Universiteit
Leiden
The Netherlands

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

Hennivanda, H.

Citation
Hennivanda, H. (2020, September 10). *Applications of natural deep eutectic solvents to extraction and preservation of biomolecules*. Retrieved from https://hdl.handle.net/1887/136537

Version:	Publisher's Version
License:	Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden
Downloaded from:	https://hdl.handle.net/1887/136537

Note: To cite this publication please use the final published version (if applicable).

Cover Page

Universiteit Leiden

The handle http://hdl.handle.net/1887/136537 holds various files of this Leiden University dissertation.

Author: Hennivanda
Title: Applications of natural deep eutectic solvents to extraction and preservation of biomolecules
Issue date: 2020-09-10

Applications of Natural Deep Eutectic Solvents to Extraction and Preservation of Biomolecules

Hennivanda

Hennivanda

Applications of Natural Deep Eutectic Solvents to Extraction and Preservation of Biomolecules.

Ph.D Thesis, Leiden University, The Netherlands

Printed by Printsupport4you

ISBN: 978-94-92597-42-7

Applications of Natural Deep Eutectic Solvents to Extraction and Preservation of Biomolecules

PROEFSCHRIFT

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof.mr. C.J.J.M. Stolker,
volgens besluit van het College voor Promoties
te verdedigen op donderdag 10 September 2020
klokke 15.00 uur

door

Hennivanda

Banda Aceh (Indonesia)

In 1975

Promotor:	Prof. Dr. P.G.L. Klinkhamer
Copromotor:	Dr. Y.H. Choi

Promotiecommissie:	Prof. Dr. G. van Wezel
	Prof. Dr. R. Offringa
	Prof. Dr. M. Frederich (University of Leige, Belgium)
	Dr. A. Misan (Institute of Food and Technology, Serbia)

Contents

CHAPTER 1

General introduction

Natural deep eutectic solvents (NADESs) are Ionic Liquids (ILs) and Deep Eutectic Solvents (DES) obtained by mixing two or more solid compounds. In the search for environmentally friendly solvents, chemists designed ILs by combining an organic cation (usually imidazolium-based cations) with a wide variety of anions. The ILs represent a new class of potential solvents used in chemical or enzymatical reactions. The advantage of the ILs is that they have a vapor pressure of virtually zero, i.e, they are not volatile at all, reducing the risks of fire and explosions in industrial processes and they can be tailor made for certain applications. But later research has confirmed the toxicity and poor biodegradability of these solvents, as well as the high price of common ILs, which hamper their commercial emergence.

In order to overcome the problems with ILs, a new generation of solvents, named Deep Eutectic Solvents (DES) was developed. These solvents can be obtained by simply mixing together two or three components that are capable of forming a eutectic mixture. DES is composed of a wide range of quaternary ammonium salts with carboxylic acid. The physical properties of DES are similar to that of ILs, except that DES are nontoxic, cheap, and biodegradable

(Abbot et al. 2004; Zhang et al. 2012; Dai et al. 2013[a]).

In order to expand the candidates of these promising solvents, researchers have given attention to natural products as new potential chemicals. Natural products such as amino acids, organic acids, sugars, sugar alcohols, choline, betaine, and urea are ideal components of DES due to their biodegradable properties, chemical diversity, and pharmaceutical acceptable toxicity. Based on these reasons, the term NADESs was introduced (Choi et al. 2011; Dai et al. 2013[a]; Dai et al. 2013[b]).

Natural deep eutectic solvents are obtained by mixing the type of solid natural compounds mentioned above in certain molar ratios. These liquids have a melting point far below room temperature and even at temperatures far below 0 ^{o}C, are characterized by strong intermolecular hydrogen bonding, and are usually viscous and of an intermediate polarity. NADESs show high solubilization ability of both polar and nonpolar compounds (Choi et al. 2011; Dai et al. 2013[b]). Though originally thought to be universal solvents for medium polar compounds, further research showed that in fact they are highly selective solvents (Dai et al. 2013[c]). They are also capable of dissolving macromolecules (Choi et al. 2011; Lores et al. 2017). These characters make NADESs potential solvents for food, cosmetics and pharmaceutical industry (Dai et al. 2013[a]; Paiva et al. 2014; Vanda et al. 2018). NADESs can also be applied in diverse aspects of research, such as secondary metabolite extraction (Dai et al. 2013[c]; Dai et al. 2016; Paradiso et al. 2016; Gonzales et al. 2018), chemical and enzymatic reactions (Durand et al. 2012; Kumar et al. 2016; Yang et al. 2017), pharmaceutical industry (Rozema et al. 2015; Faggian et al. 2016; Shamseddin et al. 2017; Sut et al. 2017) and agricultures (Huang et al. 2018; Zahrina et al. 2018).

The most interesting aspect of the NADESs is the hypothesis of Choi et al. (2011) that everywhere in Nature NADESs occur as a third liquid phase, explaining many biochemical and biological processes involving e.g. biosynthesis of non-water-soluble compounds, survival under cold or dry

conditions, high levels of dissolved secondary metabolites in flowers and glandular hairs. The other aspect is that a NADES can be diluted with water, which will give better solubility of various compounds. Adding water also could activate enzymes which are not active in a NADES. NMR spectrometric studies of NADES provide evidence that extensive hydrogen-bonding interactions are involved in this process (Choi et al. 2011; Dai et al. 2013[b]; Vanda et al. 2018). Besides their environmental advantages, NADES have specific physicochemical properties, such as very low melting points, adjustable viscosity, extremely low vapor pressure with very high flash points. NADESs also have a high solubilizing capacity and high selectivity of a wide polarity range of compounds. (Dai et al. 2013[b]; Vanda et al. 2018).

Aim of the thesis

The aim of this thesis is to probe hypothetic applications of NADESs in Nature:

- Explaining the development of ILs, DES, and NADESs, and their applications in diverse fields.
- Explaining the mechanism of catching insects by *Drosera* plant species and the components of the trapping exudate.
- The protective role of NADESs for proteins, RNA and DNA, including their enzymatic biosynthesis and breakdown in (diluted) NADESs.

In order to address these hypotheses, the following objectives for this thesis are formulated to:

1. Determine a method for extraction and analysis of the compounds in the exudate of *Drosera* species for further analysis using Gas Chromatography-Mass Spectrometry and Nuclear Magnetic Resonance spectroscopy.
2. Identify protease enzymes in the exudate of *Drosera* after stimulation with several components (chitin, jasmonic acid) added to the leaves.

3. Analyse bromelain enzyme in *Ananas comosus* L. Merr. juice and develop methods for application of NADESs in protease-catalysed reactions, and explore the effect of water concentration on the enzyme activity.
4. Determine the solubility and stability of RNA and DNA in various NADESs using *Drosophila melanogaster* as object. Explore the effect of water concentration in protecting or dissolving RNA and DNA.

Outline of experiments

The thesis begins with an introduction (Chapter 1), followed by a comprehensive review on development of ILs, DES, and NADESs (Chapter 2). Chapter 3 is about NADESs, from their discovery to their applications, especially related to macromolecules. The chemical profiling of *Drosera* exudate is presented in Chapter 4. The application of NADESs in protease-catalyzed reactions, the kinetics in NADESs with different water concentrations is described in Chapter 5. In this chapter, several NADESs were tested in order to find the best solvents for protease reactions, and also to find the optimal water concentration in order to optimize the reaction. At the same time NADESs were identified that stabilize proteins and which thus could play a role in Nature to conserve an organism under drought conditions. In Chapter 6, the application of NADESs to protect RNA and DNA from degradation is described. Finally, a general conclusion and a perspective related to NADESs applications are presented (Chapter 7).

References

Abbott, A.P., Boothby, D., Capper, G., Davies, D.L., Rasheed, R.K., J. Am. Chem. Soc. 126 (2004) 9142-9147.

Choi, Y.H., van Spronsen, J., Dai, Y., Verberne, M., Hollmann, F., Arends, I.W.C.E., Witkamp, G.-J., Verpoorte, R., Plant Physiol. 156 (2011) 1701-1705.

Dai, Y., van Spronsen, J., Witkamp, G.-J., Verpoorte, R., Choi, Y.H., J. Nat. Prod. 76 (2013[a]) 2162-2173.

Dai, Y., van Spronsen, J., Witkamp, G.-J., Verpoorte, R., Choi, Y. H., Anal. Chim. Acta 766 (2013[b]) 61-68.

Dai, Y., Witkamp, G.-J., Verpoorte, R., Choi, Y.H., Anal. Chem. 85 (2013[c]) 6272-6278.

Durand, E., Lecomte, J., Baréa, B., Piombo, G., Dubreucq, E., Villeneuve, P., Process Biochem. 47 (2012) 2081-2089.

Faggian, M., Sut, S., Perissutti, B., Baldan, V., Grabnar, I., Dall'Acqua, S., Molecules 21 (2016) 1531-1542.

González, C.G., Mustafa, N.R., Wilson, E.G., Verpoorte, R., Choi, Y.H., Flavour Fragr. J. 33 (2018) 91-96.

Huang, Y., Feng, F., Jiang, J., Qiao, Y., Wu, T., Voglmeir, J., Chen, Z.-G., Food Chem. 221 (2017) 1400-1405.

Kumar, A.K., Parikh, B.S., Pravakar, M., Environ. Sci. Pollut. Res. 23 (2016) 9265-9275.

Lores, H., Romero, V., Costas, I., Bendicho, C., Lavilla, I., Talanta 162 (2017) 453-459.

Paiva, A., Craveiro, R., Aroso, I., Martins, M., Rei, R.L., Duarte, A.R.C., ACS Sustain. Chem. Eng. 2 (2014) 1063-1071.

Paradiso, V.M., Clemente, A., Summo, C., Pasqualone, A., Caponio, F., Food Chem. 212 (2016) 43-47.

Rozema, E., Van Dam, A.D., Sips, H.C.M., Verpoorte, R., Meijer, O.C., Kooijman, S., Choi, Y.H., RSC Adv. 5 (2015) 61398-61401.

Shamseddin, A., Crauste, C., Durand, E., Villeneuve, P., Dubois, G., Durand, T., Vercauteren, J., Veas, F., Eur. J. Lipid Sci.Technol. 119 (2017) 1700171.

Sut, S., Faggian, M., Baldan, V., Poloniato, G., Castagliuolo, I., Grabnar, I., Perissutti, B., Molecules 22 (2017) 1921-1932.

Vanda, H., Dai, Y., Wilson, E.G., Verpoorte, R., Choi, Y.H., C. R. Chim. 21 (2018) 628-638.

Zahrina, I., Nasikin, M., Krisanti, E., Mulia, K., Food Chem. 240 (2018) 490-495.

Zhang, Q., De Oliveira Vigier, K., Royer, S., Jérôme, F., Chem. Soc. Rev. 41 (2012) 7108-7146.

C H A P T E R 2

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

Henni Vanda [a, b], Yuntao Dai [c], Erica G. Wilson [a], Robert Verpoorte [a], Young Hae Choi [a]

[a] Natural Products Laboratory, Institute of Biology, Leiden University, Sylviusweg 72 2333BE Leiden, The Netherlands

[b] Faculty of Veterinary Medicine, Syiah Kuala University, Banda Aceh, 20111, Indonesia

[c] Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences,100700, No. 16 Nanxiaojie, Dongzhimennei Ave, Beijing, 100700, China

Abstract

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

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

Introduction

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

ILs are undeniably attractive solvents, particularly because of their low

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

Concept of NADESs and their applications

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

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

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

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

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

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

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

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

Table 1. Application of NADESs in natural product extraction.

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

NADESs as media for enzymatic processes

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

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

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

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

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

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

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

Table 2. Application of NADESs as media in enzymatic reactions

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

Application of NADESs to solubilize macromolecules

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

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

Table 3. Application of NADESs to solubilize macromolecules

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

Application of NADESs to pharmaceuticals, cosmetics, and food

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

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

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

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

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

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

activity.

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

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

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

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

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

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

Occurrence of NADESs in nature

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

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

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

Conclusions and perspectives

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

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

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

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

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

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

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

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

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

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

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

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

References

Abbott, A.P., Boothby, D., Capper, G., Davies, D.L., Rasheed, R.K., J. Am. Chem. Soc. 126 (2004) 9142-9147.

Abe, M., Fukaya, Y., Ohno, H., Green Chem. 12 (2010) 1274-1280.

Abo-Hamad, A., Hayyan, M. AlSaadi, M.A., Hashim, M.A., Chem. Eng. J. 273 (2015) 551-567.

Ailing, W., Xueliang, Z., Zhuangzhi, Z., Changping, L., Xuefang, Z., Prog. Chem. 26 (2014) 784–795.

Armstrong, D.W., He, L., Liu, Y.-S., Anal. Chem. 71 (1999) 3873-3876.

Bakirtzi, C., Triantafyllidou, K., Makris, D.P., J. App. Res. Med. Aromat. Plants 3 (2016) 120-127.

Berthod, A., Carda-Broch, S., Anal. Bioanal. Chem. 380 (2004) 168-177.

Berthod, A., Carda-Broch, S., J. Liq. Chromatogr. Relat. Technol. 26 (2003) 1493-1508.

Berthod, A., He, L., Armstrong, D.W., Chromatographia 53 (2001) 63-68.

Bosiljkov, T., Dujmić, F., Bubalo, M.C., Hribar, J., Vidrih, R., Brnčić, M., Zlatic, E., Redovniković, I.R., Jokić, S., Food Bioprod. Process. 102 (2017) 195-203.

Bubalo, M.C., Ćurko, N., Tomašević, M., Ganić, K.K., Redovniković, I.R., Food Chem. 200 (2016) 159-166.

Byrne, N., Wang, L.-M., Belieres. J.-P., Angell, C.A., Chem. Commun. (2007) 2714-2716.

Cao, J., Yang, M., Cao, F., Wang, J., Su, E., ACS Sustain. Chem. Eng. 5 (2017) 3270-3278.

Cao, X., Ye, X., Lu, Y., Yu, Y., Mo, W., Anal. Chim. Acta 640 (2009) 47-51.

Carriazo, D., Serrano, M.C., Gutiérrez, M.C., Ferrer, M.L., del Monte, F., Chem. Soc. Rev. 41 (2012) 4996-5014.

Chen, L., Mullen, G.E., Le Roch, M., Cassity, C.G., Gouault, N., Fadamiro, H.Y., Barletta, R.E., Brien, R.A. O., Sykora, R.E., Stenson, A.C., West, K.N., Horne, H.E., Hendrich, J.M., Xiang, K.R., Davis Jr., J.H., Angew. Chem. Int. Ed. 53 (2014) 1-5.

Chiappe, C., Neri, L., Pieraccini, D., Tetrahedron Lett. 47 (2006) 5089-5093.

Chiappe, C., Rajamani, S., D'Andrea, F., Green Chem. 15 (2013) 137-143.

Choi, Y.H., van Spronsen, J., Dai, Y., Verberne, M., Hollmann, F., Arends, I.W.C.E., Witkamp, G.-J., Verpoorte, R., Plant Physiol. 156 (2011) 1701-1705.

Dai, Y., Rozema, E., Verpoorte, R., Choi, Y.H., J. Chromatogr. A 1434 (2016) 50-56.

Dai, Y., van Spronsen, J., Witkamp, G.-J., Verpoorte, R., Choi, Y. H., Anal. Chim. Acta 766 (2013[a]) 61-68.

Dai, Y., Witkamp, G.-J., Verpoorte, R., Choi, Y.H., Anal. Chem. 85 (2013[b]) 6272-6278.

Dai, Y., van Spronsen, J., Witkamp, G.-J., Verpoorte, R., Choi, Y.H., J. Nat. Prod. 76 (2013[c]) 2162-2173.

Dai, Y., Verpoorte, R., Choi, Y.H., Food Chem. 159 (2014) 116-121.

del Monte, F., Carriazo, D., Serrano, M.C., Gutiérrez, M.C., Ferrer, M.L., ChemSusChem. 7 (2014) 999-1009.

Ding, J., Welton, T., Armstrong, D.W., Anal. Chem. 76 (2004) 6819-6822.

Du, F.-Y., Xiao, X.-H., Li, G.-K., J. Chromatogr. A 1140 (2007) 56-62.

Du, F.-Y., Xiao, X.-H., Luo, X.-J., Li, G.-K., Talanta 78 (2009) 1177-1184.

Durand, E., Lecomte, J., Baréa, B., Piombo, G., Dubreucq, E., Villeneuve, P., Process Biochem. 47 (2012) 2081-2089.

Durand, E., Lecomte, J., Upasani, R., Chabi, B., Bayrasy, C., Baréa, B., Jublanc, E., Pharmaceut. Res. 34 (2017) 1134-1146.

Durand, E., Lecomte, J., Villeneuve, P., Eur. J. Lipid Sci. Tech. 115 (2013) 379-385.

Espino, M., de los Ángeles Fernández, M., Gomez, F.J.V., Silva, M.F., Trends Anal. Chem. 76 (2016): 126-136.

Faggian, M., Sut, S., Perissutti, B., Baldan, V., Grabnar, I., Dall'Acqua, S., Molecules 21 (2016) 1531-1542.

Francisco, M., Lago, S., Soto, A., Arce, A., Fluid Phase Equilib. 296 (2010) 149-153.

Francisco, M., van den Bruinhorst, A., Kroon, M.C., Green Chem. 14 (2012) 2153-2157.

Francisco, M., van den Bruinhorst, A., Kroon, M.C., Angew. Chem. Int. Ed. 52 (2013) 3074-3085.

Fukaya, Y., Iizuka, Y., Sekikawa, K., Ohno, H., Green Chem. 9 (2007) 1155-1157.

Gomez, F.J.V., Espino, M., de los Angeles Fernandez, M., Raba, J., Silva, M.F., Anal. Chim. Acta 936 (2016) 91-96.

González, C.G., Mustafa, N.R., Wilson, E.G., Verpoorte, R., Choi, Y.H.,

Flavour Fragr. J. 33 (2018) 91-96.

Gutiérrez, M.C., Ferrer, M.L., Yuste, L., Rojo, F., del Monte, F., Angew. Chem. Int. Ed. 49 (2010) 2158-2162.

Hayyan, M., Hashim, M.A., Hayyan, A., Al-Saadi, M.A., AlNashef, I.M., Mirghani, M.E.S., Saheed O.K., Chemosphere 90 (2013) 2193-2195.

He, L., Zhang, W., Zhao, L., Liu, X., Jiang, S., J. Chromatogr. A 1007 (2003) 39-45.

Hillman, A.R., Ryder, K.S., Zaleski, C.J., Ferreira, V., Beasley, C.A., Vieil, E., Electrochim. Acta 135 (2014) 42-51.

Huang, Y., Feng, F., Chen, Z.-G., Wu, T., Wang, Z.-H., Food Chem. 244 (2018) 260-265.

Huang, Y., Feng, F., Jiang, J., Qiao, Y., Wu, T., Voglmeir, J., Chen, Z.-G., Food Chem. 221 (2017) 1400-1405.

Hückel, W., Chem. Ber. 91 (1958) XIX–LXVI.

Huddleston, J.G., Willauer, H.D., Swatloski, R.P., Visser, A.E., Rogers, R.D., Chem. Commun. (1998) 1765-1766.

Ilgen, F., König, B., Green Chem. 11 (2009) 848-854.

Jeong, K.M., Ko, J., Zhao, J., Jin, Y., Yoo, D.E., Han, S.Y., Lee, J., J. Clean Prod. 151 (2017) 87-95.

Jhong, H.-R., Wong, D. S.-H., Wan, C.-C., Wang Y.-Y., Wei, T.-C., Electrochem. Commun. 11 (2009) 209-211.

Kaliszan, R., Marszałł, M.P., Markuszewski, M.J., Baczek, T., Pernak, J., J. Chromatogr. A 1030 (2004) 263-271.

Khodaverdian, S., Dabirmanesh, B., Heydari, A., Dashtban-moghadam, E., Khajeh, K., Ghazi, F., Int. J. Biol. Macromol. 107 (2018) 2574-2579.

Kumar, A.K., Parikh, B.S., Pravakar, M., Environ. Sci. Pollut. Res. 23 (2016) 9265-9275.

Lapkin, P A.A., Plucinski, K., Cutler, M., J. Nat. Prod. 69 (2006) 1653-1664.

Lee, S.H., Doherty, T.V., Linhardt, R.J., Dordick, J.S., Biotechnol. Bioeng.102 (2009) 1368-1376.

Lindberg, D., de la Fuente Revenga, M., Widersten, M., J. Biotechnol. 147 (2010) 169-171.

Liu, P., Hao, J.-W., Mo, L-P., Zhang, Z.-H., RSC Adv. 5 (2015) 48675-48704.

Liu, X., Ahlgren, S., Korthout, H.A.A.J., Salomé-Abarca, L.F., Bayona, L.M., Verpoorte, R., Choi, Y.H., J. Chromatogr. A 1532 (2018) 198–207.

Liu, Y., Wang, Y., Dai, Q., Zhou, Y., Anal. Chim. Acta 936 (2016) 168-178.

Lores, H., Romero, V., Costas, I., Bendicho, C., Lavilla, I., Talanta 162 (2017) 453-459.

Lu, Y., Ma, W., Hu, R., Dai, X., Pan, Y., J. Chromatogr. A 1208 (2008) 42-46.

Ma, W., Lu, Y., Hu, R., Chen, J., Zhang, Z., Pan, Y., Talanta 80 (2010) 1292-1297.

Malaquias, J.C., Steichen, M., Dale, P.J., Electrochim. Acta 151 (2015) 150-156.

Mamajanov, I., Engelhart, A.E., Bean, H.D., Hud, N.V., Angew. Chem. Int. Ed. 49 (2010) 6310-6314

Mondal, D., Sharma, M., Mukesh, C., Gupta, V., Prasad, K., Chem. Commun. 49 (2013) 9606-9608.

Moriel, P., García-Suárez, E.J., Martínez, M., García, A.B., Montes-Morán, M.A., Calvino-Casilda, V., Bañares, M.A., Tetrahedron Lett. 51 (2010) 4877-4881.

Morrison, H.G., Sun, C.C., Neervannan, S., Int. J. Pharm. 378 (2009) 136–139.

Mustafa, N.R., de Winter, W., van Iren F., Verpoorte, R., Nature Protocols, 6 (2011)715-742

Nam, M.W., Zhao, J., Lee, M.S., Jeong, J.H., Lee, J., Green Chem. 17 (2015) 1718-1727.

Oliveira, F.S., Pereiro, A.B., Rebelo, L.P.N., Marrucho, I.M., Green Chem. 15 (2013) 1326-1330.

Paiva, A., Craveiro, R., Aroso, I., Martins, M., Rei, R.L., Duarte, A.R.C., ACS Sustain. Chem. Eng. 2 (2014) 1063-1071.

Pandey, S., Anal. Chim. Acta 556 (2006) 38-45.

Paradiso, V.M., Clemente, A., Summo, C., Pasqualone, A., Caponio, F., Food Chem. 212 (2016) 43-47.

Plechkova, N.V., Seddon, K.R., Chem. Soc. Rev. 37 (2008) 123-150.

Radošević, K., Bubalo, M.C., Srček, V.G., Grgas, D., Dragičević, T.L., Redovniković, I.R., Ecotoxicol. Environ. Saf. 112 (2015) 46-53.

Radošević, K., Ćurko, N., Srček, V.G., Bubalo, M.C., Tomašević, M., Ganić, K.K., Redovniković, I.R., LWT-Food Sci.Technol.73 (2016) 45-51.

Rozema, E., Van Dam, A.D., Sips, H.C.M., Verpoorte, R., Meijer, O.C., Kooijman, S., Choi, Y.H., RSC Adv. 5 (2015) 61398-61401.

Ruß, C., König, B., Green Chem.14 (2012) 2969-2982.

Shamseddin, A., Crauste, C., Durand, E., Villeneuve, P., Dubois, G., Durand, T., Vercauteren, J., Veas, F., Eur. J. Lipid Sci.Technol. 119 (2017) 1700171.

Stalcup, A.M., Cabovska, B., J. Liq. Chromatogr. Relat. Technol. 27 (2004) 1443-1459.

Stott, P.W., Williams, A.C., Barry, B.W., J. Control. Release 50 (1998) 297-308.

Sun, N., Rahman, M., Qin, Y., Maxim, M.L., Rodríguez, H., Rogers, R.D., Green Chem. 11 (2009) 646-655.

Sut, S., Faggian, M., Baldan, V., Poloniato, G., Castagliuolo, I., Grabnar, I., Perissutti, B., Molecules 22 (2017) 1921-1932.

Swatloski, R.P., Spear, S.K., Holbrey, J.D., Rogers, R.D., J. Am. Chem. Soc. 124 (2002) 4974-4975.

Sze, L.L., Pandey, S., Ravula, S., Pandey, S., Zhao, H., Baker, G.A., Baker, S.N., ACS Sustain. Chem. Eng. 2 (2014) 2117-2123.

Tan, S.S.Y., MacFarlane, D.R., Upfal, J., Edye, L.A., Doherty, W.O.S., Patti, A.F., Pringle, J.M., Scott, J.L., Green Chem.11 (2009) 339-345.

Vaher, M., Koel, M., Kaljurand, M., Chromatographia 53 (2001) S302-S306.

Ventura, S.P.M., e Silva, F.A., Gonçalves, A.M.M., Pereira, J.L., Gonçalves, F., Coutinho, J.A.P., Ecotoxicol. Environ. Saf. 102 (2014) 48-54.

Visser, A.E., Swatloski, R.P., Griffin, S.T., Hartman, D.H., Rogers, R.D., Sep. Sci. Technol. 36 (2001) 785-804.

Visser, A.E., Swatloski, R.P., Reichert, W.M., Griffin, S.T., Rogers, R.D., Ind. Eng. Chem. Res. 39 (2000[b]) 3596-3604.

Visser, A.E., Swatloski, R.P., Rogers, R.D., Green Chem. 2 (2000[a]) 1-4.

Wagle, D.V., Zhao, H., Baker, G.A., Acc. Chem. Res. 47 (2014) 2299-2308;

Walden, P., Bull. Acad. Imper. Sci. (St. Petersburg) 8 (1914) 405-422.

Wei, Z., Qi, X., Li, T., Luo, M., Wang, W., Zu, Y., Fu, Y., Sep. Purif. Technol. 149 (2015) 237-244.

Weiz, G., Braun, L., Lopez, R., de María, P.D., Breccia, J.D., J. Mol. Catal. B: Enzym. 130 (2016) 70-73.

Welton, T., Chem. Rev. 99 (1999) 2071-2084.

Wen, Q., Chen, J.-X., Tang, Y.-L., Wang, J., Yang, Z., Chemosphere 132 (2015) 63-69.

Wikene, K.O., Bruzell, E., Tønnesen, H.H., J. Photochem. Photobiol. B 148 (2015)188-196.

Wikene, K.O., Rukke, H.V., Bruzell, E., Tønnesen, H.H., J. Photochem. Photobiol. B 171 (2017) 27-33.

Xia, S. Q., Baker, G. A., Li, H., Ravula, S., Zhao, H., RSC Adv., 4 (2014) 10586–10596.

