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The need for a standardized protocol for the preparation of binary and ternary deep eutectic systems

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TITLE:

Preparation of Binary and Ternary Deep Eutectic Systems

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KEYWORDS:

eutectic systems, freeze-drying, vacuum evaporation, heat and stirring, characterization, water content

SUMMARY:

This protocol aims to standardize the preparation of deep eutectic systems throughout the scientific community so that these systems can be reproduced.

ABSTRACT:

The preparation of deep eutectic systems (DES) is *a priori* a simple procedure. By definition, two or more components are mixed together at a given molar ratio to form a DES. However, from our experience in the laboratory, there is a need to standardize the procedure to prepare, characterize and report the methodologies followed by different researchers, so that the results published can be reproduced. In this work, we test different approaches reported in the literature to prepare eutectic systems and evaluated the importance of water in the successful preparation of liquid systems at room temperature. These published eutectic systems were composed of citric acid, glucose, sucrose, malic acid, β-alanine, L-tartaric acid and betaine and not all of preparation methods described could be reproduced. However, in some cases, it was possible to reproduce the systems described, with the inclusion of water as a third component of the eutectic mixture.

INTRODUCTION:

Deep eutectic solvents have been named the solvents for the 21st century and are considered a new generation of solvents. They are defined as a mixture of two or more chemical compounds at a particular molar ratio to result in a significant decrease in the melting temperature of the individual components, becoming liquid at room temperature[1-3]. In this sense, the preparation

of the solvents does not require any chemical reaction and hence the production yield is 100%. In 2011, Choi and co-workers reported the possibility of naturally occurring DES and named them, natural deep eutectic solvents (NADES)[3-5]. NADES can be prepared from different combinations of sugars, amino acids, organic acids and choline derivatives; and these systems prepared from natural components are inherently biocompatible and biodegradable, presenting considerably less toxicity compared to other alternative solvents (e.g., ionic liquids)[5-8]. Since 2015, the number of publications in the field has risen exponentially and the possible applications of NADES are very broad[3]. Even though many manuscripts and reviews have been published, there are fundamental questions that persist, and scientists have not yet found the answer to intriguing questions such as the mechanisms underlying DES formation. Understanding the DES formation mechanism would lead to a consolidated approach towards the development of new systems, rather than the current trial and error approach. Furthermore, the opportunities in the field are growing each day, as consumers become more aware of the sustainability of their products, not only in terms of their end-life but also in terms of processing itself[8-10]. To drive major innovations in the field of deep eutectic solvents, the standardization of the production and characterization methods is first required. The lack of reproducibility of some of the systems reported in the literature was the motivation to develop this work as we faced this issue several times. Herein, we demonstrate the need and crucial importance to accurately describe the materials and methods and show that although the preparation of DES is a simple and straightforward procedure, there are some key aspects (e.g., the presence/amount of water) that must always be discussed.

PROTOCOL:

NOTE: The NADES studied were betaine:L-(+)-tartaric acid (2:1), β-alanine:DL-malic acid (3:2), glucose:sucrose (1:1) and citric acid:glucose (2:1). These systems were prepared by different methods: freeze-drying (FD), vacuum evaporation (VE), and heat and stirring (HS) with and without water. As an example, the protocol for the system citric acid:glucose (2:1) is given. The NADES were characterized by differential scanning calorimetry (DSC), polarized optical microscopy (POM), water content and nuclear magnetic resonance (NMR) spectroscopy.

1. NADES preparation

1.1. Freeze drying

1.1.1. In separate containers, add 2 g of citric acid monohydrate and 0.9530 g of glucose monohydrate. Add 10 mL of deionized water to each and stir until the compounds are completely dissolved.

1.1.2. Mix the two solutions together and ensure the homogenization of the final solution. Place the solution in a round bottom flask.

1.1.3. Freeze it using liquid nitrogen. Place the flask in a freeze-dryer for 48 h to ensure that all the water is removed from the sample.

1.2. Vacuum evaporation

1.2.1. Weigh 2 g of citric acid monohydrate and 0.9530 g of glucose monohydrate in separate containers. Add 10 mL of deionized water to each and stir until the compounds are completely dissolved.

1.2.2. Mix the two solutions together and ensure the homogenization of the solution. Place the solution in a round bottom flask.

