

Thermal Instability of Choline Chloride-Based Deep Eutectic Solvents and Its Influence on Their Toxicity—Important Limitations of DESs as Sustainable Materials

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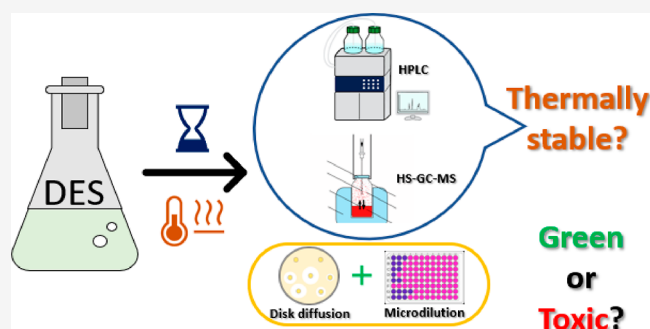
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ABSTRACT: Deep eutectic solvents (DESs) have become a hot topic in many branches of science due to their remarkable properties. They have been studied in a wide variety of applications. In particular, choline chloride (ChCl)-based DESs are one of the most commonly used representatives of these fluids. Nevertheless, in order to apply DESs in some fields, it is essential to guarantee their stability, reusability, and biocompatibility. In this context, the long-term stability of three ChCl-based DESs formed using glucose, malonic acid, and urea as hydrogen bond donors was investigated. Furthermore, the possible formation of toxic by-products during long-term heating was evaluated for the first time, and toxicological studies using three bacterial strains (*Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*) were performed. ChCl:urea DES revealed a high long-term thermal stability and was also found to be less toxic to the bacteria and thus can be considered as green solvent. ChCl:glucose DES started to decompose as a result of possible caramelization at 100 °C, and decomposition was further promoted at more elevated temperatures. Degradation of this DES did not affect greatly the toxicity toward bacteria, and low antibacterial properties were observed. The applicability of ChCl:malonic DES is not recommended as this DES was shown to be thermally unstable due to esterification and decomposition of malonic acid into acetic acid and carbon dioxide. Moreover, high toxicity of this DES in comparison to other DESs assayed in this study was reported.



INTRODUCTION

Since the first report on deep eutectic solvents (DESs) in 2003,¹ this new class of solvents has quickly attracted the scientific community. Over the years, DESs have become powerful alternatives to both organic solvents and ionic liquids (ILs),^{2–5} and they have been used in a broad variety of applications, such as biocatalysis,^{6,7} electrochemistry,^{8,9} synthesis,¹⁰ and separation and extraction processes such as CO₂ capture,¹¹ biomolecule extraction,^{12,13} desulfurization,¹⁴ azeotrope breaking,¹⁵ biomass processing,¹⁶ or gas chromatography.¹⁷ In general, DESs are defined as liquid mixtures of two or more chemical compounds resulting from the hydrogen bond interaction of a hydrogen bond acceptor (HBA) and a hydrogen bond donor (HBD), typically solids at room temperature.¹ Consequently, this eutectic mixture exhibits a significantly lower melting point compared to its pure compounds. The big advantage of DESs lies in their easy straightforward preparation, which involves simply mixing and heating HBAs and HBDs.^{18,19} Moreover, the compounds used in preparation of DESs are abundant, inexpensive, biodegradable, biocompatible, and very often come from natural sources, for example, choline chloride (ChCl), carbohydrates, amino acids, among others. All of this combined with their low

melting point and volatility, solubility for various substances, and “designer solvent” character^{20,21} contributed to their recognition as green and sustainable solvents, which are constantly sought and are becoming a corner stone of modern green chemistry according to the “Twelve Principles of Green Chemistry” established by Anastas and Warner.²²

One of the important parameters that can hinder high-temperature applications of DESs is their thermal stability. Nowadays, the thermal properties are measured using the thermogravimetric analysis (TGA), which is a powerful technique for the measurement of thermal stability of materials. In this method, changes in the weight of a compound are measured while the temperature is increased with a constant heating rate, typically 5 to 20 K/min.^{23–27} However, up to now, there have been relatively few studies on the thermal stability of DESs. Furthermore, in most of them, the onset decomposition temperatures (T_{onset}) were obtained from dynamic TGA under different experimental conditions,

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making it very difficult to compare the values obtained in different studies.^{23,27} Moreover, one should keep in mind that while performing dynamic TGA, the temperature increases rapidly at a fixed rate. Therefore, the actual critical temperature passes through the tested sample rather quickly, and thus, a noticeable and measurable mass loss is not registered. Importantly, it was previously shown that it leads to the overestimation of the onset decomposition temperature.^{28–30} From an industrial application point of view, such an overestimation of the onset decomposition temperatures of DESs could result in the use of DESs with conditions under which their thermal stability could be questioned. Having said that, it is necessary to study maximum operating temperatures in long-term scenarios, especially when DESs must endure certain high temperature for some time. Previous studies on DES thermal stability revealed that these solvents primarily decompose to HBAs and HBDs through weakening and breaking of the hydrogen bonds.²⁶ In general, two decomposition steps are observed for DESs: first, the DES component with a lower boiling point or poor stability undergoes volatilization or decomposition (usually HBD), and second, the volatilization or decomposition of the other DES component at a higher temperature takes place (usually HBA).²⁶ Furthermore, it was reported that the hydrogen bonds play a crucial role in the thermal stability of DESs since they prevent molecules from “escaping”, and thus, more energy is needed to break the bonds compared to pure HBAs and HBDs.²⁶ Overall, the more stable the DES starting materials are, the higher the T_{onset} values of the corresponding DESs could be expected.²⁶

Another important issue that should be considered is DES toxicity. Until now, there is rather scarce knowledge on this topic.^{31–35} It is mostly related to the fact that DESs are portrayed as green and non-toxic solvents because they are very often prepared using natural and biocompatible substances. Therefore, a lot of researchers simply assume that if the starting materials are non-toxic then the created DES is most likely non-hazardous to organisms without studying its effect on organisms at various trophic levels. Nevertheless, it is of pivotal importance to remember that after formation of hydrogen bonds, a new supramolecular structure is created;^{36,37} thus, possible toxicity as a result of this change may be expected and should be evaluated. Indeed, there are several studies that report the toxicity of DESs on various prokaryotic microorganisms (Gram-positive bacteria and Gram-negative bacteria) and eukaryotic organisms, including microorganisms (yeasts and molds), human and animal cell lines, and animal models (*Hydra sinensis*, *Cyprinus carpio* fish, and *Artemia salina* brine shrimp).^{31–35} Most of all, the toxicity of DESs should be controlled for DESs envisaged in applications important for the quality of life such as cosmetic, food, drug production, and medicine. Furthermore, besides the studies on toxicity of primary DES, also the toxicity of DES recovered after the process should be examined.³⁵ Very often, some of the processes require the use of characteristic conditions, for example, elevated temperatures, ultrasounds, or microwaves. Such factors can cause DES chemical instability and lead to formation of harmful byproducts. Consequently, the eco-friendly character of primary DES could be strongly affected and lead to the introduction of these byproducts to the final product.

The low number of studies on long-term thermal stability of DESs and lack of studies on toxicity of DESs that endured high

temperatures during extended time led to the current work. In this study, we have selected three choline chloride (ChCl)-based DESs. ChCl was used as a HBA because it is a quaternary ammonium salt that is non-toxic, biodegradable, and commonly used as an additive in animal feed and human nutrition.³⁸ In addition, ChCl is widely used as the HBA to form DESs due to its low price, availability, and easy connection with various HBDs.^{39,40} As a HBD, we have used urea (HBD used in pioneering work on DESs¹), glucose (representant of carbohydrates), and malonic acid (representant of carboxylic acids) since DESs derived from these compounds are employed as solvents in different separation processes.^{21,41} The temperature effect on stability of selected DESs was examined by keeping the eutectic mixtures at 80, 100, and 120 °C for 2 h. Afterward, the long-term thermal stability was assessed using high-performance liquid chromatography (HPLC) for the primary and heated DES samples. Furthermore, the possible presence of volatile compounds after the heating was also studied by performing headspace gas chromatography–mass spectrometry (HS–GC–MS). Finally, the toxicity studies [disk diffusion test and minimal inhibitory concentration (MIC) examination] of individual DES starting materials, the respective primary DESs, and DESs after long-term heating were carried out using three bacterial strains, namely, *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, and *Pseudomonas aeruginosa* ATCC 27853. The information gathered in this work is important for possible industrial applications in cosmetic, food, pharmacological, biotechnological, and biomedical sectors, in which DESs are used at high temperatures, where stability, reusability, and biocompatibility are required.

EXPERIMENTAL SECTION

Chemicals and Biologicals. ChCl and malonic acid were purchased from Sigma Aldrich (purity $\geq 99\%$ (w/w)), while D-(+)-glucose (pure) was purchased from WarChem. Acetonitrile for HPLC-Ultra Gradient (POCH, Poland) and deionized water with pH 7 (acidified with hydrochloric acid) were used as eluents and solvent in HPLC analysis. Besides eluents for HPLC, all chemicals and solvents were of analytical grade and were used without further purification.

For toxicological studies of ChCl-based DESs bacterial strains of *S. aureus* ATCC 25923 (Gram-positive), *E. coli* ATCC 25922 (Gram-negative) and *P. aeruginosa* ATCC 27853 (Gram-negative) were chosen. These strains were previously successfully used as reference strains to test the antibacterial properties of honey using the MIC method.⁴²

Preparation of DESs and Thermal Degradation Procedure. The required amounts of both chemical compounds (HBA and HBD) were weighed using an analytical balance AS.310.R2 (Redwag) in order to prepare a mixture with the desired molar ratio (ranging from 1:1 to 1:2, see Table S1 in Supporting Information). Next, the prepared mixtures were heated at 60 °C under constant stirring using a 06-MSH-PRO-T magnetic stirrer with heating (Chemland) coupled with a PT 1000 temperature sensor until a stable homogeneous liquid was formed.

To study the effect of the elevated temperature on the stability and toxicity, the DESs were prepared following the same procedure as described above. Afterward, the mixtures were collocated in closed vials and heated at different temperatures—80, 100, and 120 °C for 2 h.