Yang, T.-X., Zhao, L.-Q., Wang, J., Song, G.-L., Liu, H.-M., Cheng, H., Yang, Z., ACS Sustain. Chem. Eng. 5 (2017) 5713-5722.

Zahrina, I., Nasikin, M., Krisanti, E., Mulia, K., Food Chem. 240 (2018) 490-495.

Zeng, H., Wang, Y., Kong, J., Nie, C., Yuan, Y. Talanta 83 (2010) 582-590.

Zhai, Y., Sun, S., Wang, Z., Cheng, J., Sun, Y., Wang, L., Zhang, Y., Zhang, H., Yu, A., J. Sep. Sci. 32 (2009) 3544-3549.

Zhang, H., Wang, Y., Zhou, Y., Xu, K., Li, N., Wen, Q., Yang, Q., Talanta 170 (2017) 266-274.

Zhang, L., Geng, Y., Duan, W., Wang, D., Fu, M., Wang, X., J. Sep. Sci. 32 (2009) 3550-3554.

Zhang, L., Wang, X., J. Sep. Sci. 33 (2010) 2035-2038.

Zhang, Q., De Oliveira Vigier, K., Royer, S., Jérôme, F., Chem. Soc. Rev. 41 (2012) 7108-7146.

Zhang, W., He, L., Gu, Y., Liu, X., Jiang, S., Anal. Lett. 36 (2003) 827-838.

Zhao, H., Baker, G.A., Holmes, S., J. Mol. Catal B: Enzym. 72 (2011) 163-167.

Zhao, H., Baker, G.A., J. Chem. Technol. Biotechnol. 88 (2013) 3-12.

Zhao, H., Zhang, C., Crittle, T.D., J. Mol. Catal. B: Enzym. 85 (2013) 243-247.

Zhu, S., Wu, Y., Chen, Q., Yu, Z., Wang, C., Jin, S., Ding, Y., Wu, G., Green Chem. 8 (2006) 325-327.

C H A P T E R 3

Natural deep eutectic solvents: from their discovery to their applications

Henni Vanda [a, b], Robert Verpoorte [a], Peter G. L. Klinkhamer [c], Young Hae Choi [a, d]

[a] Natural Products Laboratory, Institute of Biology, Leiden University, Sylviusweg 72, 2333 BE Leiden, the Netherlands

[b] Faculty of Veterinary Medicine, Syiah Kuala University, Banda Aceh, 20111 Indonesia

[c] Plant Ecology and Phytochemistry, Institute of Biology, Leiden University, Sylviusweg 72, 2333 BE Leiden, the Netherlands

[d] College of Pharmacy, Kyung Hee University, 02447 Seoul, Republic of Korea

Abstract

It is generally thought that all enzyme-mediated reactions in biosynthetic pathways in living organisms occur in a medium that is basically composed of water or lipids. However, an obvious question arises: how can organisms, that produce a broad range of primary and secondary metabolites, control the biosynthetic process and substrate transport in an aqueous or lipid medium, considering the often-poor water and/or lipid solubility of these metabolites? Recently, it was found that mixtures of certain compounds in solid state that are abundant in all organisms, such as sugars, organic bases, amino- and organic acids become liquids when mixed in certain molar ratios. These liquids have been named natural deep eutectic solvents (NADESs). They were shown to be highly selective solvents for all kinds of medium polar compounds that are poorly soluble in water and lipids. NADESs are non-toxic, and environment-friendly. As they have virtually zero vapour-pressure, risks of flammability are low. These characteristics make them powerful green solvents. At present, NADESs are being extensively investigated as extraction solvents for various applications, with the first products already being on the market. Based on our hypothesis that NADESs are the third liquid phase in living organisms, a number of hypothetical roles of NADESs in cellular systems can be postulated, and also based on that applications can be developed. Examples are the preservation of biopolymers such as DNA, RNA and proteins, as well as a medium for enzymatic reactions. This paper reviews features of NADESs, and some recent applications of NADESs for small molecules, macromolecules and enzymatic reactions.

Keywords: Natural Deep Eutectic Solvents, DNA, RNA, Proteins, Enzyme Reaction, Preservation

Introduction

Ionic liquids (ILs) have been studied extensively as novel green solvents for various applications, for example, organic synthesis and enzymatic reactions. Several recent reviews deal with the development of these solvents and their uses. Choi et al. hypothesized that Nature already uses the principles of ionic liquids and deep eutectic solvents and introduced the concept of a new type of eutectic liquid, the Natural Deep Eutectic Solvents (NADESs). A comprehensive definition of NADESs includes not only their physicochemical features but above all involves the phenomena in living cells and organisms that can (at least, in part) be explained by their existence. NADESs constituents are abundant in all organisms, and based on their physicochemical characteristics these solvents may be formed locally under different conditions, and thus play various roles (Choi et al. 2011; Vanda et al. 2018). Particularly the modulation exerted by water on the characteristics of NADESs could be a major regulatory function in diverse metabolic processes in living organisms. In terms of applications, NADESs have several advantages over the first generation of synthetic ILs, such as their non-toxicity, lower corrosiveness, absence of environmental hazards, sustainable production, and last but not least lower costs. These characteristics are important aspects in green chemistry and key NADESs as green alternative for organic solvents.

Similarly, to ILs or DES, NADESs are formed by mixing two or more components in certain molar ratios; the difference is that these components are from natural sources such as sugars, sugar alcohols, polyalcohols, amino acids, organic acids, and organic bases. We view NADESs more as concept in which such mixtures may also contain water. The water content can be a part of the eutectic mixture at a certain molar ratio, from which the water cannot evaporate, explaining why aqueous plant extracts always contain some residual water. The other aspect is that a NADESs can be diluted with water, resulting in their increased solubilizing power of various compounds, or the activation of enzymes which are stable but not active in a NADESs, but which become active

upon dilution. NMR spectrometric studies of NADESs provide evidence that extensive hydrogen-bonding interactions are involved in this process (Choi et al. 2011; Dai et al. 2013[a]; Vanda et al. 2018). Different classes of NADESs can be distinguished on the basis of their components (Table 1). Besides their already mentioned environmental advantages, NADESs have very useful physicochemical properties as solvents, such as very low melting points, adjustable viscosity and extremely low vapor pressure with very high flash points. Additionally, they show a high solubilizing capacity of a wide polarity range of compounds and high selectivity (Dai et al. 2013[a]; Vanda et al. 2018).

Table 1. Classification of NADESs and their typical examples

Composition	Examples
Acid – base	Malic acid – choline chloride (1:1) Citric acid – betaine (1:1)
Polyalcohol – acid	Xylitol – citric acid (1:1) D-sorbitol – Malic acid (1:1)
Polyalcohol – amino acid	D-sorbitol –proline (1:1)
Polyalcohol – base	D-sorbitol – choline chloride (1:3)
Sugar – acid	Sucrose –malic acid (1:1) Fructose – citric acid (1:1)
Sugar – amino acid	Sucrose –proline (1:4) Glucose – proline (1:1)
Sugar – base	Sucrose – choline chloride (1:4)
Sugar – sugar	Glucose –fructose – sucrose (1:1:1)

Table 2. Examples of different combinations of natural deep eutectic solvents (Choi et al. 2011; Dai et al. 2013[a])

Component 1	Component 2	Component 3	Mole ratio
Choline chloride	Lactic acid		1:1
Choline chloride	Malonic acid		1:1
Choline chloride	Maleic acid		1:1, 2:1,
choline chloride	DL-malic acid		1:1, 1.5:1
Choline chloride	Citric acid		1:1, 2:1,
Choline chloride	D-mannose		5:2
Choline chloride	D-(+)-galactose		5:2
Choline chloride	Sucrose		4:1, 1:1
Choline chloride	Proline	DL-malic acid	1:1:1
Choline chloride	Xylitol	DL-malic acid	1:1:1
Betaine	Sucrose		2:1
Betaine	D-(+)-trehalose		4:1
Betaine	D-(+)-glucose	Proline	1:1:1
Betaine	DL-malic acid	D-(+)-glucose	1:1:1
Betaine	DL-malic acid	Proline	1:1:1
DL-malic acid	Sucrose		1:1
DL-malic acid	D-mannose		1:1
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
D/L-proline	D-sorbitol		1:1
D/L-proline	Lactic acid		1:1
D/L-proline	DL-malic acid		1:1
D-proline	D-(+)-glucose		5:3
L-proline	D-(+)-glucose		5:3
D-(+)-glucose	DL-malic acid		1:1
D-(+)-glucose	Citric acid		1:1
D-(+)-glucose	L-(+)-tartaric acid		1:1
D-(+)-glucose	D-(-)-fructose	Sucrose	1:1:1
β-alanine	Citric acid		1:1

Initially, some 50 combinations of NADESs were proposed (Choi et al. 2011; Dai et al. 2013[a]) but since then many other different NADESs have been reported, all based on combinations that can fit into the categories mentioned in Table 1. They are mixtures of two or more compounds such as sugars,

polyalcohols, amino acids, organic acids, choline chloride, and betaine (Choi et al. 2011; Dai et al. 2013[a]; Vanda et al. 2018). Some of these NADESs candidates are shown in Table 2. With the rapidly increasing interest in research associated to green technology, new candidates for NADESs are continually reported (e.g. Paiva et al. 2014; Wikene et al. 2015; Bakirtzi et al. 2016; Espino et al. 2016; Wikene et al. 2017; Huang et al. 2018).

Due to their non-toxic nature, the interest in applications of NADESs for food, agrochemicals, cosmetics and pharmaceuticals has grown exponentially since the concept was introduced (Choi et al. 2011) and patented in 2011 (Van Spronsen et al. 2016). Examples of NADESs applications are the extraction of active compounds from medicinal plants (Dai et al. 2013[b]; Dai et al. 2016), the solubilization of pharmaceuticals (Rozema et al. 2015; Wikene et al. 2015; Bakirtzi et al. 2016; Shamseddin et al. 2017; Wikene et al. 2017), the production of plant extracts as cosmetic ingredients (Jeong et al. 2017), agrochemical applications (Huang et al. 2018; Zahrina et al. 2018), and as food flavoring additives (Gonzales et al. 2018).

Besides their use as extraction solvents, many other types of applications are expected to be developed. For example, NADESs could be excellent solvents for enzymatic and synthetic reactions involving poorly water- or lipid-soluble compounds. Several studies have already shown very promising results. These applications were actually predictable considering our hypothesis of NADESs as a third liquid phase in organisms. Numerous primary and secondary metabolites are biosynthesized in nature, most of which are poorly water soluble. For example, the biosynthesis of taxol (paclitaxel) encompasses a large number of steps in which non-water soluble intermediates have to move from one enzyme to another, something which is difficult to explain in an aqueous medium. However, knowing that enzymes are quite well soluble in various NADESs (Choi et al. 2011) and assuming a NADESs environment e.g. in vesicles or metabolons, non-water soluble compounds could be processed in a multienzymes system. The biosynthesis of various non-water soluble

metabolites including macromolecules like cellulose and lignin in plants, could be easily accounted for in such a system. Learning from Nature is the way to go, and some interesting results have already been reported, such as the use of NADESs as media for enzymatic reactions (Zhao et al. 2011; Paiva et al. 2014; Yang et al. 2017; Khodaverdian et al. 2018), for the solubilisation of biomacromolecules (Kumar et al. 2016; Lores et al. 2017), and for the extraction and preservation of DNA and RNA (Mamajanov et al. 2010; Mondal et al. 2013).

In this paper, after a short general overview of NADESs as an extraction solvent, we will focus particularly on a review of the applications of NADESs involving enzymatic reactions, macromolecules and as a preservative.

Natural deep eutectic solvents is a concept based on how physicochemical features might be used for biological functions

Since the introduction of the concept of NADESs, there has been a discussion concerning their definition and various proposals for the nomenclature of deep eutectic solvents such as low-melting mixtures (Ruß et al. 2012), low transition temperature mixtures (Francisco et al. 2012), and bio-ILs (Fukaya et al. 2007) have been put forward. These names, however, are based on strict physicochemical features, whereas NADESs is a concept that goes beyond this, as it explains various functions of mixtures of NADESs components in nature. In this context, the effect of water in NADESs is of great interest.

When the concept of NADESs was introduced, they were hypothesized to constitute a third liquid phase in living organisms as an alternative to water and lipids, and thought to be vital for various biological functions (Choi et al. 2011). This alternative liquid phase could explain, for example, the biosynthesis of non-water-soluble compounds. The NMR analysis of extracts obtained from plant material using a comprehensive extraction method, (Yuliana et al. 2011) showed clearly that the compounds present in a plant represent three groups that

can be categorized as hydrophilic, medium hydrophilic and lipophilic. This would support a model in which three different liquids or solvents are present in cells.

NADESs have been proposed as solvents for the storage or solubilization of poorly water-soluble compounds (most secondary metabolites) (Dai et al. 2013[b]; Bakirtzi et al. 2016; Faggian et al. 2016; Jeong et al. 2017; Sut et al. 2017); as media for enzymatic reactions in biosynthesis (Zhao et al. 2011; Yang et al. 2017; Khodaverdian et al. 2018); to enable the transport of metabolites (Markham et al. 2000; Beaudoin and Facchini, 2014); for the preservation of biomacromolecules such as DNA, RNA or proteins (particularly for survival during extreme conditions, such as resurrection plants, cacti and lichens) (Hoekstra et al. 2001; Mamajanov et al. 2010; Mondal et al. 2013) and as media in exudates, latex or saps (Gowda et al. 1983).

All these processes are very difficult to explain considering that they all require a liquid medium and the only available "liquids" are water and lipids, the conventional natural solvents thought to be present in an organism, neither of which can readily dissolve most secondary metabolites. Thus, it was proposed that certain ionic liquids or deep eutectic solvents composed of ingredients found in Nature, i.e. NADESs, could play a role in those processes. Candidates for NADESs ingredients in various organisms were all obtained from the in-house ^{1}H NMR based metabolomics data library. The spectra of a great number of samples showed the high abundance of sugars, sugar alcohols, organic acids (e.g. malic, citric, succinic and lactic acid), bases (e.g. choline and betaine) and amino acids (e.g. proline, alanine). These were often present in certain molar ratios as could be calculated directly from the quantitation of their NMR signals (Choi et al. 2011). The first examples of NADESs (sugar and malic acid based) are shown in Figure 1 (Choi et al. 2011). Since this first discovery, the presence of NADESs ingredients in specific plant tissues and saps has been described, for example in the nectar of flowers, maple syrup, and the barley seed aleurone.

According to our hypothesis, NADESs could play a major role in the survival of plants. For example, under extreme drought conditions, when resurrection plants dehydrate, the formation of a NADESs can keep the last available water as part of the NADESs. In this liquid, enzymes remain dissolved and stabilized but are inactive, however when water becomes available, the NADESs will mix with water and at a certain water percentage the enzymes will become active again. The difference with the formation of a glass from sugar is that these do not retain water and eventually crystallize (Hoekstra et al. 2001; Choi et al. 2011). This idea is supported when considering the cryoprotectants added to plant cells to protect them during cryopreservation are all typical NADESs constituents (Mustafa et al. 2011). In fact, various studies on cold resistance of plants report compounds which are now known to be able to be part of NADESs, e.g. proline, sugars, betaine, linolenic acid, and palmitic acid (Beck et al. 2007; Xie et al. 2013; Chen et al. 2014).

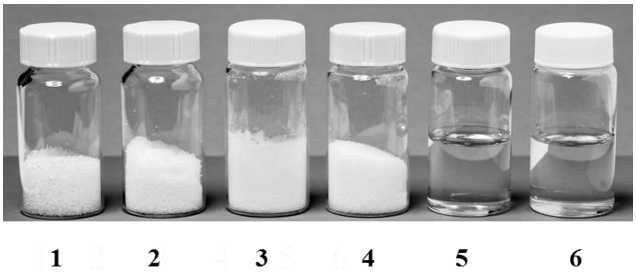

Figure 1. Typical natural eutectic solvents (NADESs) 1: sucrose, 2: fructose, 3: glucose, 4: malic acid, 5: sucrose-fructose-glucose (1:1:1, mol/mol), 6: sucrose-malic acid (1:1, mol/mol). Remade with permission from Choi et al. (2011).

Assuming that solid state biosynthesis does not occur in Nature, there must be a medium in which the enzyme-mediated reactions that produce water-insoluble small molecules and polymers such as cellulose, amylose, and lignin can proceed. In the case of macromolecules, the hydrophilic intermediates and enzymes must be in the same phase that dissolves both the precursors and the

resulting polymer. Several NADESs have indeed been found to dissolve starch and the increasing levels of water may result in the precipitation of the polysaccharide (Choi et al. 2011).

As mentioned above, cells also contain compounds like flavonoids which are not very soluble in water, yet are present in relatively high amounts. It is not only their biosynthesis that is inexplicable given their low solubility, but also their storage in very high levels in certain tissues or cells in apparently solution. In some cases, compounds are synthesized in specialized cells or tissues and then transported to another cellular compartment or other cells. Vesicles seem to be involved in the transport and storage of secondary metabolites. For example, alkaloids synthesized in sieve elements are transported to laticifers for storage in large cytoplasmic phospholipid bilayer vesicles containing a fluid which contains several common NADESs ingredients (Wink, 1993; Carqueijeiro et al. 2013; Beaudoin and Facchini 2014). Anthocyanoplasts (ACPs) and anthocyanin vacuolar inclusions (AVIs) as described by Markham (2000) might very well consist of NADESs dissolved flavonoids and anthocyanins in a phospholipids-based vesicle, providing an explanation for the presence of these compounds in cells in concentrations that are way above their solubility in water. Further supporting evidence for a biological function of NADESs is the chemical composition of the plant vacuolar content. The most abundant compounds in plant vacuoles aside from water are sucrose and malic acid (Yamaki 1984). Considering one of the roles of vacuoles, i.e., the storage of toxic defense compounds, it is likely that those with low water solubility would precipitate. Thus, NADESs constituents could play a role in increasing their solubility, either forming a layer on the inside vacuolar membrane, or alternatively, forming a complex with the metabolite that increases its water solubility, i.e., a similar principle to ion-pairing. The endoplasmic reticulum (ER) could be a NADES in which enzymes and medium polar substrates are dissolved with the ER absorbing these from the aqueous phase of the cytoplasm (Choi et al. 2011).

In connection with a possible role in plant defense, a high concentration of a soluble compound could be an advantage. For example, the solubility of rutin, the most common flavonoid in Nature (accounting, for example, for 30% of the dry-weight of *Sophora japonica* flowers) is 50 to 100 times higher in NADESs than in water (Choi et al. 2011). Moreover, it has been shown that NADESs increase the bioavailability of bioactive compounds such as antioxidants (Faggian et al. 2016) and berberine, a quaternary alkaloid (Sut et al. 2017). A similar effect could be important in plant defense against pests and diseases.

NADESs in extraction and solubilization of non-water-soluble metabolites

Since the introduction of the NADESs concept, a rapidly increasing number of applications for the extraction or solubilization of non-water-soluble metabolites have been published, and reviewed (Dai et al. 2013[a]; Dai et al. 2013[c]; Rozema et al. 2015; Dai et al. 2016; Espino et al. 2016; Huang et al. 2018; Vanda et al. 2018).

Many secondary metabolites such as taxol, ginkgolides and rutin have been found to be much more soluble in NADESs than water (Choi et al. 2011; Dai et al. 2013[a]; Faggian et al. 2016). Quercetin, a ubiquitous flavonoid in Nature, showed an enhanced solubility of up to 380% in a NADESs as compared to a buffer (Gomez et al. 2016).

As a basis for the patent of Van Spronsen et al. (2016), several applications of NADESs for extraction, including phenolic compounds from *Carthamus tinctorius* L. (Dai et al. 2013[b]), vanillin from *Vanilla planifolia* pod (Gonzales et al. 2018), and anthocyanins from *Catharanthus roseus* (Dai et al. 2016) were reported. Later, the study of the selective extraction of ginkgolides, phenolics, and ginkgolic acids from *Ginkgo biloba* as well as ginsenosides from *Panax ginseng* with NADESs from their respective plant materials was performed using a newly developed high-performance thin layer chromatography-based metabolomics method for their analysis. This method

showed to be a promising tool for the study of NADESs extracts (Liu et al. 2018).

Recently, Nam et al. (2015) published the results of a comprehensive study of the extraction of flavonoids from the traditional Chinese medicinal plant, *Styphnolobium japonicum* (L.) Schott. Quercetin, kaempferol, and isorhamnetin glycosides were extracted by ultrasound assisted extraction (UAE) using a L-proline-glycerol NADESs, giving higher yields than conventional organic solvent extraction and solid phase extraction (SPE).

Wei et al. (2015) compared the extraction of phenolics from *Cajanus cajan* leaves, with fourteen NADESs combinations. The results indicated that a combination of choline chloride-maltose (1:2, mol/mol) provided the highest extraction of both polar and weakly-polar compounds compared to conventional solvents, additionally exhibiting high linearity and recovery.

Lipophilic compounds such as plant volatiles or flavors have also been successfully extracted with NADESs (Gonzales et al. 2018; Křížek et al. 2018). For example, the extraction of cannabinoids from *Cannabis sativa* was reported by Křížek et al. (2018). Using the hydrophobic NADESs composed of menthol-acetic acid (1:1), they achieved a highly efficient extraction of tetrahydrocannabinol, tetrahydrocannabinolic acid, cannabidiol, and cannabidiolic acid with yields ranging from 118.6% to 132.6% times those obtained with organic solvents. NADESs have also been applied for the extraction of flavors. For example, the solubility in NADESs of vanillin, the most common flavor compound in the world, was tested and the combinations that proved to be most promising were then used for the extraction of the whole flavor from vanilla pods. The efficiency of the extraction with some of these NADESs proved to be higher than that obtained with conventional organic solvents or water extraction. Furthermore, the NADESs extracts proved to be suitable for flavoring, and could be marketable (Gonzales et al. 2018).

The successful application of NADESs to the extraction of natural products illustrates the potential of NADESs to replace toxic organic solvents in

large scale industrial extraction. However, their successful commercial application requires the solution of some significant drawbacks. Firstly, the vapor pressure of NADESs, which is virtually zero, means that the classical evaporation of the extraction solvent required to recover a compound(s) of interest is not feasible. On the other hand, their non-volatility can be an advantage if using the NADESs extract as such, e.g., as a food additive or in cosmetic formulations. In the cosmetic industry, NADESs have been applied to extract epigallocatechin-3-gallate (EGCG), a potent antioxidant, from green tea (*Camellia sinensis*) using ultrasound-assisted extraction, and a NADESs composed of betaine-glycerol-glucose (4:20:1) was selected, among other, for its non-toxicity and low-cost. The extraction yield was significantly higher than in classical extraction methods, with the additional advantage of an increased stability of EGCG over at least a three-week storage period (Jeong et al. 2017).

A very different application of NADESs was the removal of cadmium (Cd) from rice flour using choline chloride-tartaric acid-water (1:1:2) and choline chloride-xylitol-water (5:2:5). The removal of 51 - 96% of the Cd was achieved and the addition of 1% (w/w) of a surfactant saponin aqueous solution to the mixture increased this to 99% of the Cd without affecting the chemical composition of the flour (Huang et al. 2018).

The NADESs composed of choline chloride, betaine, glycerol, sucrose, malic acid, glucose, and urea were employed in food-grade synthesis of Maillard-type taste enhancers to reduce the use of salt, sugar, and monosodium glutamate in food. Kranz and Hofmann (2018) demonstrated that NADESs had the ability to increase the yield of the taste enhancers 1-deoxy-D-fructosyl-*N*-β-alanyl-l-histidine (49%), *N*-(1-methyl-4-oxoimidazolidin-2-ylidene) amino propionic acid (54%) and N^2-(1-carboxyethyl) guanosine 5′-monophosphate (22%), as compared to those obtained with an aqueous buffered solution, at temperatures between 80–100 °C within a two-hour reaction period. Hence NADESs may open a new direction in culinary chemistry. It might be of

interest to study the commonly applied glazing in cooking with the NADESs concept in mind.

Reading all the application papers, it is remarkable that most studies follow a trial-and-error approach when developing an application. Clearly there is still a lack of a theoretical background that would allow the prediction of which NADESs is the best for a certain application. In fact, we have observed that the solubility of a compound is not always the same as its extractability from plant material. For example, in the extraction of vanillin from vanilla pods the NADESs that showed highest yields of vanillin did not fully coincide with those that showed the highest solubility of vanillin (Gonzalez et al. 2018). It thus appears that the matrix plays a role. It is indisputable that hydrogen-bonding is the key factor in the formation of NADESs, and possibly also in its dissolving power of several compounds. Also, the role of water is interesting (Dai et al. 2015), as the addition of even small amounts produces a substantial decrease in the viscosity of the extract, but also affects the solubility of compounds in an unpredictable manner. The percentage of water in a NADESs is thus an important variable that needs to be optimized for any application.

NADESs in extraction and solubilization of macromolecules

Among the challenges faced when developing novel sustainable industrial processes, the solubilization of macromolecules is a priority. Macromolecules, such as DNA, RNA, proteins, polysaccharides and lignin, have diverse basic functions in living organisms. They differ clearly in their solubility, and particularly most polysaccharides and lignin are known to be very poorly water soluble. However, in nature these polymers must have been biosynthesized in a solution. When the NADESs concept was introduced, their possible functions in plant physiology resulting from their solubilization of macromolecules were considered. For example, in the dormancy period of seeds, NADESs could be the storage media that prevents the denaturation of proteins, DNA or RNA until germination (Choi et al. 2011).

Certain points in the physiology of the biosynthesis of polysaccharides are still unclear, particularly the chain-elongation to obtain polysaccharides must occur in a dissolved state of substrate and product before crystallization or precipitation of the end product. A NADES might be the solvent that contains the enzymes and precursors needed for the elongation. Changes in the NADESs composition, e.g. change in water content, could end the biosynthesis.

Natural polymers

The use of ILs and DES for the dissolution of natural polymers such as polysaccharides and lignin has been studied extensively. Lignin and cellulose are a source of renewable organic polymers (Financie et al. 2016). Particularly cellulose has been the subject of many studies as it is the most abundant polymer on earth. Its application, e.g. for bioethanol production, as an industrial commodity is hampered by its insolubility in water and in most other common industrial solvents.

Solubilization in ILS and DES has thus been the subject of a number of studies. It was found that cellulose could be dissolved in the synthetic IL, 1-butyl-3-methylimidazolium chloride and 1-allyl-3-methylimidazolium chloride without any derivatization. Cellulose then could be precipitated by the addition of water, ethanol, or acetone (Zhu et al. 2006). Another abundant natural polymer, lignin, is presently extracted industrially from lignocellulose in an environmentally unfriendly complex process. With the purpose of improving this process, 1-ethyl-3-methylimidazolium acetate was applied as a pretreatment solvent to provide suitable conditions for lignin degradation. The cellulose of the pretreated wood flour becomes far less crystalline without undergoing solubilization and enhanced lignin extraction (Lee et al. 2009). Another example is the pretreatment of oil palm frond biomass with a hydrophilic IL, 1-ethyl-3-methylimidazolium diethyl phosphate, which is known to solubilize lignocellulosic biomass, which resulted in an increase in the following enzymatic delignification of the biomass. The treatment lowers the lignin

content from 24.0 % to 8.5 % (w/w) (Financie et al. 2016). A series of choline based ILs was applied in pretreatment of grass lignocelluloses and eucalyptus. The treatment resulted in a significant increase in glucose yield. Choline argininate showed excellent recyclability with a total recovery as high as 75% after reuse for 8 cycles. Furthermore, rice straw pretreated by the recycled ILs remained highly digestible, and 63-75% of good glucose yields were achieved after its enzymatic hydrolysis (An et al. 2015).

