Physicochemical Properties, Cytotoxicity, and Antioxidative Activity of Natural Deep Eutectic Solvents Containing Organic Acid

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Natural deep eutectic solvents (NADES) may be considered ‘designer solvents’ due to their numerous structural variations and the possibility of tailoring their physicochemical properties. Prior to their industrial application, characterization of NADES is essential, including determination of their physicochemical properties, cytotoxicity, and antioxidative activity. The most important physicochemical properties of eight prepared NADES (choline chloride:malic acid, proline:malic acid, choline chloride:proline:malic acid, betaine:malic acid, malic acid:glucose, malic acid:glucose:glycerol, choline chloride:citric acid, and betaine:citric acid) were measured as functions of temperature and water content. In general, the structure of prepared NADES greatly influences their physical properties, which could be successfully modified and adjusted by addition of water. All tested NADES were absolutely benign and noncorrosive for investigated steel X6CrNiTi18-10. Furthermore, cytotoxicity of prepared solvents was assessed toward three human cell lines (HEK-293T, HeLa, and MCF-7 cells), and antioxidative activity was measured by the Oxygen Radical Absorbance Capacity (ORAC) method. With regard to cell viability, all tested NADES containing carboxylic acid could be classified as practically harmless and considered environmentally safe. The ORAC values indicated that the tested NADES displayed antioxidative activity.

Keywords:
antioxidative activity, corrosion rate, cytotoxicity, natural deep eutectic solvents, physicochemical characterization

Introduction

The development of new products should balance economic, social and environmental requirements and be based on green and sustainable technologies. Many industrial processes use large amounts of volatile and flammable organic solvents based on non-sustainable resources such as oil, which defines a significant portion of the environmental and economic performance of a process. The European Union Industrial Emission Directive 2010/75/EU[1] prompted growing research on the development of alternative green technologies dedicated to producing new alternatives to volatile organic solvents – green, environmentally friendly solvents that also meet technological and economic requirements.

Natural deep eutectic solvents (NADES), a promising alternative to traditional volatile organic solvents, have been dramatically increasing in popularity. NADES are generally based on mixtures of cheap and readily available components: nontoxic quaternary ammonium salts (e.g., choline chloride), and hydrogen bond donor (HBD) based on natural products (e.g., amines, sugars, alcohols, sugar alcohols, polyols, and organic acids)[2,3]. Since their emergence, NADES, a greener version of ionic liquids (ILs), have attracted attention in synthesis, electrochemistry, biochemistry, and separation[4–7].

NADES may be considered ‘designer solvents’ due to their numerous structural variations and the possibility of tailoring their physicochemical properties to be appropriate for use in different processes. In addition, NADES are a class of solvents based on compounds that are safe for human consumption, a feature that opens numerous possibilities for their applications in the life science field[8–11]. More-

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over, it has been proposed that NADES formed from compounds with proven biological activity, such as amino acid or organic acid, could also have similar properties[11,12]. A study by Radošević *et al.*[13] indicated that NADES containing malic acid could enhance the biological activity of plant extracts. Such a presumption is reasonable considering that some organic acids, as one of the hydrogen bond donors for the formation of NADES, have various pharmacological effects, indicating that not only physicochemical characteristics of the solvents, but also their biological activity, could be finely tuned[13].

Applicability of novel solvents to commercial processes is still in the initial phases of research and development. Only a few studies have reported the scale-up of processes involving NADES[14]. When deliberating scale-up possibilities, many factors need to be taken into consideration, including both economic and environmental issues. NADES, as possible alternative solvents, have many advantages, including low-cost forming components (e.g., 50 € kg^{-1} for choline chloride, 15 € kg^{-1} for urea, 25 € kg^{-1} for glycerol), simple preparation, a low or negligible toxicity profile, and sustainability in view of environment, and economic benefits[8].

Prior to their industrial application in different technological processes, it is essential that NADES be well characterized, including both their physicochemical and biological properties. We prepared and characterized eight NADES containing organic acid (malic acid or citric acid). The most important physicochemical properties of prepared NADES (density, viscosity, refractive index, conductivity, and pH values) were measured. The effects of water addition and temperature on these physicochemical properties were also evaluated. The corrosion rate of steel X6CrNiTi18-10 in prepared NADES was determined, as it is one of the critical issues in industrial application. Furthermore, cytotoxicity of prepared solvents was assessed toward three human cell lines, HEK-293T, HeLa, and MCF-7, while antioxidative activity was measured by the Oxygen Radical Absorbance Capacity (ORAC) method. Such comprehensive characterization of synthesized NADES (defined physicochemical properties, cytotoxicity on cell lines, and antioxidative properties) would enable the proper selection of the most appropriate solvent for the desired usage in the target technological process prior to possible industrial application.

Experimental section

Materials

Chemicals for NADES preparation (choline chloride, citric acid, malic acid, proline, glucose, and glycerol) were purchased from Sigma Aldrich (purity of ≥ 99 %) and used without further purification. All other chemicals were from commercial sources and of the highest purity available. The HEK-293T, HeLa, and MCF-7 cells used in this work were cultivated in Dulbecco's Modified Eagle's Medium (DMEM), and fetal bovine serum (FBS) purchased from Gibco, UK. The CellTiter 96® AQueous One Solution assay (USA) was used for determining cell viability in cytotoxicity assay.

NADES preparation

Choline chloride (ChCl) was dried in the vacuum concentrator (Savant SPD131DDA SpeedVac Concentrator, Thermo scientific, USA) at 60 °C for 24 h before use. NADES were prepared at a certain molar ratio of ChCl to hydrogen bond donor (HBD), as shown in Table 1 and described previously by Radošević *et al.*[11]. The two or more components in specific ratios with 10, 30, and 50 % (w/w) water were placed in a round-bottomed glass flask, and stirred and heated to 50 °C to obtain the following liquids at room temperature: choline chloride:malic acid (ChMa, 1:1), proline:malic acid (ProMa, 1:1), choline chloride:proline:malic acid (ChProMa, 1:1:1), betaine:malic acid (BMa, 1:1), malic acid:glucose (MaGlc, 1:1), malic acid:glucose:glycerol (MaGlcGly, 1:1:1), choline chloride:citric acid (ChCit, 2:1); betaine:citric acid (BCit, 1:1) (Table 1S, supplementary). The NADES abbreviation and corresponding mole ratios are given in parentheses. The synthesized NADES were vacuum dried prior to further use. All NADES were characterised by means of 300 MHz ^1H NMR (Bruker Avance 300, Rheinstetten, Germany) and FTIR (Bruker Vertex 70, Ettlingen, Germany) spectrometry.

Physicochemical characterization of NADES

The measured physicochemical properties (except density) were provided in temperature range from 15 to 55 °C. The temperature of the samples was controlled with thermostat Julabo CF41. The effects of water addition were also evaluated by measuring all properties for NADES with different water contents of 10, 30 and 50 % (w/w).

Measurement of viscosity

The viscosities of investigated NADES were determined using a Brookfield DV-III Ultra Programmable Rheometer. The measurements were performed with two different spindles. Spindle SC4-21 was used for low-viscosity NADES and Spindle LV62 for high-viscosity NADES. Viscosity accuracy is ± 1.0 % of full-scale range for a specific spindle running at a specific speed.

Table 1 – *Evaluated parameters of VFT and Arrhenius model for investigated NADES*

