength as acidified methanol and much higher than other NADES.

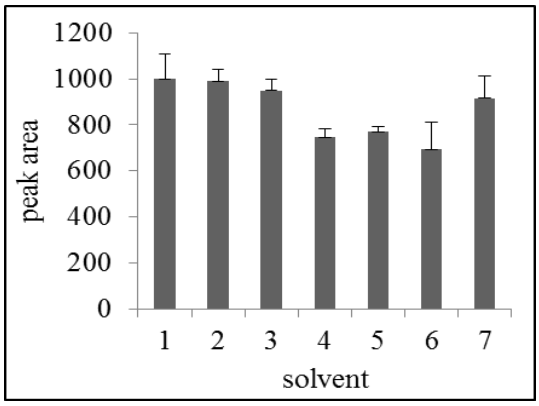

Fig. 4. The average peak area of peak (retention time at 39.9 min) of anthocyanins extracted from purple-colored petals of *Catharanthus roseus* with different solvents (the same as figure 3) in HPLC chromatogram at 520 nm. The data is expressed in mean ± SD (*n*=3).

The high extraction yield of PCH and LGH may be related to their physicochemical properties. To test the effect of viscosity, polarity, and chemical composition of NADES on their extracting characteristics, the following 6 typical NADES with different physical properties were selected for extraction: PCH, LGH, PMH, MCH, GCH, and FGSH (Dai *et al*., 2013). In the first place, the high extraction yields of anthocyanins with LGH and PCH are correlated with their low viscosity. PCH, LGH (ca. 35 mm^2/s) have the lowest viscosity among all the tested NADES, GCH (397 mm^2/s), PMH (251 mm^2/s), MCH (446 mm^2/s) (Dai *et al*., 2013). In the case of FGSH it was diluted with 25% volume of water for use, resulting in a similar viscosity as water (1 mm^2/s). Secondly, the solvent strength of NADES seems to have no direct relationship with their polarity. In the case of conventional solvents, the extraction efficiency can be estimated by polarity. However, the NADES do not show a relationship between the polarity and extraction yield. LGH and MCH have the highest polarity (44 kcal/mol), followed by PMH (48 kcal/mol), sugar/sugar alcohol-choline (49 kcal/mol); PCH has the lowest polarity (50 kcal/mol) (Dai *et al*., 2013). Lastly, no relationship between the acid or base components of NADES and their extraction capacity was observed. One method to increase the extractability of anthocyanins in conventional solvents is to add a small amount of acid such as acidified methanol (Awika *et al*., 2005). The pH values of aqueous NADES are different when diluted with 90% (v/v) of water due to the acid or base components in NADES (Dai *et al*., chapter 4). LGH and PCH with the same extractability have different pH values, while LGH and MCH have the same pH but different extraction yields of anthocyanins. All considered, as opposed to conventional solvents, the extraction efficiency of NADES is likely to be more correlated to the viscosity of NADES rather than their polarity and pH values.

3.2 Optimizing extraction and analysis method for anthocyanins with NADES

*3.2.1 Extraction methods optimization.*Three extraction methods were examined for the extraction of anthocyanins from flower petals of *C. roseus*. The extraction yield increased by 35-55% on stirring at 40 ^{o}C and only by 2-20% with sonicating at 40 ^{o}C if compared with sonication at 25 ^{o}C (Table 1). Extraction times between 30 and 90 min were examined using the stirring method at 40 ^{o}C. The result shows there is no difference in extraction yields with 30 and 60 min extraction and at 90 min the peak areas of all peaks in fact decreased (Table. 2). Long extraction time allows the dissolution of compounds but may also lead to the formation of artifacts or degradation of compounds. Thus, stirring at 40 ^{o}C for 30 min was selected for this study.

Table 1. Influence of three different extracting conditions on the relative extraction yield of anthocyanins from *Catharanthus roseus* with purple petals.

	Relative extraction yield as peak area ratio[a]		
peaks	**sonication (25 ℃)**	**sonication (40 ℃)**	**stirring (40 ℃)**
peak 1	0.6	0.8	1.0
peak 2	0.5	0.6	1.0
peak 3	0.6	0.4	1.0
peak 4	0.5	0.5	1.0
peak 5	0.4	0.3	1.0
peak 6	0.6	0.6	1.0
peak 7	0.5	0.7	1.0
peak 8	0.5	0.7	1.0
peak 9	0.5	0.7	1.0

[a]The relative extraction yield is expressed in peak area ratio of the peaks in the chromatogram of the extract as compared to the reference (40 ℃, stirring) (n=3).

Compared with conventional solvents, a disadvantage of NADES is their high viscosity, which causes slow mass transfer and decreases their extractability. In order to improve this, the above three approaches were explored with the purpose of decreasing the viscosity and improving extraction efficiency. Our previous study showed that increasing temperature leads to a decrease in viscosity of NADES (Dai *et al.*, 2013), but a high temperature can cause the degradation of anthocyanins (Kırca *et al.*, 2007). Thus, a mild temperature of 40 ^{o}C was selected. The second way to increase mass transfer and to speed up the diffusion of compounds in NADES is applying external forces such as stirring and sonication. Stirring is the simplest way to speed up the transfer rate of the compounds in the liquid. Ultrasonication combined with mild heating might be a good choice to assist extraction with NADES due to the acoustic cavitation phenomenon that leads to a disruption of cell walls causing the release of cellular contents and breaking

intermolecular interactions and therefore speeding up dissolution (Vinatoru, 2001). It has the following advantages: no limitations for solvent selection, high extraction efficiency within 30 min, and mild temperature operation favorable for thermally unstable compounds. Ultrasonication-aided extraction is widely used in the extraction of metabolites from plant materials (Ajila *et al*., 2011; Chen *et al*., 2007). However, with NADES as extraction solvent, sonication proved to be less efficient than stirring due to the high viscosity of NADES.

Table 2. Relative extraction yield of the peaks of anthocyanins from *Catharanthus roseus* with red petals at different times (30, 60 and 90 min).

	relative extraction ratio as peak area ratio[a]		
	30 min	**60 min**	**90 min**
peak 1	1.00	1.04	0.88
peak 2	1.00	1.01	0.99
peak 3	1.00	0.96	0.93
peak 4	1.00	0.97	0.95

[a]The relative extraction ratio is expressed in peak area ratio of the peaks in the chromatogram of the extract as compared with the reference (30 min) (n=3) .

3.2.2 The compatibility of NADES with reversed-phase HPLC. The chromatographic behavior of malic acid and proline in aqueous solutions and their deep eutectic mixture (PMH) were compared (Fig. 5). The same retention time of malic acid in aqueous solution and PMH was observed, and the same applied to proline. The chromatographic behaviors of malic acid in two NADES (PMH and MCH) diluted 1, 2 or 3-fold with water were compared with that of an aqueous solution of malic acid (Table 3). The same retention time and peak shape of malic acid were observed in the different dilutions of NADES, as in the aqueous solution. These results indicate that the interaction between the two components of NADES with at least 50% (v/v) water has no effect on the chromatographic behavior of components of NADES (malic acid). This is probably due to the fact that the interaction within the components of NADES could weaken with water dilution and even break completely with up to 50% (v/v) water (Dai *et al*., 2013; Gutiérrez *et al*., 2010). Additionally, the chromatographic behavior of anthocyanin extracts in methanol and different NADES were also compared. The anthocyanin extracts have the same profiles (considering the retention time and peak shape) in NADES as in methanol, and NADES with different compositions (Fig. 1), indicating that components of NADES have little effect on the chromatographic profiles of anthocyanin extracts in this study. In conclusion, it is recommended to dilute NADES extracts with 2 volumes of water for their analysis.

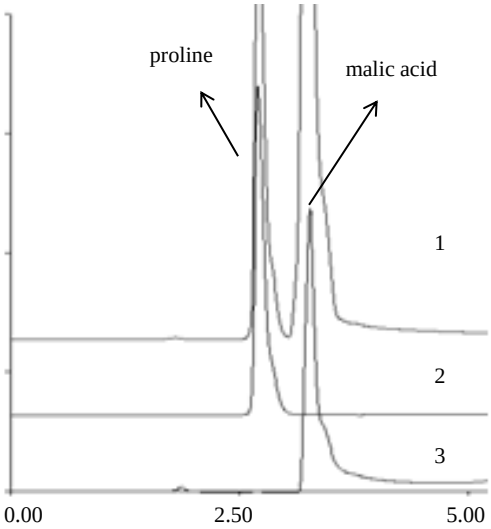

Fig. 5. HPLC-UV chromatograms of **1**) proline-malic acid-water (1:1:3) (PMH) diluted with same volume of water; **2**) proline aqueous solution; and **3**) malic acid aqueous solution at 210 nm.

Table 3. Retention time (Rt) and symmetry of the peak of malic acid in water solution (0.3 g/mL), malic acid-choline chloride-water (1:1:2) (MCH), and proline-malic acid-water (1:1:3) (PMH) with different water dilution (2-5 times) in HPLC-UV chromatograms (*n*=3).

solvents (dilution time)	Rt	symmetry
malic acid aqueous solution	4.15	0.58
MCH(2)	4.15	0.57
MCH(3)	4.15	0.57
MCH(5)	4.13	0.58
PMH (2)	4.13	0.58
PMH (3)	4.14	0.57

Regarding the chromatographic system, it is also recommendable to start with a low amount of organic solvents in the mobile phase as the initial composition of the mobile phase in gradient elution may affect the peak shape of solutes. Starting with a high amount of organic solvent will result in poor peak shape (data no shown). To ensure good chromatographic profiles of the analytes, it is thus better to set the ratio of the starting mobile phase with a high percentage of water. NADES are more polar than methanol. A high ratio of water will dilute the NADES and their hydrophillic ingredients will not be bound to the nonpolar reversed phase column, thus eluting unretained from the column.

3.3 Stability of anthocyanins in NADES

Stability of solutes should be considered during extraction and analysis, as well as during sample storage. Therefore the effects of solvent, temperature, storage time and light on the stability of anthocyanins in NADES were investigated using cyanidin in LGH as a model. To avoid the toxicity and volatility of methanol, ethanol was used in stability tests. Figure 6a-c shows the degradation curve of cyanidin at high temperature (40-80 °C) in LGH and ethanol with 3% formic acid. Cyanidin is more stable in LGH than in acidified ethanol at 60 °C and stable in both solvents at 40 °C for 1.5 hours in the dark. The degradation of anthocyanins over time follows a first-order reaction model at 60 °C and 80 °C, which is in agreement with a previous report on the degradation of anthocyanins (Kırca *et al*., 2007). The half-live time ($t_{1/2}$) of cyanidin was more than 3 times longer in LGH than in acidified ethanol at 60 °C (Table 4) and even longer at 80 °C in LGH than at 60 °C in acidified ethanol. Cyanidin is thus much more stable in LGH than in acidified ethanol at high temperature in the dark.

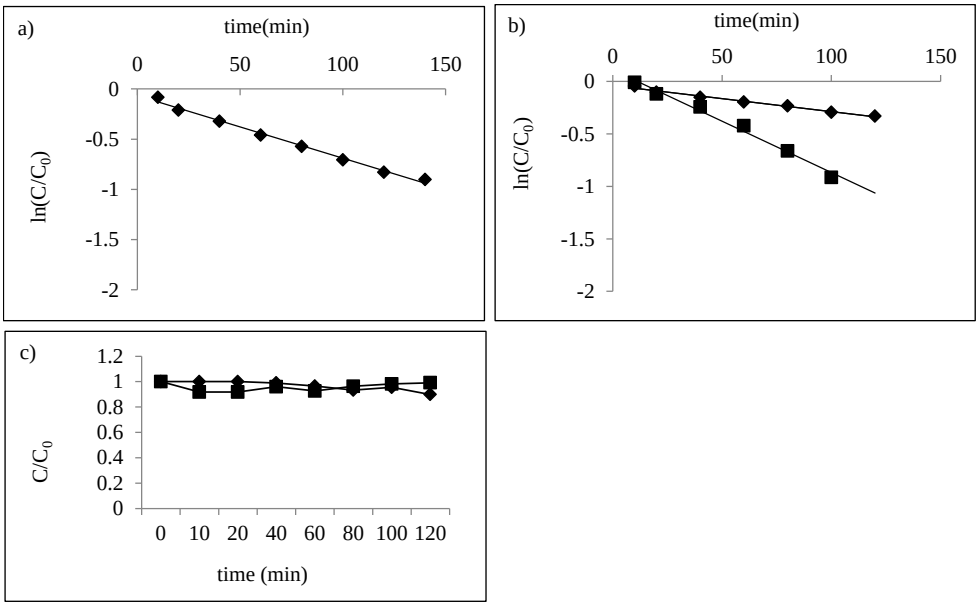

Fig. 6. Stability of cyanidin in lactic acid-glucose (LGH ♦) and ethanol with 3% formic acid (■) at **a**) 80 °C, **b**) 60 °C and **c**) 40 °C in the dark (*n*=3).

The effect of storage time and temperature on the stability of cyanidin was investigated at 25, 4 and -20 °C in the dark, both in LGH and ethanol with 3% formic acid (Fig. 7a). The different degradation curves show that cyanidin was stable at -20 °C in LGH and much more stable in LGH than in ethanol, with a similar degradation curve in LGH at 4 °C as in acidified ethanol at -20 °C. Cyanidin kept the same absorbance in LGH at -20 ℃ for 3 months, at 4 ℃ for 7 days, but degraded quickly at 25 °C, with a decrease of ca. 60% at 25 °C in 3 months, indicating that the stability of cyanidin in LGH is correlated with the temperature in the dark. Thus, samples should be stored at 4 °C in the dark and

analyzed within one week after preparation. For long-term storage, the sample can be kept at -20 ^{o}C in the dark for 3 months.

Table 4. Degradation kinetics parameters of cyanidin in lactic acid-glucose (LGH) and ethanol with 3% formic acid at high temperature including reaction rate constants (k) and half-lives ($t_{1/2}$), and the degradation functions (n=3).

t (^{o}C)	solvent	k	R^2	$t_{1/2}$ (min)	function
80	LGH	0.0062	0.9921	111.80	y = -0,0062x - 0,0653
60	LGH	0.0025	0.9844	277.26	y = -0.0025x – 0.041
	EtOH	0.0096	0.9389	72.20	y = -0.0096x + 0.1241

The effect of light was investigated at ambient conditions with sunlight compared with a control kept in the dark (Fig. 7b). In the darkness , nearly 20% cyanidin was degraded in both LGH and ethanol on day 7 while nearly 30% cyanidin in ethanol solution and 80% in the LGH solution exposed to daylight was degraded. Thus, cyanidin degrades much faster in the sunlight than in the dark both in LGH and ethanol, showing a great effect of sunlight on the stability of LGH solutions of cyanidin. Thus, cyanidin solutions should be preserved in the dark.

The higher stabilization ability of LGH for cyanidin may be correlated with the interactions between cyanidin and the molecules of LGHs. The two components of LGH may form intermolecular interactions, mainly hydrogen bonding, with cyanidin through carboxyl groups and hydroxyl groups, as in the case of quercetin in XoCH (Dai *et al*., 2013). This interaction decreases the movement of solute molecules, reduces its contact time with oxygen at the interface of NADES and air, and consequently reduces oxidative degradation, the major degradation step. It was reported that adding organic acids (such as acetic acid, citric acid, tartaric acid), sugars (such as glucose, fructose, sucrose, trehalose), and their mixtures increase color stability for anthocyanins, which is attributed to the intermolecular association (Hubbermann *et al*., 2006; Kopjar and Piližota, 2011). Cyanidin was more stable in a gel model system with pectin than with the aluminium ion, in which the stability is due to the anthocyanin-metal interaction (Buchweitz *et al*., 2012). Similarly, manually squeezed juice exhibited higher color stability than juice prepared from a concentrate, which was proposed to be due to the retention of polymeric matrix compounds in fresh juice and the interaction of this matrix with anthocyanins (Sadilova *et al*., 2009). Thus, the formation of interactions with other molecules improves the color stability of anthocyanin.