The NADESs combinations, lactic acid-choline chloride and lactic acid-betaine were shown to extract lignin of high purity (>90%) and a high yield, achieving a 60 ± 5% (w/w) separation of the biomass. The addition of 5.0% (v/v) water significantly enhanced total lignin extraction as shown by HPLC and FTIR (Kumar et al. 2016).

DNA and RNA

Several NADESs have been tested to dissolve nucleosides. The formation of at least 4 different secondary structures of nucleic acids was reported by Mamajanov et al. (2010) in a water-free DES consisting of the choline chloride-urea mixture (1:2, mol/mol). Studying the nucleic acids revealed sequence-related differences in the stability and type of secondary structure formed. These findings seem to open the way to applications of catalytic nucleic acid activities and of enzyme–nucleic acid complexes. Zhang et al. (2017) optimized the extraction of RNA in an aqueous biphase system in combination with a DES consisting of PEG and a quaternary ammonium salt. High recoveries could be obtained when a low concentration of low molecular weight PEG was used in combination with longer alkyl chain (C4) quaternary amines, and more hydrophobic inorganic salts. Mondal et al. (2013) demonstrated the high solubility and stability of DNA in the NADESs composed of choline chloride with glycerol and ethylene glycol.

A number of experiments have been performed by our group, the results of some of which will be summarized here. The stability of DNA and RNA was

tested by storing dead fruit flies (*Drosophila melanogaster*) in five different NADESs which were selected on the basis of major ingredients in cells (see Table 3.).

Table 3. Stability of DNA and RNA in dead fruit flies (*Drosophila melanogaster*), intact or as dried powder in NADESs and controls, stored for 12 months in ambient temperature, in the light, PCR analysis after 12 months

Solvent	Preserved	
	Whole fly	Dried powdered fly
N1 (sucrose-choline chloride-water, 1:4:4, mol/mol)	+	+
N2 (fructose-glucose-sucrose-water, 1:1:1:11, mol/mol)	+	+
20% water	+	+
30% water	+	+
40% water	+	+
N3 (*myo*-inositol-sucrose-$CaCl_2.2H_2O$-water, 1:2:3:5, mol/mol)	-	-
N4 (malic acid-choline chloride-water, 1:1:5, mol/mol)	-	-
N5 (sucrose-betaine-water, 1:2:5, mol/mol)	+	
Control 1 (70% ethanol)	+	+
Control 2 (water)	-	-

From the results it is clear that NADESs N1 and N2 are preserving the DNA and RNA in the original sample at least over a period of one year stored in the laboratory in the light, without any precautions to avoid decomposition. The major advantage of the storage in NADESs would be the absence of evaporation of the preserving solvent, as could happen with ethanol for example. The DNA and RNA dissolved in NADESs could be used as such for PCR analysis.

Most conventional DNA isolation protocols involve its dissolution in a buffer and storage at -20 or -80°C for preservation. Therefore, the NADESs were further tested for the stabilizing effect on DNA and RNA extracts. After

isolation of DNA using a conventional extraction, the extract was dissolved in the NADESs and left for three days in the light at room temperature. The DNA did not show any degradation in N1, N2 and N5.

In the case of long-term storage of RNA, the N5 solution of RNA proved to be the most stable after six months of storage in the light at room temperature. These preliminary results revealed the great potential of NADESs as a storage media of DNA and RNA samples. Further experiments on the stability under diverse conditions are needed to determine the long-term stability and validate NADESs as a storage media for DNA and RNA in different concentrations, i.e., in biological samples or DNA and RNA extracts.

Proteins

NADESs have been employed for solubilizing or stabilizing proteins. For example, the determination of gluten, which is a water-insoluble allergenic protein from wheat, barley, and oats, was achieved by extraction with dilute fructose-citric acid followed by analysis with ELISA. Apart from solubilizing the gluten, NADESs prevented its oxidation (Lores et al. 2017).

Li et al. (2016) used a betaine-urea NADESs in combination with an aqueous two-phase system for the extraction of proteins from calf blood sample. Betaine-urea exhibited the most suitable extractive properties with a yield of 99.8% under optimum conditions. Analysis with UV–Vis, FT-IR spectrometry and circular dichroism (CD) confirmed that the conformation of the proteins was unaltered during the extraction.

The preservation of proteins in ILs, DES and NADESs has been reported, as well as the use of these media for enzymatic reactions. In the case of plant enzymes, it was shown that laccase was easily solubilized in NADESs but was inactive. However, laccase was activated upon the addition of water (50% w/w), indirectly providing evidence, for example, of its possible role in the preservation of enzymes in NADESs in seeds of resurrection plants (Choi et al. 2011). Carnivorous plants obtain their nutrients from the digestion of trapped

insects and small animals. Proteolytic enzymes digest the prey and the resulting nutrients are absorbed by the plant. For example, *Drosera* species produce proteases in the glandular hairs of their tentacles to digest their prey and a sticky mucilage to capture them. This viscous liquid contains sugars and organic acids as their main components (Gowda et al. 1983). Analysis of the exudate of seven species of *Drosera* sp. using ^{1}H NMR spectroscopy, showed that the exudates were composed of acetoacetic acid, acetic acid, citric acid, ethanol, lactic acid, myo-inositol, glucose, sucrose, and malic acid. All these compounds are candidate constituents of NADESs that together with the enzymes attach to the prey and dissolve it, while simultaneously stabilizing the enzymes that have to be preserved for many days in the light and at high temperatures.

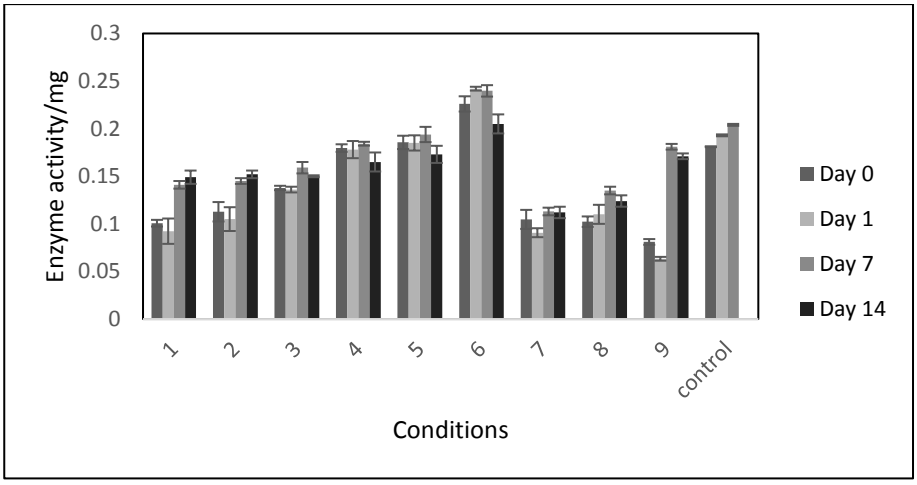

Figure 2. The effect of water on the preservation of protease activity in three NADESs and a water control. 1-3: (mannose-glucose-choline chloride, 1:2:1, mol/mol), modified with 30%, 40%, 50% water (w/w), 4-6: (fructose-glucose-sucrose, 1:1:1, mol/mol), modified with 30%, 40%, 50% water (w/w), 7-9: (sucrose-betaine, 1:2, mol/mol) modified with 30%, 40%, 50% water (w/w).

The stability of a protease from *Streptomyces griseus* has been tested in several NADESs: mannose-glucose-choline chloride, 1:2:1, mol/mol, fructose-sucrose-glucose, 1:1:1, mol/mol, sucrose-betaine, 1:2 mol/mol) with different concentrations of water (30%, 40%, and 50%, w/w). The protease activity did

not show any significant change after two weeks of storage at room temperature. Interestingly there were significant differences in activity for the enzyme in different NADESs (Figure 2).

NADESs applications in enzyme reactions

Many secondary metabolites are poorly soluble in water and this also applies to their biosynthetic precursors. Many biosynthetic pathways involve different cellular compartments and even different cells. The intra- and intercellular transport of intermediates or precursors is difficult to explain for non-water soluble compounds. Solubilization in NADESs of poorly water-soluble compound metabolons in a cytosolic pathway could provide an explanation. A metabolon is thought to be an aggregate of enzymes that can catalyze different steps in a biosynthetic pathway (Jørgensen et al. 2005). Our hypothesis proposes NADESs to be third liquid phases in cells. Furthermore, NADESs might be part of a metabolon as a factor that binds the enzymes together and form an intracellular liquid phase as a compartment where both enzymes and precursors are dissolved. In this case, poorly water-soluble compounds would be absorbed by such a metabolon. To prove the *in-situ* presence of such a third liquid phase in cells is difficult, as it might be a dynamic metastable system based on strong hydrogen bond formation in which a NADES is present in the ER, vesicles or cell membranes. This liquid phase would be in dynamic equilibrium with the water phase. The ^{1}HNMR spectra of NADESs with increasing amounts of water added, show that the signals related to hydrogen bond formation are still involved in such binding.

Based on this hypothesis NADESs would be of interest as media for (bio)synthetic reactions. Indeed, several studies already reported the occurrence of enzymatic reactions in NADESs (Table 4) which supports the hypothesis. Choi et al. (2011) showed that laccase is stabilized by dissolving in certain NADESs and that after adding water to more than 50% the enzyme becomes active. This supports the possible role of NADESs as a storage media for

proteins in nature.

Another clear support for the hypothesis is the study by Milano *et al.* that provides information about the application of choline chloride -based NADESs to photosynthetic enzymes known as reaction centers (RC). This complex membrane protein extracted from *Rhodobacter sphaeroides* bacteria was used as a model system. The results of ATR-FTIR spectroscopy showed that a complex membrane protein such as RC can work properly in a eutectic mixture (Milano et al. 2017).

Table 4. Application of NADESs in enzymatic reactions

NADESs (mole ratio)	Enzyme	Substrate	Reference
Malic acid-choline chloride (1:1)	Laccase		Choi et al. (2011)
Lactic acid-choline chloride (5:1)	Cellulase	Lignocellulose	Kumar et al. (2016)
Choline chloride–tartaric acid (1:1) Choline chloride–sorbitol (5:2) Choline chloride–lactose (4:1)	Whole cell *Lysinibacillus fusiformis*	Isoeugenol	Yang et al. (2017)
Choline chloride based NADES	Reaction centers from *Rhodobacter sphaeroides*	Quinone	Milano et al. (2017)
Glycerol-choline chloride (2:1) Glycerol-betaine (2:1)	Chondroitinases ABCI	Chondroitin-4-sulfate	Daneshjou et al. (2017)
Mannose-glucose-choline chloride (1:2:1) Fructose:sucrose:glucose (1:1:1) Sucrose-betaine (1:2)	Protease	BSA	-
Mannose-glucose-choline chloride (1:2:1) Fructose:sucrose:glucose (1:1:1) Sucrose-betaine (1:2)	Bromelain	BSA	-

The stability of enzymes can be improved with NADESs as reported by Daneshjou et al. (2017). This group demonstrated the capacity of NADESs composed of glycerol-choline chloride (2:1 mol/mol) and glycerol-betaine (2:1, mol/mol) to stabilize the chondroitinase ABCI, a clinical enzyme for treating spinal lesions. The stability of this enzyme was higher in NADESs than an aqueous buffer at −20.4 and 37 °C exhibiting activity levels of approximately 82% of the initial activity after 120 min. After 15 days, the enzyme still retained about 80% of its initial activity, but in absence of NADESs (aqueous buffer), total activity was lost after five days. Yang *et al.* demonstrated the catalytic power of NADESs as cosolvents in whole-cell biocatalysis of the reaction of isoeugenol to vanillin in *Lysinibacillus fusiformis.* NADESs enhanced the cellular membrane permeability of the bacteria (Yang et al. 2017).

Pharmaceutical applications

One of the major problems in drug development in the pharmaceutical field is the solubilization of drugs in appropriate doses in safe, non-toxic, compatible media. Considering that NADES are able to solubilize poorly water-soluble compounds (Choi et al. 2011) the idea of developing novel formulations using NADESs as solubility-enhancing excipients seems very promising.

A study by Liu et al. (2018) demonstrated the use of NADESs composed of mannose-dimethylurea-water to solubilize lipophilic molecules and load these into a hydrogel made of sodium alginate. The spontaneous separation of the hydrogel and the NADESs allowed the loaded hydrogels to be administered successfully and the lipophilic metabolites released from the hydrogel in the GI tract were readily absorbed. The delivery mechanism developed in this study could well be present in Nature, as a way of potentially improving the bioavailability of lipophilic metabolites, for example, those involved in the resistance of plants against pests and diseases.

Table 5. Application of NADESs in Pharmaceuticals

NADESs (mole ratio)	Target compounds	Result	Reference
Proline-glutamic acid (2:1) Proline-choline chloride (1:1)	Rutin	Improved solubility and bioavailability	Faggian et al. (2016)
Proline-malic acid (1:2) Proline-urea (2:1) Lactic acid-proline-malic acid-water (0.3:1:0.2:0.5)	Berberine	Improved solubility and bioavailability	Sut et al. (2017)
Mannose-dimethylurea-water (2:5:5)	Fruit extract of *Schisandra chinensis*	Improved bioavailability of gomisin A, gomisin J, and angeloylgomisin H	Liu et al. (2018)
Poly-ϵ-caprolactone polymeric blend (SPCL 30:70)	Menthol-ibuprofen (3:1)	Faster release profile	Aroso et al. (2015)
Choline chloride – oxalic acid (1:1)	Grape skin phenolic compounds	Antioxidant, antitumor	Bubalo et al. (2016)
1,2-propanediol–choline chloride – water (1:1:1)	Salsalate	Inducer brown adipose tissue (BAT)	Rozema et al. (2015)
Choline chloride–malic acid (1:1)	Grape skin extract	Antioxidant, antitumor,	Radošević et al. (2016)

A study conducted by Aroso et al. (2015) showed that bioactive or pharmaceutical ingredients themselves can form a NADESs. A starch- poly-ϵ-caprolactone polymeric blend (SPCL 30:70) with menthol-ibuprofen (3:1) in different ratios (10 and 20 %, w/w), with supercritical fluid sintering at 20 MPa and 50 ^{o}C gave a matrix that showed a faster release profile of the drug, mostly by diffusion. Another study showed that the solubility and bioavailability of both rutin and berberine were increased significantly when these compounds were administered in a NADESs, as well as their levels and permanence in plasma (Faggian et al. 2016; Sut et al. 2017), thus achieving the same

pharmacological effect with a lower dose as a result of the synergy with the NADESs.

Applications of NADESs in diverse fields have been reviewed by our group (Vanda et al. 2018) and some of the most representative applications are summarized in Table 4- 5.

Toxicity of NADESs

The assessment of toxicity is a must when developing applications for human uses. Many NADESs have already been tested for their cytotoxicity. Mbous et al. (2017) compared the cytotoxic profiles of choline chloride-glucose (2:1, mol/mol) and choline chloride-fructose (2:1, mol/mol) and the DES, *N*,*N*-diethyl ethanol ammonium chloride-triethyleneglycol (1:3, mol/mol). The cytotoxicity was tested on HelaS3, PC3, A375, AGS, MCF-7, and WRL-68 hepatic cell lines. The EC_{50} of the tested NADESs ranged from 98 to 516 mM, while the toxic profile of DES was clearly higher (34-120 mM). A glucose-based NADESs was found to be less toxic than a fructose-based NADESs due to their different metabolic pathways. Cytotoxicity of choline chloride-based NADESs was also assessed by Hayyan et al. (2013). Their antimicrobial effect was assessed using two Gram positive bacteria, *Bacillus subtilis* and *Staphylococcus aureus*, and two Gram negative bacteria, *Escherichia coli* and *Pseudomonas aeruginosa* and their cytotoxicity was evaluated with the Brine shrimp (*Artemisia salina*) test. No growth inhibition of the bacteria was observed, showing that the tested NADESs had neither toxic nor beneficial effects on these bacteria. However, they exhibited a toxic effect on brine shrimp larva. The authors considered that the observed effect should be further studied for its potential in chemotherapy. Another experiment conducted by Paiva et al. (2014) on cytotoxicity of several NADESs suggested that while choline-based NADESs are non-cytotoxic, the presence of tartaric acid affected the metabolic activity of L929 fibroblast-like cells when NADESs was administered at a concentration of 25 mg/mL.

Perspective of NADESs

In the past years, NADESs have become a hot topic in cosmetics as their use as extraction solvents opens the options to an impressive array of novel types of extracts with improved bioactivities and interesting properties for their formulation in products for skin care. It is anticipated that many new applications of NADESs in cosmetics will be developed in the near future.

Another area of application is food, particularly in flavors and fragrances, given their generally poor water solubility. NADESs may be used to obtain flavorings and aromatic extracts to be used as food additives. A major advantage for both cosmetic and food applications is the increased stability of the compounds present in the NADESs extracts that could eventually avoid the need of preservatives.

Pharmaceutical applications are not yet common, though the increase in bioavailability of drugs in NADESs has been proven and merits further research. The increased solubility of drugs in NADESs can allow the extension of the range of dose-response curves for bioassays. This could also provide a basis for the development of novel formulations including NADESs as vehicles of poorly water-soluble drugs. All these applications are directed at small molecules with interesting properties that are difficult to solubilize.

The hypothesis that NADESs are important in a variety of processes in cells and organisms is a source of inspiration to explore possible applications. Among these, their great solubilization power of macromolecules appears to be one of their most attractive features. In the first place, NADESs could play a role in nature as media for various biosynthetic reaction steps that involve water insoluble precursors. This implies that NADESs could be applied in processes involving bioconversions with diverse enzymes. As mentioned, the feasibility of several enzymatic reactions in synthetic ILs has been proved. In our experience, NADESs has shown the capacity of stabilizing enzymes that can be activated by the addition of water (e.g. 50%). Further studies in this direction

would be of interest as with much higher levels of precursors in solution the enzyme kinetics would be different.

The dissolution of DNA and RNA in NADESs has been described (Choi et al. 2011), though the stability of the resulting solutions differs considerably according to the NADESs. In some cases, stability is affected negatively while in others the stability is enhanced. Thus, further research is needed to better understand the possible relationship between RNA and DNA and NADESs in the cells. At present one may predict that the use of NADESs to store DNA and RNA samples at room temperature could be feasible. In terms of biological applications, cryopreservation seems an interesting area for NADESs, as commonly used cryoprotectants (sugars like trehalose, sugar alcohols like mannitol and proline) can actually constitute a NADESs.

Regarding the hypothesis that proposes NADESs as a third media in living cells, the evidence is so far only indirect, and its *in-situ* demonstration is a major challenge, the more so as one may expect the presence of different NADESs in different tissues and in different cellular compartments.

A major aspect of NADESs is their physical chemistry, and to date, in-depth studies on the interactions between NADESs and solutes are lacking. As a result, most investigations related to applications are based on a trial and error approach. Based on sound predictions of their behavior obtained from the physicochemical data provided by such studies, it should be possible to build models simulating the role of NADESs in biological systems and select appropriate combinations according to the application.

References

An, Y.X., Zong, M.H., Wu, H., Li, N., Bioresour. Technol. 192 (2015) 165-171.

Aroso, I.M., Craveiro, R., Rocha, Â., Dionísio, M., Barreiros, S., Reis, R.L., Paiva, A., Duarte, A.R.C., Int. J. Pharm. 492 (2015) 73-79.

Bakirtzi, C., Triantafyllidou, K., Makris, D.P., J. Appl. Res. Med. Aromat. Plants 3 (2016) 120-127.

Beaudoin, G.A., Facchini, P.J., Planta 240 (2014) 19-32.

Beck, E.H., Fettig, S., Knake, C., Hartig, K., Bhattarai, T., J. Biosci. 32 (2007) 501-510.

Bubalo, M.C., Ćurko, N., Tomašević, M., Ganić, K.K., Redovniković, I.R., Food Chem. 200 (2016) 159-166.

Carqueijeiro, I., Noronha, H., Duarte, P., Gerós, H. and Sottomayor, M., Plant physiol. 162 (2013) 1486-1496.

Chen, L.J., Xiang, H.Z., Miao, Y., Zhang, L., Guo, Z.F., Zhao, X.H., Lin, J.W. and Li, T.L., J. Agron. Crop Sci. 200 (2014) 237-245.

Choi, Y.H., van Spronsen, J., Dai, Y., Verberne, M., Hollmann, F., Arends, I.W., Witkamp, G.J., Verpoorte, R., Plant Physiol. 156 (2011) 1701-1705.

Dai, Y., Rozema, E., Verpoorte, R., Choi, Y.H., J. Chromatogr. A 1434 (2016) 50-56.

Dai, Y., van Spronsen, J., Witkamp, G.J., Verpoorte, R., Choi, Y.H., Anal. Chim. Acta (2013[a]) 766, 61-68.

Dai, Y., Witkamp, G.J., Verpoorte, R., Choi, Y.H., Anal. Chem. 85 (2013[b]) 6272-6278.

Dai, Y., van Spronsen, J., Witkamp, G.J., Verpoorte, R., Choi, Y.H., J. Nat. Prod. 76 (2013[c]) 2162-2173.

Dai, Y., Witkamp, G.J., Verpoorte, R., Choi, Y.H., Food Chem. 187 (2015) 14-19.

Daneshjou, S. Khodaverdian, S., Dabirmanesh, B., Rahimi, F., Daneshjoo, S., Ghazi, F., Khajeh, K., J. Mol. Liq. 227 (2017) 21-25.

Espino, M., de los Ángeles Fernández, M., Gomez, F.J., Silva, M.F., Trends Anal. Chem. 76 (2016) 126-136.

Faggian, M., Sut, S., Perissutti, B., Baldan, V., Grabnar, I., Dall'Acqua, S., Molecules 21 (2016) 1531-1542.

Financie, R., Moniruzzaman, M., Uemura, Y., Biochem. Eng. J. 110 (2016) 1-7.

Francisco, M., van den Bruinhorst, A., Kroon, M.C., Green Chem. 14 (2012) 2153-2157.

Fukaya, Y., Iizuka, Y., Sekikawa, K., Ohno, H., Green Chem. 9 (2007) 1155-1157.

Gomez, F.J.V., Espino, M., de los Angeles Fernandez, M., Raba, J., Silva, M.F., Anal. Chim. Acta 936 (2016) 91-96.

González, C.G., Mustafa, N.R., Wilson, E.G., Verpoorte, R., Choi, Y.H., Flavour Fragr. J. 33 (2018) 91-96.

Gowda, D.C., Reuter, G., Schauer, R., Carbohydr. Res. 113 (1983) 113-1241983.

Hayyan, M., Hashim, M.A., Hayyan, A., Al-Saadi, M.A., AlNashef, I.M., Mirghani, M.E. and Saheed, O.K., Chemosphere 90 (2013) 2193-2195.

Hoekstra, F.A., Golovina, E.A., Buitink, J., Trends Plant Sci. 6 (2001) 431-438.

Huang, Y., Feng, F., Chen, Z.G., Wu, T., Wang, Z.H., Food Chem. 244 (2018) 260-265.

Jeong, K.M., Ko, J., Zhao, J., Jin, Y., Yoo, D.E., Han, S.Y., Lee, J., J. Clean Prod. 151 (2017) 87-95.

Jørgensen, K., Rasmussen, A.V., Morant, M., Nielsen, A.H., Bjarnholt, N., Zagrobelny, M., Bak, S., Møller, B.L., Curr. Opin. Plant Biol. 8 (2005) 280-291.

Khodaverdian, S., Dabirmanesh, B., Heydari, A., Dashtban-Moghadam, E., Khajeh, K., Ghazi, F., Int. J. Biol. Macromol. 107 (2018) 2574-2579.

Kranz, M., Hofmann, T., Molecules 23 (2018) 261-273.

Křížek, T., Bursová, M., Horsley, R., Kuchař, M., Tůma, P., Čabala, R., Hložek, T., 2018. J. Clean. Prod. 193 (2018) 391-396.

Kumar, A.K., Parikh, B.S., Pravakar, M., 2016. Environ. Sci. Pollut. Res. 23 (2016) 9265-9275.

Lee, S.H., Doherty, T.V., Linhardt, R.J., Dordick, J.S., Biotechnol. Bioeng. 102 (2009) 1368-1376.

Li, N., Wang, Y., Xu, K., Huang, Y., Wen, Q., Ding, X., Talanta 152 (2016) 23-32.

Liu, X., Ahlgren, S., Korthout, H.A., Salomé-Abarca, L.F., Bayona, L.M., Verpoorte, R., Choi, Y.H., J. Chromatogr. A 1532 (2018) 198-207.

Liu, Y., Zhang, Y., Chen, S.N., Friesen, J.B., Nikolić, D., Choules, M.P., McAlpine, J.B., Lankin, D.C., Gemeinhart, R.A., Pauli, G.F., Fitoterapia 127 (2018) 212-219.

Lores, H., Romero, V., Costas, I., Bendicho, C., Lavilla, I., Talanta 162 (2017) 453-459.

Mamajanov, I., Engelhart, A.E., Bean, H.D., Hud, N.V., Angew. Chem. Int. Ed. 49 (2010) 6310-6314.
Markham, K.R., Gould, K.S., Winefield, C.S., Mitchell, K.A., Bloor, S.J., Boase, M.R., Phytochemistry 55 (2000) 327-336.
Mbous, Y.P., Hayyan, M., Wong, W.F., Looi, C.Y., Hashim, M.A., Sci. Rep. 7 (2017) 41257.
Milano, F., Giotta, L., Guascito, M.R., Agostiano, A., Sblendorio, S., Valli, L., Perna, F.M., Cicco, L., Trotta, M., Capriati, V., ACS Sustain. Chem. Eng. 5 (2017) 7768-7776.
Mondal, D., Sharma, M., Mukesh, C., Gupta, V., Prasad, K., Chem. Commun. 49 (2013) 9606-9608.

Mustafa, N.R., De Winter, W., Van Iren, F., Verpoorte, R., Nat. Protoc. 6 (2011) 715-742.

Nam, M.W., Zhao, J., Lee, M.S., Jeong, J.H., Lee, J., Green Chem. 17 (2015) 1718-1727.

Paiva, A., Craveiro, R., Aroso, I., Martins, M., Reis, R.L., Duarte, A.R.C., ACS Sustain. Chem. Eng. 2 (2014) 1063-1071.

Radošević, K., Ćurko, N., Srček, V.G., Bubalo, M.C., Tomašević, M., Ganić, K.K., Redovniković, I.R., LWT-Food Sci.Technol. 73 (2016) 45-51.

Rozema, E., Van Dam, A.D., Sips, H.C.M., Verpoorte, R., Meijer, O.C., Kooijman, S, Choi, Y.H., RSC Adv. 5 (2015) 61398-61401.