NADES	Water content % (w/w)	VFT				ARRH		
		A	B, $\cdot10^4$	T_0, K	$\sum(\ln\eta-\ln\eta_{\mathrm{VFT}})^2$	E, $\cdot10^4$, kJ kmol^{-1}	η_0, Pas	$\sum(\ln\eta-\ln\eta_{\mathrm{ARRH}})^2$
BMa	10	39.87	1.68	747.62	$4.90\cdot10^{-3}$	3.79	$2.11\cdot10^{-6}$	7.88
	30	36.17	3.62	1220.98	$3.86\cdot10^{-2}$	3.19	$1.14\cdot10^{-1}$	$1.14\cdot10^{-4}$
	50	25.97	3.59	1457.44	$3.81\cdot10^{-2}$	1.57	$1.05\cdot10^{-5}$	$4.85\cdot10^{-6}$
MaGlcGly	10	25.96	1.02	677.29	$1.01\cdot10^{-1}$	2.94	$2.88\cdot10^{-6}$	$1.07\cdot10^{-1}$
	30	22.27	1.39	847.46	$2.30\cdot10^{-2}$	2.20	$6.36\cdot10^{-6}$	$4.91\cdot10^{-4}$
	50	19.04	1.92	1102.04	$7.47\cdot10^{-3}$	2.05	$1.87\cdot10^{-6}$	$9.28\cdot10^{-7}$
ChMa	10	31.63	1.43	742.83	$2.39\cdot10^{-1}$	5.32	$3.09\cdot10^{-10}$	$1.13\cdot10^{-1}$
	30	24.87	2.77	1259.14	$1.27\cdot10^{-2}$	1.70	$1.90\cdot10^{-5}$	$1.99\cdot10^{-5}$
	50	22.02	3.21	1467.5	$8.17\cdot10^{-3}$	1.30	$2.40\cdot10^{-5}$	$8.44\cdot10^{-7}$
BCit	10	38.59	1.41	671.74	$4.80\cdot10^{-1}$	3.46	$2.94\cdot10^{-6}$	14.35
	30	31.32	2.74	1098.24	$4.53\cdot10^{-2}$	2.89	$5.00\cdot10^{-7}$	$1.99\cdot10^{-4}$
	50	27.07	3.42	1358.99	$3.65\cdot10^{-3}$	2.04	$1.62\cdot10^{-6}$	$4.25\cdot10^{-7}$
ChProMa	10	37.47	1.68	747.68	$3.87\cdot10^{-1}$	3.55	$9.05\cdot10^{-7}$	2.13
	30	28.16	3.19	1278.92	$1.02\cdot10^{-2}$	2.15	$2.09\cdot10^{-6}$	$4.73\cdot10^{-6}$
	50	26.82	3.80	1462.83	$2.02\cdot10^{-2}$	2.00	$9.05\cdot10^{-7}$	$2.71\cdot10^{-7}$
ProMa	10	36.64	1.69	759.76	$1.66\cdot10^{-2}$	3.51	$8.79\cdot10^{-7}$	$4.06\cdot10^{-1}$
	30	26.40	2.72	1186.58	$1.78\cdot10^{-2}$	2.10	$3.08\cdot10^{-6}$	$1.26\cdot10^{-5}$
	50	22.18	2.98	1371.71	$2.22\cdot10^{-2}$	1.44	$1.07\cdot10^{-5}$	$7.01\cdot10^{-7}$

Measurement of density

Densities of NADES were measured using a Mettler Toledo Densito 30PX densitometer, and for high-acidity NADES ($pH<1$) an aerometer was used. For the measurement range from 0 to 2 g cm^{-3}, density accuracy is ± 0.001 g cm^{-3}. Densities were measured at room temperature.

Measurement of refractive index

The refractive indices for all investigated samples were measured using a Refractometer Abbé, (Carl Zeiss Jena), with a precision of ± 0.0001.

Measurement of electrical conductivity

Electrical conductivity was measured using a TetraCon 325/C standard conductivity cell. The conductivity measuring range was 1 μS cm^{-1} to 2 S cm^{-1} in a temperature range of –5 to 80 °C.

Measurement of pH value

Since all DES are miscible with water and the mass portion of water was between 10 and 50 % (w/w), the procedure of pH determination was the same as for aqueous samples. Although there were some challenges during measuring, like long response time and periodic necessity of rewetting the electrode, the obtained readings were stable and repeatable. The pH values for each NADES were determined by a WTW SenTix 81 pH-electrode ATC. The pH measuring range was 0–14 in temperature range 0–100 °C. Instrument was calibrated using standard pH buffer solutions.

Corrosion activity

Weight loss measurements

The corrosion activity of the investigated NADES on steel X6CrNiTi18-10 was determined by weight loss measurements. For this purpose, the steel sheets were mechanically press-cut into coupons of dimensions 30 mm × 12 mm × 3 mm. The surface of the prepared coupons was smoothed with successively finer SiC waterproof abrasive papers (100, 600, 800, 1200, and 1500), rinsed with distilled water, and ultrasonically degreased in absolute ethanol. The coupons were stored in a moisture-free desiccator prior to use. The prepared metal coupons were weighed and immersed in a prepared

NADES with 10, 30 and 50 % (w/w) of water, in an open to air beaker. All measurements were performed at room temperature. The coupons were taken out of the NADES, and after 15 days of exposure, washed with distilled water, dried and weighed. The corrosion rate (W) was calculated and expressed as µm year^{-1}.

Electrochemical measurements

Electrochemical measurements were carried out to obtain the values of corrosion current densities of steel X6CrNiTi18-10 in investigated NADES. Potentiodynamic polarization method was performed with a scan rate of 0.5 mV s^{-1} in three electrode cylindrical glass cells with 150 mL of NADES as electrolyte at 298 K under atmospheric conditions. The electrochemical measurements were carried out in the potential range from ±150 mV with respect to the E_{ocp} in the test solution after reaching the open-circuit potential (E_{ocp}).

As a reference, auxiliary electrodes were used – saturated calomel electrode (SCE) and a graphite electrode, respectively. The working electrode was steel X6CrNiTi18-10. All measurements, performed using a potentiostat type VersaSTAT 3 (Princeton Applied Research), were controlled by a personal computer.

Evaluation of NADES toxicity

The influence of synthesized NADES on cell proliferation was examined by CellTiter 96® AQueous One Solution Assay for colorimetric analysis. Briefly, HEK-293T cells at initial concentration of $3 \cdot 10^4$ cells mL^{-1}, HeLa and MCF-7 cells at initial concentration of $5 \cdot 10^4$ cells mL^{-1} were seeded in 96-well plates. After 24 hours of growth, cells were treated with three different nominal concentrations (500 mg L^{-1}, 1000 mg L^{-1} and 2000 mg L^{-1}) of eight prepared NADES in deionized water, filtered through 0.22 µm filters, in four parallels. After 72 hours of treatment, 10 µL of CellTiter 96® AQueous One Solution reagent, a novel tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS(a)], was added to each well. The cells were further treated for 4 hours, when MTS reagent was bioreduced by living cells into a coloured formazan. The quantity of formazan product, soluble in tissue culture medium, was measured by absorbance at 490 nm on the microplate reader (Tecan, Switzerland), and was directly proportional to the number of viable cells in culture. Cell viability was calculated as described in Radošević *et al.*[11] Expressed data were the mean values of experiments performed three times.

Antioxidant activity of NADES

Oxygen radical absorbance capacity (ORAC) assay was performed based on the method described by Ninfali *et al.*[16] and the results were expressed as relative ORAC values. The assay was conducted in a quartz stone with 3 mL of reaction mixture containing 2.25 mL of fluorescein sodium salt (0.04 µmol L^{-1}) in sodium phosphate buffer (0.075 M, pH 7.0), and 0.375 mL diluted NADES or Trolox (25 µmol L^{-1}) as standard or 0.075 M sodium phosphate buffer (pH 7) as blank control. After incubation for 30 min at 37 °C, 0.375 mL of AAPH was added. Fluorescence was recorded every minute up to value zero by a Varian Cary Eclipse Spectrofluorimeter (Palo Alto, CA, USA) with 485 nm excitation and 520 nm emission. Results were analysed using the differences of areas under fluorescein decay curve between the blank and the sample, as briefly described in Jokić *et al.*[17] The results were the mean values (n = 3) and were expressed as µmol Trolox equivalent per gram of NADES (µmol TE g^{-1}).

Results and discussion

Physicochemical characterization of NADES

In order to select the most appropriate solvent for a defined process, the physicochemical properties of the solvents must be known. The most commonly used properties in solvent selection (density, viscosity, refractive indices, conductivity, and pH values) have been determined experimentally for the selected NADES containing carboxylic acid, malic or citric. These types of NADES could be used in the extraction of biological compounds, such as phenolic compounds[9,11].

FTIR and ^{1}HNMR analysis

Since all NADES were prepared as aqueous solutions, it was necessary to determine whether the addition of water influenced the structure of NADES. Therefore, FTIR and ^{1}H NMR spectra of NADES with different amounts of water were recorded. Spectra of two NADES are presented in Figs. 1 and 2. Based on the FTIR spectra of NADES (Fig. 1) prepared with different amounts of water (0 to 50 % by weight), it may be concluded that addition of water had no significant influence on the structure of the solvent. For both presented NADES, shifts in frequencies could be observed. This may be explained by the formation of new interactions and formation of additional H-bonds between components and added water. Since all NADES had been prepared from the aqueous solutions, traces of water could be found (lower than 2 %) in NADES prepared from highly hygroscopic compounds, like