1.2.3. Using a rotary evaporator, dry the sample until a clear, viscous liquid is formed.

1.3. Heating and stirring

1.3.1. Weigh 2 g of citric acid monohydrate and 0.9530 g of glucose monohydrate into the same vial. Add 278 μL of water.

1.3.2. Place the vial with a magnetic stirring bar in a 50 °C water bath.

1.3.3. Leave the sample until a clear, viscous liquid is formed.

2. NADES characterization

2.1. Polarized optical microscopy (POM)

2.1.1. Place a droplet of NADES on a microscope glass slide for observation.

2.1.2. Using the transmission mode of a microscope, perform the optical characterization of the sample at room temperature.

2.2. Karl-Fisher titration

2.2.1. Collect 100 μL of NADES in a syringe, and then clean the excess liquid on the outside.

2.2.2. Place the syringe on a scale and tare it.

2.2.3. Press **START** on the KF equipment and add a small drop of the sample to the vessel.

2.2.4. Weigh the syringe, enter the mass on the KF equipment and press **ENTER**. The result will appear on the screen in ppm of water.

2.3. Differential scanning calorimetry (DSC)

2.3.1. Place 3-10 mg of each sample in a hermetic aluminum pan with a covering lid. Close the

pan with a sample press.

2.3.2. Analyze the samples using a DSC with a temperature range of -90 °C up to the degradation temperature, with a heating rate of 10 °C/min. Perform two cycles with an isothermal hold of 2 min and analyze under a nitrogen atmosphere (50 mL/min).

2.4. Nuclear magnetic resonance (NMR)

2.4.1. Prepare a 5 mm NMR tube by dissolving 250 µL of NADES with 250 µL of dimethyl sulfoxide-d6 (DMSO-d_6).

2.4.2. Acquire the ^{1}H and NOESY spectra at 25 °C on a 400 MHz spectrometer.

2.4.3. Use an appropriate software to analyze the spectra, and use the chemical shift of DMSO-d6 (δ 2.50 ppm) to assign all the signals of each component.

REPRESENTATIVE RESULTS:

From the preparation of NADES, the results we expect to obtain are shown on **Figure 1**. A description of each system is made below. Using the freeze-drying method, the result should be a solid or a very dense paste since all the water is removed from the system. Using the evaporation method, the result should be a clear and viscous liquid. Using the heating and stirring method with the addition of small amounts of water, the result should be a clear and very viscous liquid.

The results obtained from POM can be seen on **Figure 1**. When a NADES is completely formed, we expect to see a black image, indicating that the sample is completely amorphous and that there are no crystals remaining in the system. The results obtained from KF titration are described in **Table 2**. Besides the amount of water that is added to the systems, the percentage of water of the final mixture also depends on the water content of the reagents.

Concerning the DSC, the goal of this technique is also to confirm that the system is liquid in the temperature range that it will be applied, so the expected result is to have a thermogram that shows no thermal events on the temperature range of interest (**Table 2**). The NMR technique is used to confirm the existence of hydrogen bond formation, which is the main characteristic of NADES systems. This can be confirmed by observation of the change in chemical shifts of each signal, and by analysis of the NOESY spectra, that shows spatial and intermolecular correlations (**Figure 2**).

FIGURE AND TABLE LEGENDS:

Table 1. Systems reported in literature and their preparation method.

Table 2. Water content (%) of the systems prepared by different methods.

Figure 1. **Representative results of the NADES when prepared by a) freeze-drying, b) vacuum**

evaporation and c) heating and stirring with addition of water. The picture shows that when the system is freeze-dried, the result obtained is a crystal since all the water is removed from the mixture whereas when VE and HS methods are used, the amount of water needed for the NADES to form is present and the obtained result is a homogenous liquid at room temperature.

Figure 2. **Polarized optical microscopy of CA:Glu (2:1) prepared by different methods, with cross polarizers (left image) and parallel polarizers (right image) – 100 µm (10x amplification).** The black images show that the sample is a liquid at room temperature. The FD sample is completely crystalized since the result obtained from this technique was not a liquid.

Figure 3 – a) Overlay of ^{1}H NMR spectra of the (A) NADES system citric acid:glucose:water (2:1:4), (B) glucose, and (C) citric acid; b) NOESY spectrum of the NADES system citric acid:glucose:water (2:1:4). The overlaid spectra show the difference in chemical shifts of each component upon DES formation, originated by the establishment of hydrogen bonds between them. The NOESY spectrum shows the interaction between the OH proton from citric acid with the remaining protons from both components.