Ruß, C., König, B., Green Chem. 14 (2012) 2969-2982.

Sut, S., Faggian, M., Baldan, V., Poloniato, G., Castagliuolo, I., Grabnar, I., Perissutti, B., Brun, P., Maggi, F., Voinovich, D., Peron, G., Molecules 22 (2017) 1921-1932.
Van Spronsen, J., Witkamp, G.J., Hollman, F., Choi, Y.H., Verpoorte, R., Universiteit Leiden, 2016. Process for extracting materials from biological material. U.S. Patent 9, 441,146.
Vanda, H., Dai, Y., Wilson, E.G., Verpoorte, R., Choi, Y.H., C. R. Chim. 21 (2018) 628-638.

Wei, Z., Qi, X., Li, T., Luo, M., Wang, W., Zu, Y., Fu, Y., Sep. Purif. Technol. 149 (2015) 237-244.

Wikene, K.O., Bruzell, E., Tønnesen, H.H., J. Photochem. Photobiol. B 148 (2015) 188-196.

Wikene, K.O., Rukke, H.V., Bruzell, E., Tønnesen, H.H., 2017. J. Photochem. Photobiol.B 171 (2017) 27-33.
Wink, M., J. Exp. Bot. 44 (1993) 231-246.

Yamaki, S., Plant Cell Physiol. 25 (1984) 151-166.

Yang, T.X., Zhao, L.Q., Wang, J., Song, G.L., Liu, H.M., Cheng, H., Yang, Z., ACS Sustain. Chem. Eng. 5 (2017) 5713-5722.

Yuliana, N.D., Khatib, A., Verpoorte, R., Choi, Y.H., Anal. Chem. 83 (2011) 6902-6906.

Xie, D.W., Wang, X.N., Fu, L.S., Sun, J., Guan, T. and Li, Z.F., J. Triti. Crops, 33 (2013) 746-751.

Zahrina, I., Nasikin, M., Krisanti, E., Mulia, K., Food Chem. 240 (2018) 490-495.
Zhang, H., Wang, Y., Zhou, Y., Xu, K., Li, N., Wen, Q., Yang, Q., Talanta 170 (2017) 266-274.
Zhao, H., Baker, G.A., Holmes, S., J. Mol. Catal B: Enzym. 72 (2011) 163-167.
Zhu, S., Wu, Y., Chen, Q., Yu, Z., Wang, C., Jin, S., Ding, Y., Wu, G., Green Chem. 8 (2006) 325-327.

C H A P T E R 4

Metabolic profiling of Drosera species leaves' biofluids

Henni Vanda [a, b], Natali Rianika Mustafa [a], Robert Verpoorte [a], Peter G. L. Klinkhamer [c], Young Hae Choi [a]

[a] Natural Products Laboratory, Institute of Biology, Leiden University, Sylviusweg 72 2333BE Leiden, The Netherlands

[b] Faculty of Veterinary Medicine, Syiah Kuala University, Banda Aceh, 20111, Indonesia

[c] Plant Ecology and Phytochemistry, Institute of Biology, Leiden University, Sylviusweg 72 2333BE Leiden, The Netherlands

Abstract

Many plants use biofluids as a trap to capture small animals or to hamper herbivory. Also, it serves as medium for enzymes to digest the prey. The aim of this work was to investigate if natural deep eutectic solvents (NADESs) play a role in these processes. Seven *Drosera* species were chosen for this experiment because of the sticky biofluids that contain sugars that can form NADESs. Freeze-drying showed that the biofluids mainly consisted of water. Metabolic profiling approaches based on ^{1}H NMR and GC-MS were performed on the biofluids of the *Drosera* species. The ^{1}H NMR spectra showed a high level of *myo*-inositol and sugars in the biofluids as well as some organic acids. To further confirm the identity of the sugars and to identify some further minor components, GC-MS was employed. All exudates contained sugars and organic acids, such as fructose, glucose, arabinose, sucrose, xylose, glucuronic acid, ascorbic acid, and *myo*-inositol. All compounds known to be strong hydrogen-bond donors. Based on the information from the ^{1}H NMR and GC-MS results, new NADESs compositions were hypothesized and tested, and indeed some formed liquids. However, considering that the biofluids consist mainly of water means that the stable high viscosity droplets typical for the *Drosera* species must be due to other physical-chemical forces, such as gel formation by polysaccharides with calcium ions. A possible role of the compounds found could be in stabilizing proteins, or the formation of biofilms enabling a faster digesting of the prey. The occurrence of some common products found in fermentation processes might indicate that microorganisms were present in the biofluid.

Keywords: *Drosera* exudate, biofluids, natural deep eutectic solvents, sugars, sugar alcohols.

Introduction

Carnivorous plants assimilate part of their nutrients from small animal preys. So far more than 600 species of carnivorous plants are known worldwide. Based on their trapping mechanism they are divided into five groups. Different plant species use different trapping methods, such as pitfall traps, adhesive traps, snap traps, snare traps, and sucking traps. Pitfall traps and snare traps are considered as passive traps, whilst snap traps and sucking traps are active traps. Adhesive traps are classified for both passive and active traps because some adhesive traps are passive, while other traps have the ability to move and envelop the prey. When the traps are empty, they are static, but when the prey is entrapped, the leaves can roll up to hold the insects for digesting (Müller et al. 2006; Barthlott et al. 2007).

Drosera species are probably the most well-known carnivorous plants due to the sticky exudate produced by the glands on the tentacles as the trapping mechanism. Two *Drosera* plant species are shown in Figure 1. There has been quite some research conducted on these plant species, especially related to the mechanisms of trapping insects (Schaefer and Ruxton 2008; Bennet and Ellison 2009; Jürgens et al. 2009); the enzymatic processes in prey digestion (Amagase 1972; Matusikova et al. 2005; Plachno et al. 2006; Libantova et al. 2009); and the secondary metabolites contained in the host plants and their biological activities (Xavier et al. 2010; Zehl et al. 2011, Zanasi et al. 2014). The sticky exudate acts as glue to entrap insects and other small animals. This glue seems like dew on the surface of the tentacles, and makes these plants also called sundew. Small insects are attracted and trapped because of the color and nectary composition of the exudate (Schaefer and Ruxton 2008; Bennet and Ellison 2009). Once the prey is captured, the leaves will roll-up and perform an outer stomach to cover the prey for enzymatic processes.

A B

Figure 1. Photos of typical *Drosera* species. A: *Drosera capensis* L. variety Alba (obtained from www.carnivorousplant.org)**,** B: *Drosera slackii* (Cheek) **(source** www.carnivorousokhomblogspot.com).

The enzymatic process in *Drosera* species starts when the prey is captured by the sticky exudates. The sessile glands will produce digestive enzymes such as esterases, peroxidases, phosphatases, chitinases, and proteases. All these enzymes will smother the prey, digest it, and provide small nutrient molecules for assimilation in the plant. (Matušíková et al. 2005; Barthlott et al. 2007; Takeuchi et al. 2011). The enzymatic processes will take approximately two hours up to two days to digest the prey (Pate and Dixon 1978; Barthlott et al. 2007; Schaefer and Ruxton 2008).

Despite the extensive previous research on the mechanisms for luring and digestion, the basic question on the permanent liquid character of plant exudate is still unanswered. Exudates should remain liquid and should not evaporate easily. Water does not have these characteristics. There must thus be another mechanism that keeps the exudate liquid. In this context NADESs may play a role as an alternative liquid to water or lipids. Previous experiments conducted by Choi et al. (2011) showed that many combinations of ubiquitous natural compounds present at high concentrations in all living cells can form a liquid.

In this study we want to probe our hypothesis that the mixture of components in the *Drosera* exudates are natural deep eutectic solvents (NADESs) which have the necessary physiological properties of being non-volatile and able to dissolve and stabilize enzymes for the essential digestive enzymatic processes. Compared with proteins or enzymes in the exudate, there is little known about the low molecular compounds in the *Drosera* exudates. The presence of compounds like D-mannose, D-galactose, D-xylose, D-glucuronic acid, and L-arabinose was previously reported (Gowda et al. 1982; 1983). Moreover, in the *Drosera capensis* L. biofluid also metal cations e.g. 22 mM Ca^{2+}, 19 mM Mg^{2+}, 0.9 mM K^{+}, and 0.2 mM Na^{+} were found (Rost and Schauer 1977). But the role of these inorganic ions in exudates is not yet known.

For chemical profiling of the exudate, ^{1}H NMR and GC-MS spectrophotometry were employed. ^{1}H NMR was utilized for general profiling of small molecules and GC-MS for targeted analysis based on ^{1}H NMR results.

After profiling and identification of the biofluid, the components were tested in various combinations to see if these can produce a NADESs and thus could be responsible for the permanent liquid phase required for the exudate.

Materials and Methods

Sampling and extraction of Drosera exudates

Drosera exudates were collected at the botanical garden of Leiden University (Hortus Botanicus) the Netherlands, in the period of April-June (2013). Exudates were collected from *Drosera adelae* F. Muell., *D. regia* Stephens, *D. capensis* L. variety Alba, *D. capencis* L. variety Rubra, *D. capensis* L. variety Giant, *D. binata* Labill., and *D. slackii* Cheek. The exudates of these plants were collected in 2 mL-microtubes. The microtubes were prefilled with two solvents, MeOH-aqueous KH_2PO_4 buffer pH 6 (4:1) v/v) or KH_2PO_4 buffer pH 6. When diluted using the buffer, only small amounts of sample were obtained, since the sticky exudate was very difficult to dissolve,

which might be due to resin components which are produced by the glandular hairs (Dell, 1977). With 80% MeOH, more sample was obtained, and this solvent was also able to extract the coloring component in some species. The samples obtained using buffer were freeze-dried overnight, while the samples collected using 80% MeOH were dried by using a speedvac. All the samples were further prepared for analysis by ^{1}H NMR and GC-MS.

Water content in exudates was measured by freezedrying samples of exudates to constant weight in 2 mL-microtubes, and measuring the loss of weight.

^{1}H NMR Analysis

The dried extracts from both methanol and buffer were dissolved in 500 µl of D_2O (KH_2PO_4 buffer, 1 M, pH 6.0) with 0.01% TMSP (w/v) (Kim et al. 2010) followed by ultrasonication for 10 minutes and centrifugation at 13,000 rpm for 10 minutes. An aliquot of 300 µl of sample was transferred to the 3 mm-NMR tube. ^{1}H NMR spectra 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. The resulting spectra were manually phased and baseline corrected, and calibrated to TMSP at 0.0 ppm using Topspin (Bruker).

TMS-Derivatization and GC-MS Analysis

For each sample, 100 µl of pyridine (Sigma, St Louis, MO, USA) and 100 µl of *N,O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) from Sigma (St Louis, MO, USA) were added to the dried extract in a 1 mL-glass vial and heated at 60 ^{o}C for one hour. GC-MS analysis was performed on an Agilent 7890A series gas chromatographic equipped with 7693 autosampler (Agilent Technologies, Santa Clara, CA, USA), a DB5-ms column (30 m x 0.25 mm i.d., 0.25 µm film thickness (Agilent J&W Santa Clara, CA, USA) and a single quadrupole mass spectrometer 5975C. The MS source was set at 230 ^{o}C. The

injector temperature was 60 °C with an injection volume of 1 µl, a split ratio of 1:20 and a carrier gas (He) flow rate of 3 ml/min. The oven temperature program began at 60 °C, with a ramp rate of 10 °C/min. The final temperature set at 300 °C which was held for 1 min resulting in a total run time of 25 min. The mass range analyzed by the mass spectrometer was 50-500 amu. The GC-MS was controlled by Enhanced Chemstation software version E.02.00.493 (Agilent Technologies). The NIST library version 2 (Standard Reference Data Program of The National Institute of Standards and Technology distributed by Agilent Technologies), was used for the signal identification.

Results and Discussion

Plants exudates are composed of various organic substances at high levels. Sugars or their analogues are known as main components. Of the exudate-producing plants, the *Drosera* species excrete an exudate along their tentacles, that functions as a trap for their prey. Despite of all previous research on the exudates the detailed chemical composition is still unclear. To gain insight in the character of the exudate chemical profiling of *Drosera* exudate was performed by ^{1}H NMR based metabolomics, and later followed by GC-MS for further confirmation of the identification of the compounds.

Drosera exudates play several roles in plant survival. Besides the trapping of the prey, proper functioning of enzymatic- and chemical-reactions in the exudate, liquidity is an important issue. Usually the exudates have high water content. The average water percentage ranged from 96 to 98,5% w/w (Figure 2), which makes the exudate suitable for enzymatic processes.

To identify possible NADESs components in *Drosera* exudates, the chemical composition of seven *Drosera* species was analyzed to see if there are any NADESs forming metabolites in common in the species. Chemical analysis was performed by ^{1}H NMR and GC-MS to acquire comprehensive information about the exudate's composition.

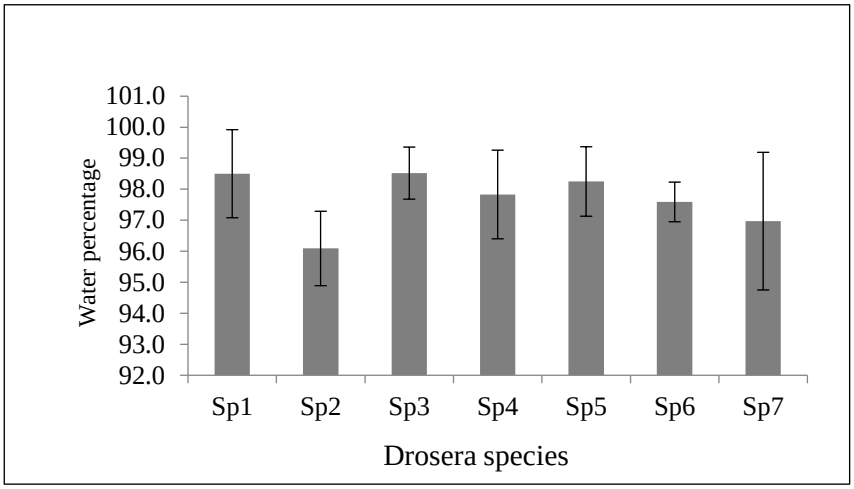

Figure 2. Water percentage (w/w) of seven *Drosera* exudates in triplicate collected in June 2013. Sp1 *Drosera adelae*, Sp2 *Drosera regia*, Sp3 *Drosera capensis* Alba, Sp4 *Drosera capensis* Giant, Sp5 *Drosera capensis* Rubra, Sp6 *Drosera binata*, Sp7 *Drosera slackii.*

^{1}H NMR analysis of Drosera exudates

^{1}H NMR spectroscopy is an attractive chemical profiling technique, because it covers a very broad range of organic compounds, moreover the quantitation of each metabolite is very simple, as for each proton the signal intensity is only dependent on its molar concentration. That means the absolute quantitation of all identified compounds can be achieved with one single internal standard. Due to these advantages ^{1}H NMR metabolomics is the first choice for a macroscopic approach, even though its sensitivity is lower than other analytical methods. But a wide range of primary metabolites including sugars, amino acid, amines and organic acids, i.e. main components of NADESs, are easily analyzed by ^{1}H NMR. To cover a wider range of metabolites, two solvents, aqueous KH_2PO_4 buffer (Figure 3) and MeOH-aqueous KH_2PO_4 buffer (4:1), were used to cover both polar (Figure 3) and medium polar metabolites (Figure 4) in the exudates.

Various NADESs can be formed with different combinations of organic acids, amines, amino acids and sugars. In the aqueous buffer the *Drosera*

exudates studied showed only a few sugars and organic acids as main metabolites, whereas amines and amino acids were not detected in any of the exudates (Fig. 3, Table 1). All the *Drosera* species showed *myo*-inositol as main ingredient. Sucrose and glucose were found in respectively 6 and 5 of the 7 studied species. The exudate of *D. capensis* Rubra was found to have a high level of oligosaccharides. Ethanol was also detected as a major ingredient in all exudates. Acetic-, acetoacetic-, and formic acid, were detected in all the species in varying concentrations. Citric acid and fumaric acid were only found in two and in one species respectively. Malic acid was found in *D. capensis* Giant and *D. slackii* at higher level than other species, at a molar concentration similar to sucrose (ca. 1:1), and in both cases also similar levels as citric acid, which was not found in the other species (Figure 5).

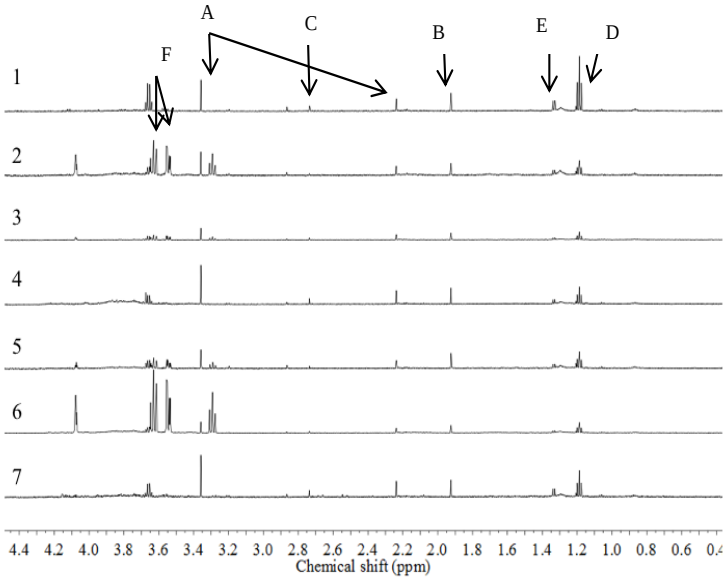

Figure 3. ^{1}H NMR spectra of seven *Drosera* species (600 MHz, KH_2PO_4 buffer containing 0.01% TMSP, w/w) in the rage of δ 0.3 – 4.5. 1. *Droserea adelae*, 2. *Drosera regia*, 3. *Drosera capensis* Alba, 4. *Drosera capensis* Giant, 5. *Drosera capensis* Rubra, 6. *Drosera binata*, 7. *Drosera slackii*
A: acetoacetic acid, B: acetic acid, C: citric acid, D: ethanol, E: lactic acid, F: *myo*-inositol

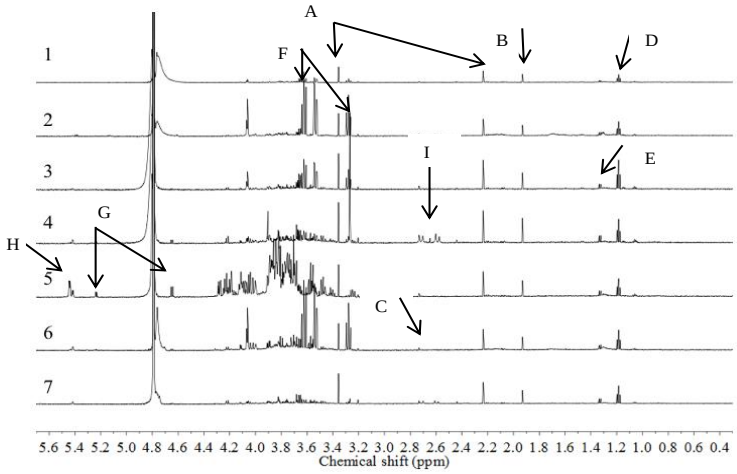

Figure 4. ^{1}H NMR spectra of seven *Drosera* species (600 MHz, CH_3OH-d_4-KH_2PO_4 buffer (8:2, v/v) containing 0.01% TMSP, w/w) in the rage of δ 0.3 – 4.5. 1. *Droserea adelae*, 2. *Drosera regia*, 3. *Drosera capensis* Alba, 4. *Drosera capensis* Giant, 5. *Drosera capensis* Rubra, 6. *Drosera binata*, 7. *Drosera slackii*.
A: acetoacetic acid, B: acetic acid, C: citric acid, D: ethanol, E: lactic acid, F: *myo*-inositol, G: glucose, H: sucrose, I: malic acid.

The presence of ethanol and some organic acids might point to a microbial fermentation in the exudates. All the identified metabolites (Table 1) are candidates for potential NADESs.

Table 1. Detected metabolites of *Drosera* exudates in ^{1}H NMR spectrum [600 MHz, CH_3OH-d_4-KH_2PO_4 buffer (8:2, v/v) containing 0.002% TMSP (w/w)]

Compound	Characteristic Chemical shift in ppm (splitting pattern and coupling constant in Hz)[1]	*Drosera* species						
		Sp 1[2]	Sp 2[3]	Sp 3[4]	Sp 4[5]	Sp 5[6]	Sp 6[7]	Sp 7[8]
Acetoacetic acid)	3.36 (s), 2.24 (s)	+	+	+	+	+	+	+
Acetic acid	1.93 (s)	+	+	+	+	+	+	+
Citric acid	2.71 (d, J = 16.3), 2.59 (d, J = 16.3)				+			+
Ethanol	3.63 (q, J = 7.1), 1.19 (t, J = 7.1)	+	+	+	+	+	+	+
Formic acid	8.5 (s)	+	+	+	+	+	+	+
Fumaric acid	6.76 (s)		+					
Glucose	5.23 (d, J = 3.9), 4.65 (d, J = 8.0)			+	+	+	+	+
myo-Inositol	3.63 (t, J = 9.54), 3.54 (dd, J = 10.1, 3.5),	+	+	+	+	+	+	+
Sucrose	5.42 (d, J = 3.9), 4.64 (d, J = 8.0)		+	+	+	+	+	+
Malic acid	2.6 (d, J = 16.9), 2.7 (d, J =16.9)				+			+
Lactic acid	1.3 (d, J = 5.64)	+	+	+	+	+	+	+
Methanol	3.35 (s)	+	+	+	+	+	+	+

[1] All the chemical shifts were calculated based on 0.00 ppm of TMSP signal, s: singlet, d: doublet, dd: double doublet, t: triplet, m: multiplet
[2]*Drosera adelae*, [3]*Drosera regia*, [4]*Drosera capensis* Alba, [5]*Drosera capensis* Giant, [6]*Drosera capensis* Rubra, [7]*Drosera binata*, [8]*Drosera slackii*. (n=3)

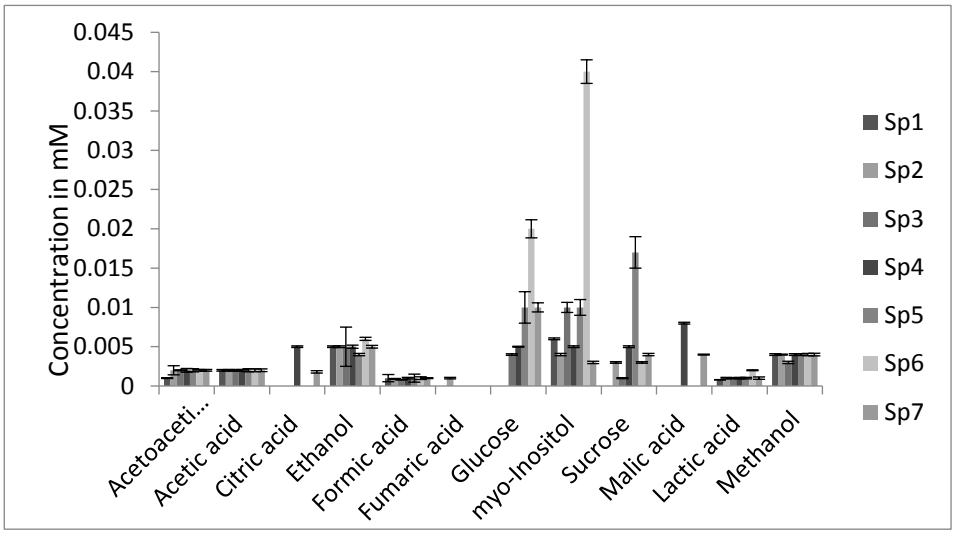

Figure 5.[1]H NMR average quantification of compounds on *Drosera* biofluids. Sp1: *Drosera adelae*, Sp2: *Drosera regia*, Sp3: *Drosera capensis* Alba, Sp4: *Drosera capensis* Giant, Sp5: *Drosera capensis* Rubra, Sp6: *Drosera binata*, Sp7: *Drosera slackii* (n=3 with standard deviation)

GC-MS analysis for Drosera exudates

[1]H NMR spectrometer showed that the main metabolites in *Drosera* species are organic acids and sugars, which might be components of NADESs. To analyze the chemical composition of the exudates in more detail, gas chromatography-mass spectrometry (GC-MS) was employed.

Table 2. Detected metabolites of MeOH and buffer extracts of *Drosera* exudates in GC-MS analysis

Compound	Retention Time	MeOH Extract							Buffer Extract						
		Sp[1]	Sp[2]	Sp[3]	Sp[4]	Sp[5]	Sp[6]	Sp[7]	Sp[1]	Sp[2]	Sp[3]	Sp[4]	Sp[5]	Sp[6]	Sp[7]
Mannose	13.15	+	+	+		+	+	+			+				+
myo-inositol	17.26	+	+	+	+	+	+	+	+	+	+	+	+	+	+ +
Glucose	15.30; 16.16	+	+	+	+	+	+	+						+	
Fructose	14.37		+	+	+	+		+			+				
Sorbose	14.50		+	+		+	+	+							
Arabinose	14.80	+		+	+	+	+	+		+		+		+	
Galactose	15.40	+	+				+	+						+	+
Ascorbic acid	15.85		+							+					
Glucuronic acid	16.25					+			+					+	+
Xylose	16.39		+	+	+	+	+	+	+	+					+ +
Sucrose	21.7	+	+	+	+	+	+	+	+	+			+		

[1]*Drosera adelae* , [2]*Drosera regia,* [3]*Drosera capensis* Alba, [4]*Drosera capensis* Giant, [5]*Drosera capensis* Rubra, [6]*Drosera binata* , [7]*Drosera slackii*

Gas chromatography-mass spectrometry is more sensitive than NMR, however, it is restricted to volatile compounds, requiring derivatization of compounds to make them volatile. Moreover, the sensitivity is quite different for individual compounds. The differences could be orders of magnitude. The separation of the compounds means that it is easier to identify and quantify minor compounds. Though for absolute quantitation calibration curves are needed for each individual compound. To identify minor sugars and to confirm the major ones already identified by NMR, GC-MS was performed. This resulted in the identification of arabinose, fructose, mannose, galactose, sucrose,

glucose, xylose, *myo*-inositol, ascorbic acid, glucuronic acid, and sorbose. In all the exudates, *myo*-inositol was detected as the main carbohydrate in both buffer and MeOH extract by GC-MS. Mannose and glucose were found in all exudates of MeOH extract. In buffer extract, fewer compounds were able to detect, as most of the sugars are bind to resin produced by the glandular hairs of the plants. The non-soluble resin fraction possibly helps keeping up the water content in the exudate and prevents evaporation (Dell, 1977). These results show that all the exudates of the seven species of *Drosera* have very similar sugar compositions. GC-MS confirmed the presence of *myo*-inositol in the exudate of all seven *Drosera* species (Table 2). Previous experiments on *D. capensis* (Rost and Schauer, 1977; Gowda et al 1982) reported that the exudates contained arabinose, mannose, galactose, xylose, and glucuronic acid, which is similar to our experiment.