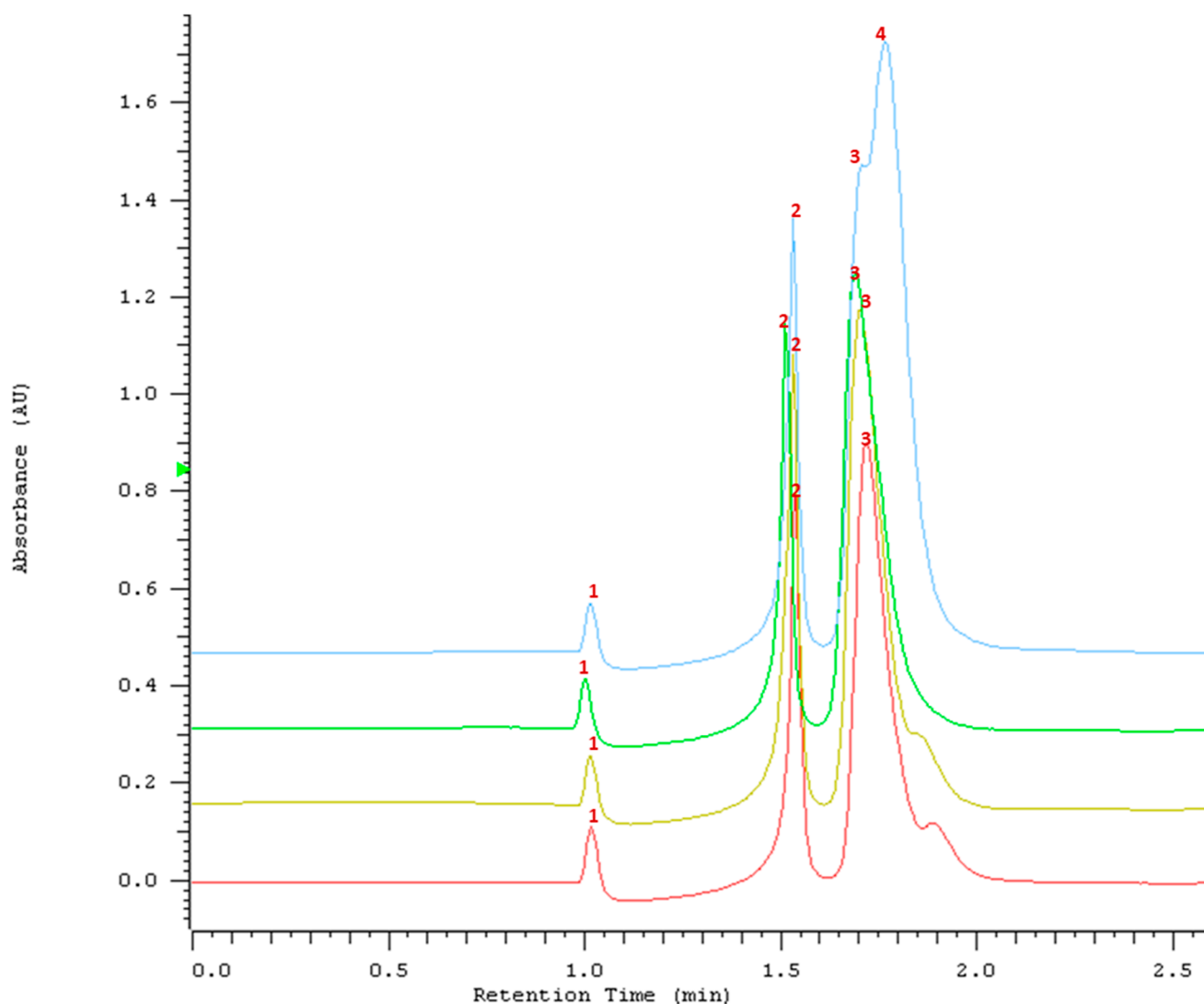


Figure 1. HPLC-UV chromatograms of ChCl:urea DES prepared via the heating method at 60 °C (red line) and after heating for 2 h at 80 °C (yellow line), 100 °C (green line), and 120 °C (blue line). Peaks 1 and 2 refer to ChCl, peak 3 refers to urea, and peak 4 refers to the unidentified product of urea decomposition.

Hydrophilic Interaction Liquid Chromatography. A Merck-Hitachi gradient liquid chromatograph (Germany) equipped with a four-channel low-pressure gradient system, a L-6200 pump, a Rheodyne Rh7161 (USA) injector with a 20 μL sample loop, a thermostat, a diode array detector (DAD) model 7455, a model 1037A refractive index detector, and HSM software was used for identification of possible temperature degradation intermediates. A LiChrospher 100 CN column with a total length of 250 mm, a diameter of 4.0 mm, and packed with 5.0 μm particles (Hewlett Packard) was used as an analytical column, and the chromatographic analysis was performed in the isocratic elution mode. Sample analysis was performed at a flow rate of 1.0 mL/min, and the mobile phase were acetonitrile 95% [v/v] and 5% [v/v] deionized water with pH 7.

Headspace Gas Chromatography–Mass Spectrometry. Headspace gas chromatography–mass spectrometry was performed using a model QP2010 GC single-bond MS SE gas chromatograph-mass spectrometer (Shimadzu, Japan) equipped with a combi-PAL AOC 5000 autosampler (Shimadzu, Japan) and a DHA Restek column with a total length of 100 m,

thickness of 0.50 μm , and diameter of 0.25 mm. LabSolutions software (Shimadzu, Japan) with NIST 14 and Wiley 8.0 mass spectrum libraries were used for data management and identification of the formed byproducts. For this, samples of the studied DESs (ca. 1 mL) were heated in 22 mL capped headspace vials at 80, 100, and 120 °C for 2 h. Next, 0.5 mL headspace samples were collected using a heated gastight syringe. The samples were immediately injected into the injection port of the gas chromatograph. The following chromatographic conditions were applied: temperature program: 50 °C (5 min)—ramped at 5 °C/min to 115 °C (0 min) and then ramped at 10 °C/min to 250 °C (10 min); injection port temperature 250 °C; split injection mode 10:1; transfer line temperature 250 °C; ion source temperature 220 °C; and the carrier gas was helium (1 mL/min). The mass spectrometer was operated in the SCAN mode in the mass range of 33–350 m/z .

Disk Diffusion Test for Toxicity Studies Against Selected Bacterial Strains. Initially, the toxicity of primary DES and heated DES samples was screened out using disc diffusion assay. To prepare the bacterial inoculum used in disc

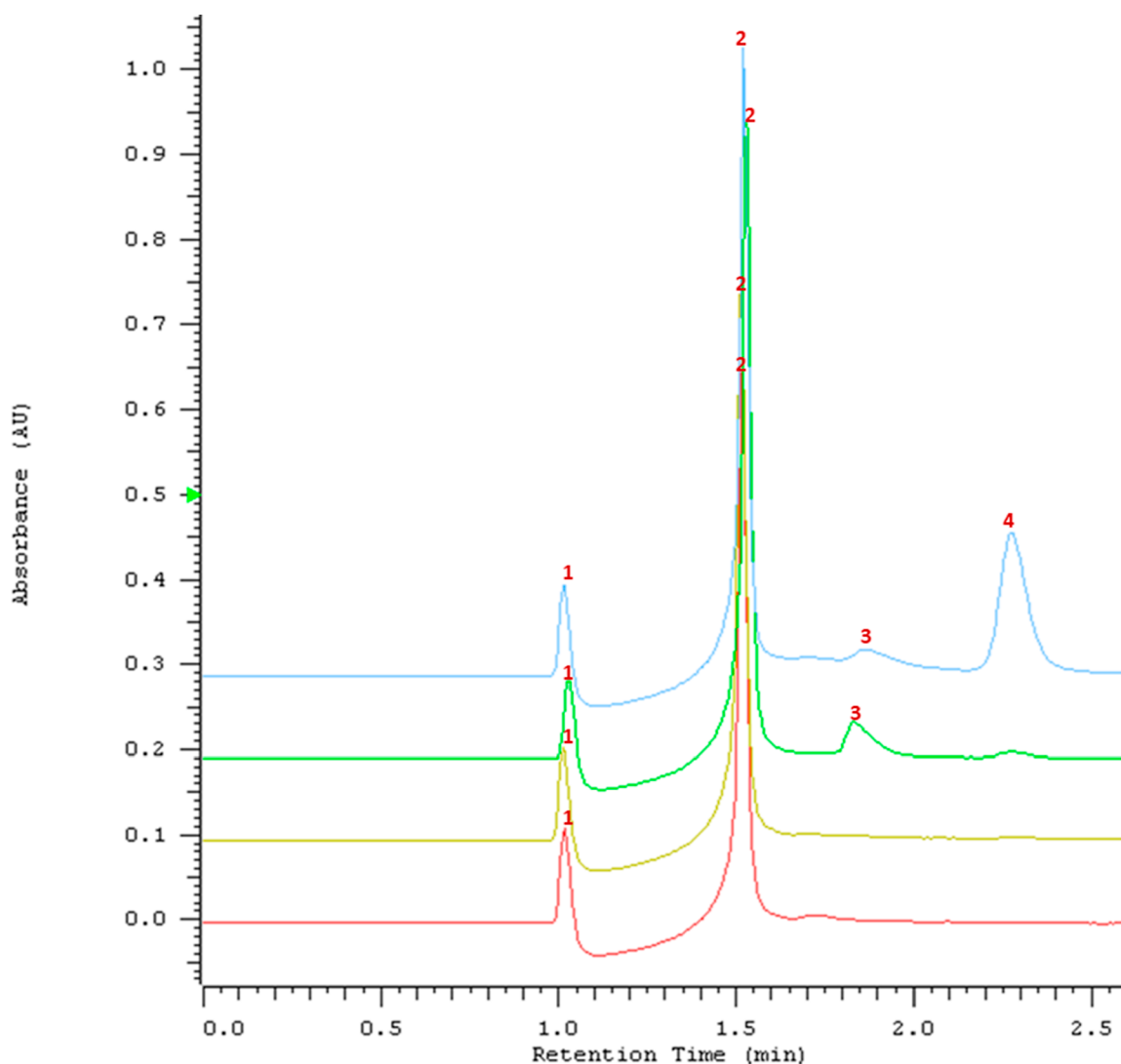


Figure 2. HPLC-UV chromatograms of ChCl:glucose DES samples prepared via the heating method at 60 °C (red line), after heating for 2 h at 80 °C (yellow line), 100 °C (green line), and 120 °C (blue line). Peaks 1 and 2 refer to ChCl, peak 3 refers to the unidentified product of glucose decomposition, and peak 4 refers to 5-HMF.

diffusion tests, Petri dishes with LB agar medium (A&A Biotechnology) were inoculated with reference bacteria strains and then incubated at 37 °C for 16–18 h. Then, using a sterile spatula, the bacteria were transferred to sterile PBS (Sigma-Aldrich) and suspended in 5 mL of sterile PBS. In each case, an additional volume of fresh PBS was added to obtain bacterial suspension with the optical density of the solution (OD_{600}) in the range of 0.1–0.12 ($\sim 1 \times 10^6$ CFU/mL). Afterward, the bacteria were collected from the prepared suspensions with a sterile ear stick and inoculated in Petri plates with fresh and sterile LB agar medium. For each type of DESs, exposed and not exposed to temperature, 10 μ L of the sample was applied to a sterile paper disc with a diameter of 5 mm (BioMaxima). After 15–30 min (the time needed for the disc to be evenly wetted with DES), each disc was placed with sterile tweezers on LB agar previously seeded with one of the three bacterial strains, that is, *E. coli* ATCC 25922, *S. aureus* ATCC 25923, and *P. aeruginosa* ATCC 27853. Thereafter, the inoculated plates with attached paper discs (control discs and discs with samples of analyzed DESs) were incubated for 24 h at 37 °C. After the incubation, the presence or absence of zones of

bacterial growth inhibition around discs with samples of tested DESs was checked. If there was a bacterial growth inhibition zone around the paper disc, its diameter was measured to assess the antimicrobial properties of each tested sample.

