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Structural elucidation and long-term stability of synthesized NADES: A detailed physicochemical analysis

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ABSTRACT

The use of sustainable solvents is gaining momentum and contributing to the development of more environmentally friendly extraction methods. However, there is a lack of solvents that can cover a wide range of polarities, as is the case with conventional solvents. Therefore, this study focuses on the preparation and comprehensive characterization of different Natural Deep Eutectic Solvents (NADES) encompassing both hydrophilic and hydrophobic types. NADES were prepared through the heating and stirring method and using various natural components (betaine, amino acids, sugars, polyalcohol, fatty acids). The synthesized NADES were evaluated for their physicochemical properties, including density, pH, viscosity, conductivity, and polarity, immediately after preparation (time zero) and after a twelve-month storage period to assess their long-term stability. Nuclear Magnetic Resonance and Fourier-transform infrared spectroscopy were utilized to elucidate the structural configurations of the NADES mixtures. These techniques confirmed the formation of hydrogen bonds among the NADES components, except for the one composed only with sugars (glucose, sucrose) and water, thereby validating the successful preparation of the target solvents. Moreover, the impact of dilution on the properties of NADES was investigated, demonstrating that 30 % water addition is optimal, maintaining the desirable properties of the solvent while enhancing certain characteristics like viscosity and density, making them more suitable for extraction purposes and subsequent analysis. The findings from this study offer interesting understanding into the structural and physicochemical properties of NADES, suggesting future applications as green solvents for lab scale extractions but also in various industrial field.

1. Introduction

Natural Deep Eutectic Solvents (NADES) have emerged as alternatives to conventional organic solvents due to their eco-friendly nature, low toxicity, improved safety, enhanced solubility and selectivity, energy efficiency and wide applicability across research fields and industrial applications such as pharmaceuticals, food, and cosmetics [1]. Inspired by the remarkable properties of biological systems, NADES represent a new class of solvents formed by the complexation of two or more naturally occurring components, typically including hydrogen bond donors (HBD) and hydrogen bond acceptors (HBA), at specific molar ratios. Sugars, organic acids, and alcohols are natural compounds widely used as the HBD. NADES are formed through molecular interactions such as hydrogen bonding, van der Waals interactions, electrostatics and dispersion forces. NADES exhibit a lower melting point than their individual starting materials (HBA and HBD). This phenomenon results from charge delocalization that arises during hydrogen bond interactions [2]. Due to their natural origin, NADES have lower toxicity than organic solvents and are easy to prepare, making them a promising substitute to traditional solvents in different sectors such as food, nutraceutical, pharmaceutical, and cosmetic industries. However, optimizing the preparation process is crucial to ensure reproducibility

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and scalability, which remain significant challenges in this area [3]. Betaine has recently been employed as HBA in the preparation of NADES, replacing choline chloride (ChCl). Derived from renewable resources and as a by-product of sugar production, betaine offers advantages over ChCl due to its high biodegradability and low toxicity [4]. Betaine-based NADES have been effectively used to extract natural compounds from plant materials [5]. Additionally, their potential is being investigated in diverse fields, including biomedical science, chemical reactions, catalysts, nanomaterials, gas separation, cosmetic and nutraceutical industries. These varied applications highlights the importance of betaine-based NADES as innovative, eco-friendly and economical solvents [6]. Additionally, other NADES types, including those based on organic acids, sugars, urea and amino acids have shown significant potential across various application, such as sample treatment to optimize the extraction of bioactive compounds [7] and contaminants [8]. Furthermore, NADES based on terpenes and medium-long chain fatty acids, known as hydrophobic NADES are gaining interest [9]. Hydrophobic NADES are particularly valued for their potential for the extraction of non-polar compounds [10]. Even though the multidisciplinary applications of NADES are abundantly reported in the literature, comprehensive data on the physicochemical properties and long-term stability, are still lacking. As solvents, the most important physicochemical properties that require characterization are viscosity, density, and polarity. Furthermore, the pH, electrical conductivity and their water content are also of significance. These physicochemical properties will determine the behavior and performance of the NADES [11,12,13]. Moreover, analytical techniques, including nuclear magnetic resonance (NMR) spectroscopy [13,14] and Fourier-transform infrared (FT-IR) spectroscopy [15], have been employed to investigate the molecular structure and dynamics of NADES. In this framework, the main purpose of this work is to study the formulation of different NADES with a broad range of polarity, spanning from 0.34 to 1.29 E_{NR} (kcal/mol). By investigating these solvents with diverse physicochemical properties, we aim to develop a comprehensive selection of solvents suitable for extracting a variety of compounds, offering a sustainable alternative to traditional organic solvents. Nine hydrophilic betaine-based NADES, eight hydrophilic NADES made with sugars, amino acids and polyols and four hydrophobic NADES were included in the study. Many combinations of HBA and HBD were sourced from the literature, while the appropriate formulation to prepare each type if NADES was optimized in the study. However, their structural elucidation has not yet been reported. Additionally, most of the physicochemical properties and long-term stability were not reported. Therefore, physicochemical properties, including viscosity, density, polarity, pH, electrical conductivity, and prediction of water content were assessed. NMR, and particularly the Nuclear Overhauser enhancement spectroscopy (NOESY), as well as FT-IR were employed for their structural elucidation. In addition, the effect of water addition (dilution) of one representative NADES was evaluated. All the properties were tested at time zero, the day after the preparation, and after twelve months to study their stability over time. As a result, we obtain a list of different characterized NADES that can be used individually as solvent and as starting eutectic mixtures to create custom NADES (e.g. water addition) tailored to specific application. The results from this work will significantly contribute to the efficient design of these versatile solvents guiding the selection of suitable NADES and enhancing the understanding of the relationship between physicochemical properties and the performance of NADES in a specific application.

2. Materials and methods

2.1. Chemical and reagents

For NADES preparation, betaine anhydrous (>97 %, CAS 107-43-7) was obtained from TCI (Tokyo, Japan), sucrose (CAS 57-50-1) and D-(+)-glucose (CAS 50-99-7) was purchased from Cmd Chemicals (Funchal, Portugal). Choline chloride (≥98 %, CAS 67-48-1), glycerol (≥99.5 % CAS 56-81-5), DL-proline (99 %, CAS 609-36-9), citric acid (>99.5 %, CAS 77-92-9), (±)-1,3-butanediol (CAS 107-88-0), urea (CAS 57-13-6), menthol (CAS 1490-04-6), thymol (CAS 89-83-8), decanoic acid (CAS 334-48-5), dodecanoic acid (CAS 143-07-9) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Deuterium oxide and deuterium chloroform were obtained from CortecNet (Voisins-Le-Bretonneux, France). Bi-distilled water was obtained using Milli-Q System (Millipore, Bedford, MA, USA).