Natural deep eutectic solvents formation with the ingredients detected in *Drosera* exudates.

From the results it is clear that the exudates consist of more than 95% of water, which makes it unlikely that the NADESs are responsible for the high viscosity of the biofluid. However, the very strong hydrogen bonding capacities of the sugars and some of the acids, might serve other roles, like producing nanoparticles that contain the various enzymes present in the exudate, or to encapsulate proteins that might be more stable than in normal water solutions. To see if any of the ingredients may form NADESs we tested some potential NADESs, which could be formed by the small molecules present in the *Drosera* biofluids.

Myo-inositol was found as the main ingredient in all *Drosera* biofluids both in ^{1}H NMR and GC-MS analysis. Therefore, combinations were designed using this compound with various sugars and acids as found in the *Drosera* samples. As none of the combinations tested did give a liquid, the effect of

non-organic ingredients was explored. As reported before, Ca^{2+} is the main cation found in *D. capensis* and *D. binata* (Rost and Schauer, 1977). Thus $CaCl_2.2H_2O$ was chosen as a potential non-organic NADESs component in combination with *myo*-inositol and sucrose. All these components were mixed together according to Choi et al. (2011) with different molar ratios of *myo*-inositol, $CaCl_2.2H_2O$, and sucrose, as sucrose was detected in six species of MeOH extract. This resulted in complex mixtures with a high-water content, of which, for example, the molar ratio 1:2:2:20 did give a homogenous liquid. The water concentration of this mixture is around 31%. Other combinations were made of a number of sugars in combination with organic acids, which refers to previous experiments by Gowda et al. (1982, 1983). The combinations included arabinose, xylose, galactose, mannose, glucuronic acid, and water with a molar ratio of 3.6:1:4.9:8.4:8.2:80, with a water concentration of about 23%. A different molar ratio 8.4:1:9.6:18.3:17.1:60 gave also a liquid which contained 10% of water. The combinations of compounds for the formation of NADESs are shown in Table 3. The combinations which gave liquids will stay liquid even after freeze-drying. The viscosity of the fluid decreased when more water was added. Sugar-based NADESs usually will have thicker and more viscous consistency (Dai et al. 2013; Hayyan et al. 2013), therefore choline chloride and betaine were chosen for NADESs compositions as they are hydrogen-bond donors and would give less-viscosity of NADESs.

Table 3. Composition and ratios of compounds tested for the formation of NADESs (based on the analysis of the *Drosera* samples: Gowda et al. 1982, 1983, this chapter)

Composition	Mole Ratio	Result
Myo-inositol : sucrose : water	1 : 2 : 5	Solid
Myo-inositol : sucrose : $CaCl_2.2H_2O$: water	1 :2 : 2 : 20	Liquid
D-mannose : glucose : choline chloride : water	1 : 2 : 1 : 2	Liquid
D-mannose : D-glucuronic acid : betaine : water	1 : 1 : 1 : 4	Liquid
Myo-inositol : malic acid : water	1 : 2 : 2	Solid
Myo-inositol : glucose : malic acid : choline chloride : water	1 : 1 : 1 : 2	Solid
Arabinose : D-mannose : *Myo*-inositol : water	2 : 1 : 1 : 5	Solid
Myo-inositol:acetic acid : water	1 : 1 : 5	Solid
Myo-inositol : formic acid : water	1 : 2 : 5	Solid
D-arabinose:D-xylose :D-galactose : D-mannose : D-glucuronic acid:water	8.4 : 1 : 9.6 : 18.3 : 17.1: 60	Liquid
D-arabinose:D-xylose :D-galactose : D-mannose : D-glucuronic acid:water	3.6 : 1 : 4.9 : 8.4 : 8.2 : 80	Liquid

Conclusions

^{1}H NMR and GC-MS were used to analyze metabolites in the exudates of seven *Drosera* species. NMR is a fast, highly selective and reproducible method for analysis of wide range of compounds in a sample. The NMR results were confirmed by the results of GC-MS, which also resulted in the identification of some minor sugars in all exudates. The *Drosera* biofluids contained 95-99% water, and their viscosity and stability are preserved without water loss over prolonged periods. As NADESs seem not to be a primary factor in this special behavior of the *Drosera* biofluids, other explanations need to be explored in future research. The role of the compounds found could be in the attraction of their preys, stabilization of the proteins or the formation of biofilms around the caught preys enabling a faster enzymatic digesting of the prey. Calcium alginate is a well-known example of a gel used to immobilize enzymes or cells in

various biotechnological applications. Taking that as an example the combination of polysaccharide and/or proteins with metal cations might play a role in keeping the *Drosera* biofluids in a highly viscous liquid state. Finally, the occurrence of ethanol, acetoacetic acid, acetic acid and lactic acid as typical products of various fermentation processes may point to the presence of one or more microorganisms in the biofluid.

References

Amagase, S., J. Biochem.72 (1972) 73-81.

Barthlott, W., Porembski, S., Seine, R., and Theisen, I., Portland, Timber Press (2007).

Bennett, K. F., Ellison, A. M., Biol. Lett. 5 (2009) 469-472.

Choi, Y. H., van Spronsen, J., Dai, Y., Verberne, M., Hollmann, F., Arends, I. W. C. E., Witkamp, G.-J., Verpoorte, R., Plant Physiol. 156 (2011) 1701-1705.

Dai, Y., van Spronsen, J., Witkamp, G.J., Verpoorte, R., Choi, Y.H., Anal. Chim. Acta (2013[a]) 766, 61-68.

Dell, B., J. R. Soc. West. Aust. 59 (1977) 119-123.

Gowda, D. C., Reuter, G., Schauer, R., Phytochemistry 21 (1982) 2297-2300.

Gowda, D. C., Reuter, G., Schauer, R., Carbohydr. Res. 113 (1983) 113-124.

Hayyan, A., Mjalli, F.S., AlNashef, I.M., Al-Wahaibi, Y.M., Al-Wahaibi, T. and Hashim, M.A., J Mol. Liq. 178 (2013) 137-141.

Jürgens, A., El-Sayed, A.M. and Suckling, D.M., Funct. Ecol. 23 (2009) 875-887.

Kim, H.K., Choi, Y.H., Verpoorte, R., Nat. Protoc. 5 (2010) 536-549.

Libantová, J., Kämäräinen, T., Moravčíková, J., Matušíková, I., Salaj, J., Mol. Biol. Rep. 36 (2009) 851-856.

Matušíková, I., Salaj, J., Moravčíková, J., Mlynárová, L., Nap, J-P., Libantová, J., Planta 222 (2005) 1020-1027.

Müller, K.F., Borsch, T., Legendre, L., Porembski, S. and Barthlott, W., Plant Biol. 8 (2006) 748-757.

Pate, J., Dixon, K., Aust. J. Bot. 26 (1978) 455-464.

Płachno, B. J., Adamec, L., Lichtscheidl, I. K., Peroutka, M., Adlassnig, W., Vrba, J., Plant Biol. 8 (2006) 813-820.

Rost, K., Schauer, R., Phytochemistry 16 (1977) 1365-1368.

Schaefer, H. M., Ruxton, G. D., Biol. Lett. 4 (2008) 153-155.

Takeuchi, Y., Salcher, M.M., Ushio, M., Shimizu-Inatsugi, R., Kobayashi, M.J., Diway, B., Von Mering, C., Pernthaler, J. and Shimizu, K.K., PLoS One, 6

(2011) e25144.

Xavier, C., Costa, P., Romano, A., J. Med. Plants Res. 4 (2010) 1601-1608.

Zanasi, A., Mazzolini, M., Tursi, F., Morselli-Labate, A.M., Paccapelo, A., Lecchi, M., Pulm. Pharmacol. Ther. 27 (2014) 102-108.

Zehl, M., Braunberger, C., Conrad, J., Crnogorac, M., Krasteva, S., Vogler, B., Beifuss, U., Krenn, L., Anal. Bioanal. Chem. 400 (2011) 2565-2576.

C H A P T E R 5

Stability of enzymes in natural deep eutectic solvents

Henni Vanda [a, b], Natali Rianika Mustafa [a], Robert Verpoorte [a], Peter G. L. Klinkhamer [c], Young Hae Choi [a]

[a] Natural Products Laboratory, Institute of Biology, Leiden University, Sylviusweg 72, 2333 BE Leiden, the Netherlands

[b] Faculty of Veterinary Medicine, Syiah Kuala University, Banda Aceh, 20111 Indonesia

[c] Plant Ecology and Phytochemistry, Institute of Biology, Leiden University, Sylviusweg 72, 2333 BE Leiden, the Netherlands

Abstract

Combinations of a certain natural small molecules e.g. sugars, amino acids, organic acids and bases in certain ratios can form eutectic mixtures, which are known as natural deep eutectic solvents (NADESs). The NADESs may play a role as alternative media to water for many non-water- or lipid-soluble metabolites in organisms. Not only small molecules but also biopolymers such as DNA, RNA, proteins and polysaccharides were shown to be solubilised by NADESs. It is essential that the integrity of these biopolymers be protected in extreme conditions such as drought or sub-zero temperatures. It was hypothesized that NADESs could preserve DNA, RNA and proteins, keeping them in solution when water is lost by the plant due to drought or water is frozen. In this study, the ability of NADESs to stabilize and preserve macromolecules such as proteins (enzymes) was explored. Enzymes are proteins which can denature easily when exposed to high temperatures and pH changes, requiring specific conditions for optimum activity. Proteases were selected as model enzymes to investigate the interaction between proteins and NADESs. Proteases are found, among others, in carnivorous plants playing an important role in the digestion of the trapped insects. The production of these plant proteases is triggered either by the presence of a prey or by stimulating agents such as chitin and jasmonic acid. These two compounds were used in this study to induce protease activity in *Drosera capensis* L. variety Rubra glands. Bromelain is a protease present in the stem and flesh of pineapple fruit. In this study, the activity of both fresh protease from *Drosera* sap and bromelain-containing pineapple juice were determined. Commercially obtained protease and bromelain were used to test the stabilization and preservation of enzymes in NADESs. For this, both protease and bromelain enzymes were stored and kept in NADESs, and their activity was examined over time. NADESs used in this experiment were Na1 (mannose-glucose-choline chloride-water), Na2 (fructose-sucrose-glucose-water), and Na3 (sucrose-betaine-water). The results showed that the NADESs tested in this study could stabilize the

enzymes preserving their activity as shown by their efficiency with substrates, even after 14 days of storage at room temperature. Based on the results it was concluded that certain NADESs might be candidates for enzyme preservation.

Keywords: Enzyme stabilization, proteases, bromelain, NADESs, preservation.

Introduction

Cell metabolism is a complex network of a wide range of different molecular interactions, e.g. signaling, gene activation, transcription, translation, synthesis, catabolism, transport, and regulation. All cells in living organisms synthesize a huge variety of molecules of diverse masses and polarities, ranging from simple molecules, such as sugars, amino acids or nucleotides to their corresponding polymers e.g. polysaccharides, proteins, DNA and RNA. Proteins are in fact the basis of the cellular machinery, functioning as catalysts, signals, receptors, and transporters. While most proteins have a highly specific function some have a more general function as for example in plants where oxidases and proteases play essential physiological roles. Proteases are also involved in the digestion of food in mammals, and even in carnivorous plants. Enzymatic reactions require very specific conditions such as a determined temperature and pH range and the presence of co-factors. This is because the function of a protein is very much dependent on its configuration and conformation. Thus, changes in the inter- and intracellular conditions of cells may damage the proteins resulting in the loss of activity. This might eventually result in cellular death.

Under extreme conditions, such as drought and cold, resistant organisms appear to form a liquid phase consisting in low-molecular weight organic compounds to store and protect essential macromolecules such as proteins and cellular membranes from decomposition and damage, respectively. In methanogenic organisms (Archaea), cyclic-2,3-bisphosphoglycerate is produced, whilst di-*myo*-inositol-phosphate is found in *Pyrococcus woesei*, *Metanococcus igneus*, and *Pyrococcus furiosus* as thermoprotectants in high temperatures. Di-*O*-β-mannosyl-di-*myo*-inositol-phosphate and di-*myo*-inositol-phosphate are found at supra optimal growth temperatures (8 ^{o}C above optimal growth) in *Thermatoga maritima* and *Thermatoga neapolitana*. In thermophilic bacteria, 2-*O*-β-mannosylglycerate is accumulated as a protectant when submitted to hot thermal and osmotic stress (Rudnick et al. 1990; Martins et al.

1996; Ramos et al. 1997). Mannosylglycerate has been evaluated as a cryopreservation agent for the freeze-drying of enzymes isolated from mesophilic (moderate temperature), thermophilic, and hyper thermophilic resources. The results indicated that mannosylglycerate effectively stabilizes enzymes (Ramos et al. 1997). Trehalose is also effective for the preservation of dehydrated enzymes without loss of activity after prolonged storage (Colaco et al. 1992).

The existence of a third type of liquid phase in nature (cells) has been hypothesized by Choi et al. (2011). This phase could have various functions that cannot be explained in an aqueous or lipid phase. These hypothetical solvents were named natural deep eutectic solvents (NADESs). They are composed of various compounds mixed in specific molar ratios, e.g. sugars, polyalcohols, amino acids, organic acids, and organic bases (Dai et al. 2013; Paiva et al. 2014; Vanda et al. 2018). It has been suggested that NADESs play an important role in the survival of plants during drought, desiccation or cold conditions as well as constituting a media for the biosynthesis and storage of non-water soluble metabolites. Among other properties, may be able to stabilize enzymes in nature. Much research has been reported on enzymatic activities in synthetic ionic liquids or deep eutectic solvents. Park and Kazlauskas (2003) reported that hydrolase and oxidoreductase types of enzymes retain their activity in ionic liquids, and Ninomiya et al. (2012) showed that ionic liquids can be applied in the enzymatic hydrolysis of cellulose. Deep eutectic solvents are also suited for enzymatic reactions, for example in epoxide-hydrolysis (Lindberg et al. 2010), transesterification of cross-linked subtilisin and α-chymotrypsin (Zhao and Baker, 2013). Other examples of recent uses of NADESs as media for various enzymatic reactions, are biodiesel synthesis (Durand et al. 2013), saccharification of rice straw (Kumar et al. 2016), and whole-cell biocatalysis of isoeugenol to vanillin in *Lysinibacillus fusiformis* (Yang et al. 2017). The use of NADESs as a stabilizing solvent was reported for chondroitinases ABCI, a clinical enzyme for treating spinal lesions (Daneshjou

et al. 2017).

Insect-eating plants are very interesting organisms. Either alone or in symbiosis with microorganisms, the plant is able to use insects as its energy source. The plant exudate attracts the prey. Once the prey is trapped, digestive enzymes are produced and released into the exudate. These enzymes, mostly proteases, are active for days, digesting the prey. Protease production can also be induced by simulating the presence of a prey with agents such as chitin, gelatin, or salicylic acid, which mimic the presence of a prey (Matušíková et al. 2005). In this study, the stimulating effect of jasmonic acid was tested as it acts as a plant hormone. There are two systems for attracting the prey. One of them occurs in beaker-like flowers that have a liquid exudate that contains the enzymes that digest the insect. If pure water were the basis of the liquid exudate, its stability could be problematic, due to the evaporation of the water when exposed to the high ambient temperatures or to the sunshine. However, were the exudate a water-containing NADESs, this would not occur.

The other mechanism for trapping insects is with a glue on the surface of leaves. For example, in *Drosera* species, the glue must maintain its liquid state on the leaves in all conditions, pointing to the option of this exudate being a NADESs. Both types of carnivorous plants might thus combine proteases as digestive enzymes and NADESs components to solubilise the enzymes and possibly the prey. This is an interesting model for further studies.

Proteases have been investigated by researchers for centuries due to their important roles in biological systems, especially related to digestive purposes, extracellular modelling, protein-protein interaction, processing of cellular information, and many more. Proteases are present in all living organisms, having important physiological functions such as the digestion of proteins and more specifically, in regulated processes such as blood coagulation, fibrinolysis, and transport of secretory proteins across membranes (Neurath 1984).

Proteases can be found in bacteria, algae, viruses, plants, and animals. In carnivorous plants, proteases break down the digestible material from the prey

to smaller molecules that can be absorbed by the plants. In *Drosera* plants, proteases are produced in the glandular hairs, where the prey (an insect usually) is captured by the sticky mucilage produced by these glands in the tentacles of the leaves. After the insect is trapped, the leaf will roll-up, enveloping the prey, and proteolytic enzymes will subsequently be released for its digestion (Król et al. 2011; Poppinga et al. 2010); Nakamura et al. 2013). Several enzymes have been detected in *Drosera* e.g. proteases, acid phosphatase, chitinase, peroxidase, and esterase. The process of digestion and absorption of the prey takes approximately 48 hours (Amagase 1972; Matušíková et al. 2005; Libantová et al. 2009; Król et al. 2011). The sap from the fruits of A*nanas comosus* (L.) Merr. was used in our study, as a model. Pineapple fruit are well-known to contain bromelain, a protease which has various medicinal and food applications. Bromelain is used as an anti-inflammatory agent, and in recent years, researchers have also investigated its possible anti-cancer activity. Bromelain has been proved to stimulate autophagy activity in mammary carcinoma cells (Bhui et al. 2010). It shows anti-proliferative and pro-apoptotic effects in colorectal carcinoma cells (Romano et al. 2014), and significantly inhibits cell proliferation in human gastrointestinal carcinoma cell lines. In combination with *N*-acetylcysteine the effect was found to be increased (Amini et al. 2014). Bromelain was thus used as a model for proteases in our studies.

To investigate the possible role of NADESs, particularly in enzyme activity, sugar-based NADESs were employed. Several sugars, including mannose, galactose, xylose, arabinose, and glucuronic acid have been detected in *Drosera capensis* exudates by Gowda et al. (Gowda et al. 1982; 1983), and our group has detected acetoacetic acid, acetic acid, *myo*-inositol, glucose, sucrose, lactic acid using ^{1}H NMR, and mannose, fructose, arabinose, galactose, and glucuronic acid using GCMS. All these components are possible NADESs constituents. If proteases were dissolved in NADESs, these could contribute to capture the prey because of their stickiness, and also provide suitable conditions for the enzymatic reactions. We thus hypothesized that given the chemical

composition of both pineapple sap or the sap of carnivorous plants, these could actually be natural deep eutectic solvents (NADESs) and could stabilize enzymes such as proteases in nature.

NADESs are a new generation of natural ionic liquids. Ionic liquids have been a hot topic in research when attempting to reduce or replace toxic organic solvents used in chemical syntheses (Abbot et al. 2004). There are also numerous examples of their use as media for enzymatic reactions such as the enzymatic transesterification of triglyceride with methanol for biodiesel (Zhao and Baker 2013), the hydrolysis of cellulose (Ninomiya et al. 2012), the lipase-catalyzed epoxidation and Baeyer-Villiger oxidation (Kotlewska et al. 2011). However, many synthetic components of ionic liquids are actually relatively toxic (Zhao et al. 2007; Romero et al. 2008). NADESs on the other hand, can be considered as "green" solvents because their components are found commonly in our food, their production is sustainable and they are easily biodegradable. Toxicity and cytotoxicity of some NADESs have been assessed, showing that NADESs have a lower toxicity and cytotoxicity profile even compared to DES (Mbous et al. 2017; Hayyan et al. 2013).

Thus, NADESs are promising alternative solvents to the synthetic ILs. Their application to enzymatic reactions however, has scarcely been evaluated. In the search for evidence of the role of NADESs as a third liquid phase in cells for the storage of enzymes, for example, or in the regulation of enzyme activity we considered that the insect-trapping plant system could be an interesting target for this.

The aim of this study was to determine if NADESs plays any role in stabilizing and preserving proteases enzymes as shown in the prey-digesting process in *Drosera capensis* and in pineapple sap. To answer this question, the activity of protease from different parts of the prey-catching leaves, and the activity of bromelain from pineapple juice was determined.

The commercially available *Streptomyces griseus* protease and the pineapple protease, bromelain, were used as models in the study of activity and

stability in sugar-based NADESs applications. The stabilizing and activity-preservative capacity of NADESs as a media for enzymes were studied to prove that they might function as a third liquid phase in cells for the storage of enzymes and in the regulation of enzyme activity.

Materials and Methods

Plant materials

Drosera capensis L. variety Rubra was collected at Hortus Botanicus Leiden and *Ananas comosus* (L) Merr. fruits were purchased from a local store in Leiden during spring time.

Chemicals

D-fructose, D-glucose monohydrate, and sucrose were purchased from Boom B.V. (Meppel, The Netherlands), and D-mannose, choline chloride, betaine, and jasmonic acid from Sigma Aldrich (The Netherlands). Protease (isolated from *Streptomyces griseus*) and bromelain (from pineapple) were purchased from Sigma Aldrich (catalog number of P8811 and B4882, respectively). A protease-kit was acquired from Pierce Biotechnology (Thermo Fisher Scientific).

Determination of protease activity in Drosera materials

The protease production in the tentacles of *D. capensis* L. Rubra was induced by spreading 0.5-1.0 mg of chitin powders on the surface of leaves for 24 hours (Matusikova et al. 2005). Leaves without any treatment were used as a negative control. Jasmonic acid, a plant hormone, was used as a positive control by placing one drop of jasmonic acid solution (10 mg/mL in ethanol 40% v/v) on the leaf surface. After 24 hours, the remaining inducer was removed by gently flicking the leaves. Samples of leaves, hairs (tentacles) and exudate were taken from three leaves of *D. capensis* L. Rubra plants, (n = 3) collected separately in 2 mL-micro-tubes. After harvesting exudates (approximately 60

µL), 100 µL of glycine-HCL buffer was added to each sample to have a sufficient volume for measurement. After collecting the exudates, the tentacles were harvested after rinsing twice with buffer solution to remove any residual exudate, and subsequently removed from the leaves using a scalpel. The tentacle- and leaf materials (10 mg) were ground and extracted with 150 µL buffer. These buffer extracts were kept for further experiments. The presence of protease in the samples was analyzed following a method described by Bohak (1970) and Matušíková et al. (2005). A volume of 150 µl of each sample was incubated with 150 µl of substrate (2% bovine serum albumin (BSA) in 200 mM glycine-HCl pH 3.0) at 37ºC for one hour after which trichloroacetic acid (450 µL) was added to stop the reaction. Samples were left on ice for 10 min and subsequently centrifuged at 20.000 g for 10 min. The absorbance of the supernatant was measured at 280 nm with reference to the blank (buffer only). One unit of enzyme activity is defined as the amount of enzyme required to cause a unit increase in absorbance at 280 nm (Bohak 1970; Matusikova et al. 2005).

Determination of protease activity after storage of the enzyme in natural deep eutectic solvents (NADESs)

In this experiment, the ability of some NADESs to dissolve and stabilize protease (P-8811) from *Streptomyces griseus, (*Sigma Aldrich, St. Louis, MO, USA) was determined by measuring the activity of the enzyme after storage in different NADESs during 2 weeks (0, 1, 7, and 14 days). The NADESs chosen in this study contained some compounds that were found in *Drosera* exudate and in pineapple sap, such as mannose, sucrose, glucose, and fructose, thus resembling the NADESs proposed to exist in nature. The NADESs were prepared as described by Dai et al. (2013), with a water content that varied between 30%, 40%, and 50% (w/w) for each NADESs. The composition of the tested NADESs were as follows: Na1 (mannose-glucose-choline chloride, 1:2:1, mol/mol), Na2 (fructose-sucrose-glucose, 1:1:1, mol/mol), Na3 (sucrose-

betaine, 1:2, mol/mol), buffer was used as positive control.

The test solutions were prepared by mixing 1 ml of each NADESs with 1 mg of protease in a 2 mL-plastic microtube. The mixtures were homogenized using a vortex until complete dissolution of the enzyme in the NADESs. If necessary, mixtures were additionally sonicated for 2-3 minutes. The mixtures were subsequently stored at room temperature (RT) in the day light for 14 days. Sampling followed a time course (0, 1, 7 and 14 days), and the enzyme activity was evaluated using the previously described method.

Determination of protease activity after storage in low-water containing NADESs

This experiment aimed to investigate the effect of low-water containing NADESs on the preservation of protease activity. The enzyme (1 mg) was first dissolved in a water-containing NADESs (40% water, w/w), after which the solutions were freeze-dried for 24 hs to eliminate the water. Sampling of this enzyme-NADESs solution was done by weighing, instead of pipetting, due to the high viscosity of the mixture. Protease activity of the sample was determined using the same protocol as described previously on days 0, 1, 5, and 7.

Determination of bromelain activity of pineapple juice

Pineapple juice was collected by expression of the fresh fruit, and directly used for bromelain activity test using the protease activity test.

Metabolic profiling of pineapple juice by ^{1}H NMR

Pineapple was peeled, cut in small pieces and crushed to obtain juice that was then freeze-dried for 48 hours. The dried juice (20 mg DW) was dissolved in 500 µl of deuterated solvent (D_2O KH_2PO4 buffer 1 M, pH 6) with 0.005% TMSP (w/v) (Kim et al. 2010). The samples were ultra-sonicated for 15 minutes followed by centrifugation at 13.000 rpm for 10 minutes. An aliquot of 300 µL of each sample was transferred to a 3-mm NMR tube and their ^{1}H-NMR

spectra were recorded at 25 ºC on a 600 MHz Bruker DMX-600 spectrometer (Bruker, Karlsruhe, Germany). The resulting spectra were manually phased and baseline corrected, and calibrated to TMSP at 0.0 ppm using Topspin (Bruker).

Determination of bromelain activity after the storage of the enzyme in natural deep eutectic solvents (NADESs)

Solutions containing 1 mg of bromelain powder (B4882 from pineapple stem, Sigma Aldrich) in 1 ml of NADESs with different water concentrations (30%, 40%, and 50% w/w) were prepared. The NADESs used for this experiment were the same as those used for the protease analysis, that is: Na1 (mannose-glucose-choline chloride, 1:2:1, mol/mol), Na2 (fructose-sucrose-glucose, 1:1:1, mol/mol), Na3 (sucrose-betaine, 1:2, mol/mol). The samples were stored at room temperature in the day light, and tested on day 0, 1, 7 and 14 after preparation for their bromelain activity using the Protease Assay Kit (Thermo-scientific USA).

Determination of bromelain activity after the storage of the enzyme in low water-containing NADESs

This experiment was conducted to examine the effect of low water-containing NADESs on the preservation of bromelain enzymatic activity. For this, 1 mg of bromelain powder was dissolved in 1 ml of the different NADESs (containing 40% water), after which the solutions were freeze-dried for 24 hs to eliminate the water. The samples were stored at room temperature in day light, and collected on day 0, 1, 5, and 7 days after freeze-drying to determine bromelain activity as described previously.

Microscopic Observation

This experiment aimed at examining a possible effect of microorganism growth on the enzyme-NADESs mixture observed in the samples. Mixtures of protease-NADESs and bromelain-NADESs were incubated at 37 ºC for seven

days, using a buffer-enzyme mixture as a positive control and NADESs and buffer samples without enzyme as controls. On day 3 and day 7, 300 uL of all samples were pipetted and transferred separately in 8-well plates and observed under a Zeiss Observer laser scanning microscope equipped with fluorescence filters at x63.

