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

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Natural deep eutectic solvents as media for DNA and RNA preservation

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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).

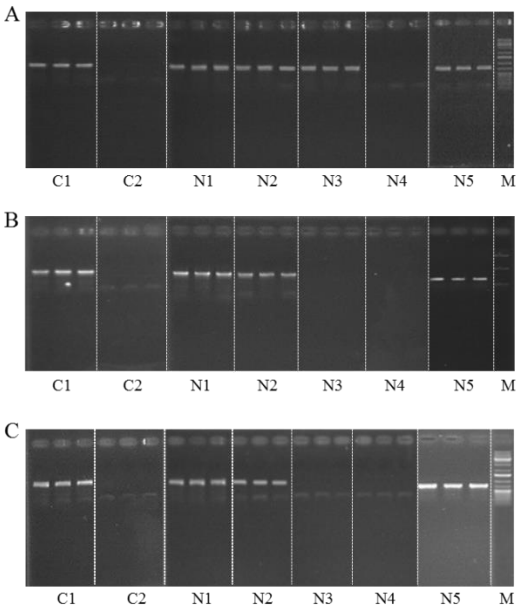

Figure 1. PCR results of intact *Drosophila melanogaster* DNA kept in five NADESs and two control solvents for 3 months (A), 9 months (B) and 12 months (C). N1 (sucrose-choline chloride-water, 1:4:4, mol/mol), N2 (fructose-glucose-sucrose-water, 1:1:1:11, mol/mol), N3 (*myo*-inositol-sucrose-$CaCl_2.2H_2O$-water 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

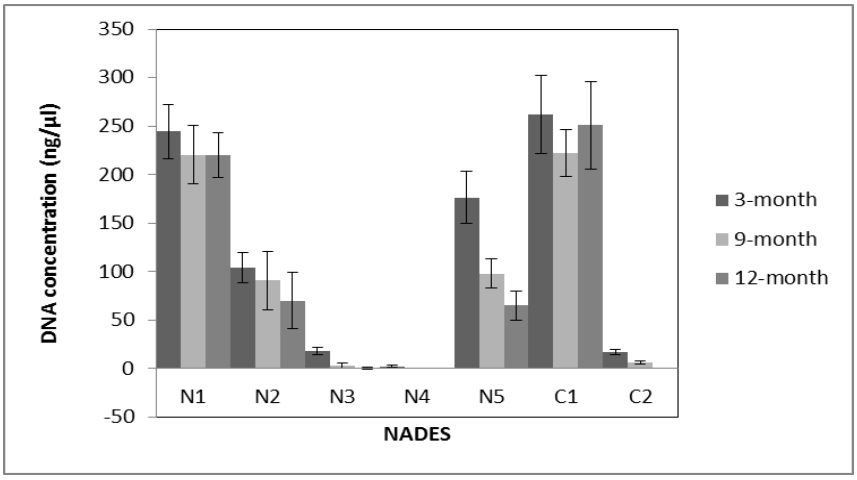

Figure 2. The concentration (ng/µl) of intact *Drosophila melanogaster* DNA analysed by Nanodrop™ 1000 Spectrophotometer at 230 nm.

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.

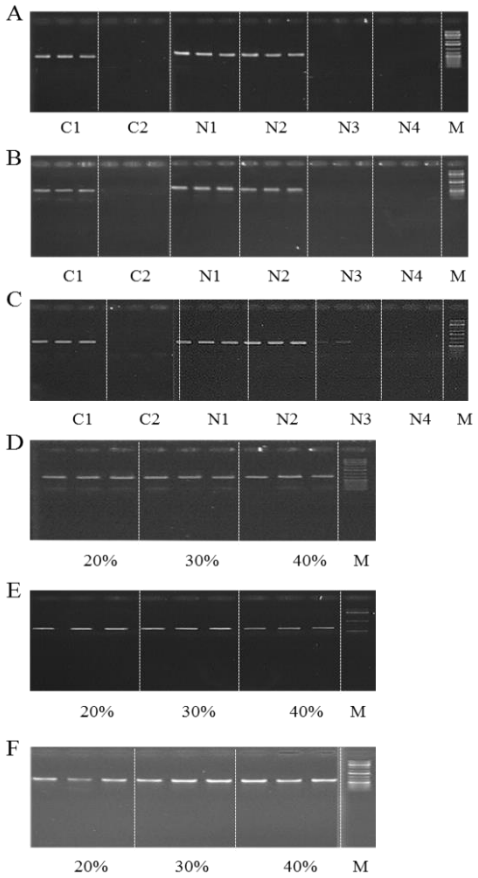

Figure 3. PCR results of *Drosophila melanogaster* dried powder kept in four NADESs and two control solvents for 3 months (A), 9 months (B) and 12 months (C) and water effect (20%, 30%, and 40%, w/w) on NADESs for preservation of *Drosophila melanogaster* using modified NADESs N2 for 3 months (D), 9 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.