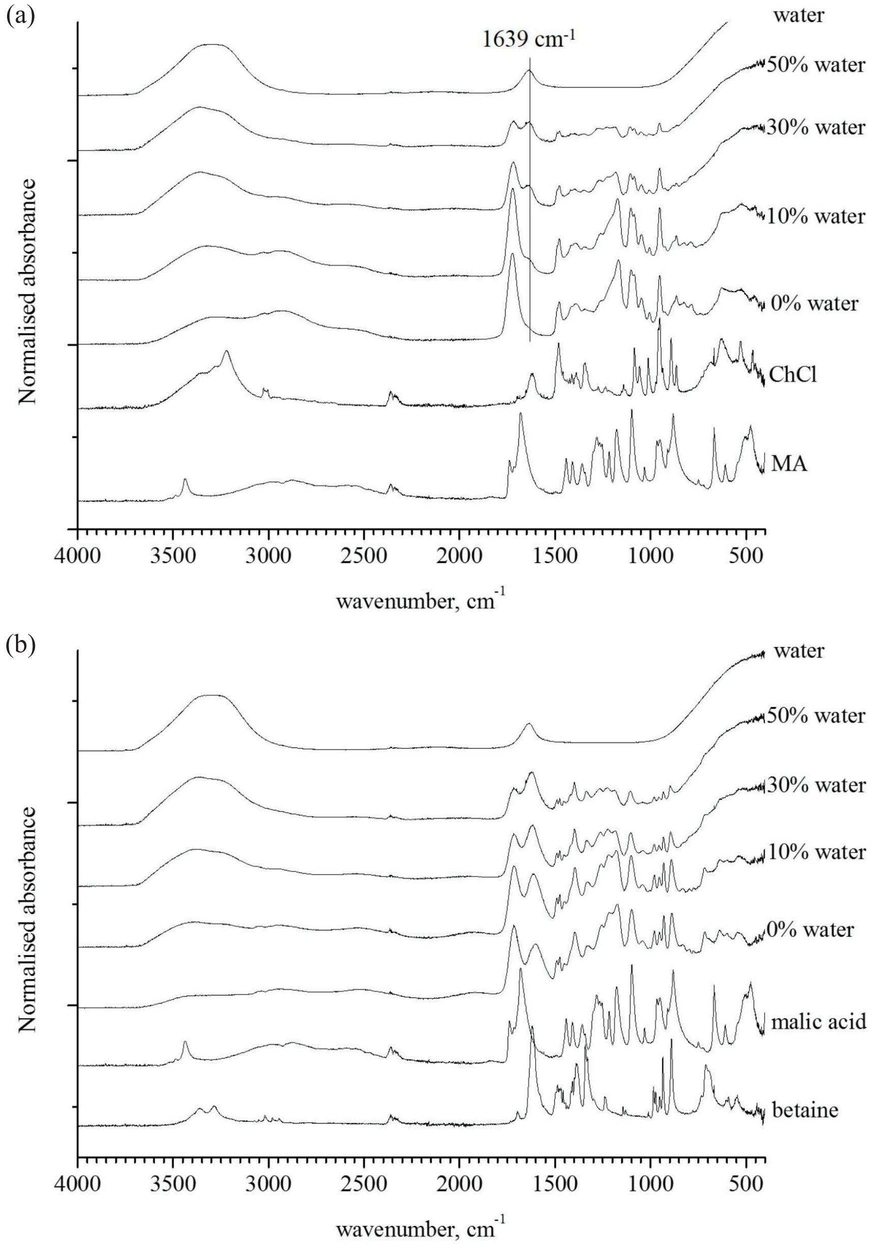
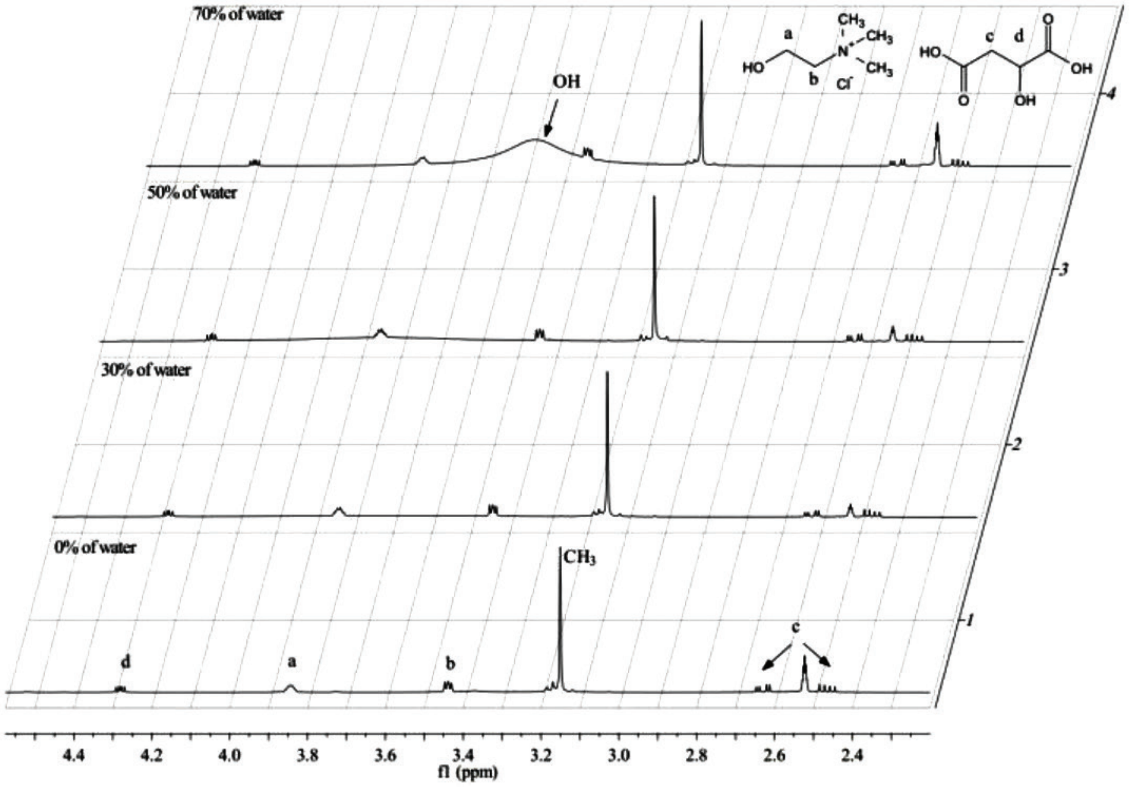


Fig. 1 – *FTIR spectra of ChMa (a) and BMa (b) prepared with different amounts of water (0 to 50 % by weight)*

choline chloride or betaine. Spectrum of BMa exhibits frequencies of FTIR spectra of betaine and malic acid, except for some frequency shifts due to formation of hydrogen bonds between betaine and malic acid. On FTIR spectrum of both components, frequencies attributable to the presence of water could be observed[18,19]. The peak at 1714 cm^{-1} is assigned to C=O stretching of malic acid, while the peak at 1603 cm^{-1} is a combination of COO^{-} stretching and O–H bending[20] of small amounts of residual water in betaine. Addition of water resulted in reduced intensities of bands belonging to BMa without water, and blueshifts of bands assigned to COO– stretching (from 1603 cm^{-1} to 1625 cm^{-1}), N–C–H bending (from 1327 cm^{-1} to 1336 cm^{-1}), C–O stretching (from 1256 cm^{-1} to 1266 cm^{-1}; from 1217 cm^{-1} to 1225 cm^{-1}; from 1174 cm^{-1} to 1188 cm^{-1}) and C–OH stretching (from 1099 cm^{-1} to 1106 cm^{-1} and from 888 cm^{-1} to 896 cm^{-1}). It was previously observed that addition of water causes blueshifts of some functional groups due to formation of stronger H-bonds involving water instead of HBA[21]. Similarly, spectrum of ChMa is a combination of FTIR frequencies of choline chloride[20] and malic acid[18]. Addition of water to ChMa resulted in redshift of C=O stretching band from 1723 cm^{-1} to 1717 cm^{-1}, and blueshift of C–O stretching bands (from 1259 cm^{-1} to 1275 cm^{-1}; from 1168 cm^{-1} to 1187 cm^{-1}; from 1101 cm^{-1} to 1107 cm^{-1}). Redshift could be explained by the formation of more stable

(a)



(b)