2.2. NADES preparation

The proposed eutectic mixtures were prepared by mixing HBD and HBA in the appropriate amounts to obtain the desired molar ratio. Combinations of HBA and HBD were sourced from the literature and for NADES preparation, heating and stirring method was used [16]. The binary and ternary component mixture with specific molar ratio were placed in a bottle with a stirring bar and cap and heated in a water bath at 50 °C, 70 °C and 80 °C, depending on the NADES, with agitation till a clear and homogeneous liquid was formed, within a time range of 25–90 min depending on the type of NADES. The mixtures were then allowed to cool down naturally at room temperature. In order to confirm the formation of an homogeneous liquid, the mixtures were observed over 24 h to ensure that no re-crystallization has occurred [9]. Details of preparation and the molar ratio used are reported in Table 1. The assigned nomenclature corresponds to the abbreviations of the two main components of the mixture. The hydrophilic nature of NADES and the addition of water to the mixture is represented by adding “H” at the end of the nomenclature.

2.3. Detailed methodology for the physicochemical characterization of NADES

Density tests were performed using a density meter (Anton Paar, model: DMA 5000, Austria) at 25 °C. Conductivity test was recorded at room temperature on a 856KF coulometer (Metrohm, Belgium) equipped with 728 stirrer and 756 KF coulometer keyboard. Viscosity was measured at 25 °C and 40 °C using a viscosimeter equipped with a CC17 cylinder (model: rotational rheometer, ReholabQC, Anton Paar, Austria). pH was recorder using a pH meter (Delta 320, Mettler Toledo, Shanghai). Polarity measurement was done with Nile red as a solvatochromatic probe. Standard solutions of Nile Red in ethanol at 0.1 % w/v were prepared. The measurement was then carried out by diluting the stock solution 1:200 in the various NADES. The λ_{max} was determined with a UV/Vis spectrophotometer (Cary WinUV Software with the Cary 60 UV–Vis Spectrophotometer, Agilent) and calculated in the following formula to obtain E_{NR}: E_{NR} (kcal/mol) = $hcNA/\lambda_{max}$ = $28{,}591/\lambda_{max}$, which was then normalized based on Reichardt’s method [17]. All the measurements were carried out in triplicate and expressed as mean ± standard deviation.

2.4. NADES structure elucidation

2.4.1. Fourier transform infrared spectroscopy

FT-IR spectroscopy was used to characterize the prepared NADES. The primary goal of using FT-IR was to analyze the functional group present and to check the preservation of their characteristic bands with up and/or down shifts, which reflect the interactions among NADES components. An FT-IR spectrometer (Agilent Cary 630 FT-IR Spectrometer) equipped with a Quest single reflection diamond attenuated total reflectance (ATR) accessory, in real time mode with a resolution of 4 cm^{-1} was used. The spectra of NADES were collected over the range from 4000 to 400 cm^{-1} at room temperature (25 °C).

Table 1
Nomenclature, composition, preparation and visual aspect of NADES.

Type		Component 1	Component 2	Water	Molar ratio	Preparation	Visual aspect
	BH	betaine	water		1:1	heating and stirring at 80 °C	transparent liquid
	BH	betaine	water		1:5		transparent liquid
	BH	betaine	water		1:10		transparent liquid
	BG	betaine	glycerol		2:1	heating and stirring at 80 °C	transparent viscous liquid
	BGH	betaine	glycerol	yes	2:1:10.2		transparent liquid
	BBG	betaine	1,3-butanediol		1:3	heating and stirring at 80 °C	transparent viscous liquid
	BBGH	betaine	1,3-butanediol	yes	1:3:10.6		transparent liquid
	BLA	betaine	lactic acid		1:1	heating and stirring at 80 °C	transparent viscous liquid
	BLAH	betaine	lactic acid	yes	1:1:3.5		transparent liquid
Hydrophilic	**GCAH**	citric acid	glucose	yes	1:1:3	heating and stirring at 80 °C	pale yellow viscous liquid
	GCAH	citric acid	glucose	yes	1:1:6		pale yellow liquid
	PCAH	proline	citric acid	yes	1:1:1	heating and stirring at 70 °C	pale yellow viscous liquid
	PCAH	proline	citric acid	yes	1:1:4.4		pale yellow liquid
	UG	Urea	glycerin		1:3	heating and stirring at 50 °C	transparent viscous liquid
	UGH	Urea	glycerin	yes	1:3:2.5		transparent viscous liquid
	GSH	Glucose	sucrose	yes	1:1:10	heating and stirring at 80 °C	transparent viscous liquid
	TM	thymol	menthol		1:1		transparent liquid
	OAM	oleic acid	menthol		2:1		pale yellow liquid
Hydrophobic	**DAM**	dodecanoic acid	menthol		1:4	heating and stirring at 70 °C	transparent liquid
	DAT	decanoic acid	thymol		1:2		transparent liquid

2.5. ^{1}H NMR and 1D-NOESY experiments

Proton NMR (^{1}H NMR) and one-dimension NOESY (1D-NOESY) experiments were conducted to confirm the formation of the supramolecular structure of the NADES by analysing the interactions and spatial proximity of molecules. For that, the presence of Nuclear Overhauser Effect (NOE) between the constituents of the NADES was studied, following the method reported by Carbonell. et al. [13]. The in-house synthetized NADES were stored in dark glass bottles before analysis at room temperature. Then, 100 μL of each NADES were placed in a 2 mL Eppendorf tube and 500 μL of deuterated solvent (i.e., water or chloroform depending on the NADES) was added. The mixture was vortexed until its homogenization. The resulting solution was placed into a 5 mm NMR tube for its analysis. ^{1}H NMR and NOESY experiments were carried out on a Bruker Avance III HD 400 MHz spectrometer (399.59 MHz proton frequency/9.38 T) equipped with a PI HR-BB0400S1 5 mm probe. During the experiments, the temperature was held stable at 25 °C. For ^{1}H NMR and NOESY spectra a total of 64 K data points were collected covering a spectral window of 20 ppm (10,000 Hz). A standard one-pulse sequence with 30° flip angle was used for excitation with an inter-scan delay of 1.0 s. 16 transients were recorded for ^{1}H NMR, while 32 transients were obtained for NOESY spectra. The NOESY spectra were performed by irradiating the particular proton signal under study at a mixing time of 0.5 s. Shimming was performed on each sample prior to data acquisition using the TopShim automatic shimming method from Bruker BioSpin software. The spectra were manually phased, baseline-corrected and integrated using TOPSPIN software (version 3.6.4). The spectral data were imported and processed using MestReNova software version 14.2 (Mestrelab Research SL, Santiago de Compostela, Spain).

2.6. ATR-IR spectroscopy for rapid quantification of water

The water content of NADES was quantified following the method previously described [18] using IR. IR spectra were acquired using a spectrometer (Agilent Cary 630 FT-IR Spectrometer) equipped with a Quest single reflection diamond ATR accessory. Spectra were recording following the method reported by Elderderi et al. [18]. For each sample, three deposits have been measured and three spectra per drop have been collected. Spectra from pure compounds have also been collected using same parameters. BG, GCAH and BBG were selected as model systems, each over a range of concentration between 0 % (w/w) and 50 % (w/w) of water.

