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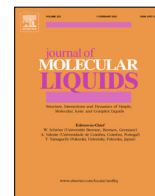
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Structural properties and stability of the Betaine-Urea natural deep eutectic solvent

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ABSTRACT

This work focuses on the stability and supramolecular structure of the betaine-urea-water (B:U:W) natural deep eutectic solvent. Solutions spanning a range of molar ratios of betaine, urea, and water were prepared, varying the temperature and preparation times, and were analyzed by attenuated total reflection Fourier-transform infrared spectroscopy and Nuclear Magnetic Resonance. Density Functional Theory and the Natural Bond Orbital analysis were employed to obtain the most stable conformations for each mixture. The experimental results show that, in non-anhydrous conditions, betaine:urea (1:1), a minimum of two moles of water are needed to form a metastable transparent liquid, and a minimum of three moles of water is required to have a stable NADES. Comparison of the ¹³C NMR spectra of B:U:W 1:1:2 and 1:1:3 shows for the latter that the carbonyl groups of betaine and urea form stronger hydrogen bonds with water, and that the CH₃ group of betaine becomes more deprotected by the addition of the extra water molecule, making 1:1:3 a more stable solution. Our experimental and computational results show that water is of crucial importance to the NADES supramolecular structure and stability. A better understanding of the structural characteristics of NADES can lead to better envisage applications for these green solvents.

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1. Introduction

Natural deep eutectic solvents (NADES) are mixtures of two or more components at a certain molar ratio, which are generally natural primary metabolites, e.g. organic acids, sugars, polyalcohols, amines, and amino acids [1,2]. These solvents are characterized for having a melting point considerably lower than that of their individual components [2,3]. Since the discovery of NADES in nature in 2011 [4], researchers have found numerous applications for these green solvents as alternatives to replace organic solvents, which have the characteristics of being toxic, flammable, volatile, and harmful to the environment [5,6]. One of the biggest advantages of these green solvents is that by a simple adjustment of the mixture molar ratio some properties such as viscosity, solute

solubility and application (e.g. biomolecules extraction), can radically change [5,7,8]. For example, Setiawan et al. used different NADES to extract brazilin from Sappan wood. They showed that out of nine different NADES, betaine-lactic acid was the one that extracted the highest amount of dye [9]. Other studies focused on using these green solvents to extract lignin and holocellulose from biomass rice straw [10] or from other types of sources such as *Eucalyptus globulus* wood [11]. Even though in both studies the extraction of lignin was tested, they found that for the rice straw, the most effective NADES was lactic acid-choline chloride, while for the *Eucalyptus globulus* wood the most efficient one was propionic acid-urea [10,11]. The above mentioned studies highlight the paramount importance between NADES composition and how it relates to solute solubility and liquid extraction.

Most of the current research involving NADES has dealt mainly with applications rather than the thermodynamic stability and supramolecular structure. A NADES stable intramolecular structure is dependent on the hydrogen bond donors or acceptors groups of the components, their position and their spatial structure [1,12,13]. It has been shown that components that have a higher presence of hydroxyl or carboxyl groups form more hydrogen bonds leading to

Abbreviations: NADES, Natural deep eutectic solvents; B:U:W, Betaine:Urea:Water; ATR-FTIR, Fourier-transform infrared spectroscopy; NMR, Nuclear Magnetic Resonance; DFT, Density Functional Theory; BO, Bond Orbital; NBO, Natural bond orbital; PCM, Polarizable continuum model.

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a better stability of the liquids [1,14]. The specific properties of each NADES are highly dependent on the intramolecular hydrogen bonding of the components as well as the cohesive energy of the supramolecular structure [15]. Understanding the NADES supramolecular structure will give insights on the type of interactions that these solvents can have with a specific type of molecule, e.g. proteins. Therefore, there is a need to better understand their physical properties, molecular interactions and supramolecular structures to disclose their full potential as green solvents.