DISCUSSION:

The different methodologies reported in the literature for the preparation of NADES are a heating and stirring method (HS), vacuum evaporation (VE), and freeze-drying (FD). The systems we have prepared in this work are described by different authors in the literature[4-6,10,11]. **Table 1** lists the components of each mixture, as reported in the original manuscript as well as their preparation method.

Upon our investigations to reproduce the systems described, we realized that in some cases it was not possible to achieve a similar NADES, as a clear, viscous, liquid sample at room temperature. Preparing a NADES relies on many factors. Some can be easily controlled, but others are more difficult to standardize. The most important thing to consider is that the final product cannot rely on external factors such as the equipment used.

The systems prepared by different methods were then characterized. With polarized optical microscopy (POM), it was observed that with the HS method without water, even at different temperatures, the NADES did not form a clear and viscous liquid. However, a homogeneous and clear, viscous liquid was observed as represented in **Figure 1** when applying the HS method with small amounts of water and the VE method for the preparation of the NADES.

DSC was used to determine the thermal events of the mixture. The results showed that the system is liquid at room temperature and up to 130 °C, since the thermogram shows no thermal events. The water content of each sample was measured by Karl-Fischer titration, and the results are represented in **Table 2**. The water content of the systems must be reported, since it is the parameter that most influences the properties of the obtained liquid, such as viscosity and polarity. These changes have great impact on the outcome of the application for which the NADES is designed.

NMR was also used to confirm the formation the mentioned NADES systems, through the formation of hydrogen bonds between the molecules of each system. One example is given in **Figure 2** for the NADES system citric acid:glucose (2:1) with 17% water obtained by HS where the proton spectrum of this NADES and the starting materials (citric acid and glucose) are overlaid (**Figure 2a**). From this, it is possible to observe changes in the chemical shifts of some protons from each molecule. The major change is the shifting of the OH proton from citric acid. Originally, this signal appears at 5.16 ppm, but this signal shifts to 6.22 ppm because of the formation of hydrogen bonds. This is confirmed by the NOESY spectrum (**Figure 2b**), where the strong interaction between the OH from citric acid and the remaining protons is visible. A similar interaction was observed for the other NADES systems.

In this study we observed that the description of the preparation method for eutectic systems reported in literature sometimes are incomplete, due to the lack of information regarding the water content of most systems. In the VE method, the water is added by preparing solutions of different components and mixing at a temperature that leads to the formation of eutectic systems; however, we cannot be sure of the minimum required water content. The knowledge of percentage of water needed to form the systems is considered hence, a crucial point that should always be reported, for others to be able to reproduce the preparation of the different eutectic mixtures.

The best method to use is the HS method with water added as it takes less time to prepare, for cases where the water content is already described. However, if this information is not available, the easiest method is the VE method, where all the available water is removed and only the water interacting with the NADES components remains in the system. In any case, researchers should let the systems evaporate for enough time to ensure that free water is removed from the system. This timing is dependent on the equipment and therefore it is not enough to describe in the materials section the duration of the VE method, but the water content has always to be reported.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