2.7. Preprocessing and PLSR analysis for prediction of water content

The statistical relevancy of quantitative analysis performed has been evaluated through cross validation procedures. The Leave K-Out Cross Validation (LOKCV) approach has been used, with 2/3 of the samples used as calibration and 1/3 remaining as validation. No pre-processing was applied before subjecting the spectra to PLSR analysis. Data preprocessing and analysis were performed using R-Studio and following the model previously developed by Elderderi et al. [18].

3. Result and discussion

3.1. NADES preparation

Twenty mixtures with varying composition and molar ratios were prepared using the heating and stirring method [16] and are listed in Table 1. As detailed in section 3, the preparation of GSH was not successful. Heating and stirring method has been selected because is the simplest method available for NADES preparation that minimize the complexity of the set-up, making it easier to scale up. Unlike other methods such as vacuum drying or microwave-assisted preparation, heating and stirring requires less specialized equipment (heating plate with magnetic stirrer for lab scale, jacketed vessels with temperature control and mechanical stirrers for industrial scale). The controlled conditions of the process (moderate temperatures and no extreme conditions) make it energy-efficient compared to other methods. Furthermore, the application of heat reduces the viscosity, enhancing the miscibility of the components and resulting in homogeneous final product. During the preparation, the mixtures gradually transformed into liquids upon heated and mixed, resulting in clear, homogeneous solutions that remained stable without re-crystallization upon cooling. Typically, the eutectic mixture formed at certain eutectic composition established a balanced intermolecular force between the HBA and HBD through hydrogen bonding interactions. These interactions stabilized the molecular structure within the eutectic system, effectively preventing crystal formation [19,20].

3.2. NADES characterization

3.2.1. Nuclear Overhauser effect in NADES

In the initial phase, NADES were examined using ^{1}H NMR to identify hydrogen atoms suitable for NOE experiments which will be later irradiated. NOE consists of a phenomenon in NMR that provides information about the spatial proximity of atoms within a molecule or between

molecules. Thus, the selected protons to be irradiated need to have chemical shifts sufficiently distinct (at least 0.3 ppm) from the protons belonging to the other molecule present in the mixture [21]. This criterion ensures that the irradiated protons respond in an isolated spectral region, preventing magnetization of other protons and avoiding incorrect analysis conclusions. NADES were subjected to ^{1}H NMR and 1D-NOESY experiments, spectra are reported in Figs. 1 and 2 and **Fig. S1** (Supplementary Information). The ^{1}H NMR and 1D-NOESY for TM (1:1), DAM (1:4), GCAH (1:1:3) and GCAH (1:1:6) were not performed as the formation of the supramolecular was previously confirmed, as reported in literature [13,22,33]. For NADES containing betaine and lactic acid such as BLA and BLAH, the NOE effect was evaluated as shown in Fig. 1**A**. The doublet of lactic acid at 1.23 ppm appears clearly separated from the singlet of betaine at 3.1 ppm, therefore, that signal was selected for the NOE experiment. For BLA (1:1) and BLAH (1:1:3.5) the existence of the eutectic mixture was confirmed by the occurrence of a signal at 3.1 ppm from betaine at the corresponding NOESY spectra. Analogously, in the case of the NADES containing proline and citric acid, the double doublet from citric acid at 2.61 ppm was selected for irradiation. In this way, for PCAH (1:1:1) and PCAH (1:1:4.4) (Fig. 1**B**), when the selected signal was irradiated, the existence of the NOE effect was confirmed by the occurrence of the signals of the at 3.8 ppm from proline. Similarly, as shown in **Fig. S1** panel A, (Supplementary information) for those NADES prepared with betaine and 1,3-butanediol such as BBG and BBGH when the selected signal at 1.09 ppm from 1,3-butanediol was irradiated, NOE effect was confirmed by the occurrence of signals at 3.2 and 3.8 of betaine. While, for NADES UG and UGH, (**Fig. S1** panel B) the signal at 3.52 ppm from glycerin was irradiated and the existence of the eutectic mixture was confirmed by the occurrence of signals at 5.6 ppm from urea. Two different NOE experiments were carried out for OAM composed by oleic acid and menthol (Fig. 2, panel A). The signals corresponding to the protons of menthol at a chemical shift of 3.4 ppm, as well as the one at 5.4 ppm from oleic acid were selected and irradiated leading to two different NOESY spectra. Unfortunately, we were unable to observe NOE effects. We expected to see the signals derived from the NOE at 3.4 ppm when 5.4 ppm was irradiated and vice versa, but NOE only occurred in the chemical shift range from 0.7 ppm to 2.4 ppm, which is the region shared by oleic acid and menthol. Therefore, the existence of a supramolecular structure through the 1D-NOESY experiment was not confirmed. However, as shown later, the existence of hydrogen bonds was confirmed via FT-IR analysis reported in section 3.2.3, Fig. 4 panel D. Previous studies have also reported the existence of bonds within these two components, as discussed by Dehury et al [23]. It is likely that this mixture is a NADES, as it is composed of an HBA (menthol) and an HBD (oleic acid). Additionally, it remains liquid at room temperature, which implies a depression of the melting point, and it is stable for a year after its preparation (stability in terms of physicochemical properties). For the proposed mixture containing glucose and sucrose (GSH) (Fig. 2**B**), no NOE effect was observed when the signal at a chemical shift of 5.41 ppm from sucrose was irradiated. This result is consistent with the FT-IR findings, indicating that a supramolecular structure was not formed in this mixture. The most probable reason is that both components of this mixture act primarily as hydrogen bond donors (HBD) with insufficient hydrogen bond acceptor (HBA) capacity, preventing the formation of stable hydrogen bonds necessary for supramolecular structure formation.

For NADES containing betaine and water such as BH (1:1), BH (1:5) and BH (1:10), the elucidation of the structure was not possible by NOESY experiments. The use of deuterated water (D_2O) as a solvent was deemed unsuitable due to the inherent water component of the NADES, which could interfere with the deuterium signal and affect the NMR readings due to overlapping. To address this, we initially explored the use of deuterated acetonitrile (CD_3CN) and deuterated methanol (CD_3OD) as alternative solvents. However, these solvents presented significant challenges as they were immiscible with these NADES, leading to phase separation. The non-miscibility and/or partial miscibility resulted in the inability to maintain a distinct NADES phase, which is critical for the integrity of the NOESY experiments. This led to altered chemical environments and interactions within the sample, affecting the reliability of the NOESY data. Therefore, for these betaine based-NADES the structure elucidation was only possible with FT-IR, as shown in section 3.3, Fig. 4 panel A. We acknowledge the challenge of conclusively distinguishing natural hydrogen bonding (e.g., due to the solubility of betaine in water) from hydrogen bonding that contributes to eutectic formation, particularly in binary mixtures involving water. However, the classification of BH as a NADES is consistent with findings from other binary systems studied (e.g. ChCl:water) using advanced techniques such as Differential Scanning Calorimetry (DSC) and Brillouin spectroscopy [24].