One way to uncover the potential of the NADES as a solvent is to understand the properties and characteristics of the NADESs single components. Using a betaine:urea NADES as an example, betaine is involved in protein and energy metabolism, and is an excellent protein stabilizer in various organisms to counteract harsh environmental conditions [16]. On the other hand, urea is a non-protective osmolyte that denatures proteins by degrading their functions and damaging the protein structure [16,17]. Previous studies with molecular dynamics simulations of the mixture of betaine-urea (B:U) highlighted that the protective role that betaine plays for the protein structures is enhanced by the urea interactions and how the mixture significantly counteracts the urea destabilizing effect [18]. However, there is still a need to experimentally understand how each component is arranged within the B:U mixture, and which molar ratio enhances the NADESs properties.

There are several known methods to prepare NADES at adequate molar ratios of hydrogen bond donor and hydrogen bond acceptor [1]. The two most used procedures are: i) the dissolution of the components in water followed by vacuum evaporation, and ii) heating the components in the desired molar ratios at a constant agitation of the components [1,14,19]. However, it is still unclear how these procedures affect the NADES supramolecular structure, properties and stability. Additionally, the water content and its role on the overall stability of the NADES and supramolecular structure has not yet been thoroughly explored.

The aim of the present study is to examine the significance of water content in betaine:urea:water (B:U:W) stability as well as the role of water in the supramolecular structure of this NADES. The betaine-urea mixture was selected due to their synergistic behavior, their capability for stabilizing proteins and its potential for biofilm treatment [17,2022]. We evaluated the stability and the interactions established within the NADES components by ATR-FTIR and ^1H and ^{13}C NMR and compared them with density functional theory calculations. From the results obtained in both experimental and theoretical studies, we analyzed the role of water in the supramolecular structure of the B:U:W NADES.

2. Materials and methods

2.1. Betaine:Urea:Water

Betaine (CAS Registry No. 10743-7), urea (CAS Registry No. 57 13-6), and ultrapure water (CAS Registry No. 773218-5) with a conductivity of $0.055\ \mu\text{S cm}^{-1}$, were purchased from Sigma-Aldrich (St. Louis, Missouri, USA) and used without further purification. The betaine and urea used for these experiments were anhydrous components. First, the B:U:W NADES was prepared with different water molar ratios (1:1:0, 1:1:1, 1:1:2, 1:1:3, 1:1:4, and 1:1:5) to observe the minimum water content needed to prepare a NADES that would not crystallize with time, in other words a stable NADES. All NADES were prepared in a water bath at 50°C at 600 rpm. For further studies, B:U:W (1:1:2) was selected to see if a stable NADES can be prepared by increasing the formation temperature. B:U:W (1:1:2) were prepared in silicon oil baths at six different temperatures (50, 60, 70, 80, 90, and 100°C) at 600 rpm, until a transparent solution was observed and kept at the mentioned temperatures for

extra 15 min. After the temperature study, 100°C was selected to study the stability of the NADES by leaving the samples 0, 5, 10, and 15 min longer at 100°C after a clear solution was observed. All metastable or stable B:U:W samples were analyzed by attenuated total reflection Fourier-Transform Infrared spectroscopy (ATR-FTIR) (PerkinElmer Inc., Waltham, Massachusetts, USA).

2.2. Structural variation of stable NADES

The following studies were done with B:U:W (1:1:2 and 1:1:3) mixtures to pinpoint the structural variation that led to their difference in stabilities. 600 MHz Nuclear Magnetic Resonance Spectroscopy (NMR) (Bruker, Billerica, Massachusetts, US) was performed at 21°C without the addition of deuterated solvent. All the chemical shifts of ^1H NMR and ^{13}C NMR were calibrated to that of the CH_2 peak at 3.6 ppm and 63.0 ppm, respectively. ATR-FTIR studies were made on the two different B:U:W molar ratios (1:1:2 and 1:1:3) to observe the impact of water addition on the B:U:W structure.

2.3. Modeling

Density functional theory (DFT) was employed at the B3LYP/6 311 + g(d,p) level of theory [23] to minimize the energy of multiple B:U:W conformations and the Natural Bond Orbital (NBO) [24] analysis was subsequently used to calculate the bond order (BO) parameter and quantify the hydrogen bond strength on the most stable structures. All of the DFT calculations and the NBO analysis (version 3.1.) were performed with the Gaussian 16 software [25]. The aqueous environment was taken into account with the polarizable continuum model (PCM) [26]. The stability of all the optimized structures was confirmed by frequency calculations obtaining 0 imaginary for each of them.