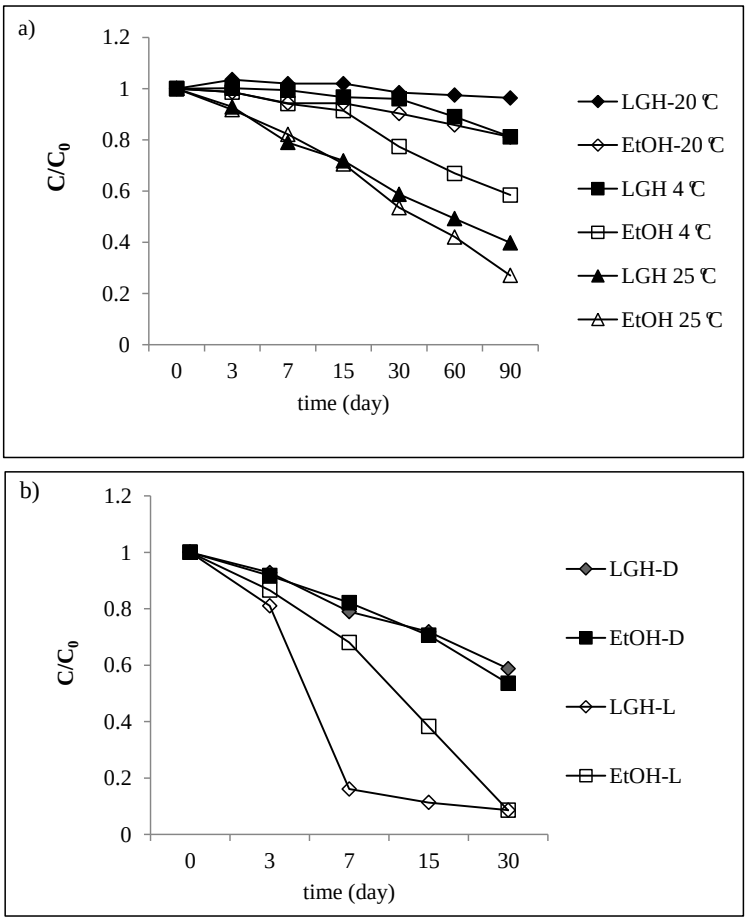

Fig. 7. Storage stability of cyanidin in lactic acid-glucose (LGH) and ethanol with 3% formic acid at **a**) -20 ℃, 4 ℃ and 25 ℃ in the dark for 3 months and **b**) at 25 ℃ with sunlight (L) and in the dark (D) for one month (*n*=3).

4. Conclusions

A green, simple, and effective extraction method using natural deep eutectic solvents (NADES) was established as well as an HPLC method to analyze the anthocyanin extracts. Under the optimized conditions, some NADES give the same yields as with acidified methanol. However, cyanidin is much more stable in some tested NADES than in acidified ethanol. NADES are alternative green solvents to harmful organic solvents for the extraction and storage of anthocyanins from plant material.

This study provides evidence on the extraction capacity, stabilization ability of NADES, and the compatibility of NADES extracts with reversed phase liquid chromatography. Based on these characteristics, NADES seem very promising for various applications in human health-related areas e.g. food, cosmetics and

pharmaceuticals. For designing application of NADES one should keep in mind their viscosity. For example, the viscosity instead of polarity plays an important role for NADES as extraction solvents. The high stability of cyanidin in NADES may be correlated with the viscosity via the establishment of hydrogen- bonds between cyanidin and the NADES molecules.

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Chapter 7

Natural Deep Eutectic Solvents as new extraction media for phenolic metabolites in *Carthamus tinctorius* L.

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Abstract

Developing green solvents with low toxicity and low cost is an important issue for chemical industry, particularly for pharmaceuticals, food and cosmetics. Synthetic ionic liquids and deep eutectic solvents have received considerable attention due to their negligible volatility at room temperature, high solubilization ability and selectivity. However, the potential toxicity of the synthetic ionic liquids and solid state at room temperature of most deep eutectic solvents hamper their applications as extraction solvents. In this study a wide range of recently discovered natural ionic liquids and deep eutectic solvents, composed of primary metabolites of living organisms, called Natural Deep Eutectic Solvents (NADES), were investigated for the extraction of phenolic compounds of diverse polarity. NADES present many advantages including low cost, simple preparation, low or negligible toxicity profile and sustainability in view of environment and economics benefits. They are stable liquids even below zero ^{o}C, and show high solubilization strength for a wide range of compounds, especially for poorly water-soluble compounds due to their unique physicochemical properties. All those features suggest their potential as green solvents for extraction. Safflower (*Carthamus tinctorius* L.) was selected as a case study because its aromatic pigments covers a wide range of polarities. Experiment with different NADES and multivariable data analysis demonstrated that the extraction ability for both polar (such as hydroxysafflor yellow A, cartomin) and less polar compounds (carthamin and five stereoisomers of tri-*p*-coumaroylspermidine) was greater with NADES than conventional solvent. Water shows high ability for extraction of polar compounds and ethanol for less polar ones. A parameters optimization study reveals that the water content in NADES has the greatest effect on the yield of phenolic compounds. A high concentration of NADES is suitable for less polar

compounds but for more polar compounds low concentration is preferable. Most major phenolic compounds were recovered from NADES with a ratio around 75%-97%, with similar chemical profiles of phenolic compounds as the 40% ethanol extract. Compared with conventional organic solvents, this study provides a greener approach for the extraction of phenolic compounds, implying that NADES have the potential to replace conventional organic solvents in health related areas such as food, pharmaceuticals, and cosmetics.

Key words: *Carthamus tinctorius* L., Asteraceae, natural ionic liquids and deep eutectic solvents (NADES), extraction ability, recovery, phenolic compounds

1. Introduction

Conventional organic solvents are widely used in preparation bioactive components from natural products resources in pharmaceutical, food, and cosmetic industry. The wide range of polarities and physical properties of cellular metabolites make it impossible to extract all metabolites from biomass in a one-step process with one single solvent (Verpoorte et al., 2006). Thus, a wide range of solvents of different polarities is required for the extraction, separation, purification, and administration of various chemicals. So far, alcohols, chloroform, and ethyl acetate are generally applied to this purpose. However, the use of large amounts of organic solvents may cause severe pollution of the environment, and result in organic impurities in extracts, requiring special assays in quality control of extracts (Puranik *et al*., 2009).

With the aim of developing environmental friendly solvents, ionic liquids (ILs) have received increasing attention because they have negligible vapor pressure at room temperature (Welton 1999; Visser *et al*., 2000). Compared with molecular liquids, ILs are a class of organic salts with a low melting point (<100 ^{o}C). Synthetic ILs possess attractive properties such as negligible volatility, tunable physicochemical properties (Huddleston *et al*., 2001), ability to dissolve a wide spectrum of solutes, and special tailor-made selectivity in extraction and separation (Yao *et al*., 2009; Ragonese *et al*., 2011). They have mainly been used in organic chemistry (Welton, 1999) and electrochemistry (Macfarlane *et al*., 2007). However, the application of synthetic ILs as solvents for extraction in pharmaceutical industry is limited because of high toxicity of some ingredients (Docherty and Kulpa, 2005; Quijano *et al*., 2011), their irritation properties, high costs of synthesis of the components, and tedious preparation procedures. Ionic liquids have been used for extracting some active compounds from plant materials including alkaloid (Ma *et al.*, 2010), phenolic compounds (Du *et al.*, 2007), essential oils (Bica *et al.*, 2011) and shikimic acid (Usuki *et al*., 2011).

Deep eutectic solvents (DES) are another type of solvents with similar physical properties (Abbott *et al*., 2007). The solvents are composed of a

mixture of organic compounds and have a melting point much lower than either of the individual component. A series of DES are reported with different components, such as choline, urea (Abbot *et al*., 2003), organic acids (Abbot *et al*., 2004), sugars (Imperato *et al*., 2005). Compared with ILs, DES show some advantages, especially considering their lower environmental and economic impact, e.g. biodegradablity, pharmaceutical accepted toxicity, low cost, and simple preparation methods. They have already been used as solvents to extract DNA and for enzymatic reactions (Gorke *et al*., 2008; Zhao *et al*., 2011). However, the high viscosity and solid state of most DES at room temperature hamper their applications for extraction (Abbott *et al*., 2003, 2004; Imperato *et al*., 2005) and there is still no report on their use for extracting active compounds from plant materials.

In previous studies, we have found a series of natural ionic liquids and deep eutectic solvents, composed of primary metabolites common in living cells, called natural deep eutectic solvents (NADES). In certain Molar ratios, these NADES (e.g. equimolar) show strong intermolecular interactions and may sometimes include water as its ingredients (Choi *et al*., 2011). Apart from sharing all the advantages of reported ILs and DES, NADES possess better properties for extraction, e.g., liquid state even below 0 ^{o}C, adjustable viscosity, and last but not least, sustainability (Dai *et al*., 2013). NADES contain components abundant in our daily foods and thus they are cheap, sustainable, and safe. Interestingly, some NADES show very high solubilization ability for both non-polar and polar compounds, and some metabolites show significantly higher solubility in NADES than in water (Choi *et al*., 2011; Dai *et al*., 2013). This indicates the great potential for NADES as solvents in the extraction of valuable secondary metabolites for their application in the food or pharmaceutical industry

Despite the extensive research on NADES, there is still a lack of information on practical issues related to their application as extraction solvent, such as their effiency, the optimal concentration of NADES, the recovery of compounds from NADES extracts. The latter is particularly challenging considering the inherent low vapor pressure of NADES which makes it difficult to recover solutes from the NADES solution. In this paper, we deal with three aspects of NADES as an extraction solvents: i) the extractability of NADES for natural dyes (phenolic compounds) of diverse polarities, ii) the optimization of the extraction parameters for these phenolic compounds with three typical NADES, and iii) the recovery of the phenolics from the NADES extract. As an example, safflower (Flos carthami), the corolla from *Carthamus tinctorius* L. (Asteraceae), was selected because of its high amount of phenolic compounds of a broad range of polarities and also its application as medicine for promoting blood circulation (Kazuma *et al*., 2000; Zhou *et al*., 2008). It contains yellow (hydroxysafflor yellow) and red pigments. Hydroxysafflor yellow A (HSYA), cartormin and carthamin are the main pigments in safflower used as dye for food and cosmetics. HSYA is also the major active component of safflower

(Watanabe *et al*., 1997; Jin *et al*., 2008). Another group of compounds present in safflower with anti-human immunodeficiency virus infection and anti-depressive activities are tri-p-coumaroylspermidines (Ma *et al*., 2001; Jiang *et al*., 2008; Zhao *et al*., 2009). In addition, different flavonoids, including rutin, quercetin, and their glucosides are reported (Wang *et al*., 2005; Kazuma *et al*., 2000). To investigate the extractability of NADES for those diverse components, HPLC fingerprint and multivariate data analysis, particularly principal components analysis (PCA) were employed to obtain an overview of extract profiles.

2. Material and methods

2.1 Plant material

Safflower was bought from Xinjiang province in China. The plant material was identified by one of the authors, Dr. Young Hae Choi, and a voucher specimen (NPL-carthamus-0913) was deposited in the Natural Products Laboratory, Institute of Biology, Leiden University. The dry plant material was ground into powder in a blender with liquid nitrogen.

2.2 Chemicals and reagents

Ethanol of analytical grade and acetonitrile of HPLC grade were purchased from Biosolve BV (Valkenswaard, The Netherlands). Water was deionized water. Malic acid, lactic acid, proline, sucrose, glucose, fructose, 1,2-propanediol, sorbitol, and choline chloride were purchased from Sigma (St. Louis, MO, USA). Macroporous resin Diaion® HP-20 from Supelco (Bellefonte, PA, USA) was used in this study. Silica gel from Sigma-Aldrich (St. Louis, MO, USA) and Sephadex^{TM} LH-20 from GE Healthcare Bio-Sciences (AB, Uppsala, Sweden) were used.

2.3 Natural deep eutectic solvents preparation

All NADES including lactic acid-glucose (LGH); proline-malic acid (PMH); sucrose-choline chloride (SuCH); glucose-choline chloride (GCH); sorbitol-choline chloride (SoCH); 1,2-propanediol-choline chloride (PCH); and fructose-glucose-sucrose (FGSH) were prepared with our reported method in chapter 2.

2.4 Extraction with different solvents

Extraction was performed in a sealed bottle with 100 mg plant material and 1.5 mL solvent, heating and stirring at 40 ^{o}C for 1 h. The sample was transferred into an eppendorf tube and centrifuged at 1300 rpm for 20 min. Then the suspension solution was filtered through a 0.45 μm cellulose acetate filter and diluted with same volume of water. Each extraction was in triplicate.

2.5 Extraction parameter optimization

The extraction parameters of the three NADES with high extractability, SUCH, LGH, and PMH, were optimized. These includes ratio of material weight to NADES volume (mg/mL) (40:1, 30:1, 20:1, and 10:1), the water content in NADES (0%, 10%, 25%, 50%, and 75%), and extraction time (30, 60, 90, 120, and 180 min).

2.6 Recovery of compounds from NADES extracts

Samples of 400 mg of powdered plant material was extracted by 6 mL SuCH, PMH and 40% ethanol with the above mentioned extraction method. The extraction solution was divided into two parts, one for HPLC-DADanalysis as the reference, and one for recovery tests of phenolic compounds from the NADES extracts. For the recovery tests, the SuCH and PMH extracts were submitted to the following procedures: 1 mL of the extracts was diluted with 10 mL of deionized water (for SUCH extract, 1% formic acid was added), loaded on an HP-20 column 60 g (height 50 cm), and eluted with enough deionized water (for SUCH extract, 1% formic acid was added) till all the NADES were washed away. The sample was then eluted with 130 mL of 50% ethanol and 260 mL of ethanol. The two ethanolic fractions were combined, dried with a vacuum evaporator, and dissolved with 3 mL of 50% methanol. An aliquot of 1 mL of the diluted solution was analyzed with HPLC-DADand compared with the equally diluted initial extract. Another 2 mL of the extract were dried, dissolved in 0.4 mL methanol-d_4 (99.80% from Cambridge Isotope Laboratories, Andover, MA) and 0.4 mL phosphate buffer (KH_2PO_4, pH 6.0) in deuterium oxide (CortecNet, Voisins-Le-Bretonneux, France), and analyzed by ^{1}H NMR together with 40% ethanol extract with the same dilution. The above experiments were performed by triplicate.

2.7 Isolation of pure compounds

A sample of 100 g of dry ground material was sonicated with two 800 mL portions of methanol for 1 h, filtered and dried with a rotary evaporator. The residue (20.7 g) was partitioned with 90% methanol and n-hexane twice, and the aqueous methanolic fraction was evaporated under vacuum, obtaining a residue of 18 g. This was fractionated on a middle pressure column with 180 g of silica gel (pore size 60 Å, 230-400 mesh) and eluted with 500 mL of *n*-hexane: chloroform (1:1), 1000 mL of chloroform, 500 mL of chloroform: methanol (10:1) and 2000 mL methanol. Each fraction (100 mL) was pooled based on its thin layer chromatography (TLC) profile [stationary phase: 60F254 plate (Merk, Darmstadt, Germany); mobile phase: chloroform-MeOH (8.5:1.5)]. Three fractions containing phenolic compounds - 26 (40 mg), 29-30 (56 mg), 33-37 (127 mg) - were collected. Each combined fraction was purified on a column with 55 g of Sephadex LH-20, eluted with methanol and further purified with semi-preparative HPLC, using a Phenomenex Luna C18 (250×10 mm, 5 μm, Torrence, CA, USA) column and acetonitrile–water (23:77, 3 mL/min) as a

mobile phase. The isolated compounds were dissolved in 1.0 mL methanol-d4 for measurement for their structural elucidation.