Results and Discussion

This study was carried out to analyze the preservation and stabilization of proteases enzymes in NADESs. Protease activity in the exudate of *D. capensis* L. Rubra after induction with chitin and jasmonic acid, as a model for the prey-digesting processes was determined, as well as bromelain activity in pineapple sap. Additionally, the stability and activity of *Streptomyces griseus* protease (SG-protease) and bromelain (from *Ananas comosus* (L.) Merr.) stored in several NADESs were also examined.

For this, the leaves of the plant were treated with chitin or jasmonic acid (JA) to mimic the prey-catching process, allowing the study of the following digestion of the prey. The levels of protease activity in the *Drosera* exudates and tentacles after a chitin induction were similar to the negative controls (without chitin induction), whilst in the samples treated with JA, the levels were much higher. No protease activity was detected in the leaf material (Figure 1).

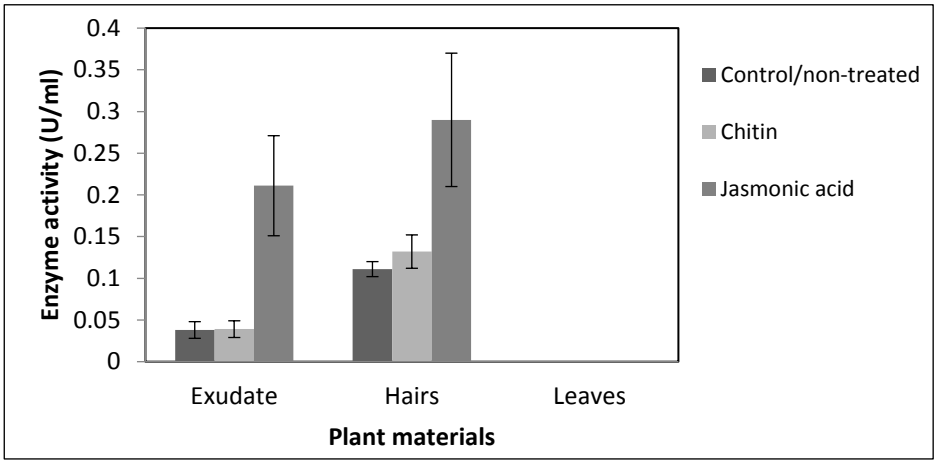

Figure 1. Protease activity of *Drosera capensis* L. variety Rubra leaves exudate induced with chitin and jasmonic acid.

Jasmonic acid is a plant hormone which is involved in the bending movement of the tentacles of *Drosera*, that signals the initiation of the prey-digesting stage (Nakamura et al. 2013). When the plant is induced with insect or insect-derived oral secretion, JA production increases, inducing both the plant defense system against wounding and the prey-digesting process (Mithöfer et al. 2014). Thus, the presence of a prey on a leaf stimulates leaf-bending to fully trap the prey and allow the subsequently produced and excreted enzyme to digest the prey. When JA is used as an induction agent, it triggers the transcription of the gene encoding proteases (Herrmann et al. 1989; Mueller 1997). The JA treatment is thus a useful model system to study the prey-digesting process. The next question to be answered was whether NADESs could play a role in the digesting process itself. The role could be two-fold, i.e., to act as a stable medium for prey-digestion, stabilizing the enzymes and ensuring that the medium does not dry up under the ambient conditions including strong sunlight and heat. The other function is to dissolve the prey, particularly to solubilize the macromolecules, making them available for the digesting process.

To study the stability of proteases, we used two commercially available

proteases, one from *Streptomyces griseus* (SG-protease) and bromelain from pineapple fruit. To validate our protease assay we determined the bromelain in fresh pineapple juice (Figure 2). The ^{1}HNMR spectra of the juice, revealed the presence of sucrose-glucose-fructose in about equal amounts. This is, clearly, the basis of a NADESs in the otherwise aqueous juice.

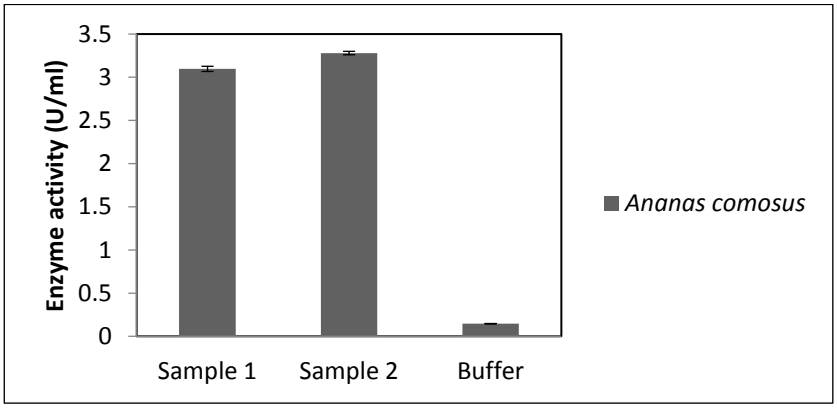

Figure 2. Bromelain Activity in *Ananas comosus* L. Merr. juice.

The SG-protease, dissolved in all three tested NADESs with different levels of water, was relatively stable after 14 days of storage at room temperature and in the daylight, showing high activity (Figure 3). In the buffer solution of the protease, enzymatic activity was significantly higher than in NADESs Na1 and Na3 on day 0 and 1. However, no activity was detected after 14 days.

Interestingly NADESs Na1 (30%, 40% water) and Na3 (50% water) showed a significantly strong increase in activity after day 1 ($p<0.05$), and stayed high until day 14. Comparing the three NADESs, the Na2 type show highest activities. The more concentrated Na3 type seems to have lowest activity, though with 50% of water after 1 day the activity raises to similar levels as the others.

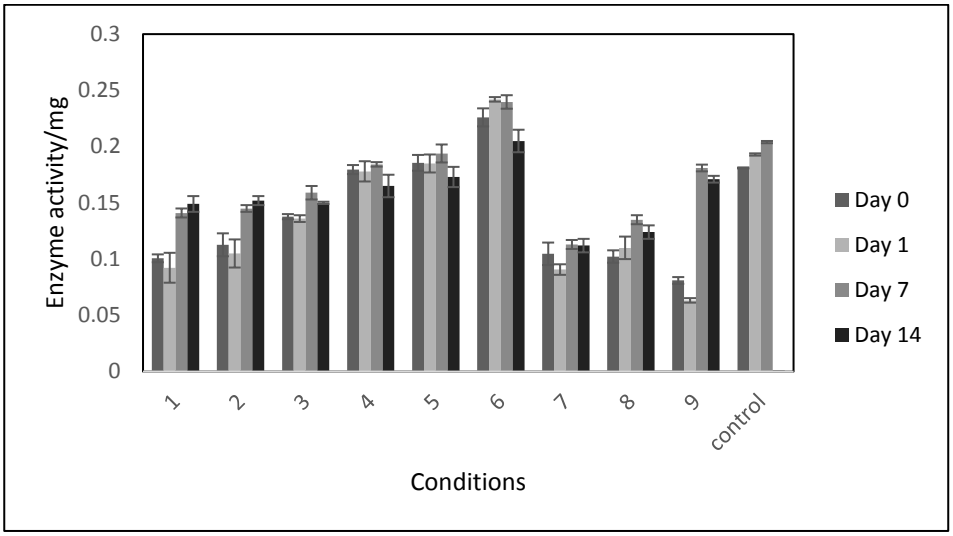

Figure 3. The effect of water on the preservation of SG-protease in three NADESs with different water content stored under ambient conditions in daylight. 1-3: Na1 (mannose-glucose-choline chloride, 1:2:1, mol/mol) with 30%, 40%, 50% water (w/w), 4-6: Na2 (fructose-glucose-sucrose, 1:1:1, mol/mol) with 30%, 40%, 50% water (w/w), 7-9: Na3 (sucrose-betaine, 1:2, mol/mol) with 30%, 40%, 50% water (w/w).

In order to determine whether the presence/absence of water in NADESs influenced the stability of the enzyme, the enzyme was dissolved in the NADESs with 40% water and then freeze-dried to remove the water. After freeze-drying, the enzyme-NADESs solutions became very viscous and sticky, except for Na3, which still had a low viscosity. The most viscous and sticky solution was Na2. The freeze-dried Na3 preparation had the highest activity among the NADESs and was still active at day 7. The lowest activity was observed for the Na2 solution of SG-protease, which had the most viscous consistency. The level of protease activity in all NADESs was lower than that determined for the same NADESs containing water (Figure 4). The highest activity was 0.127 U/mg enzyme as indicated in Na3. It was thus concluded that the higher concentration of NADESs ingredients could affect the activity in the protease assay.

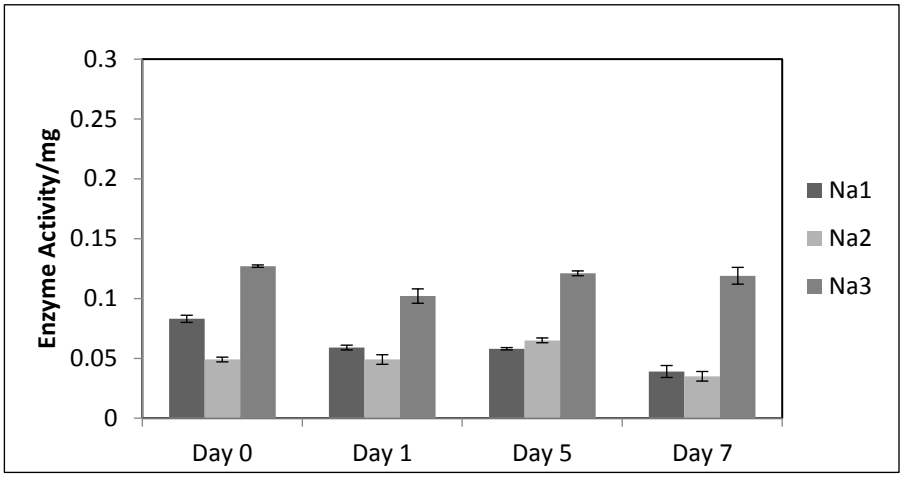

Figure 4. The stability of freeze-dried low water containing NADESs with SG-protease stored at room temperature in the light during 7 days, as measured by enzymatic activity.

^{1}H-NMR-based metabolomics was performed on dried pineapple juice. The ^{1}H-NMR showed that the main compounds were fructose {4.16 (d), 4.01 (d), 4.0 (t), 3.7 (d) ppm}, sucrose {5.42 (d), 3.7 (s), 3.6 (s), 3.5 (dd) and 3.4 (t) ppm}, and glucose {5.23 (d), 4.6 (d), 3.9 (dd), 3.8 (m), 3.2 (m) ppm} (Figure 5) in approximately equal molar quantities, i.e. the basis of a NADES.

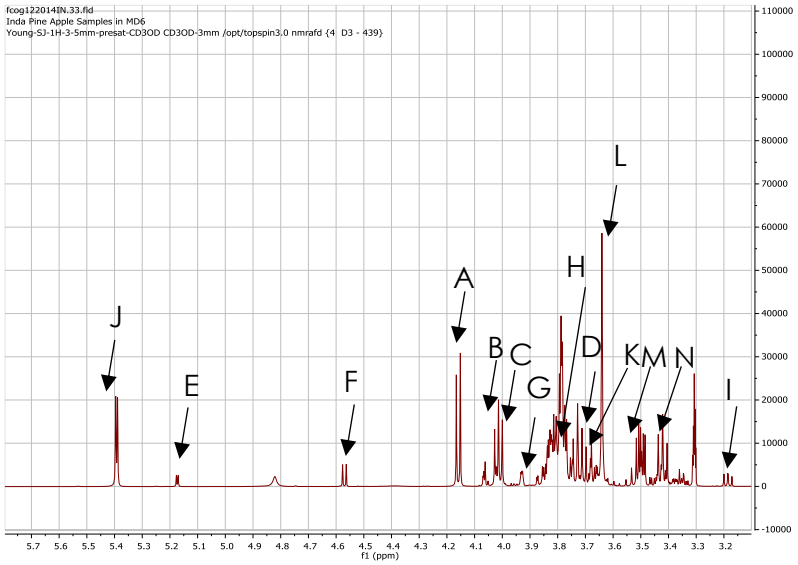

Figure 5. [1]H NMR spectra of pineapple juice. Chemical shifts for fructose are at A. 4.16 (d), B. 4.01 (d), C. 4.0 (t), D. 3.7 (d) ppm; glucose at E. 5.23 (d), F. 4.59 (d), G. 3.9 (dd), H. 3.8 (m), I. 3.2 (m) ppm; and sucrose at J. 5.42 (d), K. 3.7 (s), L. 3.6 (s), M. 3.5 (dd) and N. 3.4 (t) ppm

Commercial bromelain was dissolved in water-containing NADESs and the activity of the enzyme was measured at defined time points during a 14-day storage period at room temperature in the daylight. Activity was measured with a Protease Assay Kit (Thermo-scientific, USA). The highest bromelain activity was detected for reactions in buffer solutions and Na1 with 50% of water on day 0, decreasing significantly throughout day 1-14 ($p<0.05$). For 30% of water it was Na2 that gave the highest activity. In Na1 30%, day 0 and 1 exhibited high activity, decreasing significantly at days 7 and 14 ($p<0.05$). In Na3, the activity was also the highest on day 0, decreasing significantly after day 1. For all NADESs, the enzymatic activity decreased during storage, but in the case of the buffer no activity at all was observed at day 14 (Figure 6). It is important to note that the buffer solution was heavily contaminated with microorganisms. To measure the effect of a concentrated NADESs, water-containing solutions of bromelain in the three types of NADESs were freeze-dried. The enzyme activity in these low water-containing NADESs solutions was monitored during a 7-day

storage period in ambient conditions and exposed to daylight. At all measuring points, Na3 solutions exhibited the highest activity, whereas Na2 showed the lowest activity at day 0 and no activity on day 7 (Figure 7).

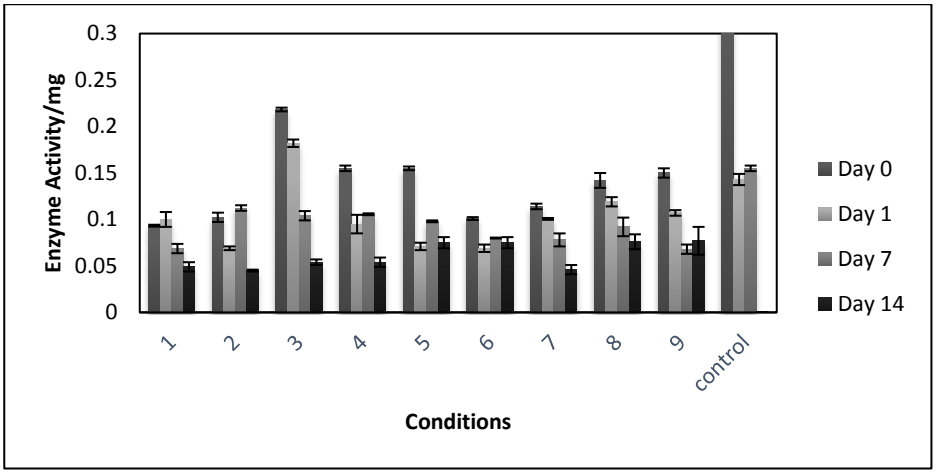

Figure 6. The effect of water on the preservation of bromelain enzymatic activity in three NADESs solutions vs an aqueous solution of bromelain. 1-3: Na1 (mannose-glucose-choline chloride, 1:2:1, mol/mol) with 30%, 40%, 50% water (w/w), 4-6: Na2 (fructose-glucose-sucrose, 1:1:1, mol/mol) with 30%, 40%, 50% water (w/w), 7-9: Na3 (sucrose-betaine, 1:2, mol/mol) with 30%, 40%, 50% water (w/w).

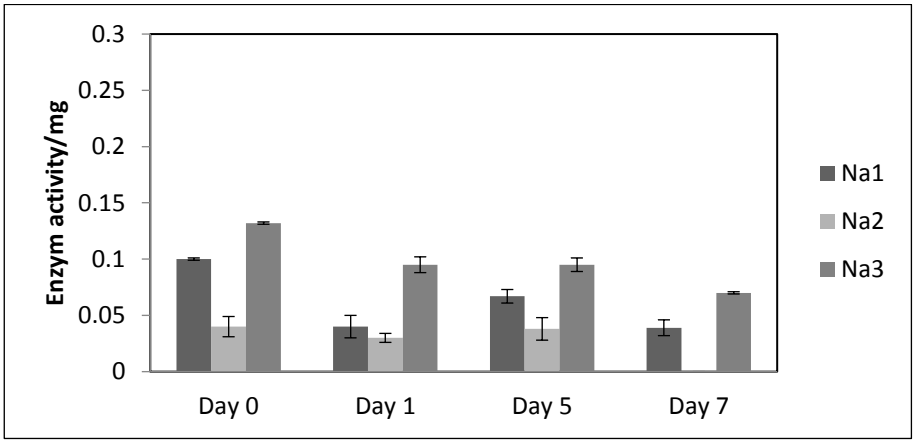

Figure 7. The ability of some low water-containing NADESs to stabilize bromelain stored at room temperature in daylight during a time course, determined by it enzymatic activity.

Microscopy

Microscopy was used to determine microbial growth in NADESs-enzyme and buffer-enzyme solutions. The solutions of SG-protease were analysed on day 3 and 7 after their preparation. Microscopic observation of all the NADESs-protease mixtures on day 3 and 7 showed the absence of any microbial growth. However, a high bacterial count was registered in the buffer-protease solution on day 3 and after 7 days, the count had increased significantly, and fungi and yeast were also detected.

Microscopy of all NADESs-bromelain mixtures did not show any microorganism growth on day 3. However, a small number of bacteria were observed in Na1 with 30% water on day 7. On day 7, bacteria and also fungi were observed in all NADESs-bromelain solutions containing 50% water, while in buffer solutions, there were large amounts of microorganisms including bacteria, fungi, and yeast.

No microorganisms were detected in NADESs containing no enzymes, only some possible microorganism debris was detected in Na1 and Na2. Some fungi were detected in the buffer solutions containing enzymes, proving that this buffer was a favourable media for microbial growth (Figure 8-9). It also shows that enzymes in NADESs provided a good source of nutrients for microorganism growth.

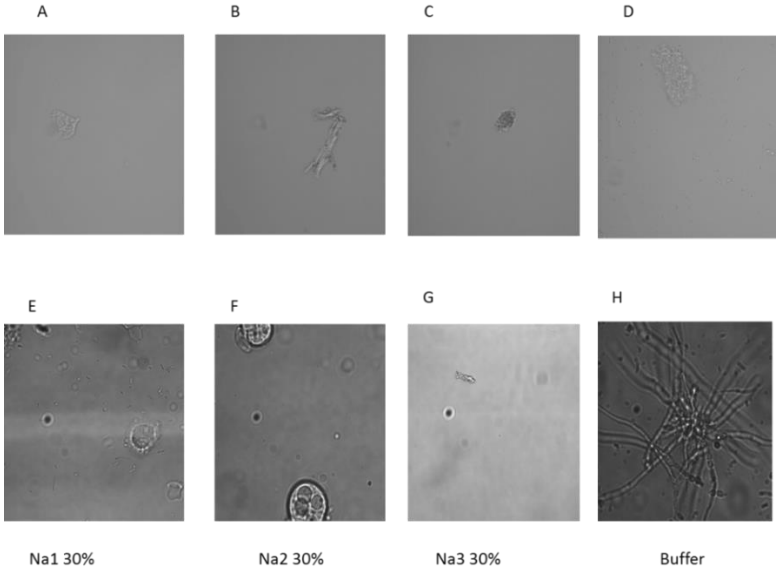

Figure 8. Microscopic observation of SG-protease and bromelain dissolved in three NADESs containing 30% of water (w/w) stored at room temperature. A-D: day 3. E-H: day 7. A: SG-Protease in Na1, B: SG-protease in Na2, C: SG-protease in Na3, D: SG-protease in buffer, E: bromelain in Na1, F: bromelain in Na2, G: bromelain in Na3, H: bromelain in buffer.

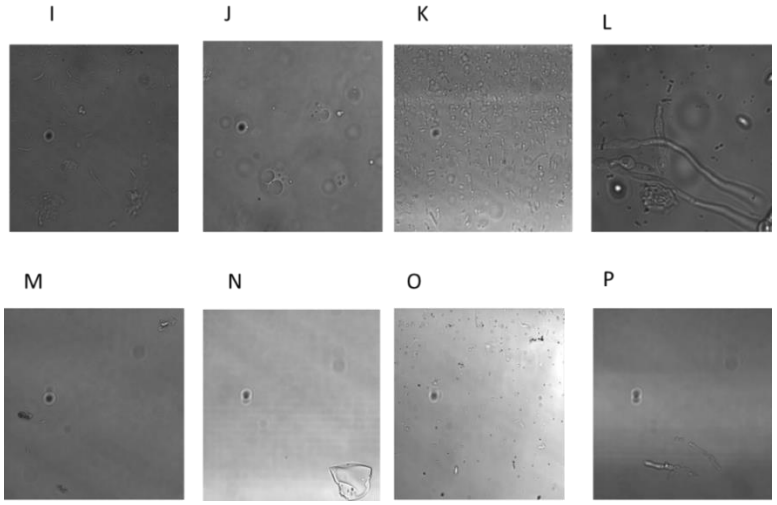

Figure 9. Microscopic observation of bromelain dissolved in three NADESs containing 50% of water (w/w) stored at room temperature on day 7. I: bromelain in Na1, J: bromelain in Na2, K: bromelain in Na3, L: bromelain in buffer. As control, NADESs with 50% water (w/w) without enzyme, M: Na1, N: Na2, O: Na3, P: buffer.

Conclusions

Protease enzymes are produced by plants for specific functions, e.g. in catalysis reactions, as receptors, in degradation processes, and other cellular metabolisms. To evaluate the potential role of NADESs in preserving and stabilizing proteases enzymes, the stability and activity of two known proteases in NADESs solutions was studied.

All NADESs composed of mannose-glucose-choline chloride-water (Na1), fructose-sucrose-glucose-water (Na2) and sucrose-betaine-water (Na3) with different percentages of water stabilized SG-protease. The highest activity was found in Na2 with 50% of water. Bromelain showed the best activity in Na1 with 50% of water. The SG-protease appeared to be more stable in NADESs than bromelain, since this enzyme lost more than 50% of its activity in 14 days. All NADESs performed better in stabilizing the enzymes than the conventional buffer tested, possibly due to the almost inexistent or total absence of microorganisms in the case of NADESs, whereas the buffer solution showed a high microorganism load after 14 days and the enzymes were totally inactive.

The results do thus point to a stabilizing effect and preservation of enzymatic activity in water-diluted NADESs supporting the hypothesis of the role of NADESs in plants. Further experiments with the native enzymes of the plant in an exudate-mimicking NADESs to evaluate the role of NADESs in other organisms are needed to further prove this hypothesis.

References

Abbott, A.P., Boothby, D., Capper, G., Davies, D.L., Rasheed, R.K., J. Am. Chem. Soc. 126 (2004) 9142-9147.

Amagase, S., J. Biochem.72 (1972) 73-81.

Amini, A., Masoumi-Moghaddam, S., Ehteda, A., Morris, D.L., J. Exp. Clin. Cancer Res. 33 (2014) 92.

Bhui, K., Tyagi, S., Prakash, B., Shukla, Y., Biofactors 36 (2010) 474-482.

Bohak, Z., Methods Enzymol. 19 (1970) 347-358.

Choi, Y.H., van Spronsen, J., Dai, Y., Verberne, M., Hollmann, F., Arends, I.W.C.E., Witkamp, G.-J., Verpoorte, R., Plant Physiol. 156 (2011) 1701-1705.

Colaco, C., Sen, S., Thangavelu, M., Pinder, S., Roser, B., Nat Biotechnol 10 (1992) 1007-1011.

Dai, Y., van Spronsen, J., Witkamp, G.-J., Verpoorte, R., Choi, Y.H., J. Nat. Prod. 76 (2013) 2162-2173.

Daneshjou, S., Khodaverdian, S., Dabirmanesh, B., Rahimi, F., Daneshjoo, S., Ghazi, F., Khajeh, K., J. Mol. Liq. 227 (2017) 21-25.

Durand, E., Lecomte, J., Villeneuve, P., Eur. J. Lipid Sci. Technol. 115 (2013) 379-385.

Gowda, D.C., Reuter, G., Schauer, R., Carbohydr. Res. 113 (1983) 113-124.

Gowda, D.C., Reuter, G., Schauer, R., Phytochemisty 21 (1982) 2297-2300.

Hayyan, M., Hashim, M.A., Hayyan, A., Al-Saadi, M.A., AlNashef, I.M., Mirghani, M.E., Saheed, O.K., Chemosphere 90 (2013) 2193-2195.

Herrmann, G., Lehmann, J., Peterson, A., Sembdner, G., Weidhase, R.A., Parthier, B., J. Plant Physiol.134 (1989) 703-709.

Kim, H.K., Choi, Y.H., Verpoorte, R., Nat. Protoc. 5 (2010) 536-549.

Kotlewska, A.J., van Rantwijk, F., Sheldon, R.A., Arends, I.W.C.E., Green Chem. 13 (2011) 2154-2160.

Król, E., Płachno, B.J., Adamec, L., Stolarz, M., Dziubińska, H., Trębacz, K., Ann. Bot. 109 (2011) 47-64.

Kumar, A.K., Parikh, B.S., Pravakar, M., Environ. Sci. Pollut. Res. 23 (2016) 9265-9275

Libantová, J., Kämäräinen, T., Moravčíková, J., Matušíková, I., Salaj, J., Mol. Biol. Rep. 36 (2009) 851-856.

Lindberg, D., de la Fuente Revenga, M., Widersten, M., J. Biotechnol. 147

(2010) 169-171.

Martins, L.O., Carreto, L.S., Da Costa, M.S., Santos, H., J. Bacteriol. 178 (1996) 5644-5651.

Matušíková, I., Salaj, J., Moravčíková, J., Mlynárová, L., Nap, J-P., Libantová, J., Planta 222 (2005) 1020-1027.

Mbous, Y.P., Hayyan, M., Wong, W.F., Looi, C.Y., Hashim, M.A., Sci. Rep. 7 (2017) 41257.

Mithöfer, A., Reichelt, M., Nakamura, Y., Plant. Biol. 16 (2014) 982-987.

Mueller, M.J., Physiol. Plant. 100 (1997) 653-663.

Nakamura, Y., Reichelt, M., Mayer, V.E., Mithöfer, A., Proc. R. Soc. B, 280 (2013) 20130228.

Neurath, H., Science, 224 (1984) 350-357.

Ninomiya, K., Kamide, K., Takahashi, K., Shimizu, N., Bioresour. Technol. 103 (2012) 259-265.

Paiva, A., Craveiro, R., Aroso, I., Martins, M., Rei, R.L., Duarte, A.R.C., ACS Sustain. Chem. Eng. 2 (2014) 1063−1071.