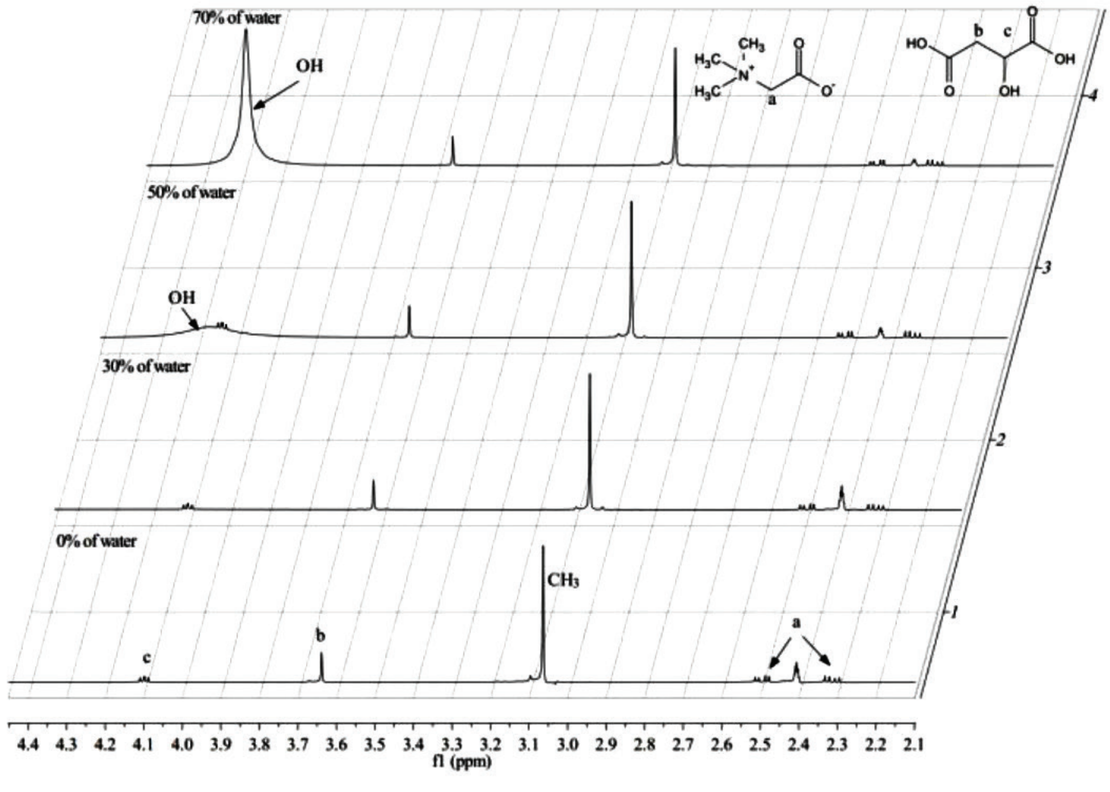


Fig. 2 – *NMR spectra of ChMa (a) and BMa (b) prepared with different amounts of water (0 to 70 % by weight)*

hydrogen bonds[22]. Appearance of a new peak with its increasing intensity with the amount of water at 1639 cm^{-1} corresponds to H–O–H bending of water. The vibrational bands from 3600 cm^{-1} to 2700 cm^{-1} refers to O–H stretching (water, alcohol, carboxylic acid), while the band at around 2950 cm^{-1} refers to aliphatic C–H stretching[23]. Blueshifts of H–O–H stretching of water in FTIR spectra of both shown NADES with increasing amount of water, together with widening and increasing band intensity indicate formation of additional hydrogen bonds[24]. At the same time, intensity of C–H stretching band was lowered due to the increasing concentration of water. Intensity of broad bands below 800 cm^{-1} that corresponds to wagging, twisting, and rocking of water molecules also increased with addition of water. In general, it may be concluded that by increasing concentration of water, the absorbance intensity of OH-stretching, H-bond libration overtones, H_2O libration and H–O–H bend increase, while the intensities of bands related to NADES decrease. However, shifts of some frequencies indicate formation of new hydrogen bonds[25].

^{1}H NMR spectra of all NADESs were measured in DMSO, since according to the literature[26], NADES conserves its structure despite dilution in DMSO. Similarly, no significant differences in ^{1}H NMR spectra of NADES prepared with different amounts of water were observed (Fig. 2). Chemical shifts, characteristic for NADES without water, were found on ^{1}H NMR spectra of all aqueous samples, except for the signal that corresponded to proton in CH group, which disappeared at the highest concentration of water (betaine:malic acid). Broad proton signal of water was observed for both types of NADES. Intensity of this signal increased with increasing water concentration. Addition of water had no influence on the structure of the prepared NADES.

Density of NADES

Density is an important property for chemical materials and their processing[27]. A high density of NADES usually has a negative impact on handling or mixing in chemical processes. However, it may have a positive effect on the extraction processes, since a relatively large difference in the densities between the raffinate and extract phases is necessary to assure phase separation. The densities were determined at 25 °C for eight NADES with different water contents, and their values varied between 1.13 g cm^{-3} and 1.50 g cm^{-3} depending on water content and type of components used. Density is a property that shows an additive relationship among the forming components, so the value of a mixture's density lies between the density of water and the density of pure NADES. Addition of water lowers the density of NADES, as shown in Fig. 3.

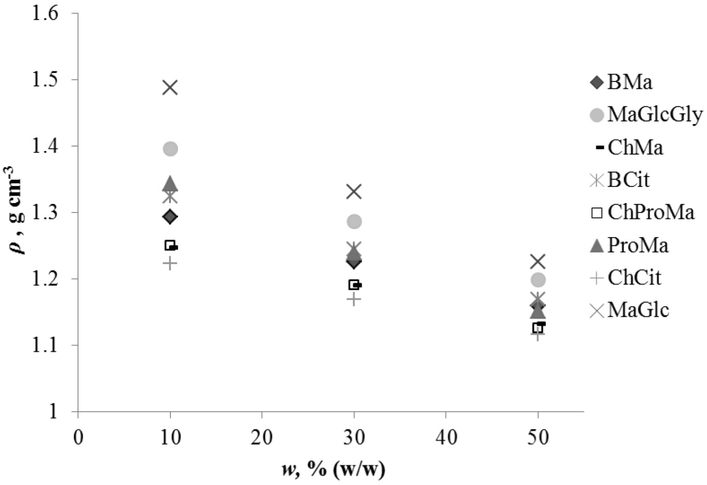


Fig. 3 – *Densities of NADES as function of water content at 298 K*

On the other hand, NADES are composed of holes and empty vacancies, so their physical properties also depend on their packing and molecular organisation[3]. Although it is known that a longer carbon chain of HBD reduces hydrogen bond interactions due to steric hindrance, which lowers density drastically, glucose-based NADES with 6 carbon atoms have the highest densities[28,29]. This is due to a very high density of pure D-glucose (1.5345 g cm^{-3} at room temperature) attributed to strong intermolecular hydrogen bonds and the cyclic structure. The lowest densities are found in NADES prepared with choline chloride. Numerous studies have confirmed that NADES consisting of choline chloride and sugar show lower density values than NADES composed of organic acid and glucose[4,27,30]. Once again, the results show that lengthening alkyl chains leads to a decrease in density. NADES densities can be organised in the following order: ChCit<ChProMa<ChMa, where the last is the NADES with the highest density. Florindo *et al.*[31] reported a similar trend.

Viscosity of NADES

Liquid viscosity is significant when selecting an appropriate solvent for a defined separation process. The liquid with the lower viscosity will be dispersed easily into the other, meaning higher rates of mass transfer can be achieved[31]. Results show that all examined NADES were Newtonian fluids with relatively high viscosity. Figures in supplemental data show linear dependences of shear stress on shear rate for all NADES at 25 °C (Fig. 1S). Same dependences were obtained in the investigated range of temperature.

The high viscosities of NADES can be a restrictive factor in their use as extraction solvents, due to both their lower extraction efficiencies and

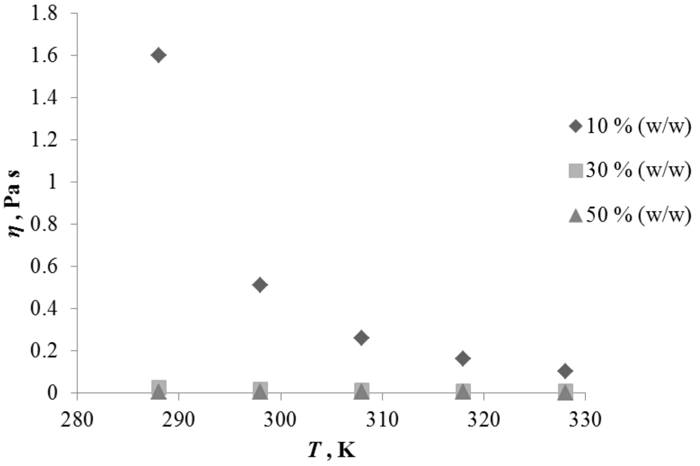


Fig. 4 – *Viscosity of ChMa with different water content as function of temperature*

the amount of energy required for stirring and pumping. On the other hand, this might be a desired characteristic, as it has been reported that the high viscosities of NADES with sugar as HBD and with low water contents allow for stable molecular interactions. Fortunately, the viscosity can be easily adjusted by operating at higher temperatures or by the addition of the optimal amount of water, which satisfies both demands, for better extraction and energy efficiencies (Fig. 4)[32]. Dilution with water leads to a great decrease in the viscosity of NADES because of the gradually weakened hydrogen-bonding interactions between the components[33,34]. Depending on type of DES and temperature, interval of obtained viscosities for DES with 10 % (w/w) water is between 59.8 – 49 400 mPa s, with 30 % (w/w) between 5.3 – 94.7, and with 50 % (w/w) between 1.5 – 10.8 mPa s. An increase in temperature causes an increase in kinetic energy and the mobility of molecules, making the interactions between them weaker.

The type of salt, type of HBD, and the strength between molecules also influence NADES viscosity. The NADES with the highest viscosity was obtained from betaine and malic acid (BMa). This fits with the theory developed by Abbott *et al.*[28] in which the structures of the HBD are an important factor in determining density and viscosity. When the number of carbon atoms increases, viscosity increases, and the density of NADES decreases. This was true for all examined NADES, except the NADES with choline chloride. It has been reported that choline chloride has the ability to disturb the intermolecular hydrogen bonding of the 3-D molecular structure of glycerol, which decreases the viscosity[28].