3.2.2. *Dilution effect*

Once we confirmed the existence of the supramolecular structure between the components of the eutectic mixture for most of the synthesized NADES, the so-called BG was selected to evaluate the dilution effects after preparation. BG was selected because it is one of the most widely used NADES, it is easy to prepare, cost-effective and therefore suited for the study. For this purpose, different dilutions of BG were analysed using 1D-NMR and NOESY, to study the effect on their intermolecular interactions. Fig. 3 shows the spectra recorded for BG and BGH at different molar ratios (panel A) and BGH (2:1) diluted with 15 %, 30 % and 50 % of water (panel B). 15 % dilution presented a low NOE effect while 30 % presented the optimal dilution in terms of recorded NOE. The supramolecular interactions appear to dissipate upon the addition of 50 % water, suggesting a dilution threshold between 30 %

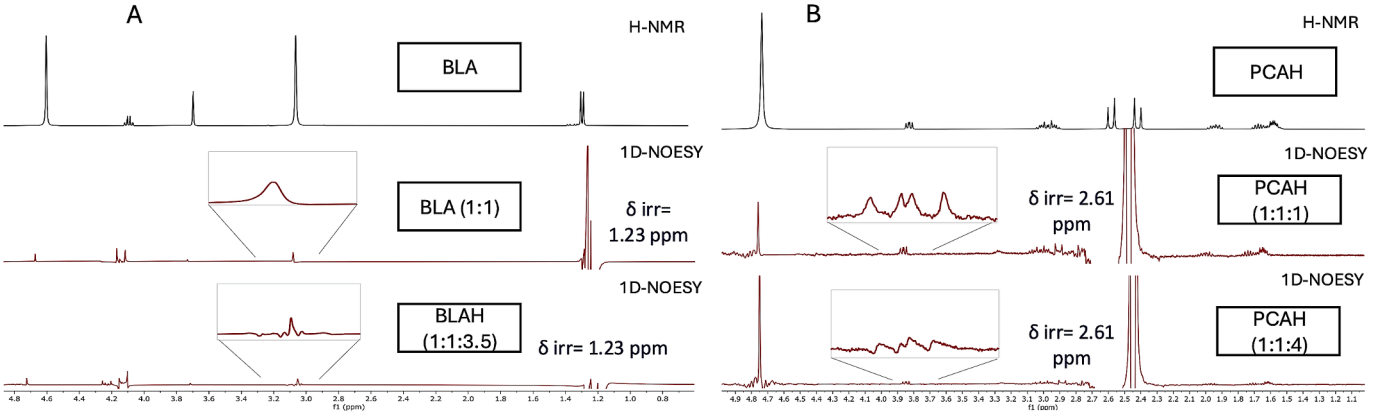

Fig. 1. ^{1}H NMR and 1D-NOESY of four of the NADES under study. **A**) BLA (1:1) and BLAH (1:1:3.5) irradiated at 1.23 ppm. **B**) PCAH (1:1:1) and PCAH (1:1:4.4) irradiated at 2.61 ppm.

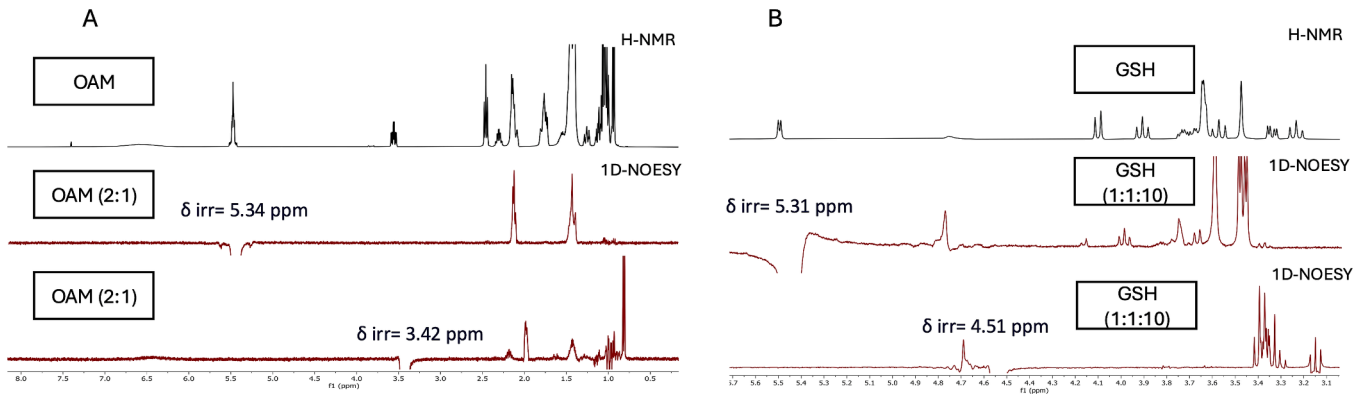

Fig. 2. [1]H NMR and 1D-NOESY of two of the NADES under study. **A)** OAM (2:1) irradiated at 5.34 and 3.42 ppm. **B)** GSH (1:1:10) irradiated at 5.31 and 4.51 ppm.