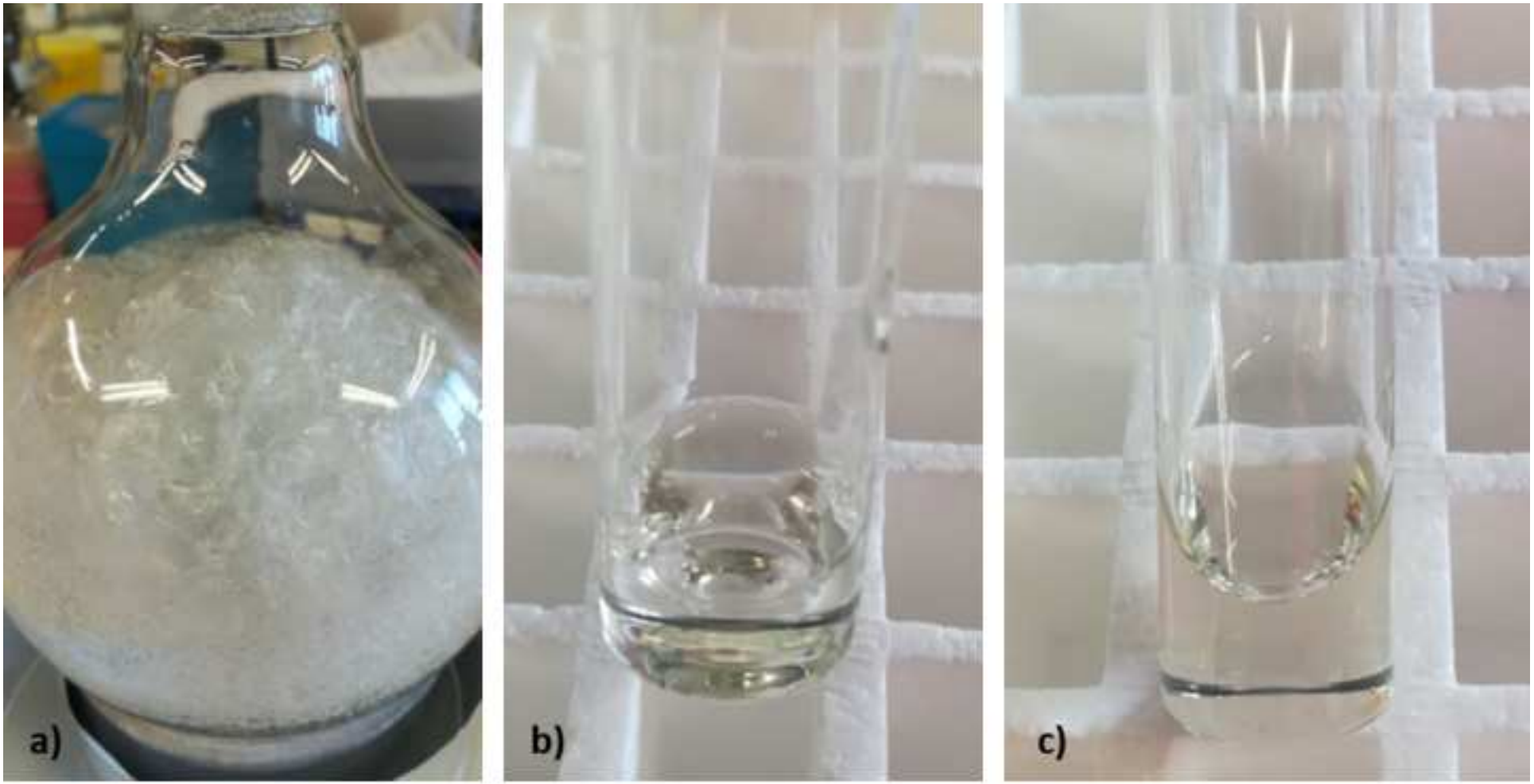

Figure 3

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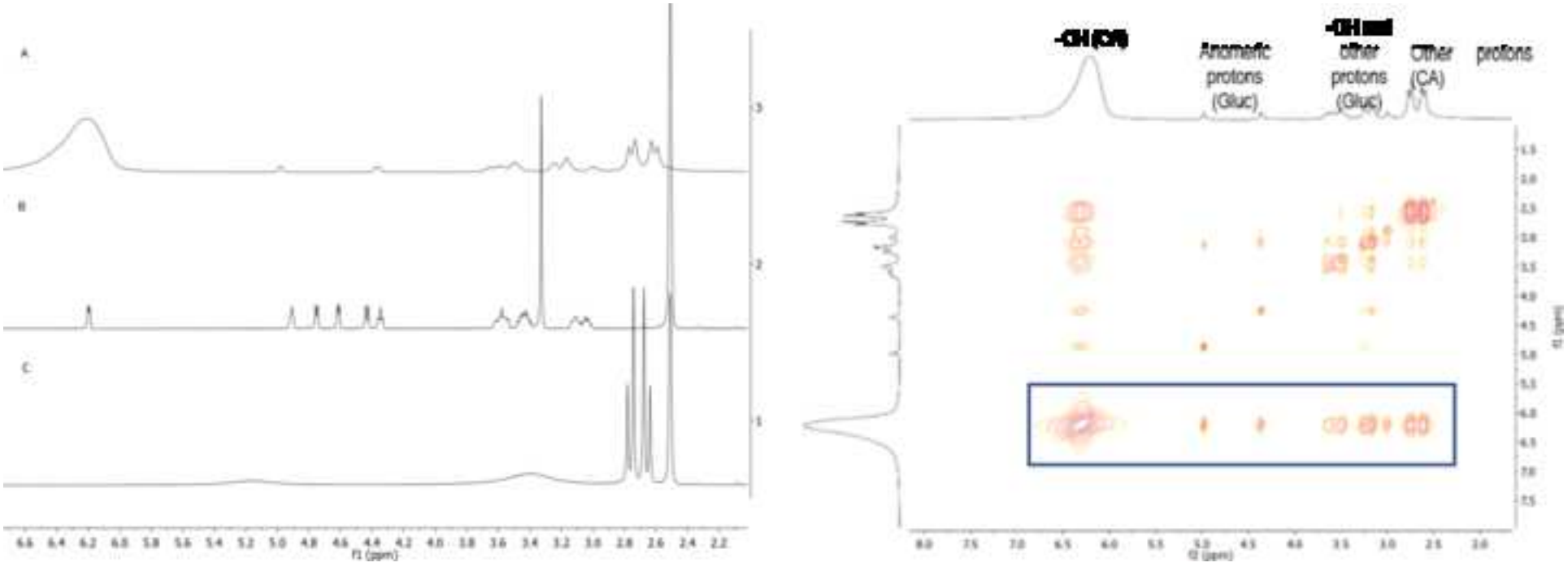

