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Hydroxyl ammonium ionic liquids as media for biocatalytic oxidations

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In this work, neoteric and biodegradable ionic liquids (ILs) based on various hydroxyl ammonium cations and formic acid anion have been used as media for biocatalytic oxidoreductions catalyzed by different metalloproteins. The effect of these ILs on the biocatalytic behavior and structure of solubilized enzymes was investigated using cytochrome c (cyt c) as a model protein. The use of ILs–based media enhances the tolerance of cyt c against the denaturing effect of H_2O_2 and increases (up to 20 fold) its catalytic efficiency compared to that observed in buffer. This beneficial effect strongly correlates with the concentration of ILs used, as well as the chaotropicity of their cations. UV–vis, circular dichroism and Fourier transform infrared (FT-IR) spectroscopic studies indicated that, the effect of ILs on the catalytic behavior of cyt c could be correlated with slight structural changes on the protein molecule and/or perturbations of the heme microenvironment. The use of hydroxyl ammonium-based ILs as reaction media increased (up to 4-fold) the decolorization activity of cyt c. All ILs used were recycled and successfully reused three times indicating the potential application of these novel ILs as environmentally friendly media for biocatalytic processes of industrial interest.

Introduction

Ionic liquids (ILs), also called molten salts, are mixtures of cations and anions that melt below 100 ^oC and have received considerable attention over the last decade as an environmentally friendly alternative to organic solvents. Due to their interesting physical and chemical properties, such as negligible vapour pressure, ability to dissolve various hydrophobic/hydrophilic compounds and excellent chemical and thermal stability, they have been widely used as "green" media for biocatalytic processes.[1-3] The first studies of enzyme-catalyzed reactions in ILs were reported in so called second generation ILs, which are mainly based on cations of heterogeneous cyclic amines, such as substituted imidazoliums and alkyl pyridiniums, as well as poor nucleophilic anions, such as $(BF_4)^-$, $(PF_6)^-$, $(CF_3CO_2)^-$,$(CF_3SO_3)^-$.[4-6]

In the last years, numerous studies of second generation ILs, in the context of biocatalysis, revealed that many enzymes exhibit excellent selectivity and activity and maintain very high thermal and operational stability in these solvents.[6-12] However, their use in large scale applications is limited due to their difficult preparation and high cost.[1] Moreover, concerns have arisen regarding the environmental toxicity and low biodegradability of commonly used second generation ILs.[13,14] Due to the above mentioned disadvantages, over the last decade significant attention has been focused on the development of novel ILs with enhanced green properties. Recently, a third generation of ILs is emerging with structures comprising of biodegradable and readily available nontoxic ions such as natural bases, amino acids, sugars and naturally occurring carboxylic acids.[15-18]

Together with this third generation ILs, deep-eutectic-solvents (DES) formed by mixture of bio-based, non-toxic, biodegradable and inexpensive salts (e.g. choline chloride and urea or glycerol), represent also a promising alternative option for using biodegradable ionic solvents in biocatalysis and biotransformations.[19-21]

A family of third generation biocompatible ionic liquids that are based on hydroxyl ammonium cation and formic acid anion was described.[22-27] These ILs display significant scientific interest due to their low cost of preparation and simple synthesis and purification methods, since they can be easily formed by the stoichiometric combination of a Brønsted acid with a Brønsted base.[22,28] Furthermore, both cation and anion exhibit a considerably low

toxicity and are biodegradable.[14,18,29] For instance, formic acid (methanoic acid), the simplest carboxylic acid that occurs widely in nature and degrades readily in the presence of oxygen, has low toxicity, hence it is used as a food additive and as a preservative and antibacterial agent in livestock feed.[29,30] Moreover, the presence of hydroxyl groups significantly decreases the toxicity (up to 100 times lower compared to imidazolium- or pyridinium- based ILs) and improves the biodegradability of quaternary ammonium cations[14,31] leading to ionic solvents that are biodegradable, recyclable and not harmful to the environment compared to conventional solvents.[18]