MIC Determination. MICs were determined using the broth microdilution method following the CLSI guidelines.⁴³ Briefly, stock solutions of primary DESs, heated DESs, ChCl, and malonic acid were dispensed in a 96-well round-bottom microtiter plate and twofold serially diluted in Müller-Hinton Broth 2 medium (MHB2, Sigma-Aldrich) to obtain a concentration range of solutions between 1.0 and 500.0 μ L of testing sample/mL. Afterward, each well containing different concentrations of tested compounds (100 μ L) was inoculated with 100 μ L of bacterial suspension (resulting in a final concentration range of solutions used in assay between 0.5 and 250.0 μ L/mL) prepared as was previously reported by Grecka et al.,⁴⁴ with one modification; in place of MHB medium, we used MHB2 medium that was previously successfully used for this purpose in the studies of Grecka et al.^{42,44} As controls, MHB2 (bacteria-free) and MHB2 (with no added tested compounds and inoculated with bacterial suspension) were



used. Furthermore, due to brown color of ChCl:glucose DES samples heated at 100 and 120 °C, uninoculated twofold serial dilutions in MBH2 were also prepared and served as a negative control during estimation of growth of the tested bacteria (an absorption assay). Each inoculated microtiter plate was incubated under aerobic conditions at 37 °C for 16–20 h. After incubation, the growth of the bacterium was followed by measuring the absorbance at 600 nm. For this purpose, each 96-well plate with analyzed samples was placed in a spectrophotometer PlateReader AF2200 (Eppendorf). MIC values were read as the lowest concentration of the analyzed DESs (primary and heated), ChCl, and malonic acid, at which growth was inhibited after incubation (concentration of an analyzed compound producing at least a 90% reduction in bacterial growth, when compared to the control growth sample). Furthermore, to confirm the obtained MIC values, resazurin sodium salt (0.015%, Acros Organics) was added to all wells (30 μ L per well) after absorbance reading, and the plates were further incubated for 2–4 h for the observation of color change. On completion of the incubation, columns with no color change (blue resazurin color remained unchanged) were marked as above the MIC value. Results were expressed as the average of the values obtained in each of two biological replicates.

RESULTS AND DISCUSSION

HILIC Study on DES Stability. The DESs selected in this work were prepared using the heating method at 60 °C, and the chosen molar ratios were used since they correspond to the most widely studied compositions.^{1,45,46} In order to investigate the effect of temperature on the long-term stability of the DESs, the prepared solvents were stirred and heated at different temperatures—80, 100, and 120 °C for 2 h. The HPLC-UV chromatograms for ChCl:urea and ChCl:glucose are shown as an example in Figures 1 and 2, respectively. These chromatograms were obtained by “extraction” of selected two-dimensional chromatograms for selected wavelengths from a 3D HPLC-UV-DAD chromatogram. In this case, 200 nm wavelength was used as all of the detected compounds with the exception of glucose absorbed UV light at this wavelength. The HPLC-UV chromatograms for ChCl:malonic acid and DES starting materials can be found in Figures S1–S2 in the Supporting Information. Note that for ChCl:malonic acid DES, only samples of primary DES and the DES heated at 80 °C were analyzed since after heating at higher temperatures, the mixtures did not maintain their liquid state, meaning that DES was decomposed.

As can be observed in Figure 1, the ChCl:urea DES remained stable after heating at 80 °C since no major changes in the chromatograms for primary DES and heated DES were detected. Please note that the peaks at around 1.00 and 1.50 min belong to ChCl and the peak around 1.70 min belongs to urea (see Figure S2). Furthermore, it shows that DES components eluted separately, further confirming the breaking of the DES supramolecular structure while in solution. On the other hand, for the mixture heated at 100 °C, some changes in the shape of the peak of urea were noted. Moreover, at 120 °C, further changes were observed, and besides the peak at around 1.70 min, a new peak at 1.80 min was detected. It seems that the changes in the shape of peak 3 (sample heated at 100 °C, see Figure 1) and appearance of new peak 4 (sample heated at 120 °C, see Figure 1) are an effect of evolution of ammonia, which after intensification of the degradation is also

accompanied by the formation of biuret (2-imidodicarbonic diamide), triuret, and ammonium isocyanate.⁴⁷ It indicates that during long-term heating at a temperature of 100 °C, urea started to decompose. Previously, the onset decomposition temperature between 172.5 and 211.0 °C was reported.^{25,26,48} Since the mentioned temperature values for the starting point of the degradation of ChCl:urea DES were obtained during dynamic TGA analysis, based on our results, it can be concluded that even at much lower temperatures than those reported in the literature and which serve as reference values for the upper temperature limit of compounds at which they can be safely applied, ChCl:urea DES starts to decompose in long-term heating scenarios. It is an important conclusion as time of DES preparation is found to have important influence on its composition. Somehow it can explain why for many DESs, reported values of physicochemical properties sometimes differ between the studies. More seriously, such composition changes can affect studies focused on evaluation of bioactive properties of DESs and extracts obtained from DESs as extractive medium. In such cases, an *in statu nascendi*-contaminated DES can cause false conclusions of many important studies.

In Figure 2, the ChCl:glucose HPLC-UV chromatograms are compared. Similar to ChCl:urea DES, the heating at 80 °C for 2 h did not have great impact on the stability of this solvent and no major changes in chromatograms for primary DES and that heated at 80 °C were observed. This scenario changed when the temperature was increased to 100 °C. The analysis of HPLC-UV chromatograms for the DES sample heated at 100 °C revealed, besides the peaks at 1.00 and 1.50 min (related to DES HBA), the presence of an additional peak at 1.80 min. On the other hand, for the DES heated at 120 °C, the peak at 1.80 min was significantly lower and another peak at 2.25 min showed up. Based on these results, it can be concluded that when heated for extended time, ChCl:glucose DES starts to decompose at 100 °C and when the time passes and temperature increases, it undergoes further decomposition. What is more, the occurring changes to the heated DES could also be observed visually since the samples kept at 100 and 120 °C changed their color from transparent to light brown and dark brown, respectively. It is well known that thermal degradation of sugars may occur either by the Maillard reaction or caramelization.^{49,50} Furthermore, in both reactions, the process of non-enzymatic browning is observed. However, for the Maillard reaction to happen, the presence of amino acids is required.^{49,50} On the other hand, the caramelization usually takes place at higher temperatures or when sugars are heated for longer time^{49,50} and is influenced by pH and sugar concentrations.⁵¹ In previous work, it was reported that ChCl:glucose DES at different molar ratios was colorless after heating at temperature up to 80 °C, which indicated that neither caramelization nor Maillard reactions occurred at these temperatures.⁵² It was confirmed in our work too since no major changes in chromatograms or visual aspect of samples heated at 80 °C were noticed. In general, thermal degradation of glucose results in formation of a mixture of different compounds including aldehydes, furfurals, phenols, acids, among others.⁵³ Among them, thermodynamically stable 5-hydroxymethylfurfural (5-HMF) is the one that is the main product of sugar degradation.^{54,55} After comparison of the UV absorption spectrum of 5-HMF and of a compound with a retention time of 2.25 min obtained with HPLC–DAD, we can confirm that it is more likely 5-HMF as two characteristic

bands (one around 230 nm and second around 284 nm) were present (for more details, please refer to Figure S3 in Supporting Information). 5-HMF is not toxic; however, it is responsible for characteristic smell, and thus in the case of large-scale industrial applications of this DES,—in case of overheating—an odor nuisance around the place of usage is caused. It is worth to mention that unintended overheating of materials is a common issue in the industry in many branches. In such cases, emission of a wide spectrum of VOCs into the environment is observed.^{56,57}

The dynamic TGA curves obtained in previous studies for ChCl:glucose DES showed two different slopes that corresponded to the two decomposition steps of glucose, while the second step also corresponded to thermal degradation of ChCl.^{25,26} Furthermore, the T_{onset} for ChCl:glucose DES ranged between 183.9 and 223.9 °C.^{25,26} Once again, it shows that lower temperatures are able to initiate thermal degradation of DESs when long-term heating is applied and that the operational temperatures for DESs should not be chosen based on T_{onset} values.

As mentioned earlier, the chromatographic analysis for ChCl:malonic acid DES was performed only for primary DES and DES heated at 80 °C. It can be observed in Figure S1 that at the lowest temperature applied, this DES seems to be thermally stable because no changes in chromatographic analysis were detected. Nevertheless, in the work of Rodriguez et al., it was concluded that during heating, esterification reaction between the hydroxyl group of ChCl and malonic acid takes place.¹⁵ What is more, it was stated that for such ChCl:carboxylic acids DESs, the decomposition had started already during preparation via the heating method (e.g., $T = 60$ °C) because all the investigated DESs were esterified to a certain extent.¹⁵ The authors further observed that during heating at 80 °C for 2 h, the decomposition of malonic acid into acetic acid and CO₂ had already started.¹⁵ This might be a case since for malonic acid individually, one peak at around 1.65 min was observed, and in the UV-HPLC chromatograms of primary and heated at 80 °C ChCl:malonic acid DES, one peak at around 1.65 min and another one at around 1.70 min were observed. It suggests that indeed the degradation of this DES had already started during preparation. These results are not in agreement with those reported by Delgado-Mellado et al., where the TGA analysis indicated that ChCl:malonic acid decomposition due to degradation of malonic acid into acetic acid started at 100 °C.²⁵ Nevertheless, the decomposition process at higher temperatures is further promoted as for the DES heated in a closed vial at 100 °C for 2 h, the presence of CO₂ bubbles was seen during the process. Moreover, the formation of acetic acid was evident after opening the vial due to the typical smell of this acid. It was assumed that the DES after heating at 100 and 120 °C for 2 h was totally decomposed because it did not maintain its liquid state. Regardless of differences in initial degradation temperature for ChCl:malonic acid DES, it is worth to mention that it is significantly lower than the temperature at which the decomposition of malonic acid into acetic acid occurs (T of 135 °C).^{58,59} In general, in previous studies where dynamic TGA analysis was performed, it was shown that DES comprising ChCl and malonic acid decomposed in the temperature range between 113.1 and 128.3 °C.^{23–27} However, as shown in this study and as it is well known, the T_{onset} may lead to an overestimation of the upper temperature stability limit^{28–30} and ChCl:malonic acid DES

degrades at significantly lower temperature than the onset temperature when heated during longer period of time.