2.8 HPLC, NMR and MS analysis

Quantitative HPLC analysis was performed on an Agilent 1200 chromatographic system with a photodiode array detector (DAD) and separated on a HPLC column, Phenomenex Luna C18 (4.6 μm x 250 mm, 5 μm). The mobile phase consisted of water with 0.5% H3PO4 (A) and acetonitrile (B) in a linear gradient program as follows: 5%-11% B (0-10 min), 11%-14% B (10-16 min), 14% B (16-23 min), 14%-20% B (23-30 min), 20%-35% B (30-70 min), 35%-60% B (70-80 min) at a flow rate of 1.0 mL/min (Wang, *et al*., 2008). Chromatograms were recorded at 520 nm, 403 nm, and 280 nm. The injection volume was 10 μL.

^{1}H NMR spectra, correlation spectroscopy (COSY), *J*-resolved spectra, heteronuclear single quantum coherence (HSQC), heteronuclear multi-bond correlation spectroscopy (HMBC), and attached proton test ^{13}C NMR (APT) of four purified compounds were recorded at 25 ^{o}C, on a 600 MHz Bruker DMX-600 spectrometer (Bruker, Karlsruhe, Germany) operating at a proton NMR frequency of 600.13 MHz (^{1}H) and 150.13 MHz (^{13}C) with MeOH-d_4 as the internal lock. All the parameters were the same as those described in our previous report (Ali *et al*., 2011).

Mass spectra were measured by an ESI/TOF/MS. The operating conditions of the ESI ion source (Jeol, Tokyo, Japan) coupled to a JMS-T100TD (AccuTOF-TLC) in the positive ion modes were a discharge needle voltage of 2000 V, nebulizing nitrogen gas flow at 1 L/min. The first orifice lens was set to 100 V and Ring lens voltage was set to 13 V. The TOFMS was set with a peak voltage of 2500 V, a bias voltage of 29 V, a pusher bias voltage of -0.76 V, and a detector voltage of 2300 V.

2.9 Data analysis

The areas of eight representative peaks in the HPLC-DADchromatograms from triplicates were subjected to PCA with the Pareto scaling method using the SIMCA-P software (verion12.1, Umetrics, Umeå, Sweden). These peaks corresponded to compounds of the whole range of polarities present in the HPLC-DAD chromatograms. Analysis of variance (ANOVA) was performed with SPSS software (version 14.0, Chicago, IL, USA) using the peak area in the HPLC chromatogram and P values ≤ 0.1 were considered as significant for comparison. The extraction yield was calculated on the basis of the peak area of the selected peak in the HPLC chromatograms of initial extracts. The recovery yield (w%) is calculated with the peak area of selected peaks in HPLC chromatograms as follows:

$W\% = (A_{rec}/A) \times 100\%$.

where A_{rec} is the peak area of a compound in the chromatogram of the recovered mixture; A is the peak area of the same compound in the chromatogram of extraction solution.

3. Results and discussion

3.1 Comparison of the extractability of safflower polyphenols with NADES, water and ethanol

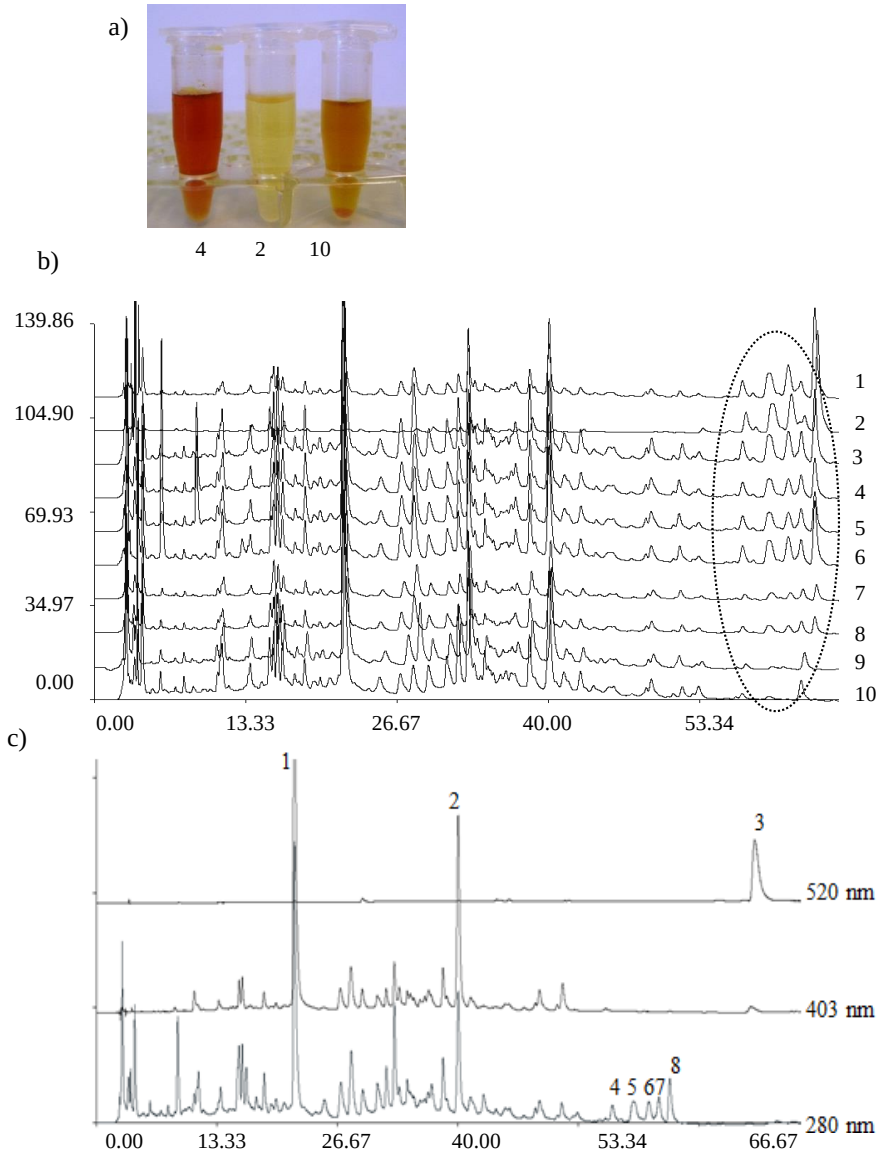

Fig. 1. Safflower extraction solutions **a**) picture of the extraction solutions; **b**) the HPLC-DAD chromatogram profiles of extractions with different natural deep eutectic solvents at 280 nm (1, 1,2-propanediol-choline chloride; 2,

ethanol; 3, 40% ethanol; 4, sucrose-choline chloride; 5, lactic acid-glucose; 6, proline-malic acid; 7, glucose-choline chloride; 8, sorbitol-choline chloride; 9, fructose-glucose-sucrose; 10, water); **c**) HPLC-DAD chromatograms of the extraction in proline-malic acid at three different wavelengths (the labeled compounds are 1, hydroxysafflor yellow A; 2, cartormin; 3, carthamin; 4, N^1, N^{10}, N^5-(*Z*)-tri-*p*-coumaroylspermidine; 5, N^1-(*E*)- N^5N^{10}- (*Z*)-tri-*p*-coumaroyl spermidine; 6, N^1, N^{10}, N^5-(*E*)-tri-*p*-coumaroylspermidine; 7, stereoisomer of tri-*p*-coumaroylspermidine; 8, N^1, N^{10}, N^5-(*E*)-tri-*p*-coumaroylspermidine).

Seven NADES with different polarity, viscosity, composition, and solubilization ability were selected in this study: LGH, PMH, SuCH, GCH, SoCH, PCH and FGSH. NADES with different compositions have different physical properties (Dai *et al*., 2013), and consequently have different solubilizing ability for phenolic compounds.

The major disadvantage of NADES is their high viscosity if compared with conventional solvents. Viscosity is known to restrict the efficiency of NADES as extraction solvents since it results in slow mass transfer. To solve the problem, extraction conditions were adjusted to reduce the viscosity of NADES and thus improve the yield. Different NADES have very different viscosity, but in all cases it can be reduced by the addition of a certain amount of water (Dai *et al*., 2013). Thus, 75% (v/v) SuCH, 75% FGSH, 75% PMH, 90% GCH were used in this study. Another variable known to affect viscosity is temperature, and using 40 ^{o}C as the extraction temperature resulted in increased yields as viscosity decreased. Lastly, in an effort to increase the diffusion rate of the compounds in the liquid with an external force, mechanical agitation was used instead of ultrasound. This resulted in a higher efficiency. Thus, phenolic metabolites were extracted from safflower by agitation of 100 mg of plant material in 1.5 mL solvent at 40 ℃ as described in the experimental section. Given the high viscosity of the resulting extracts samples were centrifuged before filtration.

Clear differences were found in the extractability of compounds from safflower with the tested NADES, water, and ethanol, which were reflected in the color of the obtained extracts and their HPLC profiles (Fig. 1). The NADES extracts exhibited the most intense color (Fig. 1a). The HPLC fingerprints of NADES extracts showed all the peaks observed in water and ethanol extracts (Fig. 1b). Three NADES, LGH, PMH, SuCH extracted polar compounds: HSYA (retention time (Tr) = 21.9 min) (Jin *et al*., 2008; Sun *et al*., 2012) and cartormin (Tr = 40.0 min) (Jin *et al*., 2008; Yin and He, 2000), as well as less polar compounds: carthamin (Tr = 72.9 min, identified with reference) and five stereoisomers of tri-*p*-coumaroylspermidine (Tr = 57.1 - 63.5 min) (Fig. 1c). The identification of each peak was confirmed by comparing NMR and MS data with previous papers and 2D NMR spectra (Jiang *et al*., 2008; Zhao *et al*., 2009). Compounds corresponding to four peaks were isolated and identified as N^1, N^{10}, N^5-(*Z*)-tri-*p*-coumaroylspermidine (peak 4), N^1-(*E*)-N^5N^{10}-(*Z*)-tri-*p*-coumaroyl spermidine (peak 5), N^1, N^{10}-(*E*)-N^5-(*Z*)-tri-*p*-coumaroyl spermidine (peak 6), N^1, N^{10}, N^5-(*E*)-tri-*p*-coumaroyl spermidine (peak 8) (Fig. 2). Peak 7

(a mixture of two compounds) and a small peak between peak 4 and 5, all have the same UV max at around 300 nm and the same molecular weight (583) as the above described tri-*p*-coumaroylspermidines, probably corresponding thus to other stereoisomers of tri-*p*-coumaroylspermidine. The ^{1}H and ^{13}C NMR spectra of coumaroylspermidines showed complex signals because of the restricted rotation around the N-C (sp^2) bond in the coumaroylspermidines (Ma *et al*., 2001). The sequence of elution of these compounds was in accordance with their polarity as can be estimated from their extraction ratios in water and ethanol. Tri-*p*-coumaroylspermidines and carthamin were highly extractable in all three NADES and ethanol, while water did not extract these less polar compounds. Moreover, LGH, PMH, SuCH were more efficient than ethanol even for the less polar compound, like carthamin. The chemical profile of some NADES extracts was qualitatively the same as 40% (v/v) ethanol extract that had been reported as the optimal solvent in extracting safflower yellow (Zhang *et al*., 2009).

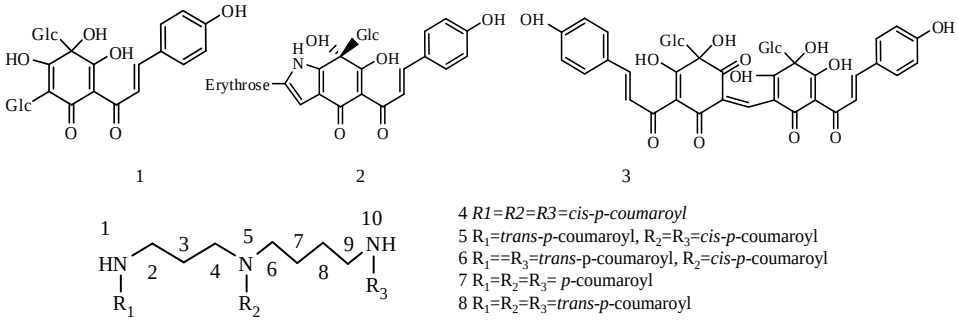

Fig. 2. Chemical structure of target phenolic compounds 1-8 in safflower (the number refers to the same compounds as that in figure 1c).

The score plot of PCA of the first two components (R^2 = 0.94 and Q^2 = 0.89) (Fig. 3a) showed a separation of the extracts into four groups. The PCA confirms the similarity between the extracts with LGH, PMH, SuCH, and the 40% ethanol (Group I), whereas the PCH is similar to the ethanol extract (group II). In the loadings plot of PCA, all metabolites selected from the chromatograms were clustered around group I (Fig. 3b), confirming that solvents in group I had a broad extraction capacity, being efficient for the extraction of both polar compounds (HSYA and cartormin) and less polar ones (carthamin and five stereoisomers of tri-*p*-coumaroylspermidine). In addition, solvents in group II (PCH, EtOH) were efficient in extracting less polar compounds (carthamin and tri-*p*-coumaroylspermidines), while those in group IV (FGSH and water) exhibited a high efficiency for polar ones (HSYA and cartormin), which is in agreement with the reports on the efficiency of water to extract the yellow pigment from safflower (Zhang *et al*., 2007). The extractability of phenolic compounds from safflower is thus higher in SuCH, PMH, and LGH.

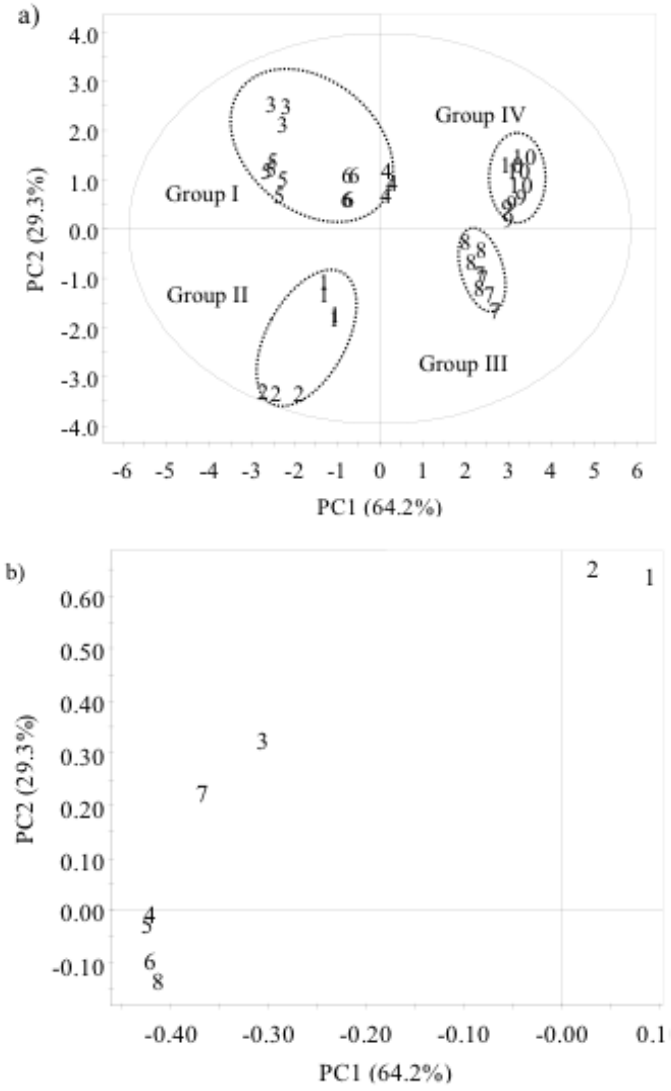

Fig. 3. Score plot **a**) and loadings plot **b**) of principal component analysis of the extracts from safflower with different solvents: (In score plot a, the number refers to the same extract in fig. 1b. In loadings plot b, the number refers to the same peak as in Fig. 1c).