A minimum of four different structural conformations were tested for each B:U:W ratio, between [04] waters. In total above 30 conformations were studied and the most stable ones were singled out. After the structures were optimized, the NMR spectra was obtained by computing ^1H NMR and ^{13}C NMR. All figures of the molecular structures and the NMR were produced with Gaussview [25].

3. Results and discussion

3.1. B:U:W formation and stability.

B:U:W mixtures were prepared varying the water molar ratio (1:1:0, 1:1:1, 1:1:2, 1:1:3, 1:1:4, and 1:1:5). The results show that even at longer times of preparation (up to 24 h) and higher temperatures (up to 100°C), the water-free B:U:W (1:1:0) and the one with one mole of water (1:1:1) turned into a turbid solution, meaning that at these water contents the NADESs formation was not achieved (Fig. 1). A clear transparent liquid was formed only after adding two or more moles of water to the mixture at 50°C (B:U:W 1:1:2; 1:1:5). After the clear liquid was obtained, these samples were placed at 21°C , to verify the formation and stability of the B:U:W NADES. After 10 min of being at 21°C , the B:U:W (1:1:2) started to crystallize while the solutions with higher water content remained as transparent liquids (no crystallization) for more than four months (Fig. 2). Following these results, different formation temperatures (50, 60, 70, 80, 90, and 100°C) were tested to see if a stable 1:1:2B:U:W could be achieved. After formation of the transparent liquid, the samples were kept at the mentioned temperatures for extra 15 min before placing them at 21°C to observe stability. The results show that the NADES formed at 50 and 60°C crystallized after 10 and 85 min, respectively. For the NADES

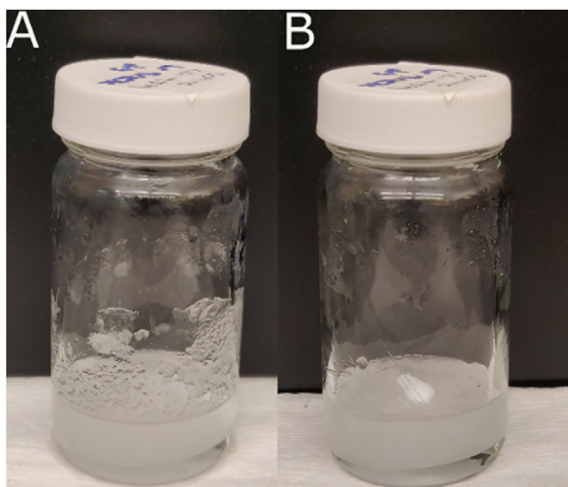


Fig. 1. Mixture water-free betaine and urea (1:1:0) (A) and that with one mole of water (1:1:1) (B). A transparent solution could not be formed at these molar ratios.

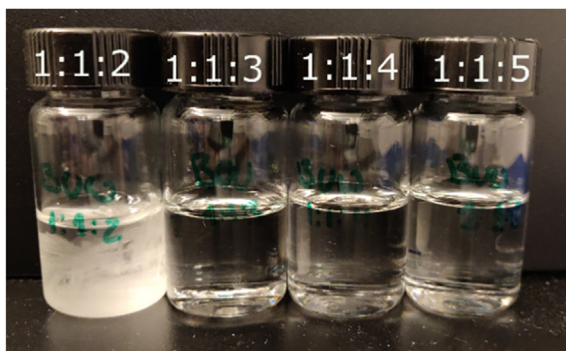


Fig. 2. Betaine:Urea:Water mixture at different water molar ratios starting from 1:1:2 up until 1:1:5. A transparent and stable solution is observed after the addition of three moles of water.

formed at 70°C and above, the B:U:W remained stable (transparent liquid) even after four months (Fig. 3).