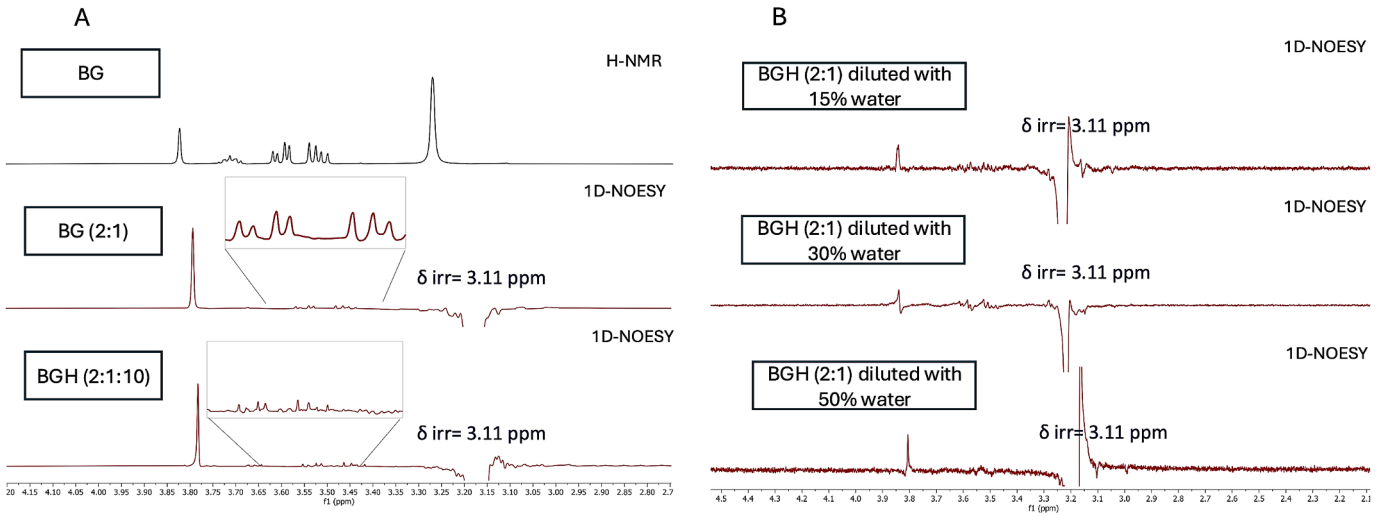

Fig. 3. [1]H NMR and 1D-NOESY of BG and its dilution. **A)** BG (2:1) and BGH (2:1:10.2) irradiation at 3.11 ppm. **B)** BG diluted with 15 %, 30 %, 50 % water.

and 50 %, as previously reported by Gabriele et al. [25]. This highlights the importance of evaluating and understanding the dilution effects of each NADES and the maximum dilution factor that can be used without breaking the interactions and hydrogen bonds between the components of the eutectic mixture, as stated by Dai et al [12].

3.2.3. *FT-IR characterization*

The formation of a eutectic solvent is caused by the hydrogen bonding between the HBA and the HBD. Herein, the structural elucidation of synthesized NADES can also be obtained by analysing the infrared spectra of the starting components (HBA and HBD) and the resulting eutectic mixture for their comparison. The preservation of their characteristic bands with up and/or down shifts, which reflect the functional groups involved in the interactions among NADES components, was observed for all NADES except for GSH (1:1:10), as shown in **Fig. S3,** panel D. Fig. 4 shows the FT-IR spectra of pure compounds betaine, water, lactic acid, glycerol, menthol, oleic acid, and their corresponding eutectic mixtures. FT-IR spectra were recorded at time zero, within a week after their preparation, and after twelve months. Fig. 4**A**, reports the spectra of betaine, water and BH (1:1) at time zero and after twelve months. Betaine showed an absorption band at 1622 cm^{-1} which represents the carbonyl (C=O) groups of the molecule and water present the characteristic peak of 3300 cm^{-1}. The spectra of BH (1:1) showed a shift of the O–H band from 3300 cm^{-1} to 3297 cm^{-1}. This indicates the formation of hydrogen bonds between HBD and HBA. According to Macchionni et al. [26], the shift of the O–H band to a lower wavenumber results from the elongation of the O–H bonds of the HBD. This is due to the O–H bond being pulled by the negatively charged O^{-} atom of the betaine molecules. Similar shift was recorded for BLAH (1:1:3.5) (Fig. 4**B**) and for BBG (1:3) (**Fig. S2**, panel D). As shown in Fig. 4**B,** shifts indicating an intramolecular interaction between COO^{-} group of betaine (from 3387 cm^{-1} to 3390/92 cm^{-1}) and the O–H group of lactic acid (from 1616 cm^{-1} and 1716 cm^{-1} to 1618 cm^{-1} and 1718 cm^{-1}) were observed, in agreement with shifts previously reported [26,27]. Moreover, the presence of an absorption band corresponding to C=O stretching vibration was observed in all NADES mixtures. This band shifted to a higher wavenumber compared to the individual components, as also shown in **Fig. S2** (panel A (UG 1:3) and B (GCAH 1:1:3)). For example, in the spectra of BG (1:2), Fig. 4**C**, the C=O band shifted from 1616 to 1628 cm^{-1}. This upward shift is due to an increase in electron density in the carbonyl oxygen, indicating the formation of a hydrogen bond in the NADES. In **Fig. S2** panel C shift in the same region was recorded from 1605 cm^{-1} of proline, 1693 cm^{-1} of citric acid and 1635 cm^{-1} of water to 1623 cm^{-1} in PCAH (1:1:1) indicating a change in the environment of C=O due to the formation of hydrogen bonds. While, for the hydrophobic NADES OAM (2:1), oleic acid showed the presence of C=O stretching bands at 1691/93 and 1703 cm^{-1} while menthol presented C–H stretch at 2923 cm^{-1} and C–H bending at 1401 cm^{-1}, as depicted in Fig. 4**D**. A displacement is observed in this mixture, where the C=O stretch of oleic acid shifts from 1706 cm^{-1} to 1708 cm^{-1} due to hydrogen bonding with menthol, similar to the shift of DAM reported in **Fig. S3** (panel A). Additionally, there is a broadening and shift of the O–H stretch, indicating the formation of hydrogen bonds between the carboxylic acid group of oleic acid and the alcohol group of menthol.