In this work, four hydroxyl ammonium based ILs, formed by different cations and the same anion (formic acid) such as: 2-hydroxyl ethylammonium formate (HEAF), 2-hydroxy-*N*-methylethanaminium formate (HMEAF), 2-hydroxy-*N*,*N*-dimethylethanaminium formate (HDMEAF) and bis(2-hydroxyethyl) ammonium formate (BHEAF), were used as media for oxidoreductions catalyzed by various biocatalysts such as cytochrome c (cyt c), peroxidase, tyrosinase, laccase and alcohol dehydrogenase. The structures of the ILs used in the present work are depicted in Fig.1. Our interest for these solvents arises from the fact that these hydroxyl ammonium-based ILs have been labeled as biodegradable, recyclable and environmentally friendly media.[18] Although the effect of different second generation ionic liquids formed with synthetic anions on the catalytic behaviour of cyt c has been recently described[32], to our knowledge, there is no published study regarding the catalytic behaviour of biocatalysts, including metalloproteins, in third generation environmentally friendly ILs, as those described in the present work. Therefore, a detailed investigation of the effect of such ILs on the catalytic and structural behaviour of biocatalysts is of great interest. In the present work,, this effect on the biochemical and structural characteristics of metalloproteins was investigated using cyt c as a model protein, since it is one of the most thoroughly physicochemically characterized metalloproteins[33,34] with biotechnological interest.[33-35] Cyt c is a hemoprotein that could catalyse peroxidase-like reactions in the presence of an electron acceptor such as hydrogen peroxide (H_2O_2). In this catalytic cycle, the reaction follows a ping pong mechanism. Firstly, hydrogen peroxide reacts with cyt c to yield an intermediate called Compound I. Reduction of Compound I leads to the formation of Compound II, while the reducing substrate is oxidized to the radical product. The reaction cycle is completed by the second reduction step, in which Compound II oxidizes another molecule of the reducing substrate.[36] Through kinetic and stability studies, as well as the application of UV-vis, ATR-FTIR and circular dichroism spectroscopic techniques, we have investigated the effect of these neoteric ionic solvents on the catalytic behaviour and structure of cyt c. Moreover, in order to estimate the environmental impact of the above mentioned ILs, their biodegradability has been assessed by measuring the Biochemical Oxygen Demand (BOD).

Experimental Section

Materials

2-(methylamino) ethanol (Alfa Aesar), 2-(dimethylamino)-ethanol (Alfa Aesar), diethanolamine (Merck), ethanolamine (Sigma Aldrich), ethanol absolute (Sigma Aldrich) and formic acid (Merck) were of the highest purity available (>99%) and were used without further purification. Cytochrome c from equine heart (>95% protein content), 552 U/mg solid (1 Unit corresponds to the amount of enzyme that causes an increase in absorbance at 470nm of 0.01 per minute at pH 7.0 and 25 °C in a reaction mixture containing guaiacol and hydrogen peroxide), peroxidase from horseradish HRP (E.C. 1.11.1.7.) ~ 66 % protein content, 261 U/mg solid (1 Unit corresponds to the amount of enzyme which produces 1mg of purpurogallin from pyrogallol in 20 s at pH 6.0 at 25 °C) (type VI), tyrosinase from mushroom *Agaricus bisporus* (EC 1.14.18.1), ~ 22 % protein content, 3933U/mg solid (1 U unit corresponds to the amount of enzyme that causes an increase in absorbance at 280nm of 0.001 per minute at pH 6.5 at 25 °C in a 3 ml reaction mixture containing L-tyrosine), laccase from *Trametes versicolor* (E.C. 1.10.3.2), ~ 8.5 % protein content, 10 U/mg solid (1 Unit corresponds to the amount of enzyme which converts 1 µmol catechol per minute at pH 4.5 and 25 °C) and alcohol dehydrogenase ADH from baker's yeast (E.C. 1.1.1.1), >90% protein content, 440 U/mg solid (1 U converts 1.0 µmole of ethanol to acetaldehyde per min at pH 8.8 at 25 °C) were purchased from Sigma Aldrich and were used without further purification. 2-methoxyphenol (guaiacol), 4-methyl-catechol (>95%) and β-nicotinamide adenine dinucleotide (β-NAD^+)

(≥99%) were obtained from Sigma. 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and hydrogen peroxide (30% w/v) were purchased from AppliChem and Fluka, respectively. H_2O_2 concentration was determined spectrophotometrically at 240 nm (ε_{240}=43.6 M^{-1} cm^{-1}).[37]

Methods

Synthesis of ILs

Hydroxyl ammonium ILs were prepared by neutralization of formic acid with different amines as described in literature.[18] 0.1 mol of amine compounds (2-(methylamino)-ethanol, 2-(dimethylamino)-ethanol, diethanolamine and ethanolamine) were placed in a two necked round-bottomed flask equipped with a reflux condenser and a dropping funnel. The flask was mounted in an ice bath due to the highly exothermic nature of the acid-base reaction. Increased heat could lead to dehydration of the salt to the corresponding amide. The formic acid (0.1 mol) was added drop wise to the flask under nitrogen atmosphere and vigorous stirring with a magnetic stirrer. Stirring was continued for 24 hours at room temperature in order to obtain a viscous clear liquid. ILs were dried at high vacuum at 40 ^oC with continuous stirring to remove the water content until no further weight loss was detected. The reaction yields for the synthesis of all ILs studied were more than 97%. The ionic liquids, when not in use, were stored at room temperature in well-sealed glass vessels in a desiccator.