To further study the temperature effect on the stability of the B:U:W system, the NADES with 1:1:2 ratio was formed at 100°C and kept at this temperature for different times (0, 5, 10, and 15 min). After the formation of a transparent liquid, the samples remained at 100°C for an extra 0, 5, 10, or 15 min. We found that leaving the sample at 100°C for 0, 5, 10 or 15 min after a transparent liquid was formed, is a determining factor for the metastability of B:U:W (1:1:2). All samples were removed from the 100°C oil bath after

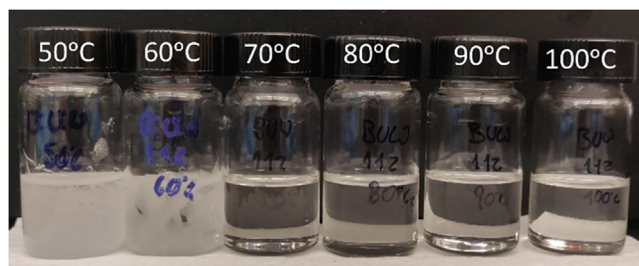


Fig. 3. B:U:W (1:1:2) prepared at different silicone oil baths temperatures (50, 60, 70, 80, 90, and 100°C). The NADES prepared at 50 and 60°C crystallized within 24 h while the NADES prepared at 70°C and above only crystallize when seeded.

their corresponding extra time and placed at 21°C to check stability. The NADES which was not kept additional time (0 min) at 100°C crystallized after 104 min. Conversely, leaving the sample for just five more minutes at 100°C, the sample crystallized only after two days of being at 21°C. The crystals were characterized by ATR-FTIR, showing that the main component of the crystals was betaine (Figure S1). The samples kept for 10 and 15 min at 100°C after the transparent liquid was formed did not crystallize and remained stable at room temperature (21°C) for the four months experimental period (Figure S2). The stability of the B:U:W (1:1:2), was evaluated by placing the non-crystallized samples at 20°C for 24 h. None of the metastable B:U:W (1:1:2) samples crystallized even after being at 20°C for 24 h. To reassure the metastability of the B:U:W (1:1:2), a seed crystal of betaine was placed in the stable B:U:W (1:1:2) and B:U:W (1:1:3). For the B:U:W (1:1:2) formation of crystals was observed almost immediately after the addition of the seed betaine crystal, while in the B:U:W (1:1:3), the crystal was dissolved and the solution remained stable (Figure S3). The same experiment was made by adding a seed crystal of urea for both ratios, however urea did not induced crystallization. These results suggest that the stability of NADES at this low water content is based on betaine stabilization in the liquid, and that water has an essential role in forming a stable NADES. Moreover, the results indicate that there should be a difference in the supramolecular structure of B:U:W 1:1:2 and B:U:W 1:1:3.

3.2. Supramolecular structures

To study the differences in the supramolecular structure formed by B:U:W 1:1:2 and 1:1:3, ^1H , ^{13}C , and ^{15}N NMR together with ATR-FTIR analyses were performed. ^1H NMR of B:U:W (1:1:2) compared with that of 1:1:3 shows that the extra water introduction had no significant impact on betaine protons (there are no observed changes in the signals generated by the resonances of the betaine protons). However, the NH of urea shifts to high field from 5.93 to 5.86, suggesting that these protons became less acidic (Fig. 4A). The analysis of B:U:W 1:1:2 ^{13}C NMR spectrum compared with B:U:W 1:1:3 shows that the introduction of the extra water caused a shift to downfield in all carbons in B:U:W 1:1:3 (Fig. 4B) except for the CH_2 group which is the reference fixed in 63 ppm. The shift to downfield of CH_3 suggests an increase of the localized charge at the nitrogen of betaine; the extra water does not accommodate the nitrogen charge. The deshielding (an increase of ppm) of the carbons of the functional groups $\text{C}=\text{O}$ (betaine and urea) proposes an increase in the participation of such groups in H-bonding, i.e., broadened electron sharing at the supramolecular interactions of the species.