3.2 Optimization of the extraction parameters for NADES with high extraction efficiency

Extraction parameters were optimized using SuCH, PMH, LGH as extraction solvents. The peak areas of HSYA, cartormin and carthamin were used as the criteria to evaluate their extractability. The areas corresponding to peaks of the five tri-*p*-coumaroylspermidines were deleted because they had a retention time close to carthamin and were very small peaks.

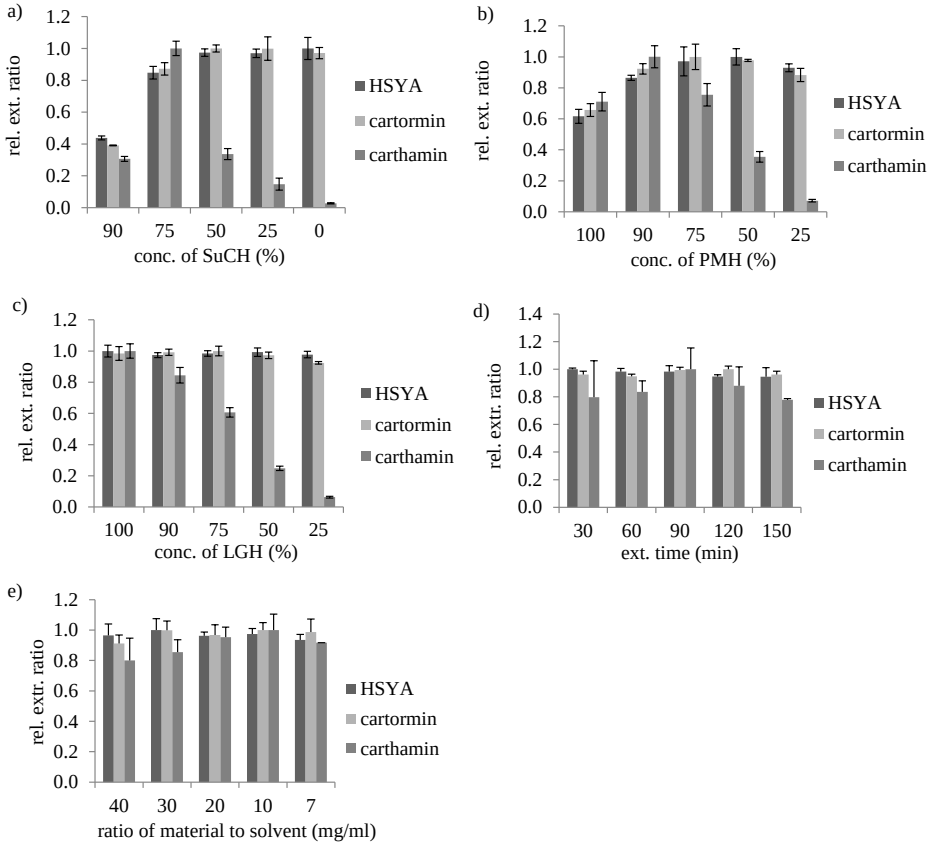

Fig. 4. The effect of water content in NADES on the extraction ability of **a**) sucrose-choline chloride (SuCH), **b**) proline-malic acid (PMH), and **c**) lactic acid-glucose (LGH) and the effect of **d**) extraction time and **e**) ratio of material weight to solvent volume of on the extraction efficiency of three typical phenolic compounds from safflower by proline-malic acid (PMH) for HSYA, cartomin and carthamin. The extraction efficiency is expressed in a relative extraction ratio with the value of a peak area divided by the biggest area of the same peak from different concentrations of the same NADES. The data is expressed in mean ± SD (n=3).

The water content in NADES has a large effect on their extract yield, varying considerably according to the target compounds and the NADES itself (Fig. 4a-c). In the case of HSYA and cartormin, the highest extraction yield was achieved with 50%-100% water in SuCH, with 25%-50% water in PMH and with no addition of water in LGH. For less polar compounds, such as carthamin, the extraction yield was also greatly affected by the water content in NADES. The highest extraction yield of carthamin was reached with 25% (v/v) water in

SuCH, 10% water in PMH, and without water in LGH. The extraction yield of carthamin in SuCH with 25% water was around three times higher than in SuCH with 10% or 50% water. So, in general, NADES with high water content are better for polar compounds and NADES with low water content are suitable for the extraction of less polar compounds. The extraction yield was not significantly affected by any of the other two studied factors: extraction time and ratio of material weight to solvent volume (Fig. 4d-e).

Table 1. The extraction yield of three representative metabolites (hydroxysafflor yellow A (HSYA), cartormin, and carthamin) from safflower with 6 different solvents (The data are the peak areas of each peak in HPLC-DADchromatogram at 403 nm for HSYA and cartormin, and 520 nm for carthamin with 10 μL injection volume, extracted from 90 mg safflower powder with 3 mL solvent and diluted with 2 times water. The data are expressed in mean ± SD (*n*=3).

	extraction yield		
solvents	**HSYA**	**cartormin**	**carthamin**
75%PMH[1]	2813±2	2925±37	134±0
75%SuCH[1]	2680±3	2591±9	152±0
LGH[1]	2244±123	2229±54	235±26
40%EtOH	2611±80	2528±79	182±42
Water	2843±28	2520±19	5±1
ethanol	30±0	13±1	12±0

[1] 75% PMH: 75% (v/v) proline-malic acid in water; 75% (v/v) SuCH: 75% sucrose-choline chloride in water, and LGH: lactic acid-glucose.

Thus, the optimized extraction conditions for these NADES were established as: 1 hour, ratio between material weight and solvent 30 mg/mL). Under these conditions, the relative extract yield of 75% PMH, 75% SuCH, and LGH was compared with that of ethanol, 40% ethanol and water (Table 1). The most efficient extraction solvent proved to be PMH (75%) for HSYA (the same as water and 8% higher than 40% ethanol) and cartormin (14% higher than water and 40% ethanol). In the case of carthamin, LGH showed the highest extraction yield (23% higher than 40% ethanol).

3.3 Recovery test of phenolic compounds from NADES

In order to recover the phenolic compounds from NADES extracts, a chromatographic resin, HP-20, was used. HP-20 (composed of styrene-divinylbenzene) can absorb phenolic compounds while the polar ingredients of NADES can be dissolved and eluted with water. Most phenolic compounds were recovered with ethanol after eluting the polar compounds with water. However, the components of NADES, highly concentrated aqueous solution of the ingredients, as the case with SuCH, can affect the separation process. Using

water, phenolic compounds were eluted together with sucrose from SuCH. However, the addition of 0.1% formic acid to the water increased the retention of phenolic compounds on the column so that they were separated from the components of the SuCH aqueous solution. This approach did not work so well with LGH, since the separation of phenolic compounds was hindered because lactic acid was also attached to the column.

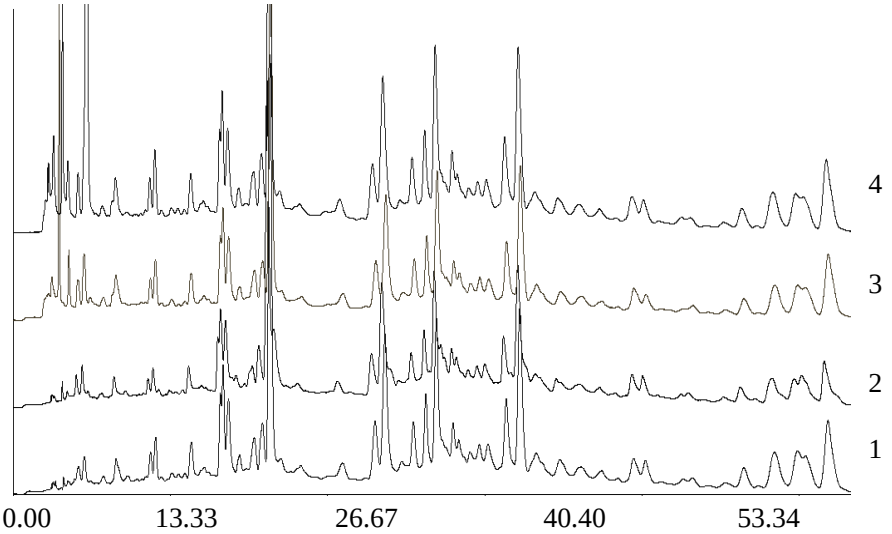

Fig. 5. HPLC chromatogram profiles at 280 nm of safflower extracts **1**) recovered with HP-20 from sucrose-choline chloride (SuCH), **2**) recovered with HP-20 from proline-malic acid (PMH), **3**) 40% ethanol extract, and **4**) PMH extract.

Table 2. The recovery yield of three representative metabolites (hydroxysafflor yellow A, cartormin, and carthamin) in safflower from two NADES (The values are mean ± SD, *n*=3).

	relative yield (%)		
	HSYA	**cartormin**	**carthamin**
75%PMH	92%±9	92%±8	84%±8
75%SuCH	71%±4	86%±4	90%±13

The chromatographic profile of the solutions containing the recovered compounds from NADES (e.g. PMH and SuCH) were qualitatively similar to the 40% ethanol extract except for the solvent peaks, as shown in the HPLC-DAD chromatogram at 280 nm (Fig. 5). The ^{1}H NMR spectra of the recovered extracts confirmed that the components from NADES were successfully separated from the targeted compounds. Furthermore, three replicates showed qualitatively the same chemical profile in HPLC-DAD chromatograms and ^{1}H

NMR spectra. Recovery rates of up to 90% were achieved for polar compounds (such as HSYA and cartormin) (Table 2). For less polar compounds such as carthamin, around 84% recovered from SuCH and 75% from PMH was obtained. It is thus evident that phenolic compounds can be recovered from NADES extracts.

3.4 NADES features affecting their extraction efficiency

The high extractability of phenolic compounds with NADES may be attributed to H-bonding interactions between molecules of NADES and phenolic compounds. In general, the functional groups involved in H-bonding are hydroxyl groups, carboxylic groups and amine groups, all of which are abundant in NADES while hydroxyl groups are obvious available in phenolic compounds. Our former studies showed that obvious interactions exist between quercetin and NADES (Dai *et al*., 2013). The phenolic compounds in safflower are *C*-glucosyl quinochalcone (Jin *et al*., 2008) and flavonoid glycosides (Kazuma *et al*., 2000). Therefore, H-bonding interactions between molecules of NADES and phenolic compounds lead to a high solubility.

The extraction capacity of NADES is also correlated with their physical properties, including polarity and viscosity. PCH has the lowest polarity among all the tested NADES and showed the lowest efficiency for polar compounds, such as HSYA and cartormin, and high extractability for non-polar compounds. Thus, the polarity of NADES has to be considered as an important property affecting their efficiency. Compared with conventional solvents, the high viscosity of NADES is an important feature but it can be decreased by water dilution (Dai *et al*., 2013). SuCH has the highest viscosity and the viscosity is so high that it is difficult to extract compounds from biomass with SuCH. However its dilution with water increased its efficiency, performing much better with a 25% water content than with 10% water content, most likely due to an increased mass transfer rate by the decreasing the viscosity of SuCH.

4. Conclusion

A mixture of natural solid compounds, NADES proved to be efficient solvents for the extraction of phenolic compounds of diverse polarities. These compounds were able to be recovered from NADES with a resin column (e.g. HP-20). These simple, low-cost, green and efficient methods can be applied to the extraction and isolation of natural products from biomaterials. This holds promise for further application of NADES in pharmaceutical, cosmetic and food industry for the extraction and isolation of natural products using fully green solvents.

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Chapter 8

Natural deep eutectic solvents in plants and plant cells: *in-vitro* evidence for their possible functions

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Abstract

The components of natural deep eutectic solvents (NADES) are abundant in plants. This led to our hypothesis that NADES may also exist in plant cells playing an important role in solubilizing, storing, and transporting poorly water-soluble metabolites in living cells, adjusting the water content of plants, and protecting cells when in harsh conditions. In order to test this, diverse plant materials were analyzed, including leaves, petals, plant secretions and seeds. Comparatively high amounts of ingredients of NADES are observed in plants. Dry mosses contain a higher amount of NADES components than fresh ones and the level of NADES components is higher in the outside layer (aleurone and seed cover) of barley, than in the inside (endosperm and embryo) layer. A high accumulation of sugars, sugar alcohols, amines, amino acids, organic acids, choline, and betaine dominate plant secretions such as sap and nectar. Beside their similar compositions, NADES showed similar physicochemical properties to that reported for plant cytoplasm. This implies the existence of NADES in plants. Experimentally, NADES and water can be mixed resulting in liquids with different compositions and properties. In the case of plants, NADES and water co-exist in the cells and may form ideal solvents for metabolites of diverse polarities and macromolecules in plants. Ingredients of NADES, as can be found in plants, are hygroscopic, providing evidence for the water-adjusting effect of NADES in plants. Most importantly, NADES may accumulate around the lipid bilayers, form intermolecular bonds with the polar heads of lipids, and

stabilize the membrane, as revealed in experiments with liposomes. This study gives *in vitro* evidence for the different roles NADES may play in living organisms, and opens perspectives for further exploring the existence and functions of NADES in plants cells.

Key words: natural deep eutectic solvents (NADES); metabolites; physicochemical properties; diffusion; hygroscopicity; water adjusting effect; membrane stabilizing effect; liposome

1. Introduction

Natural deep eutectic solvents (NADES) were proposed by our group with the purpose of extending the range of ionic liquids (ILs) and deep eutectic solvents (DES) and explore their applications in health related fields, (Choi *et al.*, 2011; Dai *et al.*, 2013). NADES are liquid supramolecules composed of natural compounds in certain molar ratios bonded by intermolecular interactions, particularly H-bonding (Choi *et al.*, 2011). The components of NADES are metabolites that are common in high amounts in living cells (sugars, sugar alcohols, organic acids, amino acids, amines) as well as water, that can also be an ingredient of NADES. NADES possess excellent properties as solvents (Dai *et al.*, 2013), e.g., negligible volatility, a very low melting point (they are liquid even below -20 ^{o}C), a broad polarity range and high solubilization power of a wide range of compounds, especially poorly water-soluble compounds (Choi *et al.*, 2011; Dai *et al.*, 2013). The high solubility of scarcely water-soluble metabolites and macromolecules (e.g. DNA, proteins, and cellulose) has been demonstrated as well as their suitability as (Mamajanov *et al.*, 2010; Dai *et al*., 2013) media for enzymatic reactions (Zhao *et al.*, 2011; Choi *et al*., 2011) and biotransformations (Gutiérrez *et al.*, 2010).