The densities of all ILs at 20^oC were measured by a SVM 3000 Stabinger Viscometer (Anton Paar).

The chemical structure of the synthesized ILs was determined by ^{1}H-NMR, ^{13}C-NMR, FT-IR and MS spectroscopy. ^{1}H-NMR spectra (300MHz) and ^{13}C-NMR spectra (75MHz) were recorded on a Varian Gemini 2000 (300 MHz) spectrometer. ILs were dissolved in DMSO and $CDCl_3$. *J* values are given in Hz.

FT-IR (ATR method) spectra were recorded by a JASCO 4200 spectrometer.

MS analysis was performed on a Varian 500 MS ion trap mass spectrometer. Instrumental control and the data processing were performed by the Varian MS workstation software. The ionization type used was electrospray ionization. Capillary voltage was 23.0 Volts. Analysis was conducted only on the positive (ESI+) mode because the instruments cut-off mass value is 50 and in the case of ILs studied, the HCOO- ion should appear at m/z 45.

2-hydroxy-N-methylethanaminium formate (HMEAF): δ_H (300MHz; $CDCl_3$) 2.66 (3 H, s, CH_3-), 3.03 (2 H, t, *J* 2.7, -O-CH_2), 3.86 (2 H, t, *J* 3.0, -CH_2-N), 7.98 (3 H, br s, -NH_2^+ & OH), 8.55 (1 H, s, H-COO).

δ_C (75 MHz, $CDCl_3$): 32.67, 50,79, 56.74, 166.74.

FT-IR (ATR) ν_{max}/cm^{-1}: 1340 ν (CN), 1469 ν_{sym} (COO^-), 1587 ν_{asym} (COO^-) & δ(NH_2^+), 2775 ν (NH_2^+), 3646 ν (OH).

MS (ESI): ES^+ m/z: 76.1 ($OHCH_2CH_2NH_2^+CH_3$, 100%)

Density (20^oC): 1.1372 g/cm^3

2-hydroxy-N,N-dimethylethanaminium formate (HDMEAF): δ_H (300MHz; $CDCl_3$) 2.74 (6 H, s, CH_3-), 3.03 (2 H , t, *J* 6.0, -O-CH_2), 3.87 (2 H , t, *J* 5.0, -CH_2-N), 8.58 (2 H, s, -NH^+ & OH), 9.48 (1H, s, H-COO^-).

δ_C (75 MHz, $CDCl_3$): 43.52, 56.42, 60.46, 168.99.

FT-IR (ATR) ν_{max}/cm^{-1}: 1340 ν (CN), 1475 ν_{sym} (COO^-), 1600 ν_{asym} (COO^-), 2775 ν (NH^+), 3632 ν (OH).

MS (ESI): ES^+ m/z: 90.1 ($OHCH_2CH_2NH_2^+(CH_3)_2$, 100%)

Density (20^oC): 1.0937 g/cm^3

bis(2-hydroxyethyl)ammonium formate (BHEAF): δ_H (300MHz; DMSO) 2.87 (4 H, t, -O-CH_2), 3.59 (4 H, t, -CH_2-N), 6.03 (4 H, br s, -NH_2^+& OH), 8.34 (1 H, s, H-COO^-).

δ_C (75 MHz, $CDCl_3$): 49.70, 57.38, 166.61.

FT-IR (ATR) ν_{max}/cm^{-1}: 1342 ν (CN), 1450 ν_{sym} (COO^-), 1587 ν_{asym} (COO^-) & δ(NH_2^+), 2798 ν (NH_2^+), 3658 ν (OH).

MS (ESI): ES^+ m/z: 106.2 $(OHCH_2CH_2)_2NH_2^+$, 100 %)

Density (20^oC): 1.1587 g/cm^3

2-hydroxylethylammonium formate (HEAF): δ_H (300MHz; DMSO-d_6): 2.81 (2 H, t, *J* 5.2, -O-CH_2), 3.56 (2 H, t, *J* 5.2, -CH_2-N), 7.41 (4 H, br s, -NH_3+ & OH), 8.41 (1 H, s, H-COO^-).