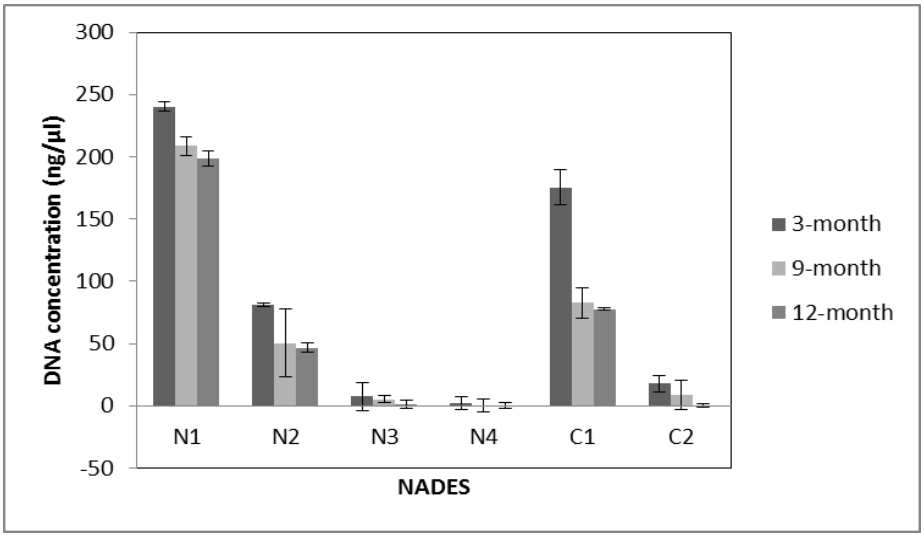

Figure 4. The concentration (ng/µl) of dried powder *Drosophila melanogaster* DNA analyzed by Nanodrop™ 1000 Spectrophotometer at 230 nm.

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.

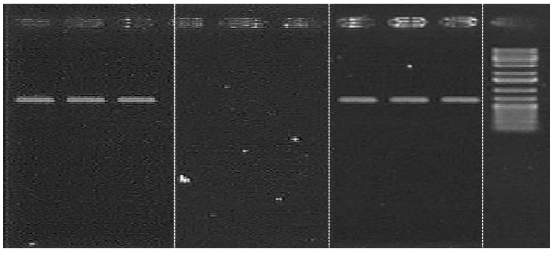

Figure 5. PCR results of isolated DNA of *Drosophila melanogaster* kept in three NADESs for 3 days at room temperature. N1 (sucrose-choline chloride-water, 1:4:4, mol/mol), N2 (fructose-glucose-sucrose-water, 1:1:1:11, mol/mol), and N5 (sucrose-betaine-water, 1:2:5, mol/mol), M: Marker (DNA ladder).

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.

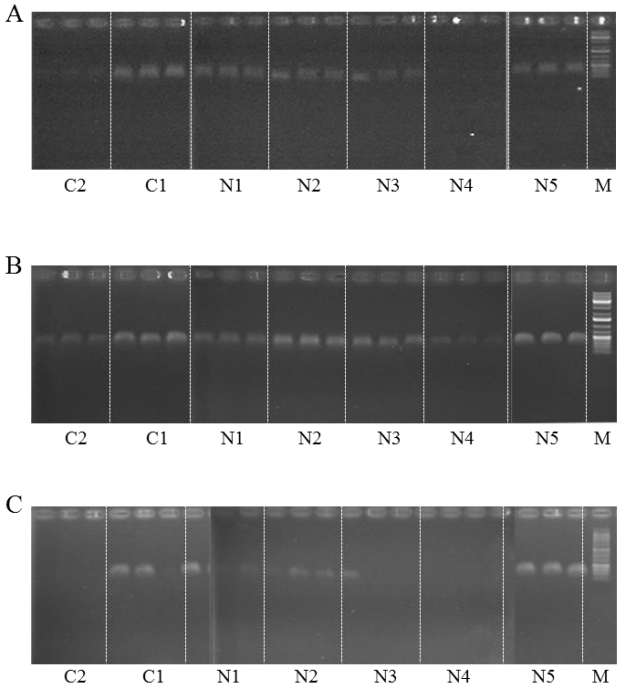

Figure 6. PCR results of isolated RNA of *Drosophila melanogaster* stored for 1 month (A), 3 months (B) and 6 months (C) in five NADESs. N1 (sucrose-choline chloride-water, 1:4:4, mol/mol), N2 (fructose-glucose-sucrose-water, 1:1:1:11, mol/mol), N3 (*myo*-inositol-sucrose-$CaCl_2.2H_2O$-water 1:2:3:5, 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.