The experimentally obtained viscosities were correlated with the Arrhenius (1) and Vogel-Fulcher-Tamman (2) (VFT) equations:

$$\eta = \eta_0 \cdot e^{E/RT} \tag{1}$$

$$\ln \eta = A + \frac{B}{T - T_0} \tag{2}$$

where η is dynamic viscosity, η_0 is the pre-exponential constant, E is the Arrhenius activation energy, R is the ideal gas constant, T is temperature in Kelvin, and A, B, and T_0 are empirical constants in eqs. 1 and 2.

The influence of temperature and water content on the evaluated model parameters was analysed. The evaluated model parameters and residual sum of squares are presented in Table 1. Table 1 contains no values for the ChCit and MaGlc NADES since their viscosities had not been measured due to high acidity (pH<1). For all tested NADES, the viscosity data was better correlated using the VFT model.

Refractive index of NADES

Refractive index is a property involved in many applications. In general, refractive indices can be used to identify substances and determine their concentration, verify their purity, or determine the concentration of a solution[27]. Refractive indices of the NADES were measured at different temperatures and with different water contents. For all investigated NADES refractive indices, n_D linearly decreased with increasing water content. Linear dependence between refractive index and density of the same NADES was observed; the reasons for this are the additive properties of both values, and the physical properties of water (lower density and refractive index) (Fig. 3 and Table 2). Furthermore, the values of refractive indices decrease with increasing temperature due to the reduction in density. No relationship was found between density and refractive index for different NADES because refractive index, except with regards to water content and temperature, depends on structure and bonds inside the NADES. At room temperature, the highest refractive index of 1.4786 was measured for NADES ChProMa with 10 % of water (w/w), and the lowest refractive index of 1.3941 was measured for NADES BCit with 50 % of water (w/w). It is worth mentioning that refractive index can be used for a quick and easy determination of water content in NADES from the known calibration curve.

pH value of NADES

The pH value is another important property, which can provide guidance in choosing the type of material and other equipment. A linear correlation between pH values and temperature was obtained (Fig. 5). The obtained pH values of NADES as a function of temperature varied in the range of

Table 2 – *Refractive indices of NADES with different water contents 10–50 % (w/w) in the temperature range 288–328 K*

NADES	*T*, K	Refractive indices			NADES	*T*, K	Refractive indices		
		water content of NADES, %					water content of NADES, %		
		10	30	50			10	30	50
BMa	288	1.4756	1.4425	1.4067	BCit	288	1.4630	1.4369	1.3951
	298	1.4734	1.4403	1.4049		298	1.4651	1.4350	1.3941
	308	1.4714	1.3885	1.4029		308	1.4599	1.4343	1.3923
	318	1.4698	1.4369	1.4016		318	1.4580	1.4309	1.3906
	328	1.4679	1.4374	1.4001		328	1.4560	1.4280	1.3888
MaGlcGly	288	1.4782	1.4433	1.4065	ChProMa	288	1.4817	1.4401	1.4034
	298	1.4769	1.4428	1.4049		298	1.4806	1.4391	1.4011
	308	1.4749	1.4402	1.4032		308	1.4786	1.4373	1.4000
	318	1.4729	1.4391	1.4023		318	1.4776	1.4366	1.3981
	328	1.4705	1.4388	1.4003		328	1.4755	1.4343	1.3978
ChCit	288	1.4762	1.4413	1.4102	ProMa	288	1.4801	1.4351	1.3969
	298	1.4742	1.4395	1.4089		298	1.4781	1.4339	1.3947
	308	1.4724	1.4378	1.4069		308	1.4768	1.4320	1.3931
	318	1.4708	1.4361	1.4054		318	1.4757	1.4306	1.3928
	328	1.4681	1.4344	1.4039		328	1.4736	1.4300	1.3900
ChMa	288	1.4749	1.4398	1.4068	MaGlc	288	1.4590	1.4473	1.4398
	298	1.4730	1.4376	1.4048		298	1.4570	1.4452	1.4380
	308	1.4712	1.4359	1.4031		308	1.4549	1.4433	1.4352
	318	1.4698	1.4339	1.4014		318	1.4519	1.4416	1.4367
	328	1.4680	1.4320	1.3994		328	1.4509	1.4404	1.4336

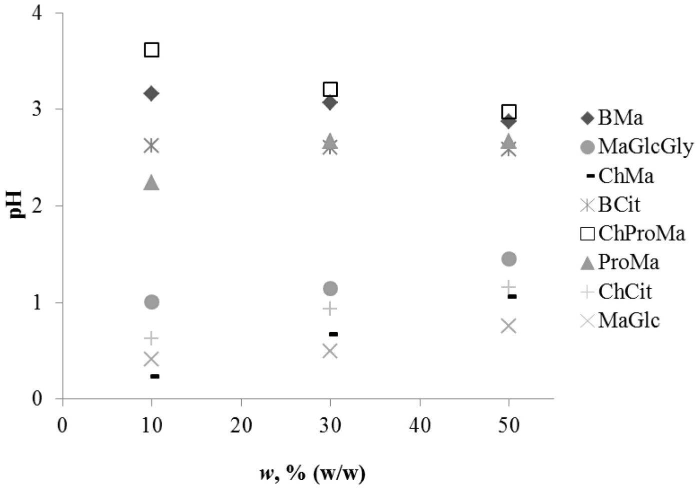


Fig. 5 – *pH values of NADES as function of water content at 298 K*

0.22–1.64 for lower acidity NADES, in and between 2.17–3.63 for higher-acidity NADES (Table 3). It is clear that pH of NADES shows no strong dependency on the temperature. On the contrary, the hydrogen bonds between HBD and hydrogen bond acceptor (HBA) seem to have a strong effect on the acidity of the NADES[34]. NADES prepared with organic acids (in a role of either acceptor or donor) and choline chloride or glucose had extremely high acidity. Increased pH values with increasing water content were visible in eutectic mixtures with extremely low pH-values, while pH values of eutectic mixtures with pH in the upper region of acidity decreased with increasing water content.

Conductivity of NADES

Conductivity depends on available ions as well as their mobility and valence. Due to their high viscosity, most NADES exhibit ionic conductivities lower than 2 mS cm^{-1} at room temperature[35]. Because composition has an impact on viscosity, conductivity is also dependent thereon[36]. As shown in Table 3, all examined NADES exhibited increasing conductivities with increasing temperatures and water content, as opposed to the trends shown in their viscosity dependences. A greater increase in conductivity with increasing water content was evident

Table 3 – *pH values and conductivity of NADES with different water contents 10–50 % (w/w) in temperature range 288–328 K*

NADES	T, K	pH			K, mS cm^{-1}		
		water content of NADES, %			water content of NADES, %		
		10	30	50	10	30	50
BMa	288	3.39	3.40	2.95	0.28	0.88	3.26
	298	3.16	3.07	2.88	0.85	1.45	4.74
	308	2.98	3.20	2.76	2.04	2.28	6.37
	318	2.83	3.01	2.61	3.37	3.29	8.35
	328	2.62	2.90	2.50	6.17	4.30	10.49
MaGlcGly	288	1.38	1.30	1.65	0.02	0.16	1.08
	298	1.01	1.15	1.45	0.05	0.25	1.43
	308	0.75	0.97	1.28	0.13	0.34	1.79
	318	0.58	0.82	1.13	0.33	0.46	2.14
	328	0.37	0.67	0.98	0.70	0.65	2.48
ChCit	288	0.63	0.88	1.11	1.54	21.70	51.60
	298	0.62	0.93	1.16	2.04	23.90	53.30
	308	0.62	0.96	1.18	2.62	26.00	54.70
	318	0.65	0.97	1.19	3.39	27.80	55.60
	328	0.67	0.98	1.18	4.33	29.30	55.70
ChMa	288	0.22	0.55	1.10	0.41	17.04	44.80
	298	0.23	0.67	1.06	0.52	19.21	46.30
	308	0.27	0.75	1.10	0.74	21.10	47.60
	318	0.31	0.77	1.11	0.88	22.50	48.60
	328	0.34	0.78	1.11	1.06	23.41	49.30
BCit	288	2.81	2.77	2.75	0.06	3.85	1.32
	298	2.63	2.60	2.59	0.16	5.40	2.18
	308	2.46	2.44	2.44	0.43	7.09	3.69
	318	2.29	2.30	2.26	1.23	10.06	5.81
	328	2.15	2.15	2.12	3.12	13.15	7.57
ChProMa	288	3.63	3.35	2.95	0.94	12.52	26.10
	298	3.62	3.21	2.97	1.32	17.71	27.80
	308	3.65	3.08	2.99	1.87	24.00	29.30
	318	3.63	2.95	3.02	2.48	31.40	30.30
	328	3.58	2.80	3.03	3.07	39.80	30.90
ProMa	288	2.17	2.87	2.86	0.15	2.77	5.27
	298	2.24	2.67	2.67	0.38	4.29	7.32
	308	2.23	2.57	2.56	0.72	6.06	9.52
	318	2.22	2.42	2.42	1.27	8.28	11.91
	328	2.19	2.29	2.28	1.93	10.56	14.59
MaGlc	288	0.37	0.45	0.76	0.02	0.07	0.43
	298	0.41	0.49	0.76	0.03	0.14	0.46
	308	0.46	0.55	0.77	0.03	0.17	0.53
	318	0.46	0.68	0.79	0.05	0.22	0.60
	328	0.46	0.67	0.81	0.06	0.25	0.64