Park, S., Kazlauskas, R.J., Curr. Opin. Biotechnol. 14 (2003) 432-437.

Poppinga, S., Koch, K., Bohn, H.F., Barthlott, W., Funct. Plant Biol. 37 (2010) 952-961.

Ramos, A., Raven, N., Sharp, R.J., Bartolucci, S., Rossi, M., Cannio, R., Lebbink, J., Van der Oost, J., De Vos, W., Santos, H., Appl. Environ. Microbiol. 63 (1997) 4020-4025.

Romano, B., Fasolino, I., Pagano, E., Capasso, R., Pace, S., De Rosa, G., Milic, N., Orlando, P., Izzo, A.A., Borrelli, F., Mol. Nutr. Food Res. 58 (2014) 457-465.

Romero, A., Santos, A., Tojo, J., Rodriguez, A., J. Hazard. Mater. 151 (2008) 268-273.

Rudnick, H., Hendrich, S., Pilatus, U., Blotevogel, K.H., Arch. Microbiol. 154 (1990) 584-588.

Vanda, H., Dai, Y., Wilson, E.G., Verpoorte, R., Choi, Y.H., C.R. Chim, 21 (2018) 628-638.

Yang, T.-X., Zhao, L.-Q., Wang, J., Song, G.-L., Liu, H.-M., Cheng, H., Yang, Z., ACS Sustain. Chem. Eng. 5 (2017) 5713-5722.

Zhao, D., Liao, Y., Zhang, Z., CLEAN – Soil, Air, Water 35 (2007) 42-48.

Zhao, H., Baker, G.A., J. Chem.Technol. Biotechnol. 88 (2013) 3-12.

C H A P T E R 6

Natural deep eutectic solvents as media for DNA and RNA preservation

Henni Vanda [a, b], Natali Rianika Mustafa [a], Robert Verpoorte [a], Peter G. L. Klinkhamer [c], Young Hae Choi [a]

[a] Natural Products Laboratory, Institute of Biology, Leiden University, Sylviusweg 72, 2333 BE Leiden, the Netherlands

[b] Faculty of Veterinary Medicine, Syiah Kuala University, Banda Aceh, 20111 Indonesia

[c] Plant Ecology and Phytochemistry, Institute of Biology, Leiden University, Sylviusweg 72, 2333 BE Leiden, the Netherlands

Abstract

All bioprocessing in living organisms is thought to necessarily occur in a water or lipidic medium. However, this is not consistent with the fact that many living organisms can adapt and survive extreme conditions such as drought or cold, in which water is not available. Despite extensive research, the mechanisms behind the drought and cold resistance are still not well understood. The hypothesis of natural deep eutectic solvents (NADESs) as a third liquid phase in cells and organisms might provide an explanation. NADESs are formed by mixing certain common cell constituents in defined molar ratios. Compounds detected often in drought and cold resistance states in living organisms have the typical chemical features required to form NADESs. To protect cells and organisms, NADESs must have the capacity to preserve macromolecules such as proteins, DNA and RNA submitted to extreme conditions such as drought and sub-zero temperatures. In this study the main goal was to determine whether NADESs could preserve DNA and RNA. A selection of NADESs was thus tested with DNA and RNA obtained from *Drosophila melanogaster*. The results showed that sugar-based NADESs, such as sucrose-choline chloride-water (1:4:4 mol/mol), sucrose-betaine-water (1:2:5 mol/mol), and fructose-sucrose-glucose-water (1:1:1:11 mol/mol) could preserve the DNA of intact- or ground fruit flies stored at room temperature, in daylight for at least 9-12 months. Furthermore, the mixture of fructose-sucrose-glucose with a water content of even 40% (w/w) preserved the DNA for at least the 9-month observation period. The mixture of sucrose-betaine-water was also able to preserve RNA. The results of this study point to some interesting applications of NADESs considering the stable storage of macromolecules.

Keywords: *Drosophila melanogaster*, DNA and RNA preservation, Natural Deep Eutectic solvents

Introduction

Living cells contain several diverse polymers, e.g. polysaccharides, proteins, ribonucleic acids (RNA), and deoxyribonucleic acids (DNA). These biopolymers are involved in important functions of living cells. Both DNA and RNA contain the genetic information to build and to maintain the organism under various conditions and DNA contains the information to transmit the genetic information to new generations of the organisms. Polysaccharides are important for energy storage and to build the supporting structures of cells, tissues and of the organisms. Proteins are required as biocatalysts and also to maintain the structure, function, and regulation of the cells. They also play a role in the response of the cells and the organisms to environmental changes, regulating its metabolic pathways which are essential for survival. This is, in part, controlled by genes coding for factors such as the stress response of cells and organisms. These biopolymers work together in a tightly controlled manner to ensure biochemical processes in the cells. In other words, polymers are the backbone of life (Lodish et al. 2008; Murray et al. 2009; Saenger 2013).

It is important to understand how living cells can produce, store, and maintain the function of polymers. For example, DNA is located in the nucleus (eukaryotic cells), and a small amount can also be found in mitochondria. Mitochondria are located in the cytosol that consists of water, salts, and organic molecules, whilst the nucleus is composed of nucleoplasm (nucleus sap) that preserves the integrity of the genetic material contained therein (Hernandez-Verdun 2006). Both the mitochondria and nuclei have double phospholipid membranes as barriers to separate the inside organelles from the outside environment in order to protect DNA and proteins from any unwanted chemical and physical damage. Besides the interference of virus, DNA can be damaged in diverse circumstances such as those caused by metabolic and hydrolytic processes, oxidative stress, radiation (ultraviolet) and exposure to toxic compounds such as pollutants (polycyclic aromatic hydrocarbons). Any damage can lead to cell death, DNA mutation and at a whole organism level, to

degenerative diseases (Cheng et al. 1992; Ballinger et al. 1999; Liang and Godley 2003; Roos and Kaina 2006).

Choi et al. (2011) introduced the concept of natural deep eutectic solvents (NADESs) as a potential third liquid phase in Nature. For NADESs to offer an alternative liquid phase to water and lipids it should for example, be able to act as a medium for the biosynthesis of the many water insoluble secondary metabolites as well as the biosynthesis of biopolymers such as cellulose and lignin. The NADESs are a new generation of eutectic mixtures that have a number of advantages over the (semi) synthetic ionic liquids (ILs) developed previously. They are biodegradable and easy to make from abundantly available natural non-toxic compounds (Dai et al. 2013[a]: 2013[b]). A NADES is a mixture of certain molar ratios of two or more naturally sourced compounds that are mostly solid at room temperature. These include sugars, organic acids, amino acids, polyalcohol, choline, betaine and urea among others, the mixture of which has a much lower melting point than that of its individual components. NADESs are proposed to be a medium for the biosynthesis of non-water soluble compounds even in extreme cold and dry conditions, thus allowing the existence of life in those conditions or to dissolve high levels of secondary metabolites in flowers and glandular hairs (Choi et al. 2011; Dai et al. 2013[a]; Vanda et al. 2018).

Water is needed to maintain fully functional cells and their organelles. In extreme conditions such as extreme drought or very low temperatures, organisms decrease their size and volume and thus surface area as an adaptation for survival in these adverse conditions. For example, resurrection plants in deserts lose almost all water under drought conditions, resulting in a cytoplasm mainly composed of sugars such as sucrose, trehalose, and amino acids such as proline and alanine. These compounds are typical candidates for the formation of a NADES that could protect the essential polymeric molecules against degradation. Similar observations were made in the composition of seeds that have to survive drought and low temperatures, including the formation of late

embryogenesis abundant (LEA) proteins that can protect all the materials in the cell, including DNA and RNA (Oliver et al. 2005; Moore et al. 2009; Dinakar et al. 2012).

When DNA and RNA are extracted from the cells, they need to be stored and kept in conditions that guarantee their long-term stability and integrity. DNA and RNA are usually stored in an ultra-cold freezers and liquid nitrogen. When these facilities are not available, for example when collecting samples in the field, organic solvents such as acetone, ethanol, and ethyl acetate provide a convenient solution (Fukatsu 1999). However organic solvents are expensive, volatile, flammable, toxic and often non-biodegradable. Consequently, NADESs was deemed to be a promising alternative to these organic solvents. They are cheap, safe, and environmentally friendly and as mentioned before, according to previous work could potentially both dissolve and preserve molecules such as DNA and RNA.

Research on NADESs applications has risen exponentially in the past years, especially for secondary metabolite extraction (Dai et al. 2013[b]; Paradiso et al. 2016; Dai et al. 2016; González et al. 2018), as a medium for enzymatic reactions (Zhao et al. 2011; Durand et al. 2013; Yang et al. 2017), pharmaceuticals and agrochemicals (Rozema et al. 2015; Wikene et al. 2015; Shamseddin et al. 2017; Huang et al. 2017; Zahrina et al. 2018), and macromolecules (Kumar et al. 2016). One of the interesting applications is their use in the preservation of the integrity and stabilization of macromolecules, especially DNA and RNA. Ionic liquids have been reported to dissolve and stabilize polymers such as proteins and DNA (Mamajanov et al. 2010; Cardoso and Micaelo 2011; Mondal et al. 2013); for example, dried DNA was preserved successfully for six months in choline-based ILs (Vijayaraghavan et al. 2010). The DNA was reported to keep its native structure in ILs with low water contents (Jumbri et al. 2014). Some ILs were also used to isolate DNA and RNA from feline caliciviruses (Fister et al. 2015).

However, little has been published on the stabilization of DNA and RNA

with NADESs (Mondal et al. 2013). In this study, the stability and integrity of DNA and RNA of *Drosophila melanogaster* in NADESs was evaluated. This species was selected because it is a favorite model for genetic research with a fully sequenced genome. NADESs were also selected and tested for their ability to preserve the isolated *D. melanoster* DNA.

The NADESs selected for this experiment are mainly composed of sugars as they are usually the main components of the cytoplasm of cells under extreme conditions. Besides sugars, the organic bases betaine and choline chloride were also used as they have been proven to stabilize dried DNA (Mondal et al. 2013).

Materials and Methods

Drosophila melanogaster flies were provided by the laboratory of Evolutionary Biology, Institute of Biology, Leiden University. Chemicals used in this experiment, D-fructose, D-glucose monohydrate, and sucrose, were purchased from Boom B.V. (Meppel, The Netherlands), malic acid, choline chloride, betaine, and *myo*-inositol were from Sigma Aldrich (The Netherlands). Calcium chloride dehydrate was purchased from Merck, Darmstadt. DNase Kit and RNase Kit were purchased from Qiagen Germany.

Preparation of NADESs

NADESs were prepared as described in Dai et al. (2013[a]). The compounds were mixed on a heated magnetic stirrer at 50 ^{o}C for about 2 hours. Once the mixture became a clear and transparent liquid it was left to cool at room temperature. The tested NADESs were N1 (sucrose:choline chloride:water, 1:4:4, mol/mol), N2 (fructose:sucrose:glucose:water, 1:1:1:11, mol/mol), N3 (*myo*-inositol:sucrose:calcium chloride dihydrate:water, 1:2:3:5, mol/mol), N4 (malic acid:choline chloride:water, 1:1:5, mol/mol), and N5 (sucrose:betaine:water, 1:2:5, mol/mol).

Determination of the efficiency of NADESs for preservation of DNA using intact fruit flies

Fruit flies were anesthetized by cooling to -2 ºC and four of these flies were placed in a micro-tube and submerged in 1 mL of NADESs. Five different NADESs (n = 3) were tested for this experiment and ethanol 70% v/v (C1) and water (C2) were used as control solvents. In order to evaluate the effect of water on the DNA preservation properties of NADESs, different amounts of water (20, 30 and 40% wt) were added to N2 (fructose:sucrose:glucose:water, 1:1:1:11, mol/mol) and tested (n=3). The samples were kept at room temperature in daylight. The DNA of the flies was isolated every three months for 12 months. The procedure for DNA isolation was conducted using the DNeasy Blood and Tissue Kit from Qiagen (Germany). Primers CO1 were used to amplify the mitochondrial cytochrome C oxidase subunit 1 gene. The isolated DNA was then multiplied by Polymerase Chain Reaction (PCR) and determined by agarose gel electrophoresis.

Determination of NADESs effect on DNA preservation using powdered fruit flies

Four flies were cooled at -2 ºC, ground to a fine powder and placed in a micro-tube and submerged in NADESs, obtaining a total volume of 1 ml. Ethanol 70% v/v (C1) and water (C2) were used as control solvents. The mixtures were stored at room temperature in daylight. DNA was isolated from the samples every three months during 12 months of storage. In this experiment, the DNA of the ground flies had direct contact with NADESs. The isolated DNA samples were compared with those of the intact flies preserved in NADESs, by gel electrophoresis.

Determination of the effect of water content on the DNA preservation capacity of N2 using intact fruit flies

The capacity of N2 containing different proportions of water to preserve

the DNA in fruit flies was tested. For this, four flies which has been cooled to -2 ^{o}C were submerged in N2 containing 20%, 30%, and 40% water as described in the previous section. The samples were stored at room temperature in daylight and DNA was isolated every three months throughout 12 months of storage, and analyzed as mentioned in the previous section.

Determination of DNA-stabilizing properties of NADESs of isolated DNA solutions

A sample of 15 µl of the DNA solution was stored in 60 µl of NADESs for three days at room temperature in the daylight. On the third day, NADESs were removed from the samples using DNeasy tubes and buffer from the DNeasy Kit with centrifugation at 8000 rpm for 3 minutes. The DNA in the filter was suspended again with buffer solution and run by PCR. The product from PCR was analyzed by gel electrophoresis.

Determination of the RNA preservation capacity of NADESs using intact fruit flies

The experimental procedure was similar to that used for DNA tests but more flies were used (5-6). The isolation of RNA was done using the RNeasy Mini Kit (Qiagen, Germany) protocol and run by reverse transcriptase PCR. Sampling was done at 1, 3 and, 6 months storage time.

Isolation of DNA

The DNA of the flies was extracted using the DNeasy Blood and Tissue Kit (Qiagen) following the manufacturer's protocol. DNA was quantified using Nanodrop^{TM}1000 Spectrophotometer at 230 nm.

Polymerase Chain Reaction (PCR)

For the PCR, 23 µl- solutions were prepared as follows: 2 µl extracted DNA, 2.5 µl PCR Buffer (10x) containing 1.5mM $MgCl_2$, 1.0 µl $MgCl_2$ (25mM), 1.0 µl forward primer (10pmol/µl), 1.0 µl reverse primer (10pmol/µl), 2.0 µl dNTP's (2.5mM), 0.25 µl Taq (5 units/µl), and 15.25 µl sterile water

(MiliQ).

The PCR procedure applied included the following steps a) Denaturation at 94 ^{o}C for 4 minutes, b). Denaturation at 94 ^{o}C for 1 minute, annealing at 50 ^{o}C for 30 seconds, extension at 72 ^{o}C for 1 minute, repeat cycle (b) 35 times, and final extension at 72 ^{o}C for 4 minutes, and pause step at 15 ^{o}C.

Agarose gel electrophoresis

Twenty percent of the amplified product was loaded on the agarose gel (10%), and electrophoresis was conducted in TBE (89 mM Tris-borate 2 mM EDTA, pH 8.0). After electrophoresis, the gel was placed in ethidium bromide (2%) solution, and the bands of DNA were visualized under UV transillumination.

Results and Discussion

Choi et al. (2011) have hypothesized that there could be a third liquid phase in nature that could solubilize compounds which are insoluble in either water or lipids. According to this hypothesis, such liquids are believed to play a role in drought and desiccation conditions in which the water in the organism dries out leaving a NADESs. This NADESs could protect the cellular membranes and keep essential proteins and other molecules dissolved, avoiding their decomposition until water becomes available again, at which time normal biochemical processes could reinitiate. The NADESs in these plants are probably sugar-based as one could learn from literature (Zhang et al. 2016; Georgieva et al. 2017). The NADESs used for this experiment were selected based on this natural phenomenon.

Determination of NADESs effect on DNA preservation using intact fruit flies

The target DNA should appear at around 1000-1500 base pairs (BP), according to 1 kb ladder plus as the gene ruler. The results of the gel electrophoresis from the 1-month samples showed that the target DNA was still

preserved; the DNA bands of all NADESs samples were clear, except for those with water, which had faded.

Analysis of the three-month samples showed that only the samples of fruit flies stored in N1, N2, N3, and N5 exhibited the bands at around 1000-1500 BP. No bands were detected in N4 and water samples, implying that the DNA was already fully degraded. After 9 months of storage, the DNA dissolved in N3 was also degraded, whereas the DNA dissolved in N1, N2, and N5, was still well preserved even after 12 months, similarly to the 70% ethanol control sample (C1) (Figure 1).

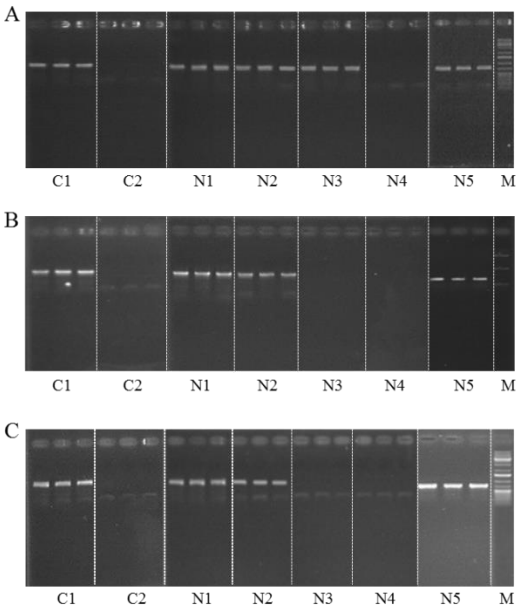

Figure 1. PCR results of intact *Drosophila melanogaster* DNA kept in five NADESs and two control solvents for 3 months (A), 9 months (B) and 12 months (C). N1 (sucrose-choline chloride-water, 1:4:4, mol/mol), N2 (fructose-glucose-sucrose-water, 1:1:1:11, mol/mol), N3 (*myo*-inositol-sucrose-$CaCl_2.2H_2O$-water 1:2:3:5, mol/mol), N4 (malic acid-choline chloride-water, 1:1:5, mol/mol), N5 (sucrose-betaine-water, 1:2:5, mol/mol), C1 (70% ethanol), and C2 (water), M: Marker (DNA ladder).

The quantitative results obtained with the Nanodrop^{TM} 1000 Spectrophotometer showed that the amount of DNA in N1 was comparable to

that in the control sample, C1, and the 3-month DNA of N1 was significantly higher than that of other NADESs ($p<0.05$)In N2 and N5, the amount was lower than in N1 ($p<0.05$), and DNA in N3, N4 and C2 was even lower (Figure 2). This is in accordance with the results of gel electrophoresis, that showed no DNA after three months in none of those samples

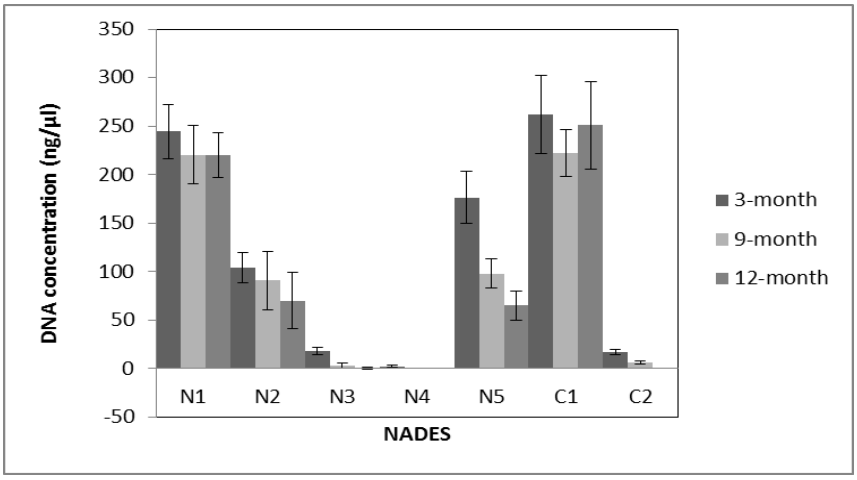

Figure 2. The concentration (ng/µl) of intact *Drosophila melanogaster* DNA analysed by Nanodrop™ 1000 Spectrophotometer at 230 nm.

Determination of NADESs effect on DNA preservation using powdered fruit flies

The ability of NADESs to preserve DNA from powdered fruit flies was compared with that of intact flies in order to determine whether or not direct contact of NADESs with DNA material would provide a better protection of the DNA. The gel electrophoresis results (Figure 3) showed some clearly visible bands of the DNA stored in N1, N2, and C1 until 12 months of storage, whilst no DNA was detected in N3, N4, and C2. Even N2 with increased water concentrations showed clear DNA bands after 12 months. Thus, the DNA was stable and preserved even in the presence of 40% (w/w) of water in N2. However, considering the possibility that higher percentages of water in

NADESs might increase the possibility of microbial growth, eventually resulting in the degradation of the DNA we conducted an experiment with N2 to determine the optimum water concentration. The results show that NADESs with up to 40% water content remained free from microorganisms while maintaining DNA preservation capacity.

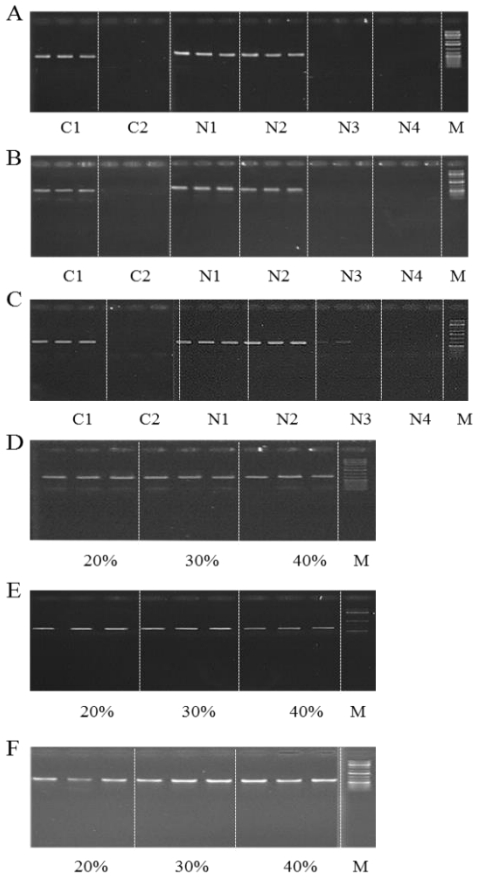

Figure 3. PCR results of *Drosophila melanogaster* dried powder kept in four NADESs and two control solvents for 3 months (A), 9 months (B) and 12 months (C) and water effect (20%, 30%, and 40%, w/w) on NADESs for preservation of *Drosophila melanogaster* using modified NADESs N2 for 3 months (D), 9 months (E) and 12 months (F). N1 (sucrose-choline chloride-water, 1:4:4, mol/mol), N2 (fructose-glucose-sucrose-water, 1:1:1:11, mol/mol), N3 (*myo*-inositol-sucrose-$CaCl_2.2H_2O$-water 1:2:3:5, mol/mol), N4 (malic acid-choline chloride-water, 1:1:5, mol/mol), modified N2 (fructose-glucose-sucrose, 1:1:1 with 20%, 30%, and 40% water, w/w), M: Marker. (DNA ladder)

Quantitative results obtained with the Nanodrop™ 1000 Spectrophotometer showed that the amount of DNA isolated from ground flies was lower than from intact flies (Figure 4). The amount of DNA in the N1 was significantly higher compared to those of other NADESs ($p<0.05$), however, the amount of DNA in N1 and N2 samples was sufficient to be multiplied by PCR giving clear bands on the agarose gel. The sugars in N1 and N2, are known to have a positive impact on DNA stability and integrity in cryopreservation of living cells and of tissue materials (Mustafa et al. 2011; Tanpradit et al. 2015). Ice formation seems to be a critical factor for the stability of DNA (Kasper et al. 2013).

NADESs as a media for short time storage of isolated DNA

The DNA isolation protocol requires isolated DNA to be dissolved in a buffer and directly stored at -20 or -80°C for its preservation. In view of the promising results regarding DNA stability obtained when storing the flies, we tested the stability of NADESs solutions of isolated DNA stored during short periods of time on the laboratory bench. After keeping the isolated DNA in the 3 tested NADESs at room temperature for 3 days, the results showed that only N1 and N5 could stabilize the DNA (Figure 5). In N2, the DNA was fully degraded as no bands could be detected on the agarose gel. Apparently those NADESs containing sucrose and an organic base such as choline or betaine are appropriate for the short-term storage of isolated DNA samples.

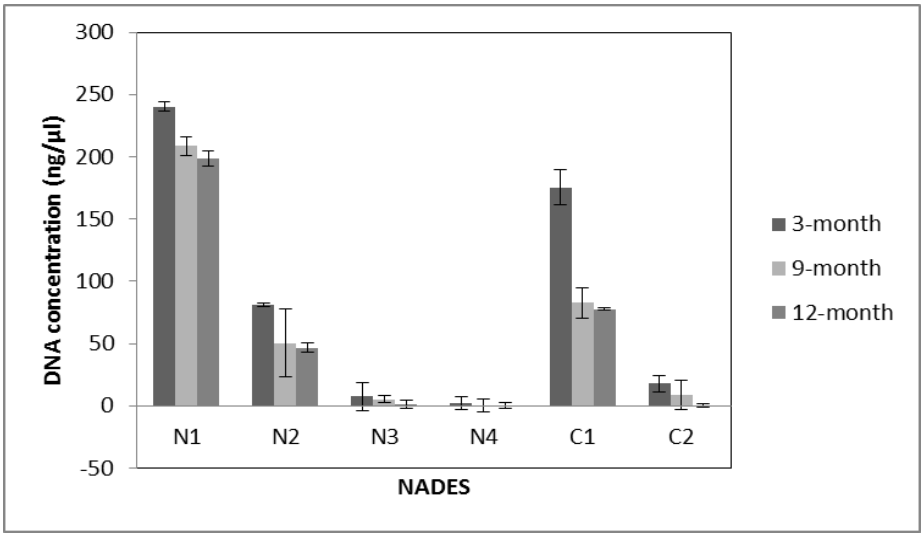

Figure 4. The concentration (ng/µl) of dried powder *Drosophila melanogaster* DNA analyzed by Nanodrop™ 1000 Spectrophotometer at 230 nm.

In previous cryopreservation studies of prokaryotes, betaine has proved to exert a positive effect on the cytoplasm of the cell and its DNA. This was attributed to the similar osmotic pressure inside and outside of the cells (Cleland et al. 2004). Microscopic observations of the spermatozoan plasmatic membranes showed a stabililizing effect for betaine (Swan and Alboghobeish, 1997). Apparently the NADESs containing both sucrose and betaine or choline seemed to combine both positive effects. The hypothesis of the existence of NADESs as a third liquid phase (Choi et al. 2011) suggests that NADESs could bind to membranes, a proposal that seems to be confirmed by observations made with the cryopreservation of various cell types. The results reported here are also in line with this, as the sucrose-organic base NADESs seem to protect the integrity of DNA, an effect that could be due to a combination of factors as mentioned.