Drought, cold, or salinity tolerance has been found to be correlated with a high level of common cell metabolites such as sugars (sucrose, trehalose, raffinose), amino acids (proline), organic acids (ascorbic acid, abscisic acid), sugar alcohols (sorbitol, mannitol), glycine betaine, and choline (Bartels *et al*., 2005). The content of proline increased over 10 times to nearly the same molar concentrations as sucrose during the natural desiccation of the resurrection plant *Selaginella bryopteris* (Pandey *et al*., 2010). Accumulation of glucose, sucrose and amino acids in *Sporobolus staofianus* (Whittaker *et al*., 2007), and sucrose and raffinose in the resurrection plant, *Xerophyta viscosa* (Peters et al., 2007) were reported. Moreover, higher amounts of sugars (fructose, glucose, raffinose), proline, and galactinol were observed in *Arabidopsis* during cold acclimation (Kaplan *et al*., 2007). In fact, the commonly used cryoprotectants

for plants are sugars, sugar alcohols, and proline. Proline is important for the cold acclimation of plants as well as in other organisms (Kovács *et al*., 2011). All these metabolites that correlate with drought, cold, or salinity tolerance have been mentioned as compatible solutes for organisms, i.e. non-toxic molecules which do not interfere with normal metabolism and accumulate predominantly in the cytoplasm at high concentration under stress conditions (Bartels and Sunkar, 2005). They accumulate in the cytoplasm to a high concentration under osmotic stress (Yancey *et al*., 1982; Bartels and Sunkar, 2005). Sugars together with other compounds may form a glass-like matrix and prevent macromolecular denaturation and loss of membrane integrity during desiccation (Moore *et al*., 2009; Hoekstra *et al*., 2001). Concerning cold resistance, it was reported that a high level of proline causes the remaining unfrozen water to undergo a glass-like transition and thus prevent cryo-injury (Kovács *et al*., 2011). The so-called compatible compounds were postulated to play a major role in the above mentioned processes. In our view, these compatible substances might be part of NADES that are formed in various cellular compartments. The strong hydrogen bonding between these compounds being the crux to explain liquid crystal formation and retaining a certain amount of water as part of the NADES. The NADES components can form liquid crystals in aqueous environments and in that way lowers the sugar content in the water and thus control osmolarity. Such liquid crystals can dissolve and stabilize macromolecules and stabilize membranes. NADES thus may explain many of the questions raised above.

More detailed measurements are needed to explore and prove the existence of NADES and their functions in plants. In this study, different plant materials and secretions were analyzed to collect more evidence for the existence of NADES; the physicochemical properties of NADES were also compared with that of cytoplasm in plants. To explore the functions of NADES related to the hygroscopicity of cells, the water absorption and desorption of seeds was analyzed. The diffusion process between water and NADES was investigated to mimic the mechanism by which metabolites of different polarity are solubilized within the cells. In order to determine the effect of NADES on membranes, the behavior of liposome bilayers in NADES media was studied.

2. Experimental

2.1 Plant materials

A dry Mexican moss (*Selaginella pallescen*) was grown with roots in water, harvested after two days, and dried in a freeze-drier. *Arabidopsis* plants were provided by Dr. Jieun Shin (Plant Developmental Biology Department, Max Planck Institute, Germany). These plants were grown under long-day conditions for one month, after which the water feeding was stopped during two weeks,

and re-initiated for one week. The control and dried plants were then collected. Barley seeds (*Hordeum distichum* L.) were obtained from Prof. Dr. Bert van Duijn from Leiden University, The Netherlands. The barley seeds were allowed to absorb water between two layers of paper (Whatman) for one day at ambient conditions. Then, the seeds were cut into two parts of which the outside (aleurone and seed cover) and inside parts (endosperm and embryo) were separated (Schuurink *et al*., 1992). The dry flower buds of *Sophora japonica* were brought from the Kyung Dong Traditional Medicine Market (Seoul, Korea Korea). C*atharanthus roseus* with purple petals, belonging to the Pacifica Orchid Halo Varitey, was bought from a flower shop in Leiden. Saps of *Cleome hasselorana* and *Drosera* from different species (*Drocera adelae*, *D. capensis*, *D. mucipula*, *D. glabripes*, *D. glabripes*) were collected into microtubes for sample preparation from the Hortus Botanicus garden in Leiden, The Netherlands.

2.2 Chemicals and reagents

Sucrose, glucose, fructose, choline chloride, 1,2-propanediol, proline, and 5-doxyl stearic acid (5-DS) were purchased from Sigma (St. Louis, MO, USA). 1,2-dioleoyl-3 trimethylammonium-propane (chloride salt), soybean L-α phosphatidylcholine, and cholesterol were purchased from Avanti Polar lipids, Inc. (Alabaster, Alabama, USA).

2.3 Sample preparation

*2.3.1 Preparation of plant material***.** All the above collected materials (mosses, *Arabidopsis* plants and barley seeds) were ground to powder with liquid nitrogen, freeze dried, and extracted with 1 mL of 50% CH_3OH-d_4 in buffer (90 mM KH_2PO_4 in deuterium oxide) containing 0.05% TMSP (trimethylsilyl propionic acid sodium salt, w/v). The mixture was vortexed at room temperature for 30 s, ultrasonicated for 20 min, and centrifuged at 30,000 rpm at 4 ^oC for 20 min. An aliquot of 700 l of the supernatant was transferred to a NMR tube for NMR analysis. Nectar and sap were added to 1 mL phosphate buffer (KH_2PO_4, pH 6) in deuterium oxide (CortecNet, Voisins-Le-Bretonneux, France), vortexed at room temperature for 30 s, centrifuged at 30,000 rpm at 4 ^oC for 20 min and the later steps are the same as above. For HR-MAS NMR analysis, the dry flower buds (50 mg) of *Sophora japonica* and the fresh petals (80 mg) of *C. roseus* with one drop of buffer containing 0.1% TSP (pH 7.0) were packed into a rotors equipped with close fitting and caps.

2.3.2 Preparation of NADES. Sucrose-choline chloride-water (1:4, molar ratio) (SuCH), glucose-choline chloride-water (1:2.5) (GCH), 1,2-propanediol-choline chloride-water (1:1:1) (PCH), Sucrose-malic acid-water (1:1) (SMH), Sucrose-proline-water (1:1) (SPH), and sucrose-fructose-glucose-water (1:1:1) (SFG) were prepared using the stirring method (Dai *et al*., 2013).

2.3.3 Preparation of liposomes. Liposomes were prepared by the film hydration-extrusion method employing a laboratory LiposoFast-Pneumatic extruder from (Avestin Inc., Ottawa, Canada). To prepare a 2 mL dispersion of liposomes, 7.8 mmol 1,2-dioleoyl-3-trimethylammonium-propane (chloride salt), 3.9 mmol soybean L-*α*-phosphatidylcholine, and 3.9 mmol cholesterol were dissolved in 2 mL chloroform to form a lipid film using a rotary evaporator. The film was hydrated with 2 mL aqueous solution (PBS phosphate buffer, PBS phosphate buffer (pH 7.0) with 35.6% (w/w) SFG, PBS phosphate buffer with 19.6% (w/w) SMH, or PBS phosphate buffer with 25.2% (w/w) SPH) and the obtained suspension was extruded successively through filters with a pore size of 200 and 100 nm. The PBS phosphate buffer contains 0.81% sodium chloride, 7.1% disodium hydrogen phosphate, 0.02 % potassium dihydrogen phosphate and 0.019% potassium chloride with phosphate sodium water.

2.3.4 Liposomes Labeling. Membrane spin probe 5-doxyl stearic acid (5-DS) (150 μg) from a stock solution in ethanol at -20 ℃ was put into a microtube, the ethanol was evaporated by blowing nitrogen gas over the solution and then 0.133 mL prepared liposome suspension (5 mg/ml, 0.0078 M) (5-DS:lipid=1:100 (molar ratio)) was added and mixed. 5-doxyl stearic acid can be easily incorporated into liposome membranes due to its high lipid/water partition coefficient. Then the final suspension was filled in to a 50 μl capillary for electron paramagnetic resonance (EPR) measurement.

2.4 Diffusion experiments

Sucrose-choline chloride-water (10 mL), or PCH (10 mL) was placed in the lower part of a tube and then 10 mL water was gently added above the NADES. A natural dye, carthamin, was added in either the water or NADES part for observation. A clear interface was observed between NADES and water in each tube. The position of the interface was marked and photos were taken every 24 hours. On the 10th day, a third layer was formed between water and SuCH layers with an interface between water and the intermediate layer (the initial position, line 1 Fig. 3) and an interface between the NADES and the intermediate layer (line 2). Samples were collected from the upper and lower part of each layer into a microtube, weighed, and freeze dried for 3 days. Then the samples were weighed and 100 mg of the sample was transferred into another microtube, dissolved with 1 mL phosphate (KH_2PO_4) buffer (pH 6.0) in deuterium oxide and analyzed by ^1H NMR. The above experiments were performed in triplicate.

2.5 Hygroscopicity test

Glucose-choline chloride-water (GCH) was used for the hygroscopicity test at 25 ^oC. Initially the sample was dried in the Q5000 apparatus (TA-Instruments, New Castle, USA) at 60 ^oC. Then humidity levels of 0, 20, 40, 60, and 80% (and

then decreasing again from 80% to 0 with the same step) were programmed with 720 min for each step. Intact barley seeds (single piece) were used for the hygroscopicity test at 25 ^{o}C. During the first 20 hours the sample was dried in the Q5000 apparatus at 60 ^{o}C. After that a relative humidity of 80 % was applied for 84 hours. The relative amount of absorbed moisture M_t is defined as

$$M_t = \frac{m_t}{m_{dry}} = \frac{\text{mass of absorbed water}}{\text{mass of dry sample}} \qquad \text{Eq.1}$$

2.6 Instruments and measurements

*2.6.1 NMR spectroscopy***.** ^{1}H NMR spectra were recorded at 25 ^{o}C on a 500 MHz Bruker DMX-500 spectrometer (Bruker, Karlsruhe, Germany) equipped with a TCI cryoprobe and Z-gradient system. All the parameters were the same as those described in our previous reports (Dai *et al*., 2013). ^{1}H NMR and 2D NOESY spectra of flower materials were recorded at 25 ^{o}C on a Bruker 400 MHz HR-MAS NMR spectrometer at a frequency of 3000 Hz.

2.6.2. Size and zeta potential measurements. The size and zeta potential of liposomes were measured on a Zeta sizer Nano-ZS 4800 (Malvern instruments Ltd., Malvern, UK). The average dynamic size was obtained with dynamic light scattering with the method of cumulants. *Zeta* potential measurements were carried out on a laser Doppler electrophoresis with a dip cell. Each measurement was repeated at least three times.

2.6.3 Electron paramagnetic resonance (EPR) spectroscopy. To monitor the structural interactions between NADES and liposomes, EPR was applied with the spin-labeled derivative of the stearic acids, 5-DS as an indicator. ESR spectra were recorded at room temperature with an X-band ESR spectrometer (Bruker EMXplus, Rheinstetten, Germany). Microwave power was 10 mW, the modulation amplitude was 5 G and the scan range was 200 G. To increase signal/noise ratio, 24 scans were accumulated.

3. Results and discussion

3.1 Different compositions of NADES in plants

3.1.1 Plants in harsh conditions. Our former experiments showed that a comparatively high amount of the components of NADES were observed in desert plants of the *Selaginela* species. In this experiment ^{1}H NMR spectroscopy showed that a high amount of sugar (glucose), organic acids (succinic acid, caffeic acid, tartatic acid, acetate, *γ*-aminobutyric acid), and amino acids (alanine, threonine, arginine) were found in dry moss (*S. pallescen*) if compared with fresh moss. The level of the primary metabolites increased in the case of water shortage even for plants, such as *Arabidopsis* which showed

increased levels of sugar (sucrose), amino acids (proline, alanine, arginine), organic acids (succinic acid, fumaric acid, malic acid), and an amine (choline) in water depleted conditions if compared with normal growing conditions. So, a relatively high amount of components of NADES exists in plants under dry conditions. However, to find direct evidence of the existence of NADES in plants, in-situ analysis of plant materials would be required instead of the indirect evidence from the contents of extracts.

3.1.2 Petals containing pigments. In cells, NADES may dissolve and preserve the compounds responsible for color that are not soluble or stable in water since NADES showed a high solubilizing and stabilizing ability for phenolic compounds (Dai *et al*., chapter 4). The dry flower buds of *Sophora japonica* contain up to 40% rutin, which is not very soluble in water (Paniwnyk *et al*., 2001). The concentration of anthocyanins in vacuolar inclusions has been reported to be higher than that expected according to their solubility in water (Markham *et al*., 2000). To get an insight into what is present in the flower, flower petals were directly measured using HR-MAS NMR. HR-MAS NMR spectroscopy is a rapid, non-destructive method and tissue can be analyzed directly without extraction. We expected to observe the components of NADES, the interactions between their components, and even the interaction between NADES and the phenolics. The ^{1}H NMR spectrum of petals is dominated by sugars and organic acids (Fig. 1). However, no signal of color compounds was observed, although a high amount of rutin was detected in HPLC-DAD with the same material.

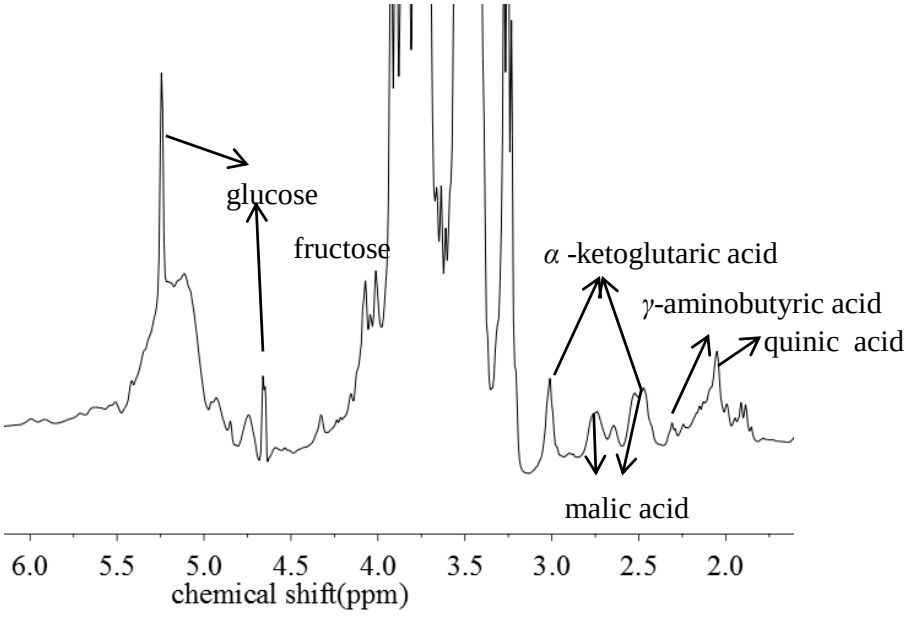

Fig. 1 ^{1}H NMR spectrum of the red petals of *Catharanthus roseus*.

It is difficult to detect NADES in plants. On one hand, the dynamic changes of NADES in cells and the presence of different potential ingredients of NADES in cells or cellular compartments, such as vacuoles, vesicles, or plastids hinder their in-situ detection. A high amount of NADES may be concentrated in anthocyanoplasts and anthocyanic vacuoles with the function of dissolving high concentrations of anthocyanins. In the analysis of the whole plant, we detected an average, diluted profile of all cell types present, and thus of all possible NADES mixtures. In the previous chapter the highest solubility may occur in NADES that contain some amount of water. This may also be the case in plant cells. One the other hand, although rutin accounts for 40% of the weight of dry flower buds of *Sophora japonica*, it was still not detected in HR-MAS HNMR. This might be due to the attachment of rutin to the cellular structures or the broadening of signal resulting from its interactions with macromolecules. This requires further study, probably with mass spectroscopy.