δ_C (75 MHz, $CDCl_3$): 49.70, 57.38, 166.61.

FT-IR (ATR) ν_{max}/cm^{-1}: 1338 ν (CN), 1400 ν_{sym} (COO^-), δ(NH_2^+), 1535 ν_{asym} (COO^-) & δ(NH_3^+), 2931-2863 ν (N^+H_3), 3623ν (OH).

MS (ESI): ES^+ m/z: 62.0 $(OHCH_2CH_2)_2NH_3^+$, 100%)

Density (20^oC): 1.2059 g/cm^3

Oxidation activity of metalloproteins

The peroxidase activity of cyt c and HRP was determined by following the color formation during guaiacol oxidation in the presence of H_2O_2. Reaction temperature was set at 30 ^oC and the increase of the absorbance at 470 nm was monitored at an interval of 2 seconds for a time period of 30 seconds as described elsewhere.[33]. Reaction conditions were adjusted according to the type of enzyme used for guaiacol oxidation. In the case of cyt c, the oxidation reaction was carried out in 50 mM sodium phosphate buffer pH 7.0 with 2 mM guaiacol, 20 mM of H_2O_2 and 13.8 U/mL of protein.[38] The concentration of ILs in the reaction medium ranged between 0-75% (v/v). When HRP (0.026 U/mL, 50 mM sodium phosphate buffer pH 6.5) was used as a catalyst, the concentration of the substrate was 20 mM and the concentration of H_2O_2 was 0.2 mM.[39]

In the case of tyrosinase, 4-methyl catechol was used as a substrate and quinone formation was monitored at 390 nm. The reaction was started by adding 23.6 U/mL of tyrosinase solution prepared in 50 mM phosphate buffer pH 6.8, containing 10 mM of the substrate at 27 ^oC.

Laccase activity was evaluated using ABTS as a substrate. Reaction mixture contained 1 mM ABTS and 0.0084 U/mL enzyme in 100 mM acetate buffer pH 4.6 at 27 ^oC and the absorbance change was measured at 415 nm.[40] For all enzymatic oxidations, the amount of oxygen has been considered in excess, since the reaction mixture was vortexed for saturation with oxygen before adding the enzyme solution.

Activity of ADH was determined by measuring the rate of reduction of β-NAD^+ by ethanol at 340 nm. 10 U/mL stock solution of enzyme was prepared immediately prior to the reaction in 10 mM sodium phosphate buffer pH 7.5. In a typical reaction mixture, 5 mM β-NAD^+ and ethanol (3.2%, v/v) were added in 50 mM sodium phosphate buffer pH 8.0. The enzymatic reaction was initiated with the addition of 8 µL of ADH solution (0.08 U/mL) to the reaction mixture at 25 ^oC. The increase in absorbance due to β-NADH formation was monitored at 340 nm.[41]

In all cases studied, the reaction mixture (1mL) was homogeneous and no precipitation was observed in the presence of all ILs. Moreover, after incubation of ILs with H_2O_2, no modification of their structure was observed by NMR analysis in all cases studied. In order to avoid the ionic liquids-induced interference to the pH, all the reaction media (buffer-ionic liquids solutions) were re-adjusted (with HCl or NaOH) to the required pH before being used in the biocatalytic reactions. All reactions were performed at the optimal pH required in each case. All experiments were performed in triplicate. Control experiments without biocatalyst were also carried out and no conversion of the substrates was observed in all cases studied. All the reaction rates were calculated from the slope of the linear portion of plots of absorbance versus time. The relative activity was expressed in each case as the ratio of activity in the presence of ILs to that observed in buffer solutions.

Kinetic study of cyt c and activation energy (E_a) determination

Guaiacol oxidation in the presence of H_2O_2 at 30 ^oC was used as a model reaction for the determination of the effect of various ILs on the kinetic constants of cyt c.[36] In a typical experimental procedure, guaiacol was added to a final concentration of 2 mM, while H_2O_2 concentration was in the range of 0.05-100 mM. The concentration of cyt c used in the reaction was 25 µg/mL. The oxidation of guaiacol was monitored at 470 nm and the extinction coefficient for the oxidation product was considered equal to ε= 26.6 mM^{-1} cm^{-1} in all cases studied.[42] The apparent kinetic parameters of maximum velocity V_{max}^{app} and Michaelis-Menten constant K_m^{app} were determined through Michaelis-Menten equation for initial reaction velocity. All the kinetic parameters were determined by non-linear regression analysis using the program Enzfitter (Biosoft, Cambridge, UK). Data reported are the mean values of three independent experiments. For the determination of the activation energy E_a for the oxidation of guaiacol catalyzed by cyt c, reactions were performed in 1 mL co-solvent mixtures of 50 mM sodium phosphate buffer pH 7.0 and 30% (v/v) aqueous solutions of ILs containing 2 mM guaiacol, 100 mM H_2O_2 and 25 µg/mL of cyt c at a temperature range from 20 to 60 ^oC. The activation energy E_a was calculated from the Arrhenius plot through linear regression analysis.