In the light of the above-described results, it can be deduced that ChCl:malonic acid DES is the least stable of all the studied solvents in this work. It is mainly due to the thermal degradation of malonic acid. ChCl:malonic acid is followed by ChCl:glucose DES, which was more stable; however, it started to decompose at 100 °C as a consequence of caramelization reaction. On the other hand, the most stable DES was ChCl:urea, which is mainly related to the high thermal stability and low volatility of urea.

Study on the Formation of Volatile Compounds during the Long-Term Heating. Headspace analysis technique coupled with gas chromatography–mass spectrometry is a suitable method for detailed characterization of volatile compounds released during thermal heating of chemicals.⁵⁷ The studies of volatile compounds by headspace with gas chromatography–mass spectrometry (HS-GC-MS) revealed some differences in volatile compound emission profiles of DES samples heated at different temperatures. The results of analysis are shown in Table 1. The presence of

Table 1. Volatile Compounds Identified by the HS-GC-MS Method

compound	peak area [arbitrary units, AU]		
	temperature		
	80 °C	100 °C	120 °C
	DES: ChCl:urea		
hydroxyurea	5,207,389	5,463,938	4,195,988
methylene chloride	8029	27,719	10,044
hexanal	55,972	85,978	87,782
ammonium carbamate	not detected	not detected	11,061,080
methylamine	not detected	not detected	59,869
	DES: ChCl:glucose		
ethylene glycol	4,729,274	4,237,650	7,187,792
methylene chloride	7681	196,011	18,487
hexanal	62,651	49,735	102,838
butyl diglycol	not detected	1,713,888	not detected
chloromethane	not detected	not detected	123,810
ethyl aldehyde	not detected	not detected	3,498,628
5-hydroxymethylfurfural	not detected	not detected	15,619
	DES: ChCl:malonic Acid		
acetic acid	1,455,931	6,200,204	9,769,011
methyl acetate	119,409	325,016	4,211,594
dimethylamine	50,113,550	68,068,579	64,529,062
chloromethane	not detected	227,179	8,975,212
acetone	not detected	9323	45,204
cynoacetic acid	not detected	not detected	87,467

different volatile compounds confirms changes in the structure of DESs and their decomposition process that occurs during the long-term heating in the applied temperature range.

One more time, it was observed that ChCl:urea DES is the most thermally stable solvent used in this work. As it can be seen in Table 1, DES samples heated at 80 and 100 °C revealed the presence of three volatile compounds—hydroxyurea, hexanal, and methylene chloride. Especially, the presence of methylene chloride is surprising because it shows that at 80 °C, some reaction between Cl[−] and the methyl group took place, suggesting that the decomposition of ChCl has already started. Furthermore, it was noticed that the total area under the individual chromatographic peak of hydroxyur-

ea increased with increasing temperature from 80 to 100 °C. On the other hand, for ChCl:urea DES heated at 120 °C for 2 h, besides hydroxyurea, hexanal, and methylene chloride, two more possible volatile compounds were characterized, namely, ammonium carbamate and methylamine. The presented results correspond with the observations made by HPLC-UV analysis, where an additional peak for the DES sample heated at 120 °C was seen. In view of these results, it can be concluded that for ChCl:urea DES, the process of thermal decomposition during long-term heating progresses with increasing temperature and is more advanced at 120 °C. Thermal decomposition is mainly a consequence of degradation and volatilization of HBD—urea.

In the case of ChCl:glucose DES, the sample heated at 80 °C revealed the presence of three volatile compounds, namely, ethylene glycol, hexanal, and methylene chloride. On the other hand, the studies for the samples heated at higher temperatures, for example, 100 and 120 °C, showed that with higher temperatures, the process of volatilization was more advanced because besides ethylene glycol, hexanal, and methylene chloride, additional volatile compounds were characterized (see Table 1, please). For DES heated at 100 °C, four compounds in total were detected including ethylene glycol, hexanal, ethylene chloride, and butyl diglycol. Moreover, for ChCl:glucose DES heated at 120 °C, additional compounds were detected. Besides the previously characterized ethylene glycol and methylene chloride, the presence of ethyl aldehyde, 5-HMF, and chloromethane was observed. The appearance of peaks that correspond to ethyl aldehyde and 5-HMF confirms the thermal degradation of glucose under such conditions because aldehydes and furfurals are one of the main products of this process.⁵³ What is more, the degradation of ChCl was further observed as chloromethane was detected along with methylene chloride. In general, it can be concluded that the detected volatile degradation products could be divided into two types of compounds. The first type are compounds emitted during thermal degradation of HBD (glucose), which are formed during Maillard reaction or caramelization. They include ethylene glycol, butyl diglycol, ethyl aldehyde, and 5-HMF. Finally, the second type are compounds emitted during the degradation of HBA (ChCl), and they include methylene chloride and chloromethane.

By performing the analysis for ChCl:malonic acid DES samples heated at 80 °C, the following volatile compounds were identified: dimethylamine, methyl acetate and acetic acid. The presence of methyl acetate confirms the observations made in the work of Rodriguez et al., where the authors showed that for ChCl:carboxylic acid DESs, the esterification reaction takes place and that it starts already during DES preparation.¹⁵ Furthermore, as also expected, the peak of acetic acid was identified. As discussed earlier, acetic acid is emitted during thermal degradation of malonic acid to acetic acid and CO₂. These results collaborate with observations made in the work of Rodriguez et al.¹⁵ and may also explain the presence of a peak at around 1.70 min in HPLC-UV chromatograms (see Figure S1 in Supporting Information). The last of the identified volatile compounds—dimethylamine—suggests that at 80 °C, the degradation of ChCl had already started. It can be a consequence of esterification reaction between the hydroxyl group of ChCl and malonic acid, making ChCl more prone to thermal degradation. For the DES samples heated at 100 and 120 °C, two more additional volatile compounds were identified, and they were acetone and chloromethane.

Furthermore, for the DES sample heated at 120 °C, the presence of cyanoacetic acid was detected. It suggests that the process of degradation and volatilization of both ChCl and malonic acid was more advanced at these temperatures. It was further noticed that concentration of volatile compounds in the gas phase increased with increasing temperature during the process of thermal degradation as reflected by the total area of individual chromatographic peaks from HS-GC-MS analysis. All of this confirms that esterification and degradation of malonic acid and ChCl progresses with increasing temperature and leads to complete degradation of DES, which is reflected by loss the liquid nature of the samples heated at 100 and 120 °C.

Herein, we showed that in order to obtain a DES that will be stable in the wide range of temperatures, it is essential to select DES starting materials with low volatility and high stability, as it was in the case of ChCl and urea. Moreover, it seems that if one of the starting materials exhibits lower thermal stability (e.g., glucose and malonic acid used in this study), it has a great effect also on the emission of volatile compounds derived from the more stable DES component, ChCl in this case. These results further confirm the previous studies, in which it was reported that DESs undergo volatilization at high temperature and/or vacuum pressure.²⁶ The formation of volatile compounds was a consequence of volatilization of HBAs and HBDs after the decomposition of DESs' cations and anions via the weakening of H-bonding interaction.²⁶ Nevertheless, the mechanism of volatilization of DESs was not clear.²⁶ Moreover, there are also examples of DESs that volatilize at ambient temperature and pressure.⁶⁰ It is an important conclusion since it further affects the applicability of these solvents and can cause environmental concerns.

Toxicity of Primary and Heated DESs. Since it is very often assumed that DESs are low toxic, making it one of the major advantages of DESs over other solvents, in this work we assessed whether the studied ChCl-based DESs can indeed be considered as biocompatible and safe. For this, the toxicity of three different DESs, their individual components, and DESs that underwent long-term heating was determined through the viability of cells after contact with aqueous solutions of primary, heated DESs, HBA, and HBDs at different concentrations. Three different bacterial strains were used: *S. aureus* ATCC 25923 (Gram-positive), *E. coli* ATCC 25922 (Gram-negative), and *P. aeruginosa* ATCC 27853 (Gram-negative). They were chosen due their previous use in antimicrobial studies⁴² and the fact that many other strains belonging to the *E. coli*, *S. aureus*, and *P. aeruginosa* genus were previously used as representative models of Gram-positive and Gram-negative species for assaying antimicrobial activity of many types of DESs and compounds used as HBAs and HBDs in their preparation.³⁵

Disk Diffusion Test. The toxicity of the compounds after contact with the bacteria was primarily determined using disk diffusion assay. In this assay, a sterile filter paper disk is impregnated with the compound to be tested and then placed on the surface of the agar plate, where tested microorganisms have been previously swabbed uniformly. After that, the plate is left to grow the tested microorganisms under optimal conditions and to allow the compound to diffuse from the disk into the agar. If the tested compound stops the microorganism growth, there will be an inhibition zone around the disk, where no colonies have grown. The advantage of such an approach in the case of DESs is the fact that here pure

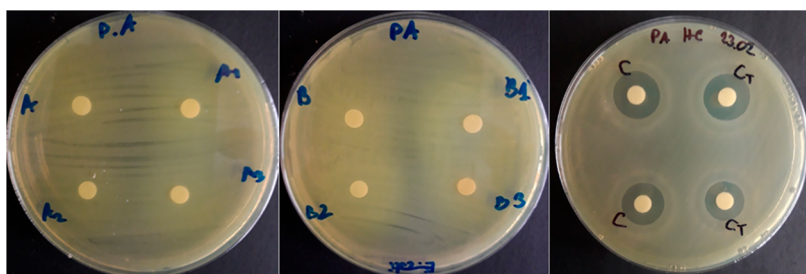


Figure 3. Effect of different DES samples on growth of *Pseudomonas aeruginosa* as determined using disk diffusion assay. (A) ChCl:urea; (A₁) ChCl:urea, 80 °C; (A₂) ChCl:urea, 100 °C; (A₃) ChCl:urea, 80 °C; (B) ChCl:glucose; (B₁) ChCl:glucose, 80 °C; (B₂) ChCl:glucose, 100 °C; (B₃) ChCl:glucose, 120 °C; (C) ChCl:malonic acid; and (C_T) ChCl:malonic acid, 80 °C.