The ^{15}N NMR shows a deshielding of the nitrogen present in the structure of betaine from 46.64 to 46.69, which is in concordance with the downfield shift observed in the ^{13}C NMR of the resonance of the CH_3 groups (Figure S4). Additional NMR spectra were made confirming the shifts observed in Fig. 4 between 1:1:2 and 1:1:3. The complementary NMR spectra of B:U:W 1:1:2 and 1:1:3 at 25°C (Figure S5) and 50°C (Figure S6), revealed that even changes in temperature maintain the supramolecular variations between both structures. A similar result is observed with B:U:W 1:1:2 and 1:1:3 with deuterated water (Figure S7). All pairs of NMR spectra showed the same tendency of chemical shifts from 1:1:2 to 1:1:3 confirming the effect of the third added water to the B:U:W system.

From the ATR-FTIR spectra of betaine-water (1:5), urea-water (1:5), and B:U:W (1:1:2 and 1:1:3), it can be observed that the $\text{C}=\text{O}$ and N-H bands from betaine and urea ($\sim 1600\text{ cm}^{-1}$) were broader in the B:U:W NADES (both 1:1:2 and 1:1:3) compared to the spectra of the single-component solutions (betaine-water and urea-water) (Fig. 5). The broadening of the peaks is ascribed to