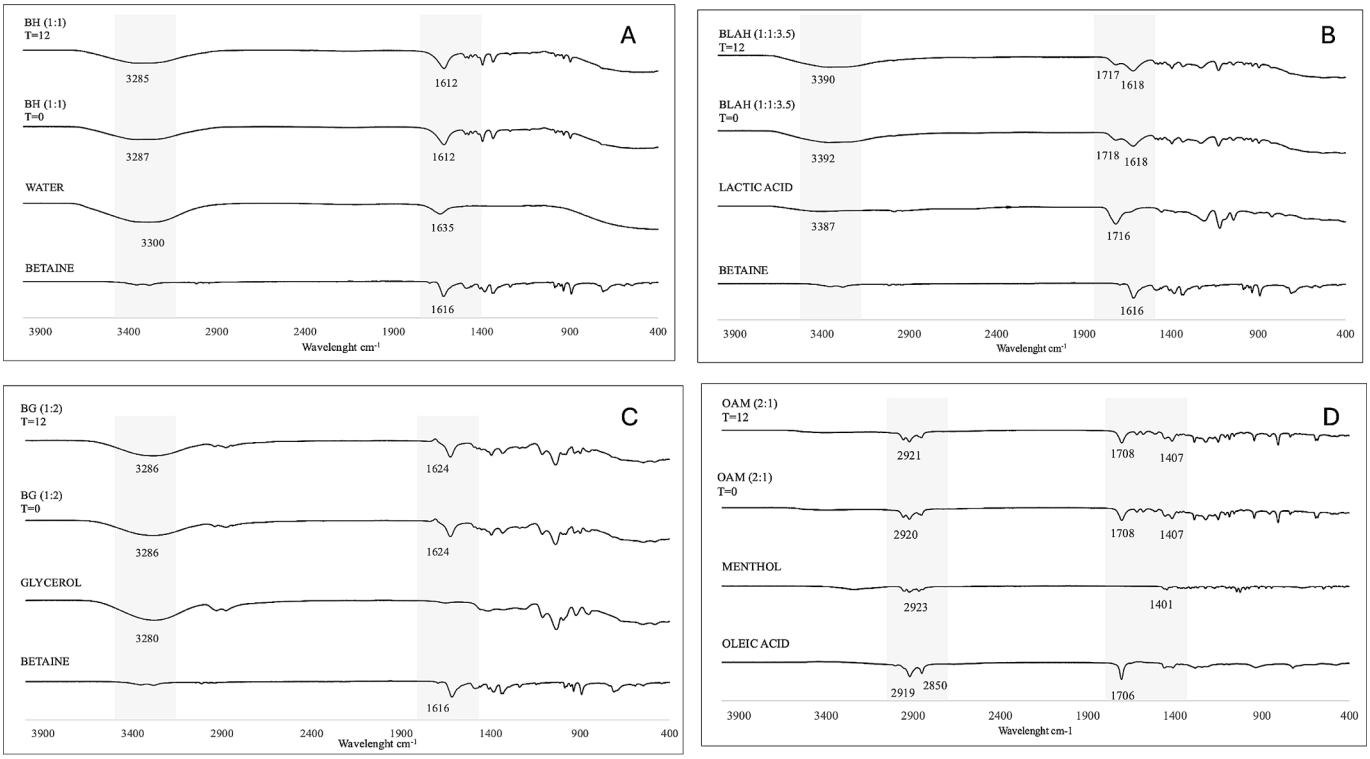

Fig. 4. FT-IR spectrum of 4 NADES recorded at time zero and after twelve months. **A**) BH (1:1) **B**) BLAH (1:1:3.5) **C**) BG (1:2) **D**) OAM (2:1).

Similar behavior was observed by Rajput et al. [28] with NADES composed by thymol and decanoic acid, as also shown in **Fig. S3**, panel C. Comparable response was observed for TM, **Fig. S3**, panel B, where the band shifts to 2919 cm^{-1} which corresponds to OH from menthol and thymol combined together. FT-IR analysis revels optimal to check the stability of the NADES after twelve months from their preparation. In all cases, the stability of the synthesized NADES was confirmed what means that these NADES could be prepared and stored for a long period of time prior their use.

3.2.4. Comparison of NOESY and FT-IR techniques for NADES characterization: advantages and limitations

The NADES can be characterized through various analytical techniques, including NMR and FT-IR. Both methods exhibit advantages and limitations. The use of NMR and the study of the relative NOE effect provide detailed information about the spatial proximity of atoms and the interactions within molecules. However, the use of NMR, particularly with deuterated solvents, makes this technique more costly and less readily accessible. Additionally, setting up 1D-NOESY experiments and interpreting the relative spectra require highly trained personnel. On the other hand, FT-IR is highly effective for identifying functional groups and analysing the chemical environment of the components of NADES. It is relatively quick and straightforward to perform, often without any sample preparation. However, it does not provide as detailed and accurate information as that provided by NOESY experiments. In conclusion, the choice between NOESY and FT-IR for NADES characterization depends on the specific information required. It is recommended to use NOESY to characterize a completely new NADES to understand the spatial relationships between the components, while FT-IR is useful for a more rapid identification of functional groups and shifts in wavelength within the mixture, to check the stability of NADES and to verify a NADES already studied or present in the literature. A combination of both techniques provides a more comprehensive characterization of NADES.

3.3. Physicochemical properties evaluation

Important physicochemical properties including density, pH, viscosity and polarity were examined and reported in Table 2. Evaluation of these properties is essential for understanding the complex nature of NADES, which can lead to new applications and also to custom-designed NADES with specific properties. Viscosity is one of the major challenges for the industrial application and scale-up of NADES. For example, in the extraction of bioactive compounds, NADES with high viscosity can hinder efficient mass transfer and solvents might not penetrate the matrix efficiently, leading to low extraction yields and slower processing times [29]. Viscosity of NADES is affected by water percentage and temperature. Thus, viscosity measurements were conducted at 25 °C and 40 °C, to study their behaviour when temperature increases. As shown in the Table 2, when temperature increases, viscosity decreases, as reported by Gabriele et al. [25] and Dai et al. [12]. In the case of UG (1:3) viscosity decreases five times the original value while heating and decreases from 885.43 ± 7.02 to 17.34 ± 1.14 mPa*s when water was used as third component of the eutectic mixture. Viscosity of BLA decreased four times when the temperature increased from 20 to 40 °C, and twenty times when water was used as third component of the mixture. Similar behaviour was observed for GCAH (1:1:3), GCAH (1:1:6) and PCAH (1:1.1), PCAH (1:1:4.4). Viscosity of NADES containing citric acid and glucose was expected to be high, as previously reported by Mohd Faud et al. [9]. This can be attributed to the presence of citric acid (higher viscosity) [15] and, the use of glucose, which has a large molecular size, and promotes the formation of a stronger hydrogen bonding with citric acid. Moreover, it is important to monitor the conductivity of NADES, as higher solvent conductivity facilitates the extraction of polar compounds [30]. Thus, NADES such as PCAH, BBGH, and BGH are suitable for the extraction of polar compounds (conductivity range from 2.2 up to 11.8 mS/cm^3). Furthermore, higher water content enhances the conductivity and should furnish better efficiencies in the extraction of polar components [31]. Conductivity of NADES also increased as a result of the decreased viscosity, due to the dilution caused by water. Within the range of our experimental analysis of binary

Table 2
Physicochemical properties of NADES under study including viscosity (20 °C and 40 °C), density, pH, conductivity and polarity, results are related to the measurements conducted at Time 0, within one-week after NADES preparation. Statistically significant differences between time zero and twelve months are marked with asterisks ($p < 0.05$* and $p < 0.01$**).