Table 2. Inhibition Zone Measurements (Diameter (mm)) for the Various Primary and Heated DESs^a

DES	<i>E. coli</i> ATCC 25922				<i>P. aeruginosa</i> ATCC 27853				<i>S. aureus</i> ATCC 25923			
	primary	80 °C	100 °C	120 °C	primary	80 °C	100 °C	120 °C	primary	80 °C	100 °C	120 °C
ChCl:glucose (1:1)	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI
ChCl:malonic acid (1:1)	13	13	NT	NT	16	15	NT	NT	13	10	NT	NT
ChCl:urea (1:2)	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI

^aResults are presented for each bacterial strain tested. NI—no inhibition and NT—Not tested because of thermal degradation at 100 and 120 °C. The presented values are average results from two experimental replicates.

solvents can be directly applied on the disks, thus avoiding the risk of breaking of the hydrogen bonds that occurs in aqueous solutions. It is of great importance to qualitatively assess the antimicrobial activity of the prepared DESs and those after long-term heating, especially envisioning their application in sectors where the biocompatibility and safety is required. The obtained results for *P. aeruginosa* against all tested samples are presented in Figure 3 and the results for *E. coli* and *S. aureus* are shown in Figures S4–S5 in Supporting Information. The inhibition zone diameters are reported in Table 2. As summarized in Table 2, ChCl:urea and ChCl:glucose DESs did not inhibit bacterial growth, while the organic acid-based ChCl:malonic acid DES had an inhibitory effect, indicating its potential application as an antibacterial agent.

In the case of ChCl:urea DES, no visible changes in growth of all three bacterial strains were observed for primary DES and the DES samples heated at 80, 100, and 120 °C for 2 h. This is in accordance with the thermal stability studies, which also showed no significant changes in the HPLC-UV chromatograms. Furthermore, our results collaborate with previous toxicological studies for this DES, in which disk diffusion tests revealed that ChCl:urea DES did not exhibit toxic effects on various microbes including Gram-positive bacteria (*Arthrobacter simplex* TCCC 11037,⁶¹ *S. aureus*,^{62–64} *Listeria monocytogenes*,⁶³ and *Bacillus subtilis*⁶²), Gram-negative bacteria (*E. coli*,^{62–64} *Salmonella enteritidis*,⁶³ *Salmonella typhimurium* 3064,⁶⁴ *Proteus mirabilis* 3008,⁶⁴ and *P. aeruginosa* 3024^{62,64}), and fungi (*Phanerochaete chrysosporium*,⁶⁵ *Aspergillus niger*,⁶⁵ *Lentinus tigrinus*,⁶⁵ and *Candida albicans* 86⁶⁴). On the other hand, relative toxic effects on the tested genus of *Candida cylindracea* were also observed for ChCl:urea DES, suggesting that antimicrobial properties seem to be species-dependent.⁶⁵

Also, ChCl:glucose DES was reported as a non-toxic solvent toward Gram-positive, Gram-negative bacteria, and fungi.^{39,63,66} This behavior was mainly ascribed to the fact that glucose as sugar was digested by bacteria due to their simple absorption and facilitated diffusion as a carbon source. Moreover, due to catabolic repression mechanisms, glucose is

generally recognized as the most preferred source of carbon for growth of bacteria or fungi, whose presence in the growth medium usually ensures high efficiency of biomass production. Looking closely at the obtained results, it can be observed that the “growth intensity” of *E. coli* and *S. aureus* appears to be more intense around the discs soaked with primary and heated ChCl:glucose DESs. Moreover, it seems that the smaller increase in “growth intensity” was for the DES sample heated at 120 °C. As discussed earlier, the HPLC analysis revealed the thermal degradation of glucose as a possible consequence of caramelization. Thus, it can be expected that the concentration of glucose for this sample was diminished compared to that for primary DES and DES heated at 80 and 100 °C. Furthermore, the changes in the viscosity of the DES after heating cannot be discarded. During the heating process, the water removal takes place and an increase in the viscosity of the samples can be expected. Such highly viscous samples do not diffuse into agar so easily, further explaining the obtained results. In the case of *P. aeruginosa* bacteria, a similar behavior was observed; however, the zone of increased growth was not uniformly present and formed a ring at some distance from the edge of the disc soaked with primary DES and DES samples heated at 80, 100, and 120 °C for 2 h.

On the other hand, the carboxylic acid-based DES (ChCl:malonic acid) displayed an inhibitory effect on growth of each of the three tested bacterial strains, which is mainly the result of the pH change to the values far below from the optimal pH for bacterial growth (pH = 6.5–7.5).⁶⁷ It was previously reported that DESs composed of acidic HBDs have toxic effects toward microorganisms due to inactivation of cells by denaturing of proteins located on the cell wall, resulting in cell collapse and death.³² For the Gram-negative strains tested (*E. coli* and *P. aeruginosa*), both primary DES and the DES after heating at 80 °C for 2 h had similar inhibitory effects and no major differences in diameter of inhibition zones were found (see Table 2). However, there were differences in the antibacterial activity, and ChCl:malonic acid DES was more toxic to *P. aeruginosa* than *E. coli* even though both strains are Gram-negative. This observation indicates that this DES's

toxicity may be species-dependent. Furthermore, the following order of increasing antibacterial activity of this DES against tested bacteria can be deduced: *S. aureus* < *E. coli* < *P. aeruginosa*. Surprisingly, the growth of the Gram-positive strain (*S. aureus*) was the least affected by primary and heated DESs. Moreover, there was a clear difference in the size of inhibition zones for primary and heated DES for *S. aureus*, and the DES heated at 80 °C inhibited the growth of this strain to a smaller extent. These observations are in contrast to what have been reported because usually lower toxicity toward Gram-negative strains was observed as a consequence of the presence of lipopolysaccharides (LPSs) on the outer membrane that prevented the DESs from penetrating into the bacterial cell envelopes.^{68,69} On the other hand, the higher toxicity toward Gram-positive strains was explained by the lack of the outer cell membrane with LPSs, so the DESs could pass through the inner membrane more easily and exert the toxic effect.^{68,69} In general, such different antibacterial activity of ChCl:malonic acid DES that underwent heating at 80 °C for 2 h against *S. aureus* may be related with the mentioned differences in the cell wall composition of Gram-negative and -positive bacteria. Based on the chromatographic analysis (see [HILIC Study in the DES Stability](#) section), we hypothesize that the lower toxicity of the heated DES can be related with the decomposition of malonic acid into acetic acid. Even though, for the samples heated at 80 °C, no changes in chromatograms were observed, it is still possible that the process of thermal decomposition of malonic acid into acetic acid and CO₂ had already started. Therefore, the lower toxicity of the heated DES may be due to the fact that acetic acid is a weaker acid than malonic acid. Furthermore, the ester formation may also have an impact on overall antibacterial properties of heated ChCl:malonic acid DES. Since the Gram-negative bacteria have a formidable barrier, which restricts the attack of DESs from penetrating into the bacterial cell envelopes, the small changes in composition of the DESs after heating did not affect the toxicity. On the other hand, for Gram-positive *S. aureus* due to lack of such a barrier and easier penetration, the formation of less-toxic compounds resulted in lower toxicity of the heated DES sample. Overall, our results collaborate with previous studies, where the toxicity of ChCl:malonic acid DES toward various microorganisms was demonstrated using disk diffusion test. For instance, the antibacterial activity against *S. aureus*, *L. monocytogenes*, *E. coli*, and *S. enteritidis* was reported in the study of Zhao et al.⁶³ Moreover, antifungal activity was observed as this DES inhibited the growth of *P. chrysosporium*, *A. niger*, *L. tigrinus*, and *C. cylindracea*.⁶⁵

Broth Microdilution. Taking into account the fact that the results from disk diffusion assay can be underestimated due to high viscosity of the DES samples and thus lower diffusivity onto agar, the antibacterial properties of primary DESs, DESs heated at 80, 100, and 120 °C, and also ChCl:malonic acid DES starting materials (the only DES that showed growth inhibition in disk diffusion test) were further evaluated using the microdilution method to determine MIC of the assayed DESs and selected starting materials of ChCl:malonic acid. [Table 3](#) summarizes the MIC values against the Gram-negative bacterial strains *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 and the Gram-positive bacterial strain *S. aureus* ATCC 25923. The MIC values are given as a measure of volume of the testing sample per final assay volume (in [$\mu\text{L}/\text{mL}$]) that correspond to an inhibitory effect. However, it should be noted that although the negative effect of high viscosity of DESs can

Table 3. Determination of MIC for Tested Bacteria Against HBA, Malonic Acid, Primary DESs, and DESs Heated at 80, 100, and 120 °C for 2 h (Final Concentration Range of Solutions between 0.5 and 250.0 μL of Testing sample/ mL)^a

target bacteria	sample	MIC [μL of testing sample/ mL]	
<i>E. coli</i> ATCC 25922	ChCl:urea	250.0	
	ChCl:urea, 80 °C	>250.0	
	ChCl:urea, 100 °C	250.0	
	ChCl:urea, 120 °C	250.0	
	ChCl:glucose	250.0	
	ChCl:glucose, 80 °C	250.0	
	ChCl:glucose, 100 °C	250.0	
	ChCl:glucose, 120 °C	250.0	
	ChCl:malonic acid	3.9	
	ChCl:malonic acid, 80 °C	3.9	
	ChCl	>250.0	
	malonic acid	3.9	
	<i>P. aeruginosa</i> ATCC 27853	ChCl:urea	250.0
		ChCl:urea, 80 °C	250.0
		ChCl:urea, 100 °C	125.0
ChCl:urea, 120 °C		125.0	
ChCl:glucose		250.0	
ChCl:glucose, 80 °C		250.0	
ChCl:glucose, 100 °C		250.0	
ChCl:glucose, 120 °C		250.0	
ChCl:malonic acid		1.9	
ChCl:malonic acid, 80 °C		1.9	
<i>S. aureus</i> ATCC 25923	ChCl	>250.0	
	malonic acid	1.9	
	ChCl:urea	>250.0	
	ChCl:urea, 80 °C	250.0	
	ChCl:urea, 100 °C	250.0	
	ChCl:urea, 120 °C	250.0	
	ChCl:glucose	>250.0	
	ChCl:glucose, 80 °C	>250.0	
	ChCl:glucose, 100 °C	250.0	
	ChCl:glucose, 120 °C	250.0	
	ChCl:malonic acid	7.8	
	ChCl:malonic acid, 80 °C	7.8	