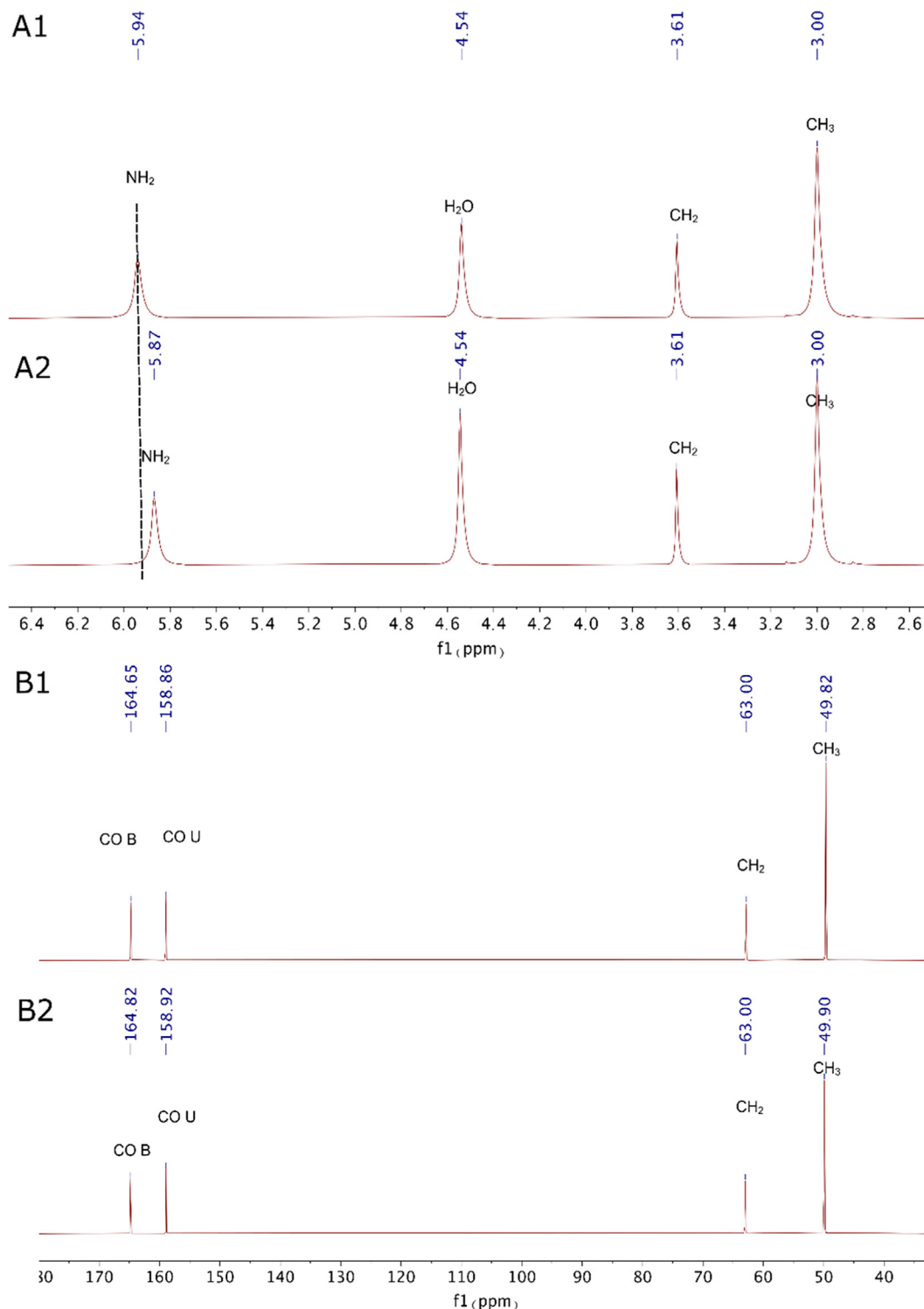


Fig. 4. ¹H NMR spectra of B:U:W (1:1:2) (A1) compared to ¹H NMR spectra B:U:W (1:1:3) (A2) and ¹³C NMR spectra of B:U:W (1:1:2) (B1) compared to ¹³C NMR spectra of B:U:W (1:1:3) (B2).

the formation of H-bonds, even though the amount of water is higher in the betaine-water and the urea-water solutions (five moles of water per mole betaine/urea). The C-N band from urea experienced a decrease in wavenumber from 1459 cm⁻¹ in urea solution to 1447 cm⁻¹ in B:U:W 1:1:2, attributed to the intermolecular H-bond formation. In 1:1:3, there was a slight increase (to 1448 cm⁻¹) of the band, suggesting an increase in the intramolecular C-N bond strength in urea, meaning that more electrons are sur-

rounding the bond, which is in concordance with the decrease of the chemical shift observed in the N-H group in ¹H NMR. The wavenumber of the carboxylate peak (COO) decreased from 1397 cm⁻¹ in betaine:water (1:5) to 1387 cm⁻¹ in B:U:W 1:1:2. This decrease indicates the formation of H-bonds between water and urea. The wavenumber increased to 1390 cm⁻¹ in 1:1:3, compared to 1387 cm⁻¹ for 1:1:2, indicates a moderate intensification in the electron density at the carboxylate bonds.

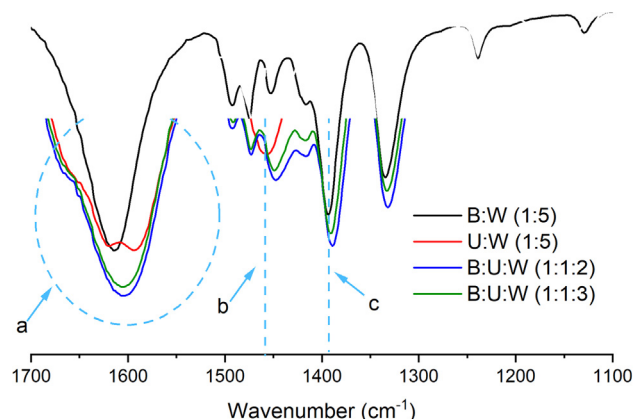


Fig. 5. ATR-FTIR spectra of Betaine:water (1:5) (black), Urea:water (1:5) (red), Betain:Urea:water (1:1:2 and 1:1:3) (blue and green respectively). C=O and N-H bands (a), C-N peak (b) and COO peak (c). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Even though the addition of the third water did not disrupt the betaine proton, there was an increase in the electron density surrounding the carboxylate group (Fig. 5). The effect in the COO observed in the ATR-FTIR was confirmed by ^1H NMR and ^{13}C NMR (Figure S8), the COO of the betaine decreased from 164.97 in B:W (1:5) to 164.65 in B:U:W (1:1:2) suggesting the formation of H-bonds. In the opposite, from 1:1:2 to 1:1:3, there is an increase in ppm from 164.65 to 164.82 which is related with an increase in the electron density.

The results of the NMR and ATR-FTIR suggest that the water plays an important role in the stability and formation of the NADES, making this a ternary mixture. This conclusion is supported by previously reported studies on the importance of water as part of the supramolecular structure of deep eutectic solvents due to the strong water interaction with the components; even at low water contents, water should be considered in the NADES structure [14,27,28].