Figure 2

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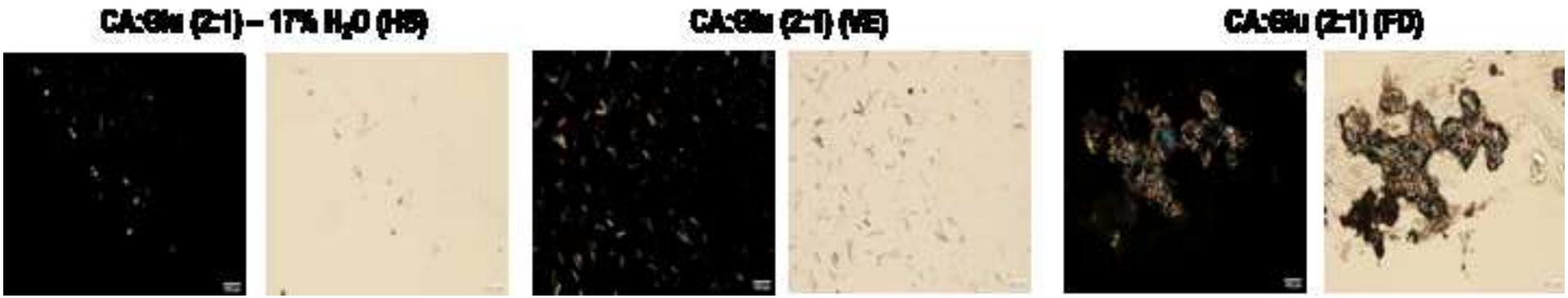

Table 1 - Systems reported in literature and their prepa

Component 1	Component 2	Preparation Method
Betaine (Bet)	L-(+)-Tartaric Acid (LTA)	Vacuum evaporating (VE)
β-Alanine (β-A)	DL-Malic Acid (MA)	Vacuum evaporating (VE)
Glucose (Gluc)	Sucrose (Suc)	Freeze-dried (FD)
Citric Acid (CA)	Glucose (Gluc)	Freeze-dried (FD)

aration method.

Reference
Dai *et al* . (2013)5 and Espino *et al* . (2016)6
Dai *et al* . (2013)5 and Espino *et al* . (2016)6
Choi *et al* . (2011)4 and Espino *et al* . (2016)6
Choi *et al* . (2011)4 and Espino *et al* . (2016)6

Table 2 – Water content (%) of the systems prepared by different methods.

NADES	Preparation Method	Water Content (%)
		Karl Fischer measure
Bet:LTA (2:1 + 20% water)	Heating and stirring, adding water	19.94 ± 1.28
Bet:LTA (2:1)	Vacuum evaporating	11.36 ± 0.78
β-A:MA (3:2 + 11% water)	Heating and stirring, adding water	11.45 ± 0.25
β-A:MA (3:2)	Vacuum evaporating	18.84 ± 1.78
Gluc:Suc (1:1 + 21% water)	Heating and stirring, adding water	20.88 ± 0.13
Gluc:Suc (1:1)	Vacuum evaporating	22.56 ± 0.48
CA:Gluc (2:1 + 17% water)	Heating and stirring, adding water	17.33 ± 0.68
CA:Gluc (2:1)	Vacuum evaporating	20.04 ± 0.26

Table of Materials

Click here to access/download;Table of Materials;JoVE_Table_of_Materials (1).xlsx

Name of Material/Equipment	Company	Catalog Number	Comments/Description
5 mm NMR tube	Norell		
Acid citric monohydrate	Sigma-Aldrich		
Advance III spectrometer	Bruker		
Deionized water			
dimethyl sulfoxide-d6	Sigma-Aldrich		
DSC Q200	TA Instruments, USA		
Freeze-dryer CHRIST ALPHA 1-4	Braun Biotec International		
Glucose monohydrate	Cmd chemicals		
Karl Fisher Coulometer	Metrohm		
Olympus BX-51 polarized optical microscope	Olympus		

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3. Please provide at least 6 keywords or phrases. There are only 5.