in NADES prepared with choline chloride (ChCit, ChMa and ChProMa). The conductivity of ChCit with 50 % (w/w) of water reached 55.70 mS cm^{-1}. It should be noted that acidity for the choline chloride NADES was very high. Meanwhile, the conductivity of NADES prepared from glucose (MaGlcGly and MaGlc) showed no considerable change when water content increased (0.02–2.48 and 0.02–0.64, respectively), although pH value of MaGlc was < 1. These moderate conductivities might result from reduced ion mobility due to ion aggregation, or from large ion size and/or ion pairing leading to a smaller amount of available charge carriers[37].

In order to discuss additionally the relationship between viscosity and molar conductivity, Walden plot is shown in Fig. 6. The Walden rule states that the product of the molar conductivity and viscosity is constant for infinitely diluted electrolyte solutions. Walden plot is particularly applicable in ionic liquids, but it can be a useful measure for analysing properties of DES/NADES. Molar conductivities are calculated from conductivity, molar mass and densities according to equation:

$$\Lambda = \frac{M \cdot \lambda}{\rho} \tag{3}$$

Ideal line with slope 1 in graph 7 represents the ideal behaviour of dilute aqueous solution of KCl, which implies equal mobility of dissociate ions. The log-log correlations between molar conductivities and reciprocal values of viscosities are linear for all NADES, regardless of the added quantity of water. It can be noticed that relations for the researched NADES show deviations from ideal line. Some of the lines lay below, while others above the ideal line[38,39]. Namely, liquids without uniform charge distribution will fall below the ideal line and they could be considered as "poor ionic liquids" in terms of ionicity[40]. On the other hand, NADES located in the upper part of the graph could be considered as "superionic liquids".

Corrosion activity of NADES

NADES can come in contact with reactors, pipelines, vessels and other various devices. Therefore, in order to turn NADES from scientific concepts to practical uses, their corrosion activity upon contact with different metals must be determined.

According to literature, interaction of NADES with metals is rarely known[41], but a large number of studies has focused on their use as alternative solvents, especially in electrochemistry[4,6,42,43].

Abbot *et al.*[42] investigated corrosion behaviour of four deep eutectic solvents (choline chloride with urea, ethylene glycol, glycerol, and oxalic acid) on steel, nickel, and aluminium, and confirmed that the NADES containing urea, ethylene glycol, and glycerol showed very low corrosion rates on investigated metals, even when NADES contained water. Only ChCl:oxalic acid showed pronounced corrosive action on steel, with a corrosion rate of 176 μm $year^{-1}$.

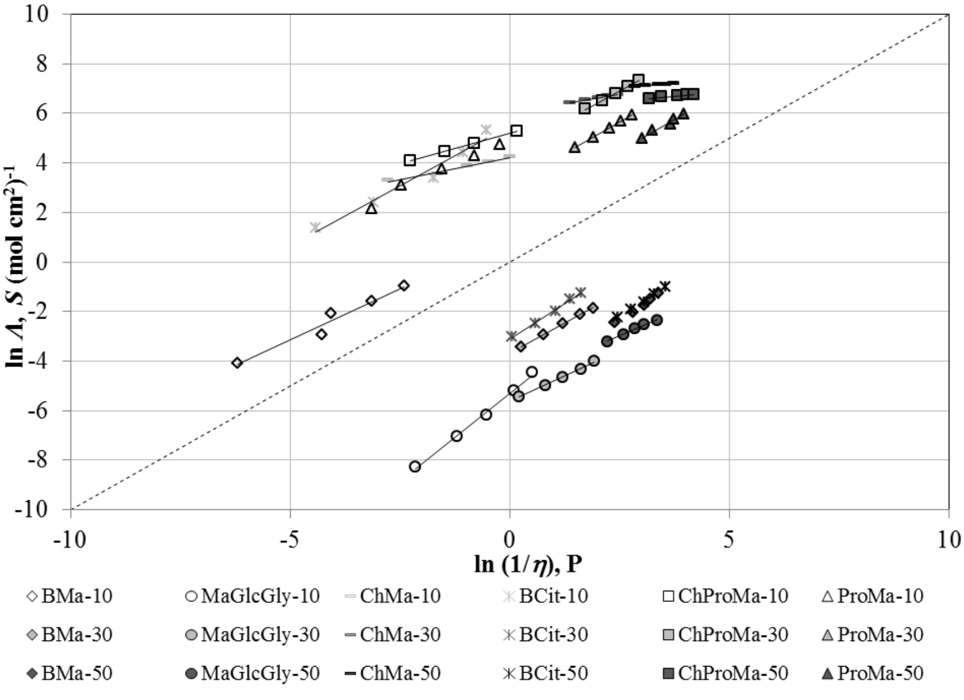


Fig. 6 – *Walden plot for NADES*

The corrosion rates (μm year^{-1}) and corrosion current densities (μA cm^{-2}) of steel X6CrNiTi18-10 in the investigated NADES with different water contents were determined (Table 4). Electrochemical measurements (potentiodynamic polarization) were performed only in NADES with 50 % (w/w) water addition. Considering that electrochemical corrosion of metals occurs when electrons from atoms at the surface of the metal are transferred to electron acceptor or depolarizer, water must be present to serve as a medium for the transport of ions. For this reason, electrochemical measurements by this method were not possible in NADES with a low water content.

As shown in Table 4, the corrosion rates obtained by weight loss measurements for all NADES ranged from 0–17.0 μm year^{-1}. It was observed that the addition of water in NADES could influence their high corrosion activity on metals[41]. We noticed that when water content in NADES increased, the corrosiveness of the resulting solution slightly increased in ProMa, MaGlcGly and BCit, while the increase of water on other investigated NADES caused a reduction in the corrosion rate for steel. Moreover, MaGlc showed absolutely no corrosion activity on the tested steel, despite the addition of water. This result is in correlation with obtained poor ionic conductivity for MaGlc.

A possible explanation for reduction in the corrosion rate on steel with increased water content in ChMa, ChProMa and ChCit could be due to the presence of chloride ions from choline chloride, as it is confirmed that some DES can dissociate in water[44].

Although it is well known that chloride ions cause pitting corrosion on metals, in this case, the chloride ions could have acted synergistically with the positively charged species present in the water solution. Namely, the steel surface was positively charged in low pH media, and chloride ions probably formed intermediate bridges between the metal surface and the positively charged species present in the solution, thus forming a layer protecting the steel surface from further corrosion[43].

Although BCit showed the highest corrosion activity on steel (17 μm year^{-1} with 50 % water addition), the rate was actually quite low, since materials with a corrosion rate of less than 20 μm year^{-1} are generally considered outstanding in relative corrosion resistance[42].

Polarisation curves obtained for steel X6CrNiTi18-10 in NADES with 50 % (w/w) water addition is given in Fig. 7. Since all investigated NADES had high acidity, the anodic reaction of corrosion was dissolution of steel, and emersion of metal ions from the metal surface into the solution. The cathodic reaction is the discharge of hydrogen ions to produce hydrogen gas or to reduce oxygen. As may be seen from Fig. 7, all NADES had influence on both anodic and cathodic reactions, which implies that corrosion is uniform without appearance of pitting corrosion.

From the obtained corrosion current densities, it is obvious that all investigated NADES showed extremely low corrosion activity on steel, since the values of current densities were between 0.0017 and 0.275 μA cm^{-2}. Although all investigated NADES had quite low pH values, from the obtained results it is evident that corrosion of steel X6CrNiTi18-10 is insignificant, and a greater influence on corrosion behaviour of steel was exhibited by structural components present in NADES.