^aIn the determination of MIC values, two replicates of each tested concentration were performed.

be reduced using the microdilution method, at the dilutions used in this work, there is a possibility that the vast network of intermolecular interactions could be weakened or totally broken as it was previously observed for DES aqueous solutions.^{39,70–73}

As it can be seen in [Table 3](#), for ChCl:urea DES, both of the tested Gram-negative bacterial strains (*E. coli* and *P. aeruginosa*) inhibited the bacterial growth at 250.0 $\mu\text{L}/\text{mL}$, the highest concentration tested. On the other hand, the growth of Gram-positive *S. aureus* was not inhibited under the conditions of the assay (presented in [Table 3](#), MIC value above 250.0 $\mu\text{L}/\text{mL}$). For the DES sample heated at 80 °C for 2 h, no changes in MIC values were observed for *P. aeruginosa* while lower toxicity was obtained for *E. coli* as the DES after

heating did not inhibit the bacterial growth at all tested concentrations. Opposite was observed for *S. aureus*, and an increase in the antibacterial properties of DES was seen with MIC value of 250.0 $\mu\text{L}/\text{mL}$. With higher temperatures applied to this DES (e.g., 100 and 120 $^{\circ}\text{C}$), there was an increase in toxicity toward *P. aeruginosa* with an MIC value of 125.0 $\mu\text{L}/\text{mL}$. The MIC values for ChCl:urea DES also revealed its increased toxicity for the other Gram-negative strain tested—*E. coli*, while no changes were seen for Gram-positive *S. aureus* as the growth was inhibited at 250.0 $\mu\text{L}/\text{mL}$. In general, these observations are in line with the HPLC and HS-GC-MS analysis, where there was no evidence of formation of significant amounts of degradation byproducts or volatile compounds. Nevertheless, it must be noted that contrary to disk diffusion test, microdilution assay revealed that primary or heated DES exert toxic effects on tested bacteria. Since ChCl is non-toxic and approved by the European Food Safety Authority⁷⁴ and was confirmed non-hazardous to the tested bacteria in this work (see Table 3), we hypothesize that some toxicity of primary and heated DES is mainly related with the toxic effect of urea, a well-known denaturing osmolyte. Our results corroborate with those of another study in which the broth dilution method was used to assay ChCl:urea DES toxicity. For instance, it was reported that ChCl:urea DES exhibited some antimicrobial properties toward bacteria and fungi.^{39,75,76} Negative impact of this DES was observed in the studies of Wen et al. and Mao et al., where relative toxic effects on the tested genus of *E. coli* DH5 α (MIC of 295.9 mM)⁷⁵ and *A. simplex* TCCC 11037 (membrane integrity decreased to 39%)⁷⁶ were reported. The same was valid in the case of *A. niger* filamentous fungi (not toxic as assayed by disk diffusion), and antifungal activity with a MIC value of 138.5 mg/mL was shown in broth dilution studies.³⁹

The microdilution assay for ChCl:glucose primary DES revealed that this DES inhibited the growth of Gram-negative bacteria (*E. coli* and *P. aeruginosa*) at 250.0 $\mu\text{L}/\text{mL}$, the highest concentration tested. On the other hand, Gram-positive *S. aureus* was found to be more resistant, and its growth was not inhibited at all the tested concentrations. Furthermore, no changes in MIC values were observed for all DES samples heated at 80, 100, and 120 $^{\circ}\text{C}$ reported for Gram-negative bacterial strains used. The same was observed for the *S. aureus* strain for the DES heated at 80 $^{\circ}\text{C}$, and the MIC above 250.0 $\mu\text{L}/\text{mL}$ was obtained. However, for this strain, with increased temperature being applied to the DES (e.g., 100 and 120 $^{\circ}\text{C}$), an increase in the antimicrobial activity was seen. The growth of *S. aureus* was inhibited at 250.0 $\mu\text{L}/\text{mL}$ for the DES samples heated at 100 and 120 $^{\circ}\text{C}$. This increase in the toxicity toward *S. aureus* may be related with increased viscosity of the samples heated at higher temperatures and with the formation of products of caramelization, as it was discussed earlier. As it was previously shown, an increase in the viscosity of DES^{62,77} results in a decrease in the cell viability due to the extensive influence of this property on the intracellular activities. Nevertheless, since the primary or heated DES was able to inhibit bacterial growth only at the highest concentration used in the assay, it can be concluded that it is a highly biocompatible solvent at concentrations up to 250.0 $\mu\text{L}/\text{mL}$. Overall, such high biocompatibility of this DES could be expected since both ChCl and glucose are considered non-toxic and can be used by microbes as sources of nitrogen and carbon, respectively.

Contrary to the studied DESs prepared with urea and glucose, the solvent in which carboxylic acid—malonic acid was used as the HBD was found highly toxic to all bacterial strains used in the assay. For the DES that was not heated after preparation, the MIC values of 1.9, 3.9, and 7.8 $\mu\text{L}/\text{mL}$ were obtained for *P. aeruginosa*, *E. coli*, and *S. aureus*, respectively. One more time, Gram-positive *S. aureus* was found more resistant to the DES than both Gram-negative bacteria tested. For all target bacterial strains, the observed MIC values for the DES that underwent heating at 80 $^{\circ}\text{C}$ were the same as for non-heated DES. Interestingly, it is not in agreement with the results obtained in disk diffusion test, where in general the zones of inhibition were smaller for the heated DES. However, such differences between results of disk diffusion test and MIC assay were previously reported for toxicity studies of other DESs.³⁵ Hence, this result seems to support our assumption that DES toxicity testing cannot be limited to the use of only one of the widely recognized methods for testing the antibacterial properties of natural and synthetic chemical compounds.³⁵ Nevertheless, looking more closely at the data from absorbance measurements collected for each well of the 96-well plate during MIC assay (data not shown), it can be indeed seen that more bacterial cells survived at the MIC value when comparing primary and heated DES samples. Furthermore, from Table 3, it can be observed that the same concentrations of malonic acid individually and DES were needed to inhibit the bacterial growth. It suggests that antibacterial properties of this DES are an effect of toxicity of malonic acid. In general, the high toxicity of ChCl:malonic acid DES may be also attributed to pH changes toward a more acidic value since the optimal pH for bacterial cell growth is around pH 6.5–7.5.⁶⁷ Hence, the tested cells became less viable in aqueous solutions of DES (pH of 2.10 at 10 mM aqueous solution⁶³) and malonic acid (pH of 2.50 at 10 mM aqueous solution), when compared to the control cells under optimal pH conditions. The results obtained in our work correspond to those previously published in the studies in which ChCl:malonic acid DES antimicrobial properties were quantitatively evaluated. For instance, complete growth inhibition of *Bacillus cereus* EMB20 at 0.5 mg/mL was observed in the work of Sadaf and co-workers.⁷⁸ The high toxic effect on the tested genus of *E. coli* (MIC of 18 mM), *L. monocytogenes* (MIC of 24 mM), *S. aureus* (MIC of 16 mM), and *S. enteritidis* (MIC of 20 mM) was also reported.⁶³

CONCLUSIONS

In this work, the long-term thermal stability of three choline chloride-based DESs has been studied. Urea, glucose, and malonic acid have been used as HBDs. The thermal stability of DESs after heating was analyzed using HPLC and HS-GC-MS. It was shown that the temperatures needed to induce DES decomposition are much lower than those reported in the literature and obtained using dynamic TGA. This is a very important observation as for some DESs even during DES preparation using the heating method, some changes in composition take place. It partially explains why for many DESs, reported values of physicochemical properties differ between the studies. Furthermore, such composition changes can affect studies focused on evaluation of bioactive properties of DESs and composition of extracts obtained by DESs as extractive medium, leading to false conclusions of many important studies. Materials synthesized in the DES environment contain pollutants that can change their physicochemical

properties and affect their further applications. In general, it was observed that the long-term thermal stability was mainly related with the thermal stability of DES starting materials. Consequently, ChCl:malonic acid DES was found to be the least stable of all solvents studied in this work due to esterification and decomposition of malonic acid to acetic acid and CO₂. ChCl:glucose DES was more thermally stable than ChCl:malonic acid and underwent decomposition most probably due to caramelization reaction at temperatures of 100 and 120 °C, respectively. Moreover, ChCl:urea DES showed high long-term thermal stability, which is mainly related to the high thermal stability and low volatility of urea in comparison to another HBDs used in this study.

The HC-GC-MS analysis of volatile compounds emitted during the heating process revealed that in order to obtain a DES that will be stable in a wide range of temperatures during long time of exposition and will not undergo volatilization, it is essential to carefully choose the DES starting materials. It was shown that the DES composed of ChCl and urea has low volatility. On the other hand, for less thermally stable glucose- and malonic acid-based DESs, higher emission of volatile compounds was observed and further some volatile compounds derived from HBA—ChCl—were characterized.

Furthermore, the toxicity of primary and heated ChCl-based DESs in three bacterial strains (*E. coli*, *P. aeruginosa*, and *S. aureus*) was evaluated qualitatively and quantitatively, and it can be concluded that both ChCl:urea and ChCl:glucose seem to be of a low toxicity and thus may be referred to as “green solvents”.⁷⁹ The heating of these DESs did not greatly affect their toxicity. On the other hand, ChCl:malonic DES was hazardous to the bacterial strains used; however, creation of a new supramolecular structure by combining malonic acid with ChCl slightly reduced antibacterial activity of the HBD. Moreover, the heating of this DES at 80 °C resulted in decreased toxicity.

The results presented in this work highlight the need to study the long-term thermal stability of DESs as it allows to obtain the real range of the temperatures, which allows industrial application of these solvents. It was shown that ChCl:urea DES and ChCl:glucose DES besides their good thermal stability are less toxic to the tested bacteria, and thus, it seems to allow their use in cosmetic, food, pharmacological, biotechnological, and biomedical industries. The applicability in these sectors of ChCl:malonic DES is rather not recommended as this DES was thermally unstable and highly toxic to tested microorganisms.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.iecr.2c01898>.