3.3. Computational modeling

DFT calculations were performed to investigate the supramolecular interactions of Betaine:Urea:Water. Firstly Gaussview [25] was employed to manually create different conformations of possible candidates for B:U:W 1:1:2 and 1:1:3 supramolecular structure (Figure S9). The conformations showed in Figure S9 are all the structural conformations that converged by computational modeling. Additional structural candidates were proposed (over 30 different conformations) but these ones were not able to converge. From the converged structures the most stable ones (including thermal corrections at 298 K) were singled

out and the calculated NMR was compared with the experimental one (Fig. 6 and Table 1). Overall, structures where water lies next to the methyl groups were higher in energy (around 10 kJ/mol) compared to those where water was forming a H-bond network to betaine or urea.

Probing the minima of the potential energy surface of any liquid is a challenging task and in the case of NADES, classical molecular dynamics might be the first choice since it can be scaled up to hundreds or thousands of molecules, reaching the bulk level. However, NADES properties such as density, viscosity and transport coefficients depend on the water content and deviate from linearity, therefore any empirical force field must be further fine-tuned to account for all of these properties so as to obtain, for example, a meaningful hydrogen bond analysis or accurate solvation free energy values for each component [15,29].

Another procedure to search for a global minima is to employ a search algorithm coupled with a minimization method. The method employed in this work is similar to a combination of a search algorithm, such as the basin hopping, coupled with DFT instead of an empirical force field. This procedure is very suitable to prove the phase-space of small clusters in the gas or liquid phase [30] and also ideal for NADES whenever the use of a macro-scale model is unfeasible or needs fine-tuning [29]. The advantage of this method is that many conformations can be sampled with the accuracy of DFT and then IR, NMR spectra can be directly compared with experiments to assess the results. We would rather obtain a molecular-scale picture of the NADES stability (at the molecular level), still a lot smaller than the supramolecular ensemble [15]. Nevertheless, qualitative conclusions on the H-bond network, bond strength and the stability of each component in the presence of water can be extrapolated to the supramolecular structure as well.

The BO parameter was calculated from the NBO analysis to assess the stability of betaine in the 1:1:2 and 1:1:3 most stable structures. The BO of the carboxylate group of betaine is impacted by the addition of the third water near the C=O of urea. In betaine, the BO of the carboxylate (COO) changed from (1.9, 1) to (1.75, 1.75), that is, from a double-single bond to a nearly double bond with more resonance. This result is in concordance with the presented NMR and ATR-FTIR experimental results, indicates an increase in the electron density in the carboxylate moiety. The NBO analysis does not directly provide the BO for each bond but it gives a summary associated to each intramolecular bond corresponding to the number of electrons in the bonding and antibonding orbital. The BO parameter is obtained by subtracting the number of electrons in the bonding and antibonding orbitals divided by two and is directly related to bond strength and therefore, molecular stability [31]. We automated the calculation process by means of a bash/python script to facilitate the analysis from the Gaussian 16 output for all of the structures. In addition, a comparison of the experimental ^{13}C NMR was made with the simulated carbon NMR (Table 1). To facilitate the interpretation

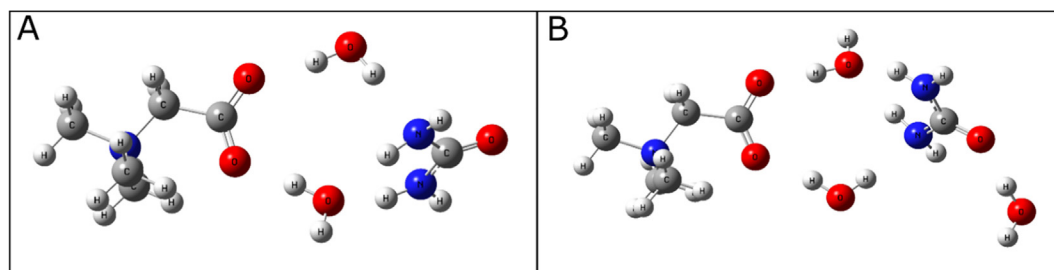


Fig. 6. Structural conformations of B:U:W 1:1:2 (A) and 1:1:3 (B) based on the minimum energy obtained by computational modelling. All the molecules are represented in different colors: oxygen (red), hydrogen (light grey), carbon (dark grey) and nitrogen (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1

NMR data comparison between the simulated supramolecular structure vs. the experimental data of B:U:W 1:1:2 and B:U:W 1:1:3.