	Viscosity [mPa·s] T = 20 °C	Viscosity [mPa·s] T = 40 °C	Density g/cm3	pH	Conductivity mS/cm^3	E_{NR}(kcal/mol)
UG (1:3)	885.43 ± 7.02	141.88 ± 4.1	1.42 ± 0.014	9	0.009 ± 0.003	1.15
UGH (1:3:2.5)	17.34 ± 1.14	8.24 ± 0.89	1.16 ± 0.012	8	0.2808 ± 0.0003	1.17
BLA (1:1)	234.6* ± 1.45	63.65 ± 1.45	1.15 ± 0.012	2.1	0.1927** ± 0.002	1.00**
BLAH (1:1:3.5)	12.3 ± 1.21	6.21 ± 1.54	1.09 ± 0.006	3.3	0.2813 ± 0.0001	1.09
BG (2:1)	197.32 ± 0.98	56.53 ± 1.59	1.22 ± 0.006	5.6	0.0072 ± 0.001	1.05
BGH (2:1:10.2)	11.01 ± 0.78	5.43 ± 0.97	1.12 ± 0.006	4.1	2.1798* ± 0.002	1.14
GCAH (1:1:3)	102.45 ± 1.54	34.78 ± 1.13	1.13 ± 0.01	2.3	0.1045 ± 0.003	1.24
GCAH (1:1:6)	12.32 ± 1.78	6.01 ± 0.56	1.08 ± 0.0011	3	1.307* ± 0.246	1.25
BH (1:1)	6.05 ± 1.09	3.64 ± 2.01	1.04 ± 0.017	6.2	0.147 ± 0.004	0.99
BH (1:5)	4.89 ± 0.14	2.71 ± 0.62	1.02 ± 0.006	6.3	0.377 ± 0.002	1.05
BH (1:10)	3.87 ± 0.16	1.93 ± 0.9	1.00 ± 0.006	6.5	0.661** ± 0.003	1.00
BBG (1:3)	10.3 ± 1.08	5.44 ± 1.34	1.09 ± 0.01	5.8	3.202 ± 0.001	1.10
BBGH (1:3:10)	5.56 ± 0.98	2.53 ± 1.05	1.02 ± 0.006	5.9	5.323 ± 0.000	1.15
PCAH (1:1:1)	107.02 ± 0.56	33.03 ± 1.03	2.21 ± 0.02	2.7	3.7697 ± 0.005	1.24
PCAH (1:1:4.4)	94.98 ± 0.78	22.84 ± 0.93	1.75 ± 0.0264	3.7	11.80* ± 0.267	1.25
TM (1:1)	47.93 ± 2.11	13.25 ± 1.32	0.93 ± 0.006	6.1[a]	0.003 ± 0.001	0.55
DAT (1:2)	16.02 ± 0.87	7.28 ± 0.98	0.91 ± 0.006	5.6 [a]	0.005 ± 0.001	0.41
OAM (2:1)	49.54 ± 0.56	17.32 ± 1.46	0.87 ± 0.006	4.1 [a]	0.006 ± 0.002	0.49
DAM (1:4)	14.72 ± 0.78	7.24 ± 1.44	0.86 ± 0.006	4.2 [a]	0.005 ± 0.002	0.34

[a] *$p < 0.05$; **$p < 0.01$; [a]expressed as potential difference generated between the NADES and the electrode.

and ternary mixtures, conductivity and viscosity exhibit a negative correlation. For example, in the case of NADES BG viscosity at 20 °C was 197.32 ± 0.98 mPa*s and conductivity 0.0072 ± 0.001 mS/cm^3 while for BGH viscosity at 20° C decreases to 11.01 ± 0.78 mPa*s while conductivity increases up to 2.1798 ± 0.002 mS/cm^3. Density is another important property that needs to be evaluated particularly for mixing and separation process. For instance, in liquid–liquid extraction, knowing the densities of the solvents is crucial to favour phase separation. The densities of all tested NADES proved to be higher than that of water, except for the hydrophobic ones (TM, DAT, OAM, DAM). Density of hydrophobic NADES ranged between 0.86 and 0.93 g/cm^3, while hydrophilic NADES like UG, PCAH (1:1:1) and PCAH (1:1:4.4) reached respectively 1.42 ± 0.014, 2.21 ± 0.02 and 1.75 ± 0.0264 g/cm^3. As expected, when water content increases in NADES composition, the density decreases in all cases. The knowledge about the polarity of a NADES can be very useful for the extraction of target compounds. Organic acid-based and amino-acid based NADES were the most polar (1.24 E_{NR}(kcal/mol)), followed by urea-based NADES (1.15 E_{NR}(kcal/mol)). NADES made with betaine and water presented a polarity closed to 1 (pure water 1 E_{NR}(kcal/mol)), while TM, OAM, DAT, DAM presented a lower polarity, ranging between 0.34 E_{NR}(kcal/mol) and 0.55 E_{NR}(kcal/mol) being the preferred in case of the extraction of non-polar compounds. Polarity comparable to the one of acetone 0.36 E_{NR}(kcal/mol) and ethanol 0.64 E_{NR}(kcal/mol). The pH is another essential characteristic that needs to be consider in developing NADES. It is known that the acidity of NADES is strongly dependent on the types of HBD used. pH of organic acid-based NADES revealed to be the between 2.1 and 3.7. The fairly low pH of these NADES can present challenges, particularly when extracting pH sensitive plant metabolite or when the resulting extracts are applied directly in pharmaceutical, nutraceutical or cosmetic formulation. Acidic condition may degrade certain bioactive compounds or limit their compatibility with final product formulations requiring neutral or slightly alkaline pH [32]. Employing additional purification steps such as liquid–liquid partitioning or membrane filtration, could help to remove or dilute residual NADES, making the extract more suitable. On the other hand, pH of UG and UGH revealed to be the highest within the NADES under study, with a value of 9.0 and 8.0, respectively. While regarding the set of hydrophobic NADES, OAM and DAM presented the lowest pH, 4.1 and 4.2 respectively. Generally, for non-aqueous solvents, pH depends on the chemical potential of hydrogen, which is affected by the presence of cations and anions [9]. For hydrophobic NADES, pH as defined by H_3O^+ concentration does not apply, instead, when measuring the pH of hydrophobic NADES, you are measuring the potential difference generated between the NADES and the electrode, which is influenced by factors such as proton transfer capacity, viscosity, and polarity. Therefore, it should not be considered an absolute value but rather an indication that can still be useful [9].

Moreover, the objective of this study was to examine the long-term stability of NADES by assessing the chemical properties outlined above at twelve months post-preparation (see **Table S1**). Until now, the long-term stability of NADES as solvents has not yet been reported in literature. Our study addresses this gap by demonstrating that the majority of NADES exhibit minimal changes in their properties over time. In most cases (14 out of 19), the properties remained stable for at least twelve months following the initial measurements. However, there are some exceptions. BLA shows significant differences (see correlation matrix with *p*-values in **Fig. S4)** in viscosity, conductivity and polarity over the twelve months period, likely due to moisture incorporation into the NADES. We believe that also the other differences in conductivity are caused by moisture absorption by the NADES over the twelve-month stability period. To ensure optimal preservation, it is recommended to store NADES in a properly sealed, airtight container.