Compositions and molar masses of ChCl-based DESs used in this work; HPLC-UV chromatograms of DES starting materials and primary and heated ChCl:malonic DES; comparison of the UV absorption spectrum of a compound with a retention time of 2.25 min obtained with HPLC—DAD with the UV absorption spectrum of 5-HMF; and disk diffusion assay results for *E. coli* and *S. aureus* (PDF)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

- (1) Abbott, A. P.; Capper, G.; Davies, D. L.; Rasheed, R. K.; Tambyrajah, V. Novel solvent properties of choline chloride/urea mixtures. *Chem. Commun.* **2003**, *1*, 70–71.
- (2) Tang, B.; Row, K. H. Recent developments in deep eutectic solvents in chemical sciences. *Monatsh. Chem.—Chem. Mon.* **2013**, *144*, 1427–1454.
- (3) Alonso, D. A.; Baeza, A.; Chinchilla, R.; Guillena, G.; Pastor, I. M.; Ramón, D. J. Deep Eutectic Solvents: The Organic Reaction Medium of the Century. *Eur. J. Org. Chem.* **2016**, 612–632.
- (4) Mouden, S.; Klinkhamer, P. G. L.; Choi, Y. H.; Leiss, K. A. Towards eco-friendly crop protection: natural deep eutectic solvents and defensive secondary metabolites. *Phytochem. Rev.* **2017**, *16*, 935–951.
- (5) Xu, P.; Zheng, G. W.; Zong, M. H.; Li, N.; Lou, W. Y. Recent progress on deep eutectic solvents in biocatalysis. *Bioresour. Bioprocess* **2017**, *4*, 34.
- (6) Lindberg, D.; de la Fuente Revenga, M.; Widersten, M. Deep eutectic solvents (DESs) are viable cosolvents for enzyme-catalyzed epoxide hydrolysis. *J. Biotechnol.* **2010**, *147*, 169–171.
- (7) Wu, B.-P.; Wen, Q.; Xu, H.; Yang, Z. Insights into the impact of deep eutectic solvents on horseradish peroxidase: Activity, stability and structure. *J. Mol. Catal. B: Enzym.* **2014**, *101*, 101–107.
- (8) Nkuku, C. A.; LeSuer, R. J. Electrochemistry in Deep Eutectic Solvents. *J. Phys. Chem. B* **2007**, *111*, 13271–13277.
- (9) Abbott, A. P.; El Ttaib, K.; Frisch, G.; McKenzie, K. J.; Ryder, K. S. Electrodeposition of copper composites from deep eutectic solvents based on choline chloride. *Phys. Chem. Chem. Phys.* **2009**, *11*, 4269–4277.
- (10) Avalos, M.; Babiano, R.; Cintas, P.; Jiménez, J. L.; Palacios, J. C. Greener Media in Chemical Synthesis and Processing. *Angew. Chem., Int. Ed.* **2006**, *45*, 3904–3908.
- (11) Harifi-Mood, A. R.; Mohammadpour, F.; Boczkaj, G. Solvent dependency of carbon dioxide Henry's constant in aqueous solutions of choline chloride-ethylene glycol based deep eutectic solvent. *J. Mol. Liq.* **2020**, *319*, 114173.
- (12) Marchel, M.; Coroadinha, A. S.; Marrucho, I. M. Novel Acidic Deep Eutectic Solvent-Based Aqueous Biphasic Systems for Efficient

Extraction of Pepsin. *ACS Sustainable Chem. Eng.* **2020**, *8*, 12400–12408.

(13) Marchel, M.; Niewisiewicz, J.; Coroadinha, A. S.; Marrucho, I. M. Purification of virus-like particles using aqueous biphasic systems composed of natural deep eutectic solvents. *Sep. Purif. Technol.* **2020**, *252*, 117480.

(14) Makoš, P.; Boczkaj, G. Deep eutectic solvents based highly efficient extractive desulfurization of fuels – Eco-friendly approach. *J. Mol. Liq.* **2019**, *296*, 111916.

(15) Rodriguez, N. R.; Requejo, P. F.; Kroon, M. C. Aliphatic–Aromatic Separation Using Deep Eutectic Solvents as Extracting Agents. *Ind. Eng. Chem. Res.* **2015**, *54*, 11404–11412.

(16) Vigier, K. D. O.; Chatel, G.; Jérôme, F. Contribution of Deep Eutectic Solvents for Biomass Processing: Opportunities, Challenges, and Limitations. *ChemCatChem* **2015**, *7*, 1250–1260.

(17) Momotko, M.; Łuczak, J.; Przyjazny, A.; Boczkaj, G. First deep eutectic solvent-based (DES) stationary phase for gas chromatography and future perspectives for DES application in separation techniques. *J. Chromatogr., A* **2021**, *1635*, 461701.

(18) Florindo, C.; Branco, L. C.; Marrucho, I. M. Quest for Green-Solvent Design: From Hydrophilic to Hydrophobic (Deep) Eutectic Solvents. *ChemSusChem* **2019**, *12*, 1549–1559.

(19) Zhang, Q.; De Oliveira Vigier, K. D. O.; Royer, S.; Jérôme, F. Deep eutectic solvents: syntheses, properties and applications. *Chem. Soc. Rev.* **2012**, *41*, 7108–7146.

(20) Francisco, M.; van den Bruinhorst, A.; Kroon, M. C. Low-Transition-Temperature Mixtures (LTTMs): A New Generation of Designer Solvents. *Angew. Chem., Int. Ed.* **2013**, *52*, 3074–3085.

(21) Smith, E. L.; Abbott, A. P.; Ryder, K. S. Deep Eutectic Solvents (DESs) and Their Applications. *Chem. Rev.* **2014**, *114*, 11060–11082.

(22) Anastas, P. T.; Warner, J. C. Green chemistry. *Frontiers* **1998**, *640*, 1998.

(23) Florindo, C.; Oliveira, F. S.; Rebelo, L. P. N.; Fernandes, A. M.; Marrucho, I. M. Insights into the Synthesis and Properties of Deep Eutectic Solvents Based on Cholinium Chloride and Carboxylic Acids. *ACS Sustainable Chem. Eng.* **2014**, *2*, 2416–2425.

(24) Skulcova, A.; Majova, V.; Haz, A.; Kreps, F.; Russ, A.; Jablonsky, M. Long-term isothermal stability of deep eutectic solvents based on choline chloride with malonic or lactic or tartaric acid. *Int. J. Sci. Eng. Res.* **2017**, *8*, 2249–2252.

(25) Delgado-Mellado, N.; Larriba, M.; Navarro, P.; Rigual, V.; Ayuso, M.; Garcia, J.; Rodriguez, F. Thermal stability of choline chloride deep eutectic solvents by TGA/FTIR-ATR analysis. *J. Mol. Liq.* **2018**, *260*, 37–43.

(26) Chen, W. J.; Xue, Z. M.; Xue, J. F.; Wang, J. Y.; Jiang, X. H.; Zhao, T. C.; Mu, T. Investigation on the Thermal Stability of Deep Eutectic Solvents. *Acta Phys.-Chim. Sin.* **2018**, *34*, 904–911.

(27) Rodriguez Rodriguez, N.; van den Bruinhorst, A.; Kollau, L. J. B. M.; Kroon, M. C.; Binnemans, K. Degradation of Deep-Eutectic Solvents Based on Choline Chloride and Carboxylic Acids. *ACS Sustainable Chem. Eng.* **2019**, *7*, 11521–11528.

(28) Crosthwaite, J. M.; Muldoon, M. J.; Dixon, J. K.; Anderson, J. L.; Brennecke, J. F. Phase transition and decomposition temperatures, heat capacities and viscosities of pyridinium ionic liquids. *J. Chem. Therm.* **2005**, *37*, 559–568.

(29) Wooster, T. J.; Johanson, K. M.; Fraser, K. J.; MacFarlane, D. R.; Scott, J. L. Thermal degradation of cyano containing ionic liquids. *Green Chem.* **2006**, *8*, 691–696.

(30) Del Sesto, R. E.; McCleskey, T. M.; Macomber, C.; Ott, K. C.; Koppisch, A. T.; Baker, G. A.; Burrell, A. K. Limited thermal stability of imidazolium and pyrrolidinium ionic liquids. *Thermochim. Acta* **2009**, *491*, 118–120.

(31) Kudlak, B.; Owczarek, K.; Namieśnik, J. Selected issues related to the toxicity of ionic liquids and deep eutectic solvents—a review. *Environ. Sci. Pollut. Res.* **2015**, *22*, 11975–11992.

(32) Juneidi, I.; Hayyan, M.; Hashim, M. A. Intensification of biotransformations using deep eutectic solvents: Overview and outlook. *Process Biochem.* **2018**, *66*, 33–60.

(33) Yang, Z., Toxicity and Biodegradability of Deep Eutectic Solvents and Natural Deep Eutectic Solvents. In *Deep Eutectic Solvents*; Wiley Online Library 2019; pp 43–60.

(34) Lomba, L.; Ribate, M. P.; Sangüesa, E.; Concha, J.; Garralaga, M. P.; Errazquin, D.; García, C. B.; Giner, B. Deep Eutectic Solvents: Are They Safe? *Appl. Sci.* **2021**, *11*, 10061.

(35) Marchel, M.; Ciesliński, H.; Boczkaj, G. Deep eutectic solvents microbial toxicity: Current state of art and critical evaluation of testing methods. *J. Hazard. Mater.* **2022**, *425*, 127963.

(36) Gutiérrez, M. C.; Ferrer, M. L.; Mateo, C. R.; del Monte, F. Freeze-Drying of Aqueous Solutions of Deep Eutectic Solvents: A Suitable Approach to Deep Eutectic Suspensions of Self-Assembled Structures. *Langmuir* **2009**, *25*, S509–S515.

(37) Gutiérrez, M. C.; Ferrer, M. L.; Yuste, L.; Rojo, F.; del Monte, F. Bacteria Incorporation in Deep-eutectic Solvents through Freeze-Drying. *Angew. Chem., Int. Ed.* **2010**, *49*, 2158–2162.

(38) Ruß, C.; König, B. Low melting mixtures in organic synthesis – an alternative to ionic liquids? *Green Chem.* **2012**, *14*, 2969–2982.

(39) Juneidi, I.; Hayyan, M.; Hashim, M. A. Evaluation of toxicity and biodegradability for cholinium-based deep eutectic solvents. *RSC Adv.* **2015**, *5*, 83636–83647.