Carbon signal(ppm)	Chemical shifts 1:1:2 [a]	Chemical shifts 1:1:2 [b]	Chemical shifts 1:1:3 [a]	Chemical shifts 1:1:3 [b]
CH₃	49.62	47.2252.5*	49.90	47.3952.97*
CH₂	63	63	63	63
CO (Urea)	158.86	159.22	158.92	160.13
CO (Betaine)	164.65	154.01	164.82	154.68

[a] Experimental values. [b] Modelling values. *The CH₃ groups in the modelling are represented in a range since the modelling shows a signal for each CH₃ groups.

of the experimental and theoretical spectra, the value of the CH₂ carbon signal was fixed as a reference. The same tendency was observed in both the experimental and theoretical studies, all carbon signals showed an increase in the chemical shift from B:U:W 1:1:2 to B:U:W 1:1:3. The slight changes observed in both NMR results (experimental and theoretical) confirms our proposed supramolecular structure.

To confirm the reliability of our conclusions, similar analyses were performed for the other calculated structures with higher energy (less stable) for 1:1:2 and 1:1:3 ratios, different from those analyzed in Table 1. The tendency observed in the ¹³C NMR was not in agreement with the experimental results (Table S1). These results confirm that the third water molecule stabilizes the supramolecular interaction between the species increasing the electron density localized at the betaine carboxylic group and the nitrogen of the urea moiety without causing a significant change in the overall chemical environment. The water molecules act as a solvent for the presented NADES and as a link of the bigger compounds, creating a stable supramolecular structure, forming a ternary mixture [14,27].

4. Conclusion

In this study, we evaluated the importance of the water content for the stability of the B:U:W NADES as well as the role of water in the supramolecular structure of this NADES. The importance of water in the stability and supramolecular structure of B:U:W system is confirmed by experimental studies and computational modeling. Different water ratios (from 0 to 5 mol) were studied in a betaine:urea (1:1) mixture. The experimental studies revealed that the minimum water content needed to prepare a metastable B:U:W NADES was in a ratio of 1:1:2 formed at 70 °C and above and remained at this temperature for 10 min or more after the NADES is formed. In addition, a minimum of three moles of water is required to form a NADES which won't crystallize even after seeded. NMR and ATR-FTIR studies demonstrated a clear difference in the hydrogen bonding and the supramolecular structure of the B:U:W system when a third mole of water was added (1:1:3) in the formation of this NADES. The addition of only one extra water molecule to the mixture creates an increase in the electron density in the moiety of the carboxyl group of the betaine. This increase in the electron density is also observed in the computational studies, where the bonds of the carboxyl group of the betaine experienced an increase in electron density as, quantified by the BO parameter, suggesting the generation of resonance. This work has demonstrated that the increase of the electron density enhances betaine's stability and the consequent stability in the B:U:W system. Understanding the structural characteristics and how the components interact in the mixture can lead to tailor-made applications of these green solvent in fields as diverse as biofouling control.

CRedit authorship contribution statement

Maria F. Nava-Ocampo: Conceptualization, Methodology, Data curation, Investigation, Software, Formal analysis, Project adminis-

tration, Writing - original draft. **Lamy Al Fuhaid:** Conceptualization, Writing - review & editing. **Adriano Santana:** Software, Formal analysis, Validation, Conceptualization, Writing - review & editing. **Szilárd S. Bucs:** Validation, Investigation, Methodology, Supervision, Conceptualization, Writing - review & editing. **Robert Verpoorte:** Writing - review & editing. **Young Hae Choi:** Writing - review & editing. **Geert J. Witkamp:** Conceptualization, Funding acquisition, Writing - review & editing. **Johannes S. Vrouwen-velder:** Funding acquisition, Writing - review & editing. **Andreia S. F. Farinha:** Validation, Investigation, Supervision, Methodology, Conceptualization, Project administration, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.molliq.2021.117655>.

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