3.4. Water quantification

The water content of NADES is of significant importance, as it has the potential to impact the physical and chemical properties of the solvent. Moreover, monitoring the water content is of paramount importance for ensuring the reproducibility and performance of the solvent. PLSR was used as tool to quantitatively correlate spectral variability with water concentration. The initial step was the selection of the latent variable to achieve the optimal predictive model. Fig. 5**A** presents the plot of the root mean square error cross validation (RMSECV) calculated from the interactions of the cross-validation. The RMSECV decreases as the number of components increases up to five components, indicating an improved predictive accuracy. Beyond the fifth, RMSECV increases indicating overfitting. Plot suggests that using five components provides a good balance between minimizing prediction error and maximizing the explained variance. The lowest RMSECV was obtained with five latent variables RMSECV = 4.4 %. Fig. 5**B**, shows the trends of the predicted (fitted) values from the model with five variables to the actual experimental values. The points are scattered around the diagonal line suggesting that the model is performing well in predicting experimental values. Table 3 reports the results expressed as % w/w of water in each

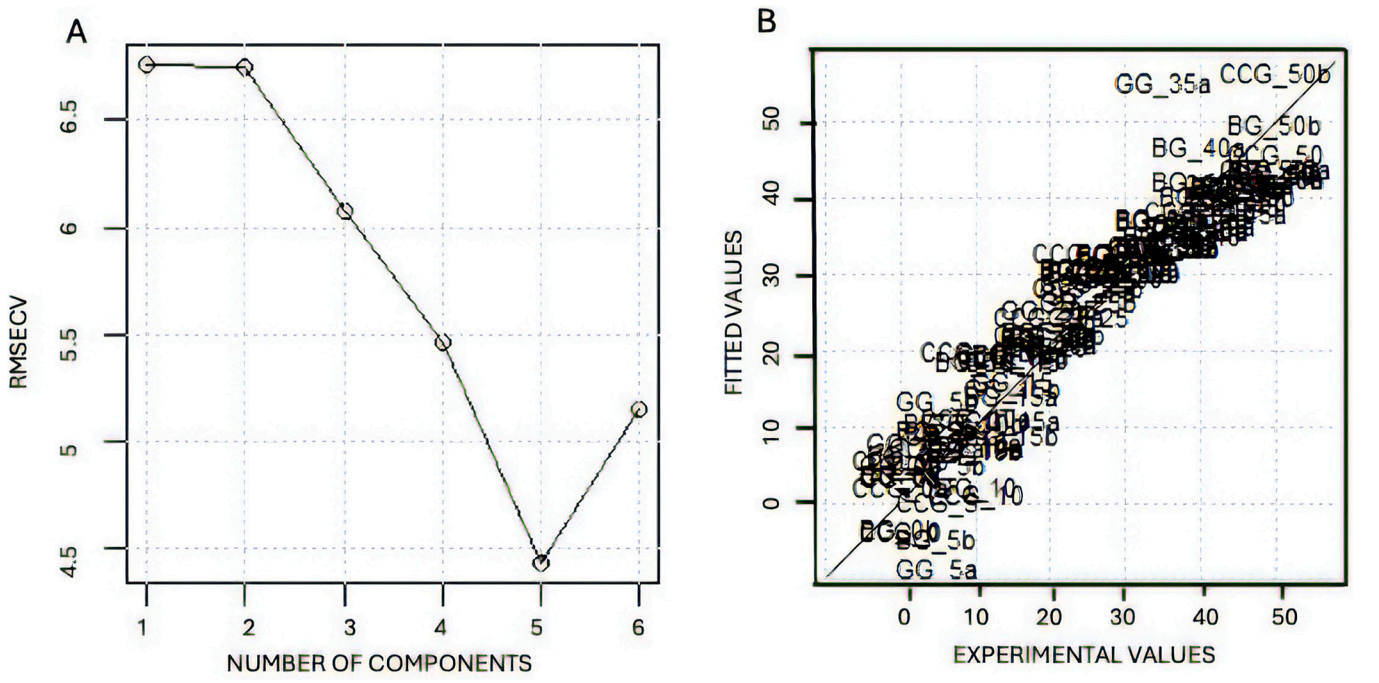

Fig. 5. RMSECV (A) and experimental value vs fitted value (B) for PLSR analysis.

Table 3
Percentage of water content in NADES (expressed as % w/w).

	% w/w water		
	time 0	time 12 months	difference btw 12 and 0
UG (1:3)	3.03	3.11	0.08
UGH (1:3:2.5)	42.04	42.12	0.08
BLA (1:1)	3.24	5.16	1.92
BLAH (1:1:3.5)	44.9	47.32	2.42
BG (2:1)	4.89	5.01	0.12
BGH (2:1:10.2)	40.01	41.18	1.17
GCAH (1:1:3)	10.94	11.32	0.38
GCAH (1:1:6)	50.84	52.66	1.82
BH (1:1)	15.88	16.11	0.23
BH (1:5)	59.13	59.32	0.19
BH (1:10)	65.04	66.12	1.08
BBG (1:3)	5.13	7.12	1.99
BBGH (1:3:10.6)	35.44	36.77	1.33
PCAH (1:1:1)	12.62	14.9	2.28
PCAH (1:1:4.4)	29.55	31.22	1.67
TM (1:1)	0.98	1.02	0.04
DAT (1:2)	1.11	1.23	0.12
OAM (2:1)	1.09	1.11	0.02
DAM (1:4)	0.96	1.12	0.16

of NADES under study. As previously reported, few NADES presented statistically significant differences in conductivity and viscosity after twelve months. Since viscosity and conductivity are physicochemical properties strongly related to water content, water quantification was performed also after twelve months in order to check if the percentage of water increased. As shown in Table 3, values highlighted a little increased of water content, probably responsible of slightly changes in physicochemical properties.

4. Conclusions

In this study, we successfully synthesized and comprehensively characterized nineteen different NADES, encompassing both hydrophilic and hydrophobic types, using a variety of natural components. The synthesis was confirmed through advanced analytical techniques such as NMR (specifically 1D-NOESY) and FT-IR, which elucidated the structural configurations and confirmed bonds formation among NADES components, except for the one composed solely of sugars. Furthermore, out of the nineteen NADES, fifteen new NADES were characterized for the first time, reporting their structural characterization and physicochemical properties for the first time. A significant contribution of our research is the comprehensive evaluation of the long-term stability of these NADES, which has not been previously documented in the scientific literature. FT-IR was employed as a rapid and effective tool for structural elucidation during the stability assessment, confirming that the molecular structure remained consistent over a twelve-month period. Furthermore, our findings indicate that a 30 % water addition was the optimal ratio for maintaining the eutectic while simultaneously enhancing the requisite characteristics for NADES utilization as solvents. Our findings make a substantial contribution to the existing body of knowledge on NADES. It is our hope that this knowledge will serve to increase the spectrum of available NADES and foster applications in various fields. The stability, adaptability, customizability and eco-friendly nature of these solvents make them candidates for sustainable and efficient solvent systems in a variety of fields, including natural product research and industrial applications.

CRediT authorship contribution statement

Chiara Spaggiari: Writing – original draft, Methodology, Data curation, Formal analysis, Investigation, Visualization, Conceptualization. **Laura Carbonell-Rozas:** Writing – review & editing, Validation, Formal analysis. **Han Zuilhof:** Writing – review & editing, Resources. **Gabriele Costantino:** Writing – review & editing, Resources. **Laura Righetti:** Writing – review & editing, Validation, Supervision, Project administration, Conceptualization, Resources.

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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 data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.molliq.2025.127105.

Data availability

No data was used for the research described in the article.

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