(40) Sun, S.; Niu, Y.; Xu, Q.; Sun, Z.; Wei, X. Efficient SO₂ Absorptions by Four Kinds of Deep Eutectic Solvents Based on Choline Chloride. *Ind. Eng. Chem. Res.* **2015**, *54*, 8019–8024.

(41) Pena-Pereira, F.; Namieśnik, J. Ionic Liquids and Deep Eutectic Mixtures: Sustainable Solvents for Extraction Processes. *ChemSusChem* **2014**, *7*, 1784–1800.

(42) Grecka, K.; Kuś, P. M.; Worobo, R. W.; Szweda, P. Study of the Anti-Staphylococcal Potential of Honeys Produced in Northern Poland. *Molecules* **2018**, *23*, 260.

(43) CLSI, *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically*. Approved Standard: Pennsylvania, 2012; pp 19087-21898.

(44) Grecka, K.; Kuś, P. M.; Okińczyc, P.; Worobo, R. W.; Walkusz, J.; Szweda, P. The Anti-Staphylococcal Potential of Ethanolic Polish Propolis Extracts. *Molecules* **2019**, *24*, 1732.

(45) Abbott, A. P.; Boothby, D.; Capper, G.; Davies, D. L.; Rasheed, R. K. Deep Eutectic Solvents Formed between Choline Chloride and Carboxylic Acids: Versatile Alternatives to Ionic Liquids. *J. Am. Chem. Soc.* **2004**, *126*, 9142–9147.

(46) Bjelić, A.; Hočevar, B.; Grilc, M.; Novak, U.; Likozar, B. A review of sustainable lignocellulose biorefining applying (natural) deep eutectic solvents (DESs) for separations, catalysis and enzymatic biotransformation processes. *Rev. Chem. Eng.* **2022**, *38*, 0077.

(47) Koebel, M.; Strutz, E. O. Thermal and Hydrolytic Decomposition of Urea for Automotive Selective Catalytic Reduction Systems: Thermochemical and Practical Aspects. *Ind. Eng. Chem. Res.* **2003**, *42*, 2093–2100.

(48) Zhao, H.; Baker, G. A.; Holmes, S. New eutectic ionic liquids for lipase activation and enzymatic preparation of biodiesel. *Org. Biomol. Chem.* **2011**, *9*, 1908–1916.

(49) BeMiller, J. N.; Whistler, R. L., Carbohydrates. In: *Food Chemistry*; Fennema, O. R., Ed. Ed.; Marcel Dekker: New York, 1996; pp 157–225.

(50) Quintas, M.; Brandão, T. R.; Silva, C. L. Modelling autocatalytic behaviour of a food model system—sucrose thermal degradation at high concentrations. *J. Food Eng.* **2007**, *78*, 537–545.

(51) Eggleston, G.; Vercellotti, J. R. Degradation of sucrose, glucose and fructose in concentrated aqueous solutions under constant pH conditions at elevated temperature. *J. Carbohydr. Chem.* **2000**, *19*, 1305–1318.

(52) Hayyan, A.; Mjalli, F. S.; AlNashef, I. M.; Al-Wahaibi, Y. M.; Al-Wahaibi, T.; Hashim, M. A. Glucose-based deep eutectic solvents: Physical properties. *J. Mol. Liq.* **2013**, *178*, 137–141.

(53) Yu, Y.; Lou, X.; Wu, H. Some Recent Advances in Hydrolysis of Biomass in Hot-Compressed Water and Its Comparisons with Other Hydrolysis Methods. *Energy Fuels* **2008**, *22*, 46–60.

(54) Kroh, L. W. Caramelisation in food and beverages. *Food Chem.* **1994**, *51*, 373–379.

(55) Yu, Y.; Wu, H. Kinetics and Mechanism of Glucose Decomposition in Hot-Compressed Water: Effect of Initial Glucose Concentration. *Ind. Eng. Chem. Res.* **2011**, *50*, 10500–10508.

(56) Boczkaj, G.; Przyjazny, A.; Kamiński, M. Characteristics of volatile organic compounds emission profiles from hot road bitumens. *Chemosphere* **2014**, *107*, 23–30.

(57) Gągol, M.; Boczkaj, G.; Haponiuk, J.; Formela, K. Investigation of volatile low molecular weight compounds formed during continuous reclaiming of ground tire rubber. *Polym. Degrad. Stab.* **2015**, *119*, 113–120.

(58) Hall, G. A. The Kinetics of the Decomposition of Malonic Acid in Aqueous Solution. *J. Am. Chem. Soc.* **1949**, *71*, 2691–2693.

(59) Wilhoit, R. C.; Shiao, D. Thermochemistry of Biologically Important Compounds. Heats of Combustion of Solid Organic Acids. *J. Chem. Eng. Data* **1964**, *9*, 595–599.

(60) Chen, Y.; Yu, D.; Lu, Y.; Li, G.; Fu, L.; Mu, T. Volatility of Deep Eutectic Solvent Choline Chloride:N-Methylacetamide at Ambient Temperature and Pressure. *Ind. Eng. Chem. Res.* **2019**, *58*, 7308–7317.

(61) Mao, S.; Li, K.; Hou, Y.; Liu, Y.; Ji, S.; Qin, H.; Lu, F. Synergistic effects of components in deep eutectic solvents relieve toxicity and improve the performance of steroid biotransformation catalyzed by *Arthrobacter simplex*. *J. Chem. Technol. Biotechnol.* **2018**, *93*, 2729–2736.

(62) Hayyan, M.; Hashim, M. A.; Hayyan, A.; Al-Saadi, M. A.; AlNashef, I. M.; Mirghani, M. E. S.; Saheed, O. K. Are deep eutectic solvents benign or toxic? *Chemosphere* **2013**, *90*, 2193–2195.

(63) Zhao, B.-Y.; Xu, P.; Yang, F.-X.; Wu, H.; Zong, M.-H.; Lou, W.-Y. Biocompatible Deep Eutectic Solvents Based on Choline Chloride: Characterization and Application to the Extraction of Rutin from *Sophora japonica*. *ACS Sustainable Chem. Eng.* **2015**, *3*, 2746–2755.

(64) Radošević, K.; Čanak, I.; Panić, M.; Markov, K.; Bubalo, M. C.; Frece, J.; Srček, V. G.; Redovniković, I. R. Antimicrobial, cytotoxic and antioxidative evaluation of natural deep eutectic solvents. *Environ. Sci. Pollut. Res.* **2018**, *25*, 14188–14196.

(65) Juneidi, I.; Hayyan, M.; Mohd Ali, O. Toxicity profile of choline chloride-based deep eutectic solvents for fungi and *Cyprinus carpio* fish. *Environ. Sci. Pollut. Res.* **2016**, *23*, 7648–7659.

(66) Huang, Y.; Feng, F.; Jiang, J.; Qiao, Y.; Wu, T.; Voglmeir, J.; Chen, Z.-G. Green and efficient extraction of rutin from tartary buckwheat hull by using natural deep eutectic solvents. *Food Chem.* **2017**, *221*, 1400–1405.

(67) Tortora, G. J.; Funke, B. R.; Case, C. L., *Microbiology: An Introduction*; Pearson: Chicago, 2019.

(68) Silva, J. M.; Silva, E.; Reis, R. L.; Duarte, A. R. C. A closer look in the antimicrobial properties of deep eutectic solvents based on fatty acids. *Sustainable Chem. Pharm.* **2019**, *14*, 100192.

(69) Teh, S. S.; Loh, S. K.; Mah, S. H. Development of choline-based deep eutectic solvents for efficient concentrating of hemicelluloses in oil palm empty fruit bunches. *Korean J. Chem. Eng.* **2019**, *36*, 1619–1625.

(70) Hammond, O. S.; Bowron, D. T.; Edler, K. J. The Effect of Water upon Deep Eutectic Solvent Nanostructure: An Unusual Transition from Ionic Mixture to Aqueous Solution. *Angew. Chem., Int. Ed.* **2017**, *56*, 9782–9785.

(71) Hayyan, M.; Looi, C. Y.; Hayyan, A.; Wong, W. F.; Hashim, M. A. In vitro and in vivo toxicity profiling of ammonium-based deep eutectic solvents. *PLoS One* **2015**, *10*, No. e0117934.

(72) Radošević, K.; Cvjetko Bubalo, M.; Gaurina Srček, V.; Grgas, D.; Landeka Dragičević, T.; Radojčić Redovniković, R. I. Evaluation of toxicity and biodegradability of choline chloride based deep eutectic solvents. *Ecotoxicol. Environ. Saf.* **2015**, *112*, 46–53.

(73) Silva, J. M.; Pereira, C. V.; Mano, F.; Silva, E.; Castro, V. I. B.; Sá-Nogueira, I.; Reis, R. L.; Paiva, A.; Matias, A. A.; Duarte, A. R. C. Therapeutic Role of Deep Eutectic Solvents Based on Menthol and Saturated Fatty Acids on Wound Healing. *ACS Appl. Bio Mater.* **2019**, *2*, 4346–4355.

(74) EFSA. EFSA Panel on Additives and Products or Substances used in Animal Feed, Scientific Opinion on the safety and efficacy of

betaine anhydrous as a feed additive for all animal species based on a dossier submitted by Trouw Nutritional International B.V. *EFSA J.* **2013**, *11*, 3211.

(75) Wen, Q.; Chen, J.-X.; Tang, Y.-L.; Wang, J.; Yang, Z. Assessing the toxicity and biodegradability of deep eutectic solvents. *Chemosphere* **2015**, *132*, 63–69.

(76) Mao, S.; Yu, L.; Ji, S.; Liu, X.; Lu, F. Evaluation of deep eutectic solvents as co-solvent for steroids 1-en-dehydrogenation biotransformation by *Arthrobacter simplex*. *J. Chem. Technol. Biotechnol.* **2016**, *91*, 1099–1104.

(77) Hayyan, M.; Hashim, M. A.; Al-Saadi, M. A.; Hayyan, A.; AlNashef, I. M.; Mirghani, M. E. S. Assessment of cytotoxicity and toxicity for phosphonium-based deep eutectic solvents. *Chemosphere* **2013**, *93*, 455–459.

(78) Sadaf, A.; Kumari, A.; Khare, S. K. Potential of ionic liquids for inhibiting the growth and β -lactamase production by *Bacillus cereus* EMB20. *Int. J. Biol. Macromol.* **2018**, *107*, 1915–1921.

(79) Chen, Y.; Mu, T. Revisiting greenness of ionic liquids and deep eutectic solvents. *Green Chem. Eng.* **2021**, *2*, 174–186.

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