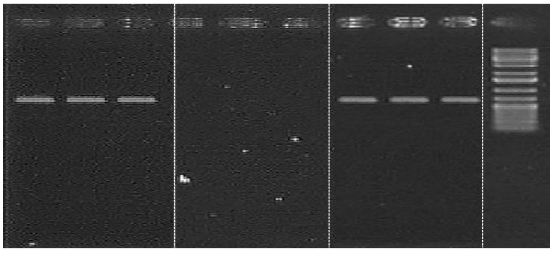

Figure 5. PCR results of isolated DNA of *Drosophila melanogaster* kept in three NADESs for 3 days at room temperature. N1 (sucrose-choline chloride-water, 1:4:4, mol/mol), N2 (fructose-glucose-sucrose-water, 1:1:1:11, mol/mol), and N5 (sucrose-betaine-water, 1:2:5, mol/mol), M: Marker (DNA ladder).

Determination of NADESs property on RNA preservation of intact fruit flies

Ribonucleic acid (RNA) is less stable than DNA being easily degraded due to the presence of the ribose group (instead of deoxyribose) that makes it more prone to hydrolysis. The results of this experiment showed that N5 was much more efficient in the preservation of RNA than the other tested NADESs (Figure 6). The bands of RNA were clear on agarose gel throughout a six-month storage period. In N2, the bands were visible but not clear, indicating partial degradation. Apparently, the type of organic base plays a role in the preservation effect, as N1 proved to be less efficient as a preservative.

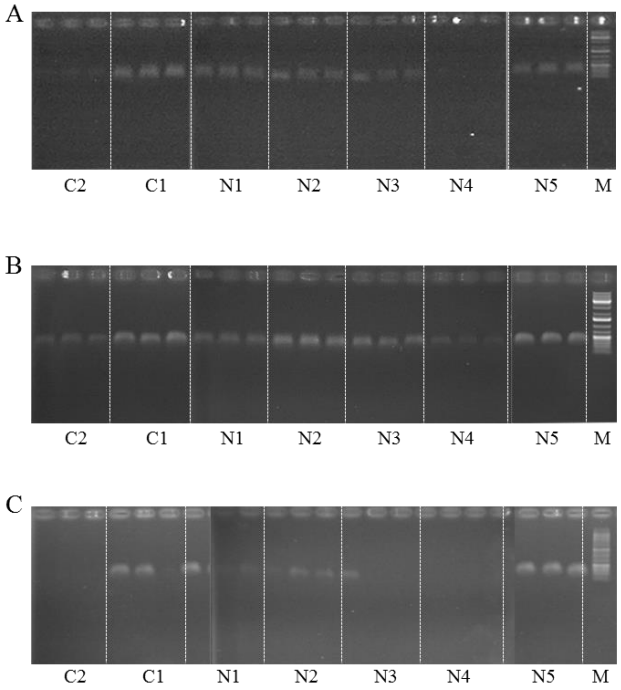

Figure 6. PCR results of isolated RNA of *Drosophila melanogaster* stored for 1 month (A), 3 months (B) and 6 months (C) in five NADESs. N1 (sucrose-choline chloride-water, 1:4:4, mol/mol), N2 (fructose-glucose-sucrose-water, 1:1:1:11, mol/mol), N3 (*myo*-inositol-sucrose-$CaCl_2.2H_2O$-water 1:2:3:5, mol/mol), N4 (malic acid-choline chloride-water, 1:1:5, mol/mol), N5 (sucrose-betaine-water, 1:2:5, mol/mol), C1 (70% ethanol), and C2 (water), M: Marker (RNA ladder).

Conclusion

These studies showed that NADESs composed of sugars, choline, and betaine can preserve DNA and RNA within biological materials. NADESs have been reported to preserve macromolecules such as proteins and in this case, we showed that it was also possible to store isolated DNA in the sucrose: organic base-type NADESs. These observations are in line with observations in cryopreservation and fits in with the hypothesis of NADESs as part of the physicochemical environment in cells, in which they are thought to be attached to cellular membranes. It also correlates well with the observations of sucrose-choline chloride being present in high levels in the aleurone of barley seeds, and

the role of sugars combined with various amino acids in resurrection plants. Thus, reproducing the natural preservation systems of living cells and their constituents seems to be a promising approach to find ways of storing DNA and biological samples for prolonged periods of time.

References

Ballinger, S.W., Van Houten, B., Conklin, C.A., Jin, G.-F., Godley, B.F., Exp. Eye Res. 68 (1999) 765-772.

Cardoso, L., Micaelo, N.M., Chem. Phys. Chem. 12 (2011) 275-277.

Cheng, K.C., Cahill, D.S., Kasai, H., Nishimura, S., Loeb, L.A., J. Biol. Chem. 267 (1992) 166-172.

Choi, Y.H., van Spronsen, J., Dai, Y., Verberne, M., Hollmann, F., Arends, I.W.C.E., Witkamp, G.-J., Verpoorte, R., Plant Physiol. 156 (2011) 1701-1705.

Cleland, D., Krader, P., McCree, C., Tang, J., Emerson, D., J. Microbiol. Methods 58 (2004) 31-38.

Dai, Y., Rozema, E., Verpoorte, R., Choi, Y.H., J. Chromatogr. A 1434 (2016), 50-56.

Dai, Y., van Spronsen, J., Witkamp, G.-J., Verpoorte, R., Choi, Y.H., J. Nat. Prod. 76 (2013[a]) 2162-2173

Dai, Y., Witkamp, G.-J., Verpoorte, R., Choi, Y.H., Anal. Chem. 85 (2013[b]) 6272-6278.

Dinakar, C., Djilianov, D., Bartels, D., Plant Sci. 182 (2012) 29-41.

Durand, E., Lecomte, J., Villeneuve, P., Eur. J. Lipid Sci.Technol. 115 (2013) 379-385.

Fister, S., Fuchs, S., Mester, P., Kilpeläinen, I., Wagner, M., Rossmanith, P., Sep. Purif. Technol.155 (2015) 38-44.

Fukatsu, T., Mol. Ecol. 8 (1999) 1935-1945.

Georgieva, K., Rapparini, F., Bertazza, G., Mihailova, G., Sárvári, É., Solti, Á., Keresztes, Á., Protoplasma 254 (2017) 193-201.

González, C.G., Mustafa, N.R., Wilson, E.G., Verpoorte, R., Choi, Y.H., Flavour Fragr. J. 33 (2018) 91-96.

Hernandez-Verdun, D., Histochem. Cell Biol.125 (2006) 127-137.

Huang, Y., Feng, F., Chen, Z.-G., Wu, T., Wang, Z.-H., Food Chem. 244 (2018) 260-265.

Jumbri, K., Rahman, M.A., Abdulmalek, M. A., E., Ahmad, H., Micaelo, N.M., Phys. Chem. Chem. Phys.16 (2014) 14036-14046.

Kasper, J.C., Pikal, M.J., Friess, W., J. Pharm. Sci. 102 (2013) 929-946.

Kumar, A.K., Parikh, B.S., Pravakar, M., Environ. Sci. Pollut. Res. 23 (2016) 9265-9275.

Liang, F.-Q., Godley, B.F., Exp. Eye Res.76 (2003) 397-403.

Lodish, H., Berk, A., Darnell, J.E., Kaiser, C.A., Krieger, M., Scott, M.P., Bretscher, A., Ploegh, H., Matsudaira, P., (2008) Macmillan.

Mamajanov, I., Engelhart, A.E., Bean, H.D., Hud, N.V., Angew. Chem. Int. Ed. 49 (2010) 6310-6314.

Mondal, D., Sharma, M., Mukesh, C., Gupta, V., Prasad, K., Chem. Commun. 49 (2013) 9606-9608.

Moore, J.P., Le, N.T., Brandt, W.F., Driouich, A., Farrant, J.M., Trends Plant Sci. 14 (2009) 110-117.

Murray, K., Rodwell, V., Bender, D., Botham, K.M., Weil, P.A., Kennelly, P.J., *28* (2009) New York: McGraw-Hill.

Mustafa, N.R., De Winter, W., Van Iren, F., Verpoorte, R., Nat. Protoc. 6 (2011) 715-742.

Oliver, M.J., Velten, J., Mishler, B.D., Integr. Comp. Biol. 45 (2005) 788-799.

Paradiso, V.M., Clemente, A., Summo, C., Pasqualone, A., Caponio, F., Food Chem. 212 (2016) 43-47.

Roos, W.P., Kaina, B., Trends Mol. Med.12 (2006) 440-450.

Rozema, E., Van Dam, A.D., Sips, H.C.M., Verpoorte, R., Meijer, O.C., Kooijman, S., Choi, Y.H., RSC Adv. 5 (2015) 61398-61401.

Saenger, W., (2013) Springer Science & Business Media.

Shamseddin, A., Crauste, C., Durand, E., Villeneuve, P., Dubois, G., Durand, T., Vercauteren, J., Veas, F., Eur. J. Lipid Sci.Technol. 119 (2017) 1700171.

Swan, M., Alboghobeish, N., J. Microsc. 187 (1997) 167-169.

Tanpradit, N., Comizzoli, P., Srisuwatanasagul, S., Chatdarong, K., Theriogenology 83 (2015) 1553-1561.

Vanda, H., Dai, Y., Wilson, E.G., Verpoorte, R., Choi, Y.H., C.R. Chim, 21 (2018), 628-638.

Vijayaraghavan, R., Izgorodin, A., Ganesh, V., Surianarayanan, M.,

MacFarlane, D.R., Angew. Chem. Int. Ed. 49 (2010) 1631-1633.

Wikene, K.O., Bruzell, E., Tønnesen, H.H., J. Photochem. Photobiol. B 148 (2015)188-196.

Yang, T.-X., Zhao, L.-Q., Wang, J., Song, G.-L., Liu, H.-M., Cheng, H., Yang, Z., ACS Sustain. Chem. Eng. 5 (2017) 5713-5722.

Zahrina, I., Nasikin, M., Krisanti, E., Mulia, K., Food Chem. 240 (2018) 490-495.

Zhang, Q., Song, X. and Bartels, D., Proteomes, 4 (2016) 40-53.

Zhao, H., Baker, G.A., Holmes, S., J. Mol. Catal B: Enzym. 72 (2011) 163-167.

C H A P T E R 7

General conclusion and perspective

1. Conclusions

Ionic liquids (ILs) have been considered as green solvents for over 20 years due to their characteristic features such as chemical and thermal stability, nonflammability, nonvolatility, and high solubilizing capacity. However, due to their toxicity and nondegradable property, have turned scientist to search for a more promising solvents, and then the term of deep eutectic solvents (DES) was introduced. DES are mixtures of a quaternary ammonium salt with hydrogen bond donors such as organic acids, urea, or glycerol, to form a complex with the halide anion of the quaternary ammonium salt. DES are believed to be generally nontoxic, easy to prepare, accessible, and cheap (Abbot et al. 2004; Jhong et al. 2009). Choi et al. (2011) proposed that Nature have applied the principles of ILs and DES, and the term of natural deep eutectic solvents (NADESs) was presented (**Chapter 2**).

Natural deep eutectic solvents (NADESs) are new generation of green solvents which are composed of natural ingredients such as sugars, amino acids, organic acids, and organic bases, and mix together under certain conditions to

become liquid. NADESs have several advantages such as low melting point, nontoxic, zero vapor pressure, nonflammability, no environmental hazards, and sustainable production. The concept of NADESs is that everywhere in Nature, there is a third liquid phase, apart from water and lipid phase, which could solubilize medium polar compounds that are poorly soluble in water or lipids. This could explain the role of NADESs in extreme conditions, e.g. cold and drought resistance, desiccation and resurrection plants, and also as media for biosynthetic pathways (**Chapter 3**). NADESs is also believed to be involved in digesting process of insect-eating plants, e.g. *Drosera* species. *Drosera* obtained their nutrients from insects that are trapped by sticky exudates produced by glands on the tentacles. Digestive enzymes are released to digest the insects, and this process could take up for two days. From ^{1}H NMR and GC-MS measurement, the exudate of *Drosera* contains several NADESs components such as glucose, fructose, *myo*-inositol, arabinose, malic acid, lactic acid, mannose, and galactose. (**Chapter 4**).

To further explore the possibility of NADESs in protein preservation, two proteases enzymes were tested, namely protease (isolated from *Streptomyces griseus),* and bromelain from pineapple fruit (**Chapter 5**). These enzymes were stored in several NADESs with different water concentration for 14 days under day light. It showed that NADESs had the ability to stabilize proteases in different water content, and NADESs also protect the enzymes from microorganism growth. Macromolecules like DNA and RNA are susceptible to degradation under certain environmental conditions, and NADESs may be involved in stabilizing these genetic materials in extreme conditions. DNA and RNA of *Drosophila melanogaster* were well preserved in NADESs even after 12 months, which could explain the role of NADESs in stabilizing and preserving macromolecules (**Chapter 6**). NADESs are also potential solvents for keeping isolated DNA for short time storage.

2. Perspectives

Natural deep eutectic solvents (NADESs) are promising green solvents which can be used in various applications, including secondary metabolite extraction, enzymatic reaction, solvents for medium polarity compounds, protein stabilizer, and media for drugs and cosmetics. The use of NADESs can be developed to a larger scale with intended to reduce the use of organic solvents in industrial fields. The combinations of NADESs can be expanded as their ingredients are abundant in Nature, and they can be tailored to a specific application.

The obstacles in using NADESs are the viscosity and nonvolatility, which makes NADESs difficult to remove from the materials. However, in food and pharmaceutical industry, NADESs can be used directly, in case of extraction process, some extra procedure is necessary to eliminate the solvents. Some methods can be applied, including supercritical CO_2 extraction, liquid-liquid and liquid-solid extraction may be employed (Dai et al. 2013).

Another important application of NADESs is in pharmaceutical fields, where NADESs can be used as media to dissolve poorly water-soluble compounds and directly administered to the patients. The natural components of NADESs make it safe for oral use, as for injection, specific NADESs combination may be applied to avoid any unwanted reactions of the body. The search for the right combinations is a challenge since injection forms need to be sterile, compatible with active compounds, hypoallergenic, and stable in body biofluids.

As for preservation of macromolecules, little information is available. The stability of DNA and RNA solution in NADESs was reported, yet the utilization of NADESs as cryoprotectants for DNA and RNA under extreme condition is need to be explored, as well as preserving bacteria in nonaqueous environment.

The invention of NADESs has contributed a great deal of diverse biotechnology or biological engineering applications. It may explain many cellular processes which may be applied in laboratory and even in industrial

scale. The limitless NADESs combinations which are found in Nature will generate various applications, and specific combination will result in more suitable conditions for desired applications.

References

Abbott, A.P., Boothby, D., Capper, G., Davies, D.L., Rasheed, R.K., J. Am. Chem. Soc. 126 (2004) 9142-9147.

Choi, Y.H., van Spronsen, J., Dai, Y., Verberne, M., Hollmann, F., Arends, I.W.C.E., Witkamp, G.-J., Verpoorte, R., Plant Physiol. 156 (2011) 1701-1705.

Dai, Y., van Spronsen, J., Witkamp, G.-J., Verpoorte, R., Choi, Y. H., Anal. Chim. Acta 766 (2013) 61-68.

Jhong, H.-R., Wong, D. S.-H., Wan, C.-C., Wang Y.-Y., Wei, T.-C., Electrochem. Commun. 11 (2009) 209-211.

Summary

The search of green solvents has brought scientist on the discovery of ionic liquids (ILs) to replace organic solvents. ILs are made of ions and short-lived ion pairs of salts that form liquid. ILs has special characteristic such as low melting point, nonflammable, low vapor pressure, thermally stable, and solvating properties for polar and non-polar compounds. The physicochemical properties of ILs can be adjusted by changing the cation and anion combination, which open various applications of ILs, from extraction, chemical synthesis, and catalysis. Despite of all preferable characteristics, ILs are considered to be toxic and non-degradable. Then deep eutectic solvents (DES) was invented as new generation of ILs with more advantages and less toxic.

Deep eutectic solvents are composed of quaternary ammonium salts with hydrogen bond donors in certain molar ratio and form a eutectic mixture. DES has similar characteristics with ILs, but components of DES are non-toxic, easy to obtain, and low cost. DES applications cover a wide range of sectors, including plant extraction, catalysis, organic synthesis, biodiesel synthesis, biomass processing, nanotechnology, and many more. However, the safety of DES ingredients is still inconclusive, that made scientist tried to find the natural origin of these components. Finally, natural deep eutectic solvents (NADESs) was introduced by Choi et al. as truly green solvents which composed of natural

ingredients found abundantly in Nature. There are about 150 combinations of NADESs has been proposed and they have been used in diverse applications, including extraction of secondary metabolites, enzymatic processes, agricultural and pharmaceutical fields, and protein stabilization (**Chapter 2**).

NADESs also play major roles in plants survival, such as under extreme drought condition, and plant resurrection. In this condition, NADESs which is in liquid form will preserve the enzymes and other macromolecules like DNA and RNA. NADESs could provide suitable condition for enzymatic reactions, and preserve macromolecules from environmental disturbance (**Chapter 3**).

Not only NADESs ingredients are safe, they also have physiological and biological role in Nature. For example, in *Drosera* species, where the plants catch insects as their source of nutrients. The plant produces sticky biofluids as entrapment and also providing suitable environment for digesting processes. To investigate the components of this biofluid, NMR and GC-MS analysis was carried out for seven species of *Drosera*. The results showed that the biofluids contained sugars, amino acids, and organic acids which were NADESs components. These components together with calcium ion would stabilize the digesting enzymes and prevent biofluids from evaporation (**Chapter 4**).

Enzymes secretion in *Drosera* can be mimicked by using plant hormone, e.g. jasmonic acid, as it stimulates plant movement, and this will trigger the plant to enter digesting stage. Mixtures of proteases enzymes are involved in digesting process of *Drosera* plant, and enzymes are easily denatured due to high temperature and pH changes. In this situation, NADESs play roles in keeping the media for enzymatic process. In **Chapter 5**, several NADESs with different water concentration are proven to be good media for protease (isolated from *Streptomyces griseus*) and bromelain (from pineapple) stability and activity. The enzymes were active for 14 days of storage. NADESs also protected the enzymes from microorganism contamination, where NADESs alone are free from microorganism growth. Therefore, NADESs had positive effect for enzymes stability and activity which may explain their possible role in prey-digesting

process.

Besides enzymes, genetic materials like DNA and RNA are also prone to degradation, and they need special condition to stabilize their macromolecules. To investigate the ability of NADESs to stabilize DNA and RNA, several NADESs were tested using *Drosophila melanogaster* which was stored in NADESs for 12 months (**Chapter 6**). The DNA and RNA were isolated in a three-month interval, and analyzed by PCR. NADESs composed of sugars, choline chloride, and betaine exhibited the ability to stabilize DNA and RNA. DNA was well preserved at least for 9 months of storage, and RNA was stable for six months of observation. This experiment may explain the importance and roles of NADESs in cell physiology, where NADESs may keep the cell functions in extreme condition such as drought and desiccation condition, when water in hardly available.

Samenvatting

De zoektocht naar natuurlijke oplosmiddelen heeft geleid tot de ontdekking van ionic liquids (ILs) als vervanger van organische oplosmiddelen. ILs bestaan uit ionen en kortlevende ionenparen van zouten die gezamenlijk organische oplosmiddelen vormen. ILs hebben speciale karakteristieken, zoals een laag kookpunt, niet ontvlambaar, verdampen onder lage druk, thermische stabiliteit en oplossend eigenschappen voor zowel polaire en niet-polaire stoffen. De fysiochemische eigenschappen van ILs kunnen aangepast worden door de combinatie van kationen en anionen te veranderen, waardoor verschillende toepassingen van ILs ontstaan, van extractie tot chemische synthese en katalyse. Ondanks alle voordelige eigenschappen, zijn ILs ook toxisch en niet-afbreekbaar. Daardoor zijn deep eutectic solvents (DES) uitgevonden, welke als volgende generatie van ILs meer voordelen hebben en minder toxisch zijn.

Deep eutectic solvents bestaan uit quartaire ammonium zouten met waterstofbinding donoren in een specifiek molair ratio en vormen een eutectisch mengsel. DES hebben gelijke karakteristieken als ILs, alleen zijn de onderdelen van DES niet toxisch, gemakkelijk te verwerven en laag in kosten. DES toepassingen bestrijken een breed spectrum van sectoren, zoals plant extracties, katalyse, organische synthese, biodiesel synthese, verwerking van biomassa,

nanotechnologie en vele anderen. Echter, omdat de veiligheid van DES ingrediënten is nog niet volledig bekend, hebben wetenschapper de natuurlijke oorsprong van de componenten te achterhalen. Ten slotte zijn natural deep eutectic solvents (NADESs) door Choi et al. geïntroduceerd al natuurlijke oplosmiddelen welke bestaan uit natuurlijk ingrediënten die veel voorkomen in de natuur. Er zijn ongeveer 150 combinaties van NADESs voorgesteld en deze hebben allen verscheidene functies, zoals extractie van secundaire metabolieten, enzymatische processen, agrarische en farmaceutische toepassingen en eiwit stabilisatie (**Hoofdstuk 2**).

NADESs spelen een rol in de overleving van de plant, zoals onder extreme droogte en wederopstanding na extreme omstandigheden. Onder deze condities zijn NADESs vloeibaar en zorgen voor het behoud van enzymen en andere macromoleculen, zoals DNA en RNA. NADESs kunnen een geschikte omgeving bieden voor enzymatische reacties en behouden macromoleculen van verstoringen door de omgeving (**Hoofdstuk 3**).

Buiten het feit dat NADESs veilig zijn hebben ze ook een fysiologische en biologische rol in de natuur. Bijvoorbeeld in *Drosera sp.*, waarin de planten insecten vangen als bron van nutriënten. De planten produceren plakkerige bio vloeistoffen om insecten te vangen en om een geschikte omgeving te creëren voor het verteringsproces. Om de componenten van deze bio vloeistoffen te onderzoeken zijn NMR en GC-MS analyses uitgevoerd op zeven soorten van *Drosera*. De resultaten toonden aan dat de bio vloeistoffen bestonden uit suikers, aminozuren en organische zuren, ook componenten van NADESs. Deze componenten tezamen met calcium ionen stabiliseren verteringsenzymen en voorkomen de verdamping van de bio vloeistoffen (**Hoofdstuk 4**).

De uitscheiding van enzyme in *Drosera* kunnen worden nagebootst door plantenhormonen te gebruiken, zoals jasmijnzuur (jasmonic acid), omdat het de beweging van planten stimuleert, wat het begin van verteringsfase inluidt. Mengsels van protease enzymen zijn betrokken bij het verteringsproces van *Drosera* planten en de enzymen zijn makkelijk te denatureren door de hoge

temperatuur en pH fluctuaties. In deze situatie spelen NADESs een rol in het behoudt van het medium voor de enzymatische processen. In **Hoofdstuk 5** bewijzen verschillende NADESs met verschillende water concentraties een goed medium te zijn voor de stabiliteit van proteases (geïsoleerd uit *Streptomyces griseus*) en bromelaïne (uit ananas). Deze enzymen waren actief gedurende de bewaring van 14 dagen. Daarnaast beschermen NADESs de enzymen voor de vervuiling van mirco-organisme, omdat mirco-organismen niet in staat zijn te groeien in NADESs. Door deze eigenschappen hebben NADESs een positief effect op enzymatische stabiliteit en activiteit, wellicht een verklaring voor hun rol in het verteringsproces van de prooi.

Buiten enzymen is ook genetisch materiaal, zoals DNA en RNA, gevoelig voor degradatie en heeft ook genetische materiaal speciale condities nog om de macromoleculen te stabiliseren. Om de eigenschap van NADESs als stabilisator van DNA en RNA te onderzoeken werden verschillende NADESs getest door middel van *Drosophila melanogaster,* welke 12 maanden opgelost waren in NADESs (**Hoofdstuk 6**). Het DNA en RNA werd geïsoleerd in intervallen van drie maanden en geanalyseerd door middel van PCR. NADESs bestaan uit suikers, choline chloride en betaïne duiden de mogelijkheid om NADESs te stabiliseren aan. Het DNA was goed bewaard gebleven voor ten minste 9 maanden en het RNA was stabiel gedurende de 6 maanden van observeren. Dit experiment verklaard het belang en de rol van NADESs in de fysiologie van de cel, waar NADESs de cel functies behouden onder extreme omstandigheden, zoals droogte en uitdrogende condities, wanneer water niet of nauwelijks beschikbaar is.

Acknowledgement

First of all, I would like to thank the Directorate General of Higher Education, Ministry of Education, Republic of Indonesia, for providing the BLN-DIKTI scholarship program to pursue my PhD study. Also, my appreciation to Faculty of Veterinary Medicine, Universitas Syiah Kuala for supporting me during my study.

My gratitude is to Prof. Robert Verpoorte and Dr. Natali Rianika Mustafa for helping me with the project and assisted me in writing, Dr. Klass Vrieling for allowing me to work in Molecular Biology Laboratory, and Rogier van Vugt for giving me permission to collect samples at botanical garden of Hortus Botanicus. I also thank Dr. Yuntao Dai for helping me in NADESs project, Dr. Justin Fischedick for introducing me to GC-MS, and Gerda Lamers for helping me in microscopy observation.

To all my colleagues and friends in Natural Products Laboratory and Plant Ecology, thank you for all your kindness and support during my study in Leiden. To my beloved family, thank you for your encouragement, support, and prayers.

Curriculum vitae

Hennivanda was born in Banda Aceh, Indonesia on 8th of September 1975. She enrolled Faculty of Veterinary Medicine, IPB University in Bogor in 1993, and obtained Doctor of Veterinary Medicine in 1999. In 2001, she was awarded BPPS-DIKTI scholarship for postgraduate study and continued her MSc in Medicinal Chemistry, School of Pharmacy, Bandung Institute of Technology (ITB). Her research project was about bacteria fusion to enhance protease production in *Bacillus licheniformis*. In 2011, she was awarded BLN-DIKTI scholarship to pursue PhD in Natural Products Laboratory, Institute of Biology, Leiden University. She was accepted as a lecture in Faculty of Veterinary Medicine, Universitas Syiah Kuala in 2006, and has become a staff member of Veterinary Pharmacology Department ever since. After finishing her PhD study, she will continue to work as a lecture at Universitas Syiah Kuala, Banda Aceh, Indonesia.

Publications

Henni Vanda, Yuntao Dai, Erica G. Wilson, Robert Verpoorte, Young Hae Choi (2018) Green solvents from ionic liquids and deep eutectic solvents to natural deep eutectic solvents. Comptes Rendus Chimie 21: 628-638.

Henni Vanda, Robert Verpoorte, Peter G. Klinkhamer, Young Hae Choi (2019) Natural Deep Eutectic Solvents: From Their Discovery to Their Applications. Deep Eutectic Solvents: Synthesis, Properties, and Applications, First Edition_Edited by Diego J. Ramon and GabrieLi Guillena. Wiley-VCH Verlag GmbH & Co. KGaA: 61-81.