3.1.3 Plants secretions and seeds. Various secretions, such as maple syrup, nectar, and mucilage are present extracellularly on the plant surface. For example, glucose: sucrose: fructose with a molar ratio around 1:1:1 was detected in the nectar of *Cleome hasselorana*, and honey is composed of glucose and fructose (1:1, molar ratio) (Fig. 1 in chapter 2). Obvious signals of *myo*-inositol and betaine were observed in the ^{1}H NMR of *Drosera* extracts from five different species (Fig. 2).

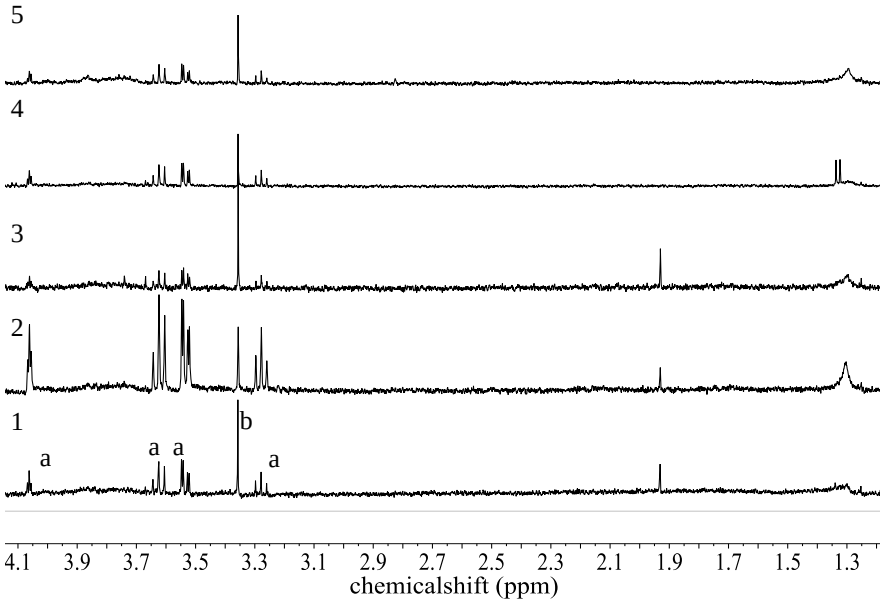

Fig. 2 ^{1}H NMR of sap from different *Drocera* species (**1**, *Drocera capensis*; **2**, *D. binata* ; **3**, *D. capensis*; **4**, *D. glabripes*; **5**, *D. adelae*) with *myo*-inositol (**a**) and betaine (**b**) in all samples.

A seed, as a temporary static system, is an interesting model to study the presence of NADES. In our view NADES could be the explanation for the cold and drought resistance of seeds and the relative ease to revive when under the right conditions for germination. NADES could keep essential enzymes dissolved and upon addition of water be activated (Choi *et al*., 2011). The ^{1}H NMR of extracts of the outside and inside parts of barley seeds revealed higher amounts of sugars (sucrose), organic acids (acetic acid, succinic acid), amino acids (threonine, alanine), alcohols (isobutanol, ethanol), and amines (choline, betaine) in the outside layer (aleurone and seed cover) of a barley seed than its inside part. During the dormant period, enzymes stored in the outside layer of the seed must survive in extremely dry conditions (Schuurink *et al*., 1992). The presence of typical NADES components in the outside layer of the seed implies that the NADES could be the solvents that protect the enzymes.

3.2 The physicochemical properties of NADES

Experiments to determine the physicochemical properties of NADES showed that the density of NADES is in the range of 1.08 -1.36 g/cm^3 (Dai *et al*., 2013) and their viscosity between 37 and 720 mm^2/s, which is much higher than water (1 mm^2/s). The viscosity increases dramatically at low temperature and with low water contents. The NADES have a T_g far below minus 50 ^{o}C, giving them a stable liquid status over a wide temperature range.

Several studies have reported the physicochemical properties of cytoplasm in cells before the intracellular molecular glass formation or glass in plant anhydrobiosis, which is in fact similar to the NADES. The cytoplasmic glass in dry cells results from a complex of sugars and other cytoplasmic components such as organic acids, amino acids, and salts (Buitink and Leprince, 2004). The viscosity of cytoplasm increases dramatically during drying-out conditions and eventually transforms into a glass state. In general, the glass has the following physical properties: high density, temperature dependent mobility and slow mobility in solid state, high viscosity, and low T_g (Dijksterhuis *et al*., 2007; Buitink and Leprince, 2008), which are typical of the physical properties of NADES (Dai *et al*., 2013). The consistency in physicochemical properties as well as in the composition of cellular cytoplasm may be explained by the existence of NADES in plant cells. The major difference is that a glass does not have an orderly structure like the NADES where a liquid crystal-like structure strongly retains water in a certain molar ratio to the NADES components.

3.3 The diffusion between NADES and water

The diffusion process between water and NADES was studied. All tested NADES are hydrophilic and miscible with water since the individual

components are polar compounds. However, the high viscosity may slow down the diffusion process. We selected SuCH from among all our different NADES combinations as it has the highest viscosity and PCH as the lowest viscosity NADES. A natural dye, carthamin, was added in either the water or NADES part for observation.The diffusion test was performed with the NADES as the lower layer, since it is denser than water. With an equal diffusion rate of the constituents in both layers one would not expect any change, except that at a certain point the interface should disappear. If the diffusion ratio were not equal, then the interface would move up (water diffuses faster into lower NADES layer, than NADES into the water layer) or down (NADES components have faster diffusion in upper layer, than water in the lower layer).

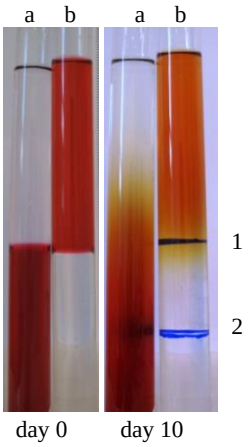

Fig.3 The picture of diffusion tests between sucrose-choline chloride-water (1:4:4) and water on day 0 and 10, and 3 layers were observed with two interfaces (line 1 and 2) labeled in blue on day 10.

Interestingly, diffusion between NADES and water behaved in an unexpected manner as three phases were formed (two interfaces marked with blue color), instead of one (Fig. 3), both in SuCH and PCH. Interface 1 is obvious in the tube b which is indicated by the position of the dye and also the chemical composition of the phases (Table 1). In tube a, the different chemical composition of the different layers is revealed by the interface 1 (line 1) between the water layer and intermediate layer, this interface is at the same height as in tube b, although the position of the dye is different in the tube a. Apparently the mass transfer of the dye through interface 1 is difficult. Interface 2 is clear in both tubes and moved down in the same rate and reached the same height over a 10-day period in both tubes. The separation into three phases is not affected by the added dye.The red dye was found to diffuse from the NADES to the intermediate layer, where a gradient in the color was observed

with the lowest concentration in the upper part. In the case of the dye in the water layer the diffusion of the dye seemed to be impeded at the interface between the upper water layer and the intermediate layer, although its solubility is higher in NADES than in water (Dai *et al*., 2013). The diffusion process between the NADES and water was monitored by recording the position of the interface with the initial interface as reference, measuring the water content in the different layers as well as the ratio of the two NADES components.

The interface (line 1 in Fig. 3) between water and intermediate layer remained more or less in the same position. The interface between SuCH and the intermediate layer (line 2 in Fig. 3) moved down in a gradually decreasing rate from 1.1 cm/day to around 0.2 cm/day in 10 days with 10 mL of each water and SuCH phase at the start of the experiment. In the other experiment the interfaces between PCH and the intermediate layer disappeared in two days. So, the dye diffusion between PCH and water was much faster than that between SuCH and water. Faster diffusion was also observed when the starting phase of SuCH was diluted with 10% (v/v) water. Thus, the high viscosity of NADES decreases the diffusion between NADES and water. Clearly, viscosity plays an important role in the phase behavior between NADES and water.

Table 1 The water weight percentage and molecular ratio of components of sucrose-choline chloride in two parts of three different layers (upper, middle and lower) after 10 days' diffusion between water and sucrose-choline chloride-water (1:4:4) (n=3).

		up1	up2	mid 1	mid 2	low	low
water w%	1	90±2.2	60.1±3.3	50.0±4.5	18.0±3.7	9.0±2.8	6.4±1.5
	2	91.9±1.7	64.2±5.7	42.1±4.5	15.3±2.7	6.4±1.0	6.3±0.9
	3	90	62	49	15	7.9	7.2
molar ratio (choline: sucrose)	1	14.5	3.2	2.8	3.1	3.5	3.7
	2	16.6	3.0	3.0	3.5	3.5	3.9
	3	10.3	3.0	2.7	3.1	3.5	3.7

1: carthamin in the sucrose-choline chloride layer; 2: carthamin in the water layer; 3 sucrose-choline chloride.

The composition of the organic components and the water content are different in the different layers. At day 10, the water content in the water layer showed a gradient from about 90% to 60% (up to down), in the middle layer 50% to 20% and more or less unchanged in the lower phase, showing that instead of complete mixing, a new metastable NADES was formed as an intermediate phase (Table 1). The ratio of the two components in NADES is

different in the layers. The molar ratio of choline chloride to sucrose in SuCH is 4 when prepared. During the diffusion process, the ratio was around 15 in the upper layer, 3 in the middle layer, and remained at the same value of 4 in the lower layer. Apparently, in the interface of SuCH and water a new phase is formed from which choline can move into the water layer whereas the sugar is kept in the middle layer by the hydrogen bonding.

This mixing behavior indicates the complexity of the physicochemical properties of the cellular contents. A change in pH, concentrations, temperature etc. may cause the formation of separate phases, experiencing a dynamic system like the endoplasmic reticulum e.g. creating the conditions for the biosynthesis of poorly water soluble compounds in a cell by creating temporarily a third phase in which enzymes are dissolved and where poorly water soluble substrates are concentrated.

3.4 Adjustment of the water content in cells and plants (the hygroscopicity of GCH and barley seeds)

The NADES in plants may play a role in absorbing water from the surroundings. Hygroscopicity measurements of GCH showed that (Fig. 4a) the water percentage in GCH increases gradually with an increasing humidity level in the surroundings from 0% to 80% and decreases when the humidity level in surroundings went down. The water percentage in GCH reached equilibrium in about 10 hours for a certain condition (Fig. 4b). So, the NADES may have the function of adjusting the water level in a plant through interaction with water vapor in the air.

To further explore the water adjustment effect of NADES in plants, the hygroscopicity level of a barley seed was tested at a moisture level of 80% at 25 ^{o}C. The relative amount of absorbed water in Fig. 5 shows a combined water absorption and desorption curve. As can be seen, most water was absorbed during the first 48 hours and the equilibrium water content for 80% external relative humidity was about 9.1 wt%. When the humidity level decreased to zero, the amount of absorbed water in the barley seed returned to the initial level within the same time interval. The observation fits our hypothesis of NADES being involved in water loss and uptake, e.g. several NADES are clearly hygroscopic and at the same time are able to strongly retain certain minimum amounts of water.

Apparently, plants can lose water in relative dry conditions and absorb water in relative humid conditions. The hygroscopicity may explain how mosses can live on rocks, without roots, and how Cactaceae can possibly live without any water absorbed by the roots. Cacti open their stomata during the night when in deserts the relative humidity may increase considerably as temperature drops. A hygroscopic NADES in the cacti could then absorb water diluting the NADES, activating the enzymes of photosynthesis that subsequently produce sugars

again during the day thus restoring a hygroscopic NADES for nightly water harvesting.

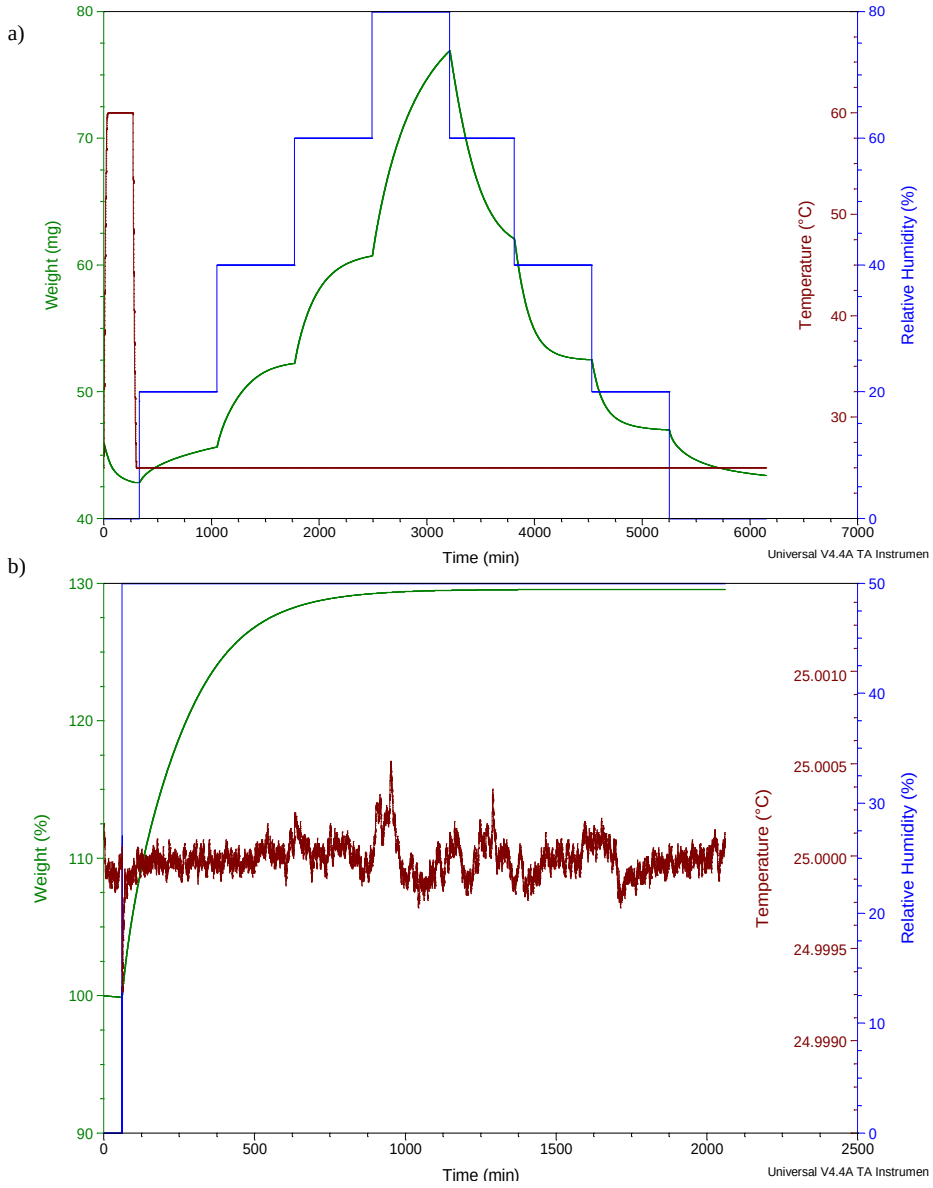

Fig. 4 Hygroscopicity test of glucose:choline chloride:water (1:2.5:2.5) at 25 ^{o}C with relative humidity steps (**a**) and 50% relative humidity level **(b)**. The green line indicates the weight of glucose:choline chloride:water and the blue line refers to the relative humidity of test conditions.