Table 4 – *Corrosion rate data (W) obtained from weight loss measurements and corrosion current densities values for steel X6CrNiTi18-10 in NADES at 25 °C*

NADES	Water content, %	Steel X6CrNiTi18-10	
		W, μm year^{-1}	I_{corr}, μA cm^{-2}
ChMa	10	8.6	–
	30	2.1	–
	50	0.93	0.026
ProMa	10	0.13	–
	30	7.5	–
	50	10.4	0.201
ChProMa	10	0.92	–
	30	0.90	–
	50	0.22	0.008
BMa	10	8.3	–
	30	6.3	–
	50	0.32	0.013
MaGlcGly	10	0.08	–
	30	0.26	–
	50	0.50	0.021
MaGlc	10	0	–
	30	0	–
	50	0	0.0017
ChCit	10	2.77	–
	30	0.11	–
	50	0.10	0.003
BCit	10	3.7	–
	30	6.3	–
	50	17.0	0.275

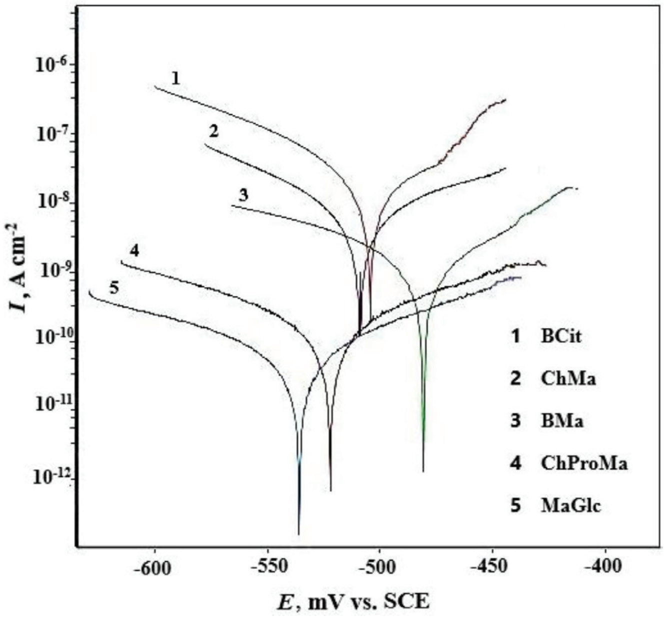


Fig. 7 – *Potentiodynamic polarization curves obtained for steel X6CrNiTi18-10 in NADES with 50 % (w/w) water at 25 °C*

Because all investigated NADES were absolutely benign and noncorrosive for investigated steel X6CrNiTi18-10, they are suitable for possible applications in similar industrial environments. It is important to emphasize that future studies are needed to evaluate the corrosion resistance at different process conditions (higher temperature), and of other metals and alloys, particularly aluminium and copper, which have a variety of uses in industry.

Evaluation of NADES cytotoxicity and antioxidative activity

Evaluation of prepared NADES by testing their effect on cell viability on three human cell lines was performed. The cell lines serve as good biological system for toxicity testing of novel chemicals, as well as for assessing potential biological activity of those compounds. The aim of such *in vitro* approach is to predict the effects these solvents might have on the environment, and to implement the REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) regulation that promotes alternative *in vitro* assays for the reduction of animal testing[3]). Mammalian cell lines are already used for ecotoxicological profiling of DES/NADES[8,11,45–47] yielding a good agreement with the *in vivo* results[34]. Therefore, in this work, we assessed the effect of eight NADES with 10 % water content on the viability of HEK-293T, HeLa, and MCF-7 cells by the colorimetric CellTiter 96® AQueous One Solution assay after a 72-h treatment with three different mass concentrations of NADES (500, 1000, and 2000 mg L^{-1}). Because there were no significant differences in observed effect toward cell viability, Fig. 8 shows only the results for the highest-tested concentration of NADES (2000 mg L^{-1}).

The results demonstrate that the tested NADES had no inhibiting effect on growth of human HEK-293T, HeLa, and MCF-7 cells, even when the highest concentration (2000 mg L^{-1}) was applied. The viability of cells ranges from 92.01 ± 5.09 (HeLa cells treated with BCit) to 111.85 ± 2.26

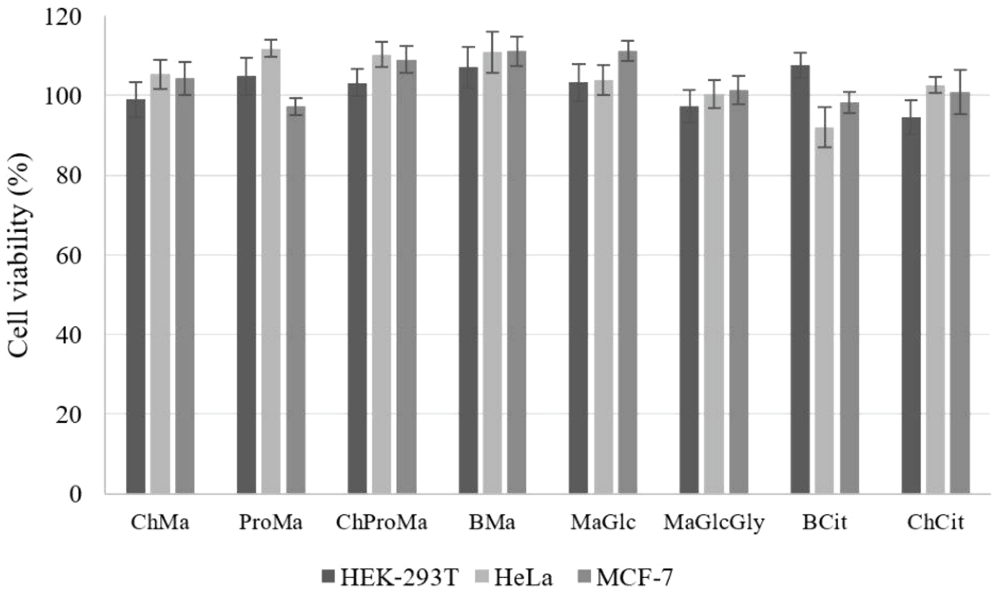


Fig. 8 – *Effect of NADES containing carboxylic acids on viability of HEK-293T, HeLa, and MCF-7 cells determined by the CellTiter 96® AQueous One Solution assay. The results for the highest-tested concentration (2000 mg L^{-1}) of NADES are shown. Cell viability (%) was expressed as percentage of treated versus control cells, and the data are the means ±S.D of four replicates for each exposure concentration.*

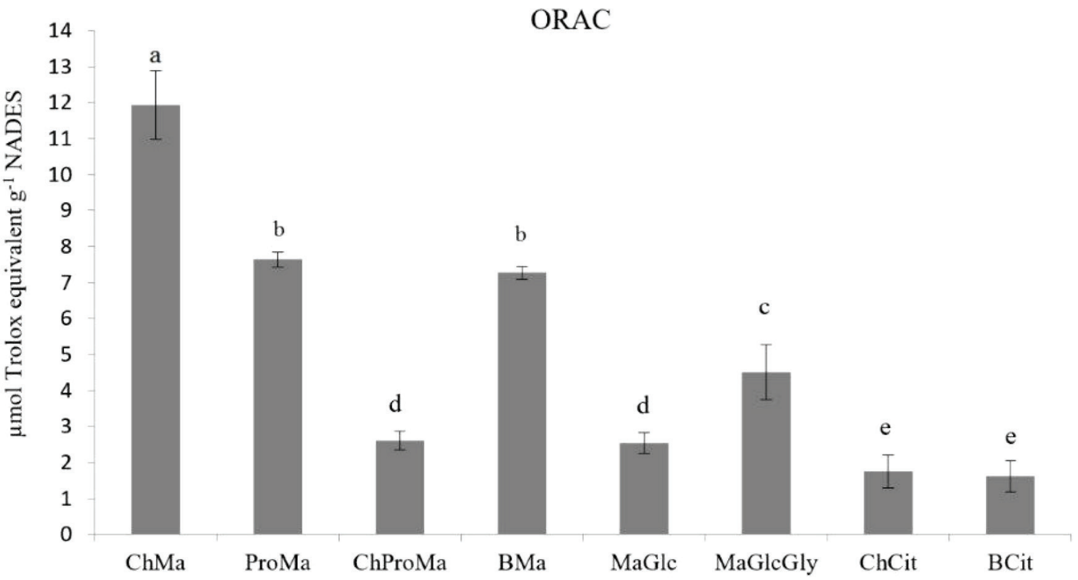


Fig. 9 – *Oxygen radical absorbance capacity (ORAC) of prepared NADES. Results (mean values, n = 3, ± SD) are expressed as µmol TE g^{-1}*