“Water content” was added as a keyword.

4. JoVE cannot publish manuscripts containing commercial language. This includes company names of an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

All commercial language was removed from the manuscript and the products are referenced in the Table of Materials and Reagents.

5. Please add a one-line space between each of your protocol steps.

It was added

6. Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file.

All the tables were removed from the manuscript and uploaded as .xlsx files.

7. Please remove the embedded figure(s) from the manuscript. All figures should be uploaded separately to your Editorial Manager account. Each figure must be accompanied by a title and a description after the Representative Results of the manuscript text.

All figures were removed from the manuscript and uploaded separately.

8. Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

The discussion was carefully reviewed, and all the topics are now included. Also citations were included where needed.

9. Please provide more references (at least 10) to support your manuscript.
The manuscript was carefully reviewed and references were added.

Reviewers' comments:
Reviewer #1:

Manuscript Summary:
The manuscript repots on different methods for preparation of DESs and how these methods affect the composition of the resulting DESs, mainly in terms of water content.

Major Concerns:
None

Minor Concerns:

I think the authors should also include a solvent-less method such as grinding the individual components in a mortar to obtain the DES.
Moreovoer, I think the authors should acknowledge who firts explore each of the methods (including the reference where each particular method was first described).

In this regard, I think Abbott and coworkers were the first describing the heating and stirring method (Chem. Commun. 2003), I think del Monte and coworkers did it with the freeze-drying method (Langmuir 2009), and I am not fully sure about the evaporation and the griding in a mortar processes (for instance, how heating and stirring and grinding in a mortar affect the composition of the resulting DESs can be found in ACS Sustainable Chem. Eng.2014, 210, 2416-2425 but I think prepared DESs using the grinding in a mortar process before 2014). I guess the authors should search in these two latter cases who first reported these methods of preparation.

We tested the heating and stirring method without adding water (which is similar to grinding the components with a mortar). The obtained result was a solid white powder, or in some cases a white paste, but never a liquid. The references for the first time each of the methods were described were included in the introduction.

Reviewer #2:

The authors proposed a standard protocol for the preparation of deep eutectic solvent. The preparation of these solvent seems simple but it indeed need some standardization as it is often difficult to reproduced experimental results. Therefore, this standardization is crucial for the future of DES.

I have only minor remarks before publication:
- line 70 author should add abbreviation after freeze drying, vacuum evaporating....

The abbreviations were added and applied to the rest of the manuscript.

- line 80: authors used solvent evaporation while in the whole manuscript they use vacuum evaporating

The title of the section was changed to vacuum evaporation

- lie 129: authors should explain how the standards are prepared for NMR studies
We have not used external standards (e.g. TMS) in DES samples. We used the chemical shift of DMSO-d6 (δ 2.50 ppm) as standard to assign all the signals in the spectra.

Reviewer #3:

Manuscript Summary:
The authors presented a protocol to synthesize DESs in order to reproduce literature data. This protocol will be helpful to the community. It can be accepted with minor revision.

Minor Concerns:

1) I understood that water is added to the DES to keep a liquid solution. However, this will change also the properties of the DES as solvent. For some applications, water has to be avoided. It should be stated clearly that the presence of water change the properties.

The presence of water and its influence in the properties of the DES was clearly stated on the discussion section.

2) If two components are mixed without adding additional water, how can be ensured how much water is present in the DES phase? It should be recommended to measure the water content of a DES, as this not only changes the viscosity or decides whether the mixture is liquid or not, it also decides about the properties. The maximum water content is the equilibrium concentration, which can be obtained by thermodynamic models or experimental data (authors might cite https://doi.org/10.1016/j.fluid.2019.02.010).

This article mentions hydrophobic systems, but in our work the systems used were hydrophilic, hence this data does not correlate. However, the water content of all the systems used was carefully measured by Karl-Fisher titration.