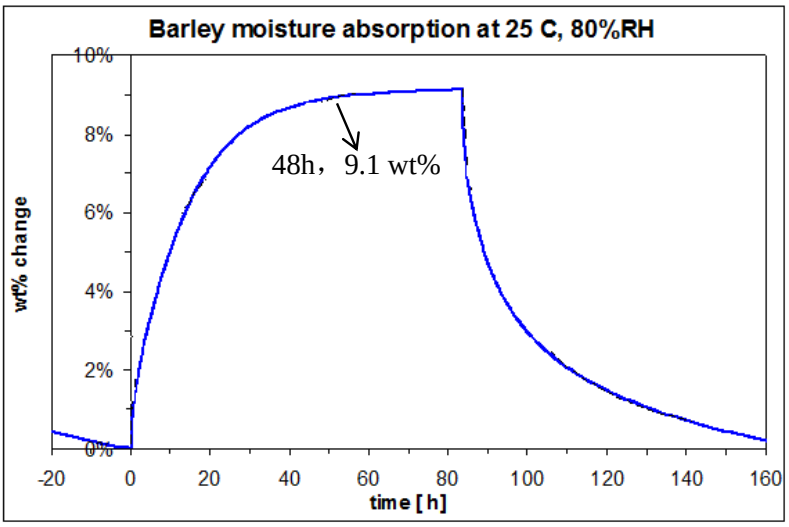

Fig. 5 The hygroscopicity test of barley seeds at 25 ^{o}C with 80% relative humidity level.

3.5 The interaction between NADES and liposomes: the membrane stabilizing effect

3.5.1 The effect of NADES on the size, zeta potential and stability of liposome. The size (hydrodynamic diameter) of liposomes was increased by 30 nm in a buffer with 35.6% sucrose:fructose:glucose (SFG), increased by 600 nm in buffer with 25.2% sucrose:proline (SPH), and decreased by 40 nm in buffer with 19.56% sucrose:malic acid (SMH), compared with phosphate buffer (Table 2). It means that NADES may form layers around liposomes, which increase the size of liposomes in the case of SFGH, SPH and decreases the size in the case of SMH. The zeta potential decreased in buffer with SFG, SPH and increased in buffer with SMH, compared with phosphate buffer. The zeta potential is widely used for quantifying the electrical charge at the double layer. The different zeta potentials confirm the interactions between NADES and liposomes and also imply that the interactions between liposomes and NADES are different. For SFGH, the sugar components may hydrogenbond to the head group of the lipids and form a film around the liposome through a hydrogen bonding network, increasing the size of liposome (Crowe et al., 1992; Christensen et al., 2007). For SMH, the negatively charged acid group of the tested malic acid may have ionic interactions with the positively charged head group of the lipids and this interaction may shrink the size of liposome due to the attraction effect. The size of liposomes in SPH increased a lot but they were physically unstable probably due to the zwitterionic form of proline. The preparation of liposomes with sucrose: choline chloride: water (SCH) in buffer (pH 7.0) was also attempted

but the hydration of the lipid film failed and the lipid precipitated in the buffer containing SuCH. This might be because of the repulsive forces between choline and the head group of the lipid (1,2-dioleoyl-3-trimethylammonium-propane). In all, the different behavior of liposomes in buffers containing NADES with neutral (SFG), acid (SMH), basic (SCH) and zwitterionic (SPH) components indicates the existence of interactions between liposomes and the molecules of NADES components.

The stability of liposomes in different media was investigated with the size and zeta potential as parameters. The size, polydispersity index, and zeta potential of liposomes were practically stable for one month at 4 ℃ in buffer and buffer with SFG or SMH, but unstable in SPH. This indicates that liposomes are stable for shape, homogeneity, and interaction between NADES and liposomes for at least one month in buffer, buffer with SFG or SMH. However, after five months at 4 ℃, the liposomes had precipitated in both buffer and SPH, while they were still in suspension in SFG and SMH. This proves the stabilizing effect of some NADES on liposomes.

Table 2. The size (hydrodynamic diameter, nm) and *zeta* potential (ZP) of liposome in three media (n=3): phosphate buffer (pH=7.0), buffer with 19.56% (w/w) sucrose:malic acid:water (SMH), buffer with 35.6% (w/w) sucrose: fructose: glucose-water (SFG) and buffer with 25.2% (w/w) sucrose-proline-water (SPH).

	Size (d,nm)			**ZP**	
	average	**PDI**[a]	**RSD%**	**average**	**RSD%**
buffer+SMH	117.2	0.124	0.60	40.5	0.65
buffer+SFG	192.2	0.175	4.85	20.4	12.70
buffer+SPH	790	0.369	25.90	15.2	34.30
buffer	162.5	0.092	1.07	27.4	1.03

[a] PDI: polydispersity index, the width of molecular size distribution.

3.5.2 The effect of NADES on the membrane dynamics of liposomes. The effect of NADES on membrane dynamics was studied with the EPR spin probe technique. Spin-labeled 5-doxylstearic acid (5-DS) was used to probe the membrane interface in liposomes. In the 5-DS molecule the nitroxide doxyl group (a stable radical) is attached in a rigid, stereospecific manner to stearic acid at the 5^{th} carbon from the COOH group. In phospholipid membranes the COOH group of spin-labeled stearic acid is inserted between the polar heads. Therefore, the nitroxide (doxyl group) of 5-DS resides in a polar area of the bilayer, which is called bilayer surface or bilayer interface. The EPR spectral shape of 5-DS depends on the motion and angular orientation of the nitroxide group with respect to the membrane lipid-water interface (Marsh, 1981). This spin label allows the probing of the motional freedom in membranes at the lipid-water interface of the bilayer.

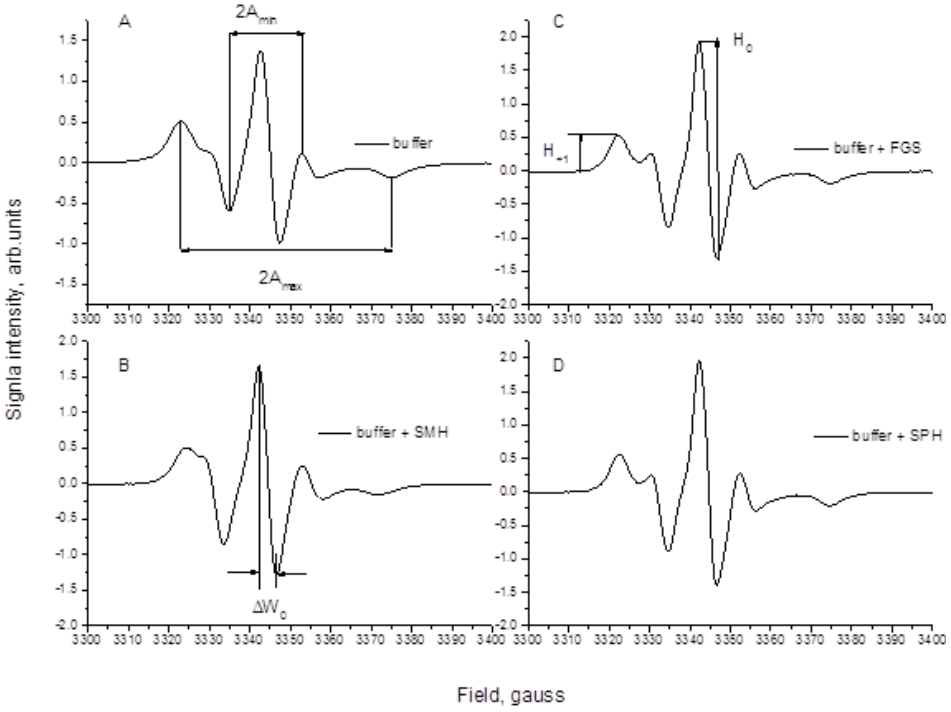

Fig. 6 Electron paramagnetic resonance spectra of 5-doxyl stearic acid in liposomes in PBS phosphate buffer (pH=7.0) (A); in PBS phosphate buffer with 19.6% (w/w) sucrose:malic acid:water (SMH) (B); in PBS phosphate buffer with 35.6% (w/w) sucrose:fructose:glucose (SFG) (C); and in PBS phosphate buffer with 25.2% (w/w) sucrose:proline:water (SPH) (D).

The anisotropic character of the spectral shape of 5-DS in liposomes in normal buffer (Fig. 6A) results from the restricted angular freedom of the radical group of 5-DS in the bilayer interface. The spectral parameters A_{max} and A_{min} indicate the outer and inner hyperfine splitting in an experimental spectrum (as shown in Fig. 6A). The membrane-order parameter S_{zz} can be calculated as the ratio between the observed hyperfine anisotropy (A_{max} - A_{min}) to the maximum theoretically obtainable value, which corresponds to the completely rigid orientation of 5-DS (Knowles *et al*., 1976). With membrane fluidization outer splitting (A_{max}) decreases and inner splitting (A_{min}) increases, so that the order parameter decreases. Later on, some corrections have been proposed to account for differences in polarity and for the range of membrane-order parameter. Here the order parameter is calculated according to the formula proposed by Marsh and Schorn (1998). This formula takes into account the

principle splitting values for 5-DS and is corrected for polarity of the spin label environment. S_{zz} = 1/7 (A_{max}+2A_{min}) – sqrt{ [1/7(A_{max}+2A_{min})]^2 -0.46(A_{max}-A_{min})+0.6}

The EPR spectra of the 5-DS incorporated into the liposome in three media were compared: phosphate buffer, phosphate buffer with SFG (35.6%) and phosphate buffer with SMH (19.56%). The data on 2A_{max} and S_{zz} are presented in Table 3. Outer splitting 2A_{max} considerably decreases in the presence of 19.6% SMH and decreases insignificantly in the presence of SFG and SPH. The order parameter S_{zz} of the EPR spectra of 5-DS in liposomes in the presence of 19.6% SMH is considerably reduced (Table 3). In the presence of SFG and SPH the order parameter slightly increases.

The decrease of outer splitting 2A_{max} and order parameter S_{zz} in the presence of SMH results from the fluidization of the membrane interface. This data is also supported by a decrease in the width of the central line (Table 3). The results are in accordance with a considerable decrease of liposome size from 162.5 to 117.2 nm (Table 2). Obviously, the decrease of liposome size inevitably causes the fluidization of the liposome bilayer outer surface due to an increased bilayer curvature and the spacing between lipids. The effect of bilayer curvature on phospholipid behavior recently has been demonstrated by molecular dynamics simulations (Risselada and Marrink, 2009). The size of liposomes in the presence of SFG and SPH considerably increases (Table 3). However, this causes only a slight ordering of the membrane interface as determined by order parameter (Table 3). If the ordering/immobilization increases slightly in the presence of SFG and SPH, an increase of the outer splitting 2A_{max} would also be expected. However, this was not the case. The slight decrease of the 2A_{max} for these cases could be explained by the decrease of the polarity in the vicinity of spin label moiety.

Table 3. Parameters of electron paramagnetic resonance spectra of 5-doxyl stearic acid in liposomes in the presence of three different natural different deep eutectic solvents (buffer with 19.56% (w/w) sucrose:malic acid (SMH), buffer with 35.6% (w/w) sucrose:fructose:glucose (SFG), and buffer with 25.2% (w/w) sucrose:proline:water (SPH), compared with phosphate buffer (pH=7.0)). The parameters include membrane-order parameter (S_{zz}), the outer hyperfine splitting (2A_{max}), the width of the central line (ΔW_0) and the ratio between the heights of the central (H_0) and low-field (H_{+1}) lines (H_0/H_{+1}).

media composition	**2A_{max},**	**S_{zz},**	**ΔW_0**	**H_0/H_{+1}**
buffer (pH=7.0)	52.08	0.617	4.6919	2.71391
buffer + 35.6% (w/w) SFG	51.99	0.629	4.30102	3.67734
buffer + 19.6% (w/w) SMH	47.45	0.507	4.30103	3.26143
buffer + 25.2% (w/w) SPH	51.87	0.622	4.30102	3.55936

The width of the central line decreases in the presence of all NADES (Table 3, Fig. 6) while the ratio between the heights of the central (H_0) and low-field (H_{+1}) lines (as indicated in Fig. 5C) increases in the presence of all NADES (Table 3, Fig. 7). There are many factorsthat determine the line width in the EPR spectra. Line broadening can be paramagnetic and motional. Line narrowing can only be explained in the case of SMH by fluidization of the membrane interface, because in this case the order parameter of the spectrum also decreases. In the case of SFG and SPH the situation is more complicated. Both spectra are identical, thus the mechanism of line narrowing for these two compounds must be the same. In these samples, line narrowing occurs without the decrease of the order parameter. The order parameter relates to the average angle of the departure of the molecular axis of the acyl chain from the normal to the bilayer surface (angular orientation or angular freedom). When the system is disordered, this angle increases and the spin label moiety experiences more freedom to move. This results in line narrowing. If order parameter does not change, this can only mean that the rate of rotation around the molecular axis of the acyl chain increases without system disordering. Usually such effect is observed in the membrane interface at higher temperature. The fact that the same phenomenon is observed in case of NADES at room temperature, is very intriguing.

If line narrowing originates from a decreased probability of interaction between the spin label moieties at the liposomal membrane (paramagnetic broadening due to dipole-dipole interaction or exchange), then such an effect may result from decreased membrane lateral diffusion (Sachse *et al*., 1987**)**. The restriction of lateral diffusion of lipids within bilayers in the presence of NADES would be one other possible mechanism for their stabilizing effect. All the parameters in EPR show an unexpected behavior of 5-DS which may probably indicate the establishment of a NADES layer around the liposomes. Generally label experiments are in aqueous surroundings, with the -COOH group of 5-DS at the polar heads of the lipid bilayer and the doxyl group in a polar area of the bilayer. If NADES builds up a shell around the liposomes, the label will be in NADES, a less polar but viscous environment, in which the 5-DS might bind more loosely with the polar heads of the bilayer as it dissolves better in the NADES than in water. In other words, the 5-DS will have a different behavior in a water-lipid system than in a water-NADES-lipid system due to a different interface at the bilayer. In the spectra of 5DS in liposomes in the presence of FGS and SPH there is a clear indication of the presence of another fraction of 5-DS, which demonstrates liquid-type behavior. This may be caused by the deeper localization of the spin-label moiety in the bilayer. Such vertical excursion can result from the lost interactions of 5-DS with the bilayer interface due to protonation at low pH (Fig. 7).

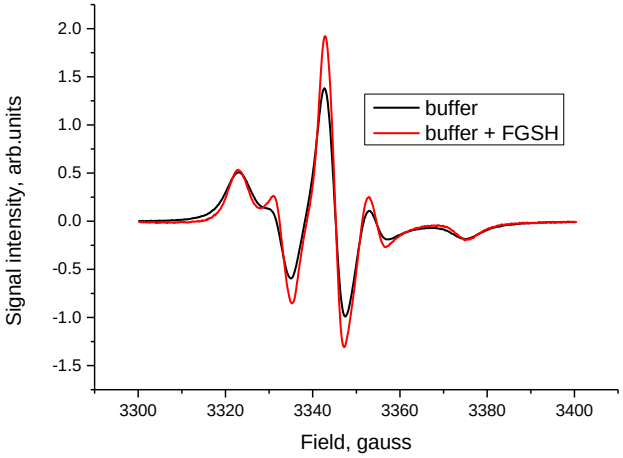

Fig. 7 Comparison of the spectral shape of 5-doxyl stearic acid in liposomes in buffer (black lines) and in buffer with SFG (red lines).