(HeLa cells treated with ProMa). Because none of the tested NADES caused 50 % growth inhibition in any of the used cell lines, the EC$_{50}$ value was declared to be higher than 2000 mg L^{-1} and NADES were considered to have low cytotoxicity (EC$_{50}$ > 2000 mg L^{-1} i.e., >5 mM). Such results were somehow expected, since NADES are presumed to be non-toxic, as they are synthetized from compounds of natural origin and safe for humans and the environment. On the other hand, many authors suggest that NADES containing organic acids as HBD (e.g., oxalic, citric, malic, or tartaric acids) exhibit greater cytotoxicity *in vitro* than NADES containing sugars as HBD (e.g., glucose, mannose, fructose, and xylose)[8,11,46–48]. NADES containing carboxylic acid produced no dramatically higher cytotoxicity than those already estimated and presented in the current literature. For example, a higher concentration of ChClCit (2500 mg L^{-1}) resulted in around 70 % survival of L929 cells[8], the same as ChClMa (2000 mg L^{-1}) in CCO cells[11]. Such inhibitory effect of ChMa (about 30 %) was not observed toward HEK-293T, HeLa, and MCF-7 cells. It is obvious that NADES may have different inhibitory effects on different cell lines[47,49], nevertheless, the overall conclusion is that the majority of NADES possess low cytotoxicity toward cell lines and have better toxicity profiles than their DES or IL equivalents[11,46]. The recent research on cytotoxic profile of NADES from Hayyan *et al.*[47] confirmed that organic acids as HBD highly contribute to the increasing toxicity of those mixtures. Among five tested solvents, NADES$_5$ (ChCl:malonic acid) was the most toxic for HelaS3, MCF-7, and B16F10 cell lines[45]. Nevertheless, among all NADES with organic acids as HDB, assessed in the literature so far, choline chloride:oxalic acid was the only one in which moderate cytotoxicity toward fish and human cell line was reported[45]. Since current knowledge on toxicity of deep eutectic solvents indicates that it is dependent on multiple variables, including the salt's cation, the counteranion species, the molar ratio, and the chemical nature of the HBD, further evaluation of cytotoxicity of NADES is needed for better insight into their possible biological effects and ecotoxicological impact, all in order to enable their large-scale utilization.

Using the ORAC assay described previously, the antioxidant capacity of prepared NADES was determined. The ORAC values of the prepared NADES were between 1.67 and 1.87 µmol TE g^{-1} dw, with the best antioxidant activity obtained for the ChMa, followed by ProMa ≈ BMa> MaGlcGyl > ChProMa≈MaGlc >BCit ≈ ChCit (Fig. 9). The antioxidative activities of these NADES were not unexpected because the forming compounds (malic acid, citric acid, prolin, and betaine) also possess antioxidative activity[50–52]. The lowest antioxidant activity was observed for NADES comprising citric acid, in contrast to those with malic acid, which corresponded to the different antioxidative activities of these organic acids. We also measured antioxidative activity of forming compounds of NADES, and malic acid showed the highest antioxidative activity among all (data not shown). It is known that malic acid is used as a food additive and has a much higher antioxidant activity than citric acid[53]. The antioxidant activity of the NADES itself, or the NADES-forming compound, was noticed previously when antioxidative activity of plant phenolic extract in NADES was measured. This discrepancy in the results was observed and explained by the reactive oxygen species scavenging activity of NADES-forming compounds[11,12]. However, antioxidative activity of NADES composed of choline chloride and hydrogen bond donors, such as polyalcohols glycerol, ethylene glycol, triethylene glycol, and amide urea, was low, indicating that those NADES could not serve as radical scavengers[47,54]. Therefore, by choosing appropriate NADES-forming compounds, a solvent with antioxidative activity could be prepared. Considering that NADES-forming compounds possess various biological traits, including anti-inflammatory response, antiplatelet aggregation, reducing cell apoptosis, and providing protective effects against myocardial ischemia/reperfusion injury, such health-beneficial properties could be also expected from NADES themselves[11,12,47,50].

Conclusions

In conclusion, the applicability of NADES to commercial processes is still in the initial phase of research and development. Prior to their industrial application, it is essential that these new solvents be well characterized, including both physicochemical and biological profiling. The structure of prepared NADES greatly influences their physical properties, which could be successfully modified and adjusted by addition of water. All tested NADES were non-corrosive for the investigated steel X6CrNiTi18-10. They possess antioxidant activity and could be defined as environmentally safe. Comprehensive physicochemical and biological characterization of NADES would contribute to the overall knowledge about these solvents. This would help in designing the best possible solvent for a certain application, which would be a great step forward in their more significant usage in real industrial processes.

Supplement information

Table 1*S* – *Used NADES and their molar ratios and molar mass*

NADES	Water content,% (w/w)	Molar ratio	Molar weight, g mol^{-1}
BMa	10	1:1:1.5	115.38
	30	1:1:6.0	93.74
	50	1:1:13.9	72.11
MaGlcGly	10	1:1.1:2.5	132.20
	30	1:1.1:9.7	106.90
	50	1:1.1:22.6	81.51
ChCit	10	2:1:3	160.98
	30	2:1:11.5	146.68
	50	2:1:26.7	118.09
ChMa	10	1:1:1.7	125.03
	30	1:1:6.5	101.25
	50	1:1:15.2	66.69
BCit	10	1:1:1.9	149.16
	30	1:1:7.4	120.02
	50	1:1:17.2	90.88
ChProMa	10	1:1.1:2.4	119.23
	30	1:1.1:9.3	96.74
	50	1:1.1:21.6	74.25
ProMa	10	1:1:1.5	114.60
	30	1:1:5.9	93.14
	50	1:1:13.8	71.68
MaGlc	10	1:1:1.9	146.25
	30	1:1:7.5	117.76
	50	1:1:17.4	89.26

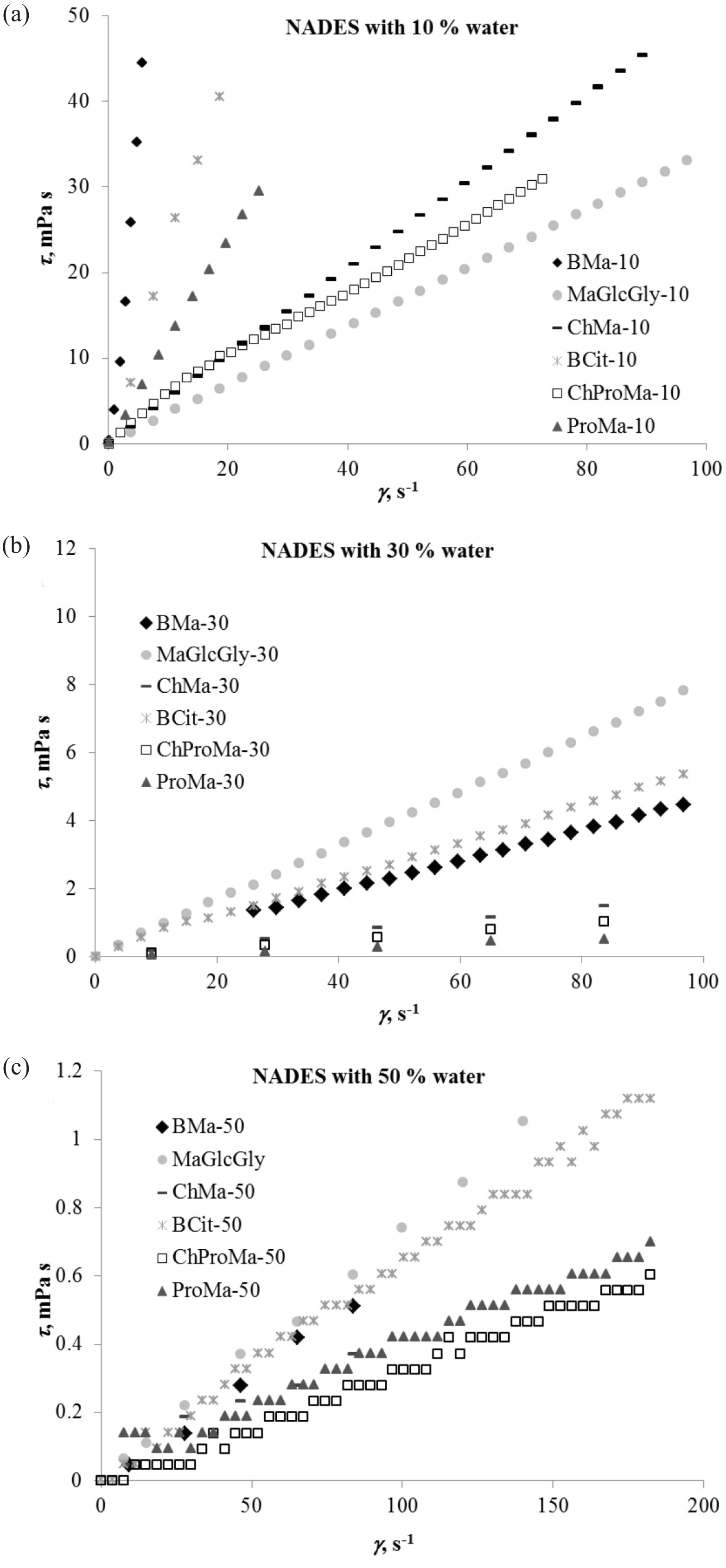


Fig. 1*S* – *Influence of shear stress, τ on shear rate, γ for NADES with 10 % (w/w) (a), 30 % (w/w) (b), 50 % (w/w) (c) water*

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