4. Conclusion

This study provides in-vitro evidence for the existence of NADES in biological systems and some of their possible functions. NADES show a similar chemical composition and physicochemical properties to cytoplasm in plants. NADES may be involved in controlling the water level in plants and even trap water from humid air. The phase separation of NADES and water is a dynamic process in which even three phases may be formed different with different solubilization properties. This may explain the phenomenon that metabolites of quite diverse polarities can be produced and stored in a plant. Furthermore, NADES may exist around cell membranes and play a role in stabilizing the lipid membrane through intermolecular interactions. The attached NADES would be the medium where biosynthetic enzymes may function in an environment in which non-water soluble intermediates are dissolved and may be transferred from one enzyme to the next in the sequence of the biosynthesis pathway. This could be a metastable NADES phase that interacts with the water phase again, and may form vesicles as vehicles for transport of poorly water-soluble compounds, explaining biosynthesis and translocation of poorly water-soluble metabolites.

NADES might be involved in the biosynthesis and storage of various non water-soluble metabolites (e.g. secondary metabolites and macromolecules) in cells and play an important role in protecting organisms from extreme conditions. To fully address this hypothesis, further experiments are required, and these may revolutionize our views on cell physiology, as well as the physiology of whole organisms. When plants are submitted to harsh conditions, such as drought, excessivesalinity or very low temperatures, NADES may protect the integrity of the cell membrane as well as the macromolecules. The biosynthesis of poorly water-soluble compounds could then occur in the NADES media in which both substrates and enzymes are dissolved. Thus, the characteristics of the enzyme might be quite different to those displayed when disolved in water, among other things because of the much higher concentration of substrates.

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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 ^{o}C. NADES are liquid supermolecules composed of natural metabolites as well as water is some cases, that are mixed in certain molar ratios and are characterized by extensive inter-molecular 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 ^{o}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 ^{o}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, asdoes 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 enatioselectivity 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 13C 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 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 ^{o}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 ^{o}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 ^{o}C, and stirring at 40 ℃. Stirring at 40 ℃ 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.

Samenvatting

De afgelopen tien jaar hebben ionische vloeistoffen (ILs) en diep eutectische oplosmiddelen (DES) veel aandacht getrokken omdat ze milieuvriendelijk en onschadelijk zijn doordat ze een verwaarloosbare dampspanning hebben. Recent zijn in de internationale wetenschappelijk literatuur ILs en DES beschreven die gemaakt zijn met biologisch hernieuwbare natuurlijke producten als uitgangsmateriaal. Voor de productie werden verlaagde potentiële toxiciteit en productiekosten en de verhoogde biocompatibiliteit gerapporteerd. Met het oog op fysisch-chemische eigenschappen werden ook specifieke ILs of DES gemaakt met een lage viscositeit en smeltpunt, en een hoge thermische stabiliteit en selectiviteit voor gewenste chemicaliën. Ze werden succesvol gebruikt voor de extractie en scheiding van verschillende natuurlijke producten evenals enzymreacties. Hieruit bleek dat deze synthetische oplosmiddelen een betere oplos capaciteit hebben dan de klassieke organische oplosmiddelen. Dit geldt voor een groot aantal verbindingen, waaronder ook macromoleculen zoals lignine en cellulose. De belangrijkste drijvende krachten voor extractie zijn moleculaire interacties, bij voorbeeld waterstof bruggen, tussen het extractie medium en opgeloste verbindingen. Deze interacties worden sterk beïnvloed door de structuur van ILs maar ook door het watergehalte, de viscositeit, pH en uit-zout effecten. Uiteraard spelen deze factoren ook een belangrijke rol bij enzymreacties die plaatsvinden in ILs of DES. Na verdunning van het IL / DES extractie medium kunnen analytische en spectroscopische technieken gebruikt worden om de extracten te analyseren zoals HPLC, NMR, IR, UV, CD en SEM (scanning electron microscope). Bovendien kunnen de geëxtraheerde verbindingen worden geïsoleerd, waarna het IL / DES extractiemedium hergebruikt kan worden (**hoofdstuk 2**). Alle extractieprocessen met ILs of DES die worden beschreven in de internationale wetenschappelijke literatuur werden uitgevoerd met imidazolium gebaseerde ILs, deze zijn echter toxisch. Vanwege een hoge viscositeit of vaste toestand bij kamertemperatuur is het daarnaast voor de meeste van deze extracties noodzakelijk om ze te verwarmen tot hoge temperaturen of een verdunde waterige oplossing te gebruiken.

Om het bereik van ILs en DES uit te breiden, in het bijzonder om goedkope en onschadelijke groene oplosmiddelen te ontwikkelen met een lage viscositeit en de mogelijkheid om deze toe te passen voor gezondheid gerelateerde werkvelden, heeft onze groep natuurlijke deep eutectic solvents (NADES) voorgesteld. Deze term wordt voor zowel natuurlijke ILs als DES gebruikt. NADES zijn vloeibare supramoleculen die zijn samengesteld uit veel voorkomende primaire metabolieten in een specifieke molaire verhouding en, in

sommige gevallen, water. Intermoleculaire interacties waaronder waterstofbruggen en ionische bindingen zijn karakteristiek voor NADES. Meer dan 100 NADES combinaties zijn gevonden en deze kunnen worden onderverdeeld in vijf groepen: ionische vloeistoffen gevormd uit een zuur en een base, op suiker gebaseerde NADES met ongeladen verbindingen, op suiker gebaseerde NADES met basen, op suiker gebaseerde NADES met zuren en op suiker gebaseerde NADES met aminozuren. Deze NADES zijn eenvoudig te bereiden door een mengsel van de verbindingen te roeren bij een temperatuur van 50 °C. Uit de fysische eigenschappen van typische NADES bleek dat NADES een dichtheid hebben die groter is dan water. Daarnaast beschikken ze over een hoge viscositeit, een lage glasovergangstemperatuur waardoor ze vloeibaar blijven tot onder 0 °C, een hoge thermische stabiliteit en een polariteits bereik hebben van meer polair dan water tot gelijk aan methanol. Qua toepassingen bleken NADES een uitstekend oplosmiddel voor een breed scala van natuurlijke producten met een lage tot gemiddelde polariteit die niet of slecht oplosbaar zijn in water. Macromoleculen zoals DNA, eiwitten en polysacchariden zijn ook oplosbaar in NADES (**hoofdstuk 3**). Door deze fysisch-chemische eigenschappen en een bijbehorend hoog oplossend vermogen zijn NADES zeer interessant voor toepassingen binnen de farmaceutische- , cosmetische- en voedingsindustrie.

Met als doel de toepassingen van NADES te vergemakkelijken en uit te breiden, werden de structuur en eigenschappen van mengsels van NADES en water onderzocht (**hoofdstuk 4**). Door met water te verdunnen, worden de waterstofbrug interacties tussen de componenten van NADES verzwakt en bij toevoeging van 50% water worden deze geheel gemaskeerd. Het watergehalte in NADES beïnvloedt niet alleen de structuur maar ook de fysisch-chemische eigenschappen. Door met water te verdunnen wordt de elektrische geleidbaarheid van NADES wel tot 100 maal groter. De wateractiviteit en dichtheid van NADES vertoonden een kwantitatieve relatie met het watergehalte in NADES. Verdunning van de NADES met een bepaalde hoeveelheid water verminderde de viscositeit van NADES sterk en veranderde de polariteit. Dit beïnvloedt ook de oplosbaarheid van sommige chemische verbindingen. Al deze effecten van water op de structuur en eigenschappen van NADES bieden een basis voor het moduleren van NADES voorspecifieke toepassingen.

Het stabiliserend vermogen is een belangrijk aspect voor het realiseren van toepassingen van NADES. Het instabiele pigment carthamin werd als model gebruikt om de stabiliteit van verbindingen in NADES te onderzoeken. In NADES die op suiker gebaseerd zijn werd een hogere stabiliteit van carthamin waargenomen dan in water of 40% ethanol oplossing. Dit geldt voor alle geteste omstandigheden zoals hoge temperatuur, blootstelling aan licht, en bewaartijd.

Zelfs in een ruw extract van carthamin, hydroxysafflor yellow A en cartormin in twee op suiker gebaseerde NADES werd een verbeterde stabiliteit waargenomen bij normale omgevings omstandigheden en in het zonlicht. Het hoge stabiliserend vermogen van NADES wordt toegeschreven aan de waterstofbrug interacties tussen opgeloste stoffen en NADES moleculen. Hierdoor wordt de beweging van opgeloste stoffen beperkt waardoor contact met zuurstof uit de gasfase als interface met de lucht word beperkt. Daarnaast word de afbraaksnelheid van opgeloste stoffen gereduceerd door de lage oplosbaarheid van zuurstof in NADES. Dit stabiliserend vermogen van NADES wordt groter naarmate de viscositeit toeneemt en kleiner naarmate het watergehalte toeneemt (**hoofdstuk 5**). Op suiker gebaseerde NADES met een hoge viscositeit zijn bijzonder efficiënt voor het stabilizeren van fenolische verbindingen.

Voor de toepassing van NADES in de extractie van fenolische verbindingen werden anthocyanen gebruikt als model om het gebruik van NADES extractie in combinatie met met reversed phase HPLC te onderzoeken, waarbij ook de stabiliteit gemeten werd. Drie extractie methodes werden vergeleken: sonicatie bij kamertemperatuur, sonicatie bij 40 ℃, en mechanisch roeren bij 40 ℃. Mechanisch roeren bij 40 ℃ gedurende 1 uur bleek het meest effectief. De verenigbaarheid van NADES met reversed phase HPLC was goed en er werd geen effect van de NADES op het chromatografische gedrag van anthocyanen waargenomen. Echter, het vooraf verdunnen van het NADES extractiemedium met water is vereist om de viscositeit dusdanig te verlagen zodat injecteren van een monster mogelijk is. Stabiliteit experimenten toonden aan dat cyanidine stabieler is in sommige NADES dan in aangezuurd ethanol. Dit maakt de bereiding en het analyse proces van anthocyanine makkelijker. Met de bovengenoemde methode werden verschillende NADES gebruikt om anthocyanen te extraheren en werd voor de analyse van anthocyanen in bloembladeren van *Catharanthus rosus* op HPLC-UV gebaseerde metabolomics toegepast. Een multivariate analyse van de data toonde aan dat melkzuur:choline chloride:water en 1,2-propaandiol:choline chloride:water eenzelfde extraheerbaarheid van anthocyanen hebben als aangezuurde methanol (**hoofdstuk 6**). De extractie van fenolen met NADES werd vergeleken met conventionele oplosmiddelen. Saffloer werd geselecteerd vanwege de aromatische pigmenten die een breed scala van polariteiten vertonen, zoals de polaire fenolische verbindingen “hydroxysafflor yellow A” (HSYA) en cartormin en de minder polaire verbindingen carthamin en tri-p-coumaroyl spermidines. Met de hierboven vastgestelde werkwijzen werden extracten van acht NADES, water en ethanol geanalyseerd met HPLC-UV. Een multivariate analyse van de data toonde aan dat NADES een hogere opbrengst en breder spectrum van zowel polaire als minder polaire verbindingen gaf dan

conventionele oplosmiddelen. De extractie opbrengst van fenolische verbindingen werd sterk beïnvloed door het watergehalte in NADES. Onder geoptimaliseerde omstandigheden werden met sommige NADES hogere opbrengsten verkregen dan met water voor polaire verbindingen en hogere opbrengsten dan met ethanol voor minder polaire verbindingen. Met Diaion hars (HP-20) werden de meeste fenolische verbindingen terug gewonnen uit de NADES, met een rendement van 75% - 97% (**hoofdstuk 7**). Deze studie laat een groene, goedkope, en efficiënte aanpak zien voor de extractie en terugwinning van natuurstoffen uit biomassa. Deze kan worden toegepast voor de extractie en isolatie van natuurstoffen uit biomaterialen ten behoeve van de farmaceutische- , cosmetische- en voedingsmiddelenindustrie.

Het bestaan van NADES en hun mogelijke functies in planten werden onderzocht (**hoofdstuk 8**). Een relatief hogere hoeveelheid van de samenstellende ingrediënten van NADES werden waargenomen in droog mos in vergelijking met vers mos en in de episperm van gerst in vergelijking met het embryo. Een hoog gehalte aan suikers, polyolen of glycine betaïne domineert in planten excreties, zoals sap en nectar. NADES vertoonden naast een soortgelijke samenstelling dezelfde fysisch-chemische eigenschappen zoals die gevonden worden in het cytoplasma van planten. Dit vormt een mogelijk bewijs voor het bestaan van NADES in planten. NADES vertoonden daarnaast een vergelijkbare hygroscopiciteit als plantaardige materialen, wat een wateraanpassings rol van NADES in planten suggereert. het mengen van NADES en water resulteerde in vloeistoffen met verschillende samenstellingen en eigenschappen, die goede oplosmiddelen zijn om metabolieten van diverse polariteiten te extraheren uit planten. Bovendien kunnen NADES zich rond membranen ophopen en ze daardoor stabiliseren, zoals blijkt uit experimenten met liposomen. Verdere studies naar de biologische rol van NADES in cellen en organismen zullen mogelijk ons beeld van de celfysiologie, evenals van de fysiologie van hele organismenveranderen. Bijvoorbeeld In planten is de resistentie tegen droogte en koude uitgebreid gerapporteerd. Hierbij zijn metabolieten betrokken die de bouwstenen van NADES vormen.

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Curriculum Vitae

Yuntao Dai was born on June 18th, 1981, in Shanxi, China. She got her Bachelor degree in the Department of Pharmacy, School of Chemistry, Shanxi University in 2005. Her undergraduate research project was about natural products isolation in the Institute of Pharmacology and Toxicology, Academy of Military Medical Sciences, Beijing, China, supervised by the researcher Dr. Qiao Shanyi. She received a recommendation to be exempted from the admission exam to her Master course which she followed in the Modern Research Center for Traditional Chinese Medicine, Shanxi University from 2005 to 2008. During the M.Sc., she studied the anti-depressive effect of Xiaoyaosan with GC-MS based metabolomics and the quality assessment of tradition herbal medicine supervised by Prof. Dr. Qin. At the end of 2008, she did research on the implementation of a 2D RP/RPLC method to separate components in *Fructus schisandrae* chinensis in the Multi-component Chinese Medicine Group, Dalian Institute of Chemical Physicals, China. In 2009, she started her PhD studies in Leiden University, the Netherlands sponsored by the Chinese Scholarship Council. Her PhD research project was "Natural deep eutectic solvents (NADES) and their application in green extraction of flavonoids" supervised by Prof. Dr. Rob. Verpoorte, Prof. Dr. Geert-jan Witkamp, and Assistant Prof. Dr. Young Hae Choi.

She is interested in NADES and their applications in the pharmaceutical industry, quality and activity of traditional Chinese medicine (TCM), extraction and isolation of biologically active natural products, and NMR- and chromatography- based metabolomics.

List of Publications

- Dai, Y.; Verpoorte, R.; Choi, Y. H., Natural deep eutectic solvents for the extraction and storage of anthocyanins. Ready for submittion.
- **Dai, Y**.; Verpoorte, R.; Witkamp, G.-J.; Choi, Y. H., Natural deep eutectic solvents providing enhanced stability of natural colourants from safflower (*Carthamus tinctorius*). Ready for submittion.
- **Dai, Y**.; Verpoorte, R.; Witkamp, G.-J.; Choi, Y. H., Tailoring the properties of natural deep eutectic solvents with the addition of water to facilitate their applications. Submitted.
- **Dai, Y**.; Spronsen, J. v.; Witkamp, G.-J.; Verpoorte, R.; Choi, Y. H., Ionic liquids and deep eutectic solvent in natural products research: a mixture of solid as an extraction solvent. *Journal of Natural Product*, Submitted.
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