Stability study of cyt c

Stability study of cyt c was performed by incubating cyt c (13.8 U/mL) in aqueous solutions of ILs containing 1 mM

H_2O_2 at 30 °C. The incubation mixture did not contain guaiacol since the presence of reducing substrates in the reaction mixture could increase the stability of cyt c against H_2O_2.[43,44] After 15 min of incubation, 200 µL of sample were removed and transferred to a 96-well microplate in order to determine the remaining peroxidase activity of cyt c using guaiacol (2 mM) as a substrate, as described before. All experiments were performed in triplicate.

UV-vis spectroscopy

A double-beam UV-vis spectrophotometer (UV-1601 Shimadzu, Tokyo, Japan) was used in order to monitor the effect of aqueous solutions of ILs on the absorption spectrum of cyt c (25 µg/mL) in a standard 1 cm path length quartz cuvette. UV-vis absorption spectra of cyt c was recorded at 30 °C.

ATR-IR Spectroscopy

Single pass attenuated total reflection Fourier transform infrared (ATR-FTIR) spectra were recorded on a Shimadzu FT-IR 8400 (Tokyo, Japan) infrared spectrophotometer equipped with a deuterated triglycine sulfate (DTGS) detector in the region of 400- 4000 cm^{-1} using a (ZnSe)-attenuated total reflection accessory. 200 scans were collected for each sample at 2 cm^{-1} resolution and 1 cm^{-1} time interval. The concentration of cyt c was 5 mg/mL in buffer and 9 mg/mL in 30% (v/v) aqueous solutions of ILs. Reference spectra under identical conditions without the presence of cyt c were also recorded. Data analysis of the Amide I region and band assignment were performed as described elsewhere.[45]

Circular Dichroism Spectroscopy

Soret region CD spectra (350-450 nm) of cyt c (200 µg/mL) in 0.5 mM sodium phosphate buffer pH 7.0 and in 60% (v/v) aqueous solution of ILs were obtained using a Jasco J-815 spectropolarimeter (Tokyo, Japan) in a 1 cm path length quartz cell. All spectra were obtained at 25 °C with a 2 nm bandwidth and a scan speed of 10 nm/min. For every medium scanned, a baseline was recorded and subtracted from the protein spectrum. All scan measurements were performed in triplicate.

Dye decolorization

The decolorization activity of cyt c was measured by following color elimination of pinacyanol chloride (1,1′-diethyl-2,2′-carbocyanine chloride) with H_2O_2 in buffer and in the presence of various amounts of ILs. The reaction mixture contained 130 µM pinacyanol chloride and 80 µg/mL cyt c. The oxidation of the dye was started by adding 0.3 mM H_2O_2 at 27 °C under stirring at 300 rpm. At predetermined time intervals 30 µL aliquots were removed from the reaction mixture and added to a 1:1 (v/v) mixture of methanol and 50 mM sodium phosphate buffer pH 7.0. The remaining concentration of the dye was monitored by measuring the absorbance at 603 nm using an extinction coefficient for pinacyanol chloride equal to ε= 82,350 M^{-1} cm^{-1}.[46]

Recycle of ILs

The enzymatic decolorization of pinacyanol chloride was further used in order to investigate the reusability of the ILs. In this case, cyt c was immobilized on celite in a similar manner as described elsewhere[47], in order to facilitate the recovery of the enzyme and the reuse of ILs. The reaction mixture (0.5ml) contained 130 µM pinacyanol chloride and 30 mg/mL of immobilized biocatalyst (containing 2 µg of cyt c per 1 mg of celite). The oxidation of the dye was started by adding 0.3 mM H_2O_2. The reaction mixture was incubated at 27 °C under stirring at 300 rpm for 3 hours. At the end of the incubation, 1 ml of water was added to the reaction mixture and the immobilized biocatalyst was filtered off. The aqueous filtrate containing the ILs was washed with ethyl acetate in order to remove any amount of the residual substrates and products and then the water was evaporated in vacuo. The residual IL was dried under high vacuum at 40 °C until constant weight. The structure and purity of the recycled IL were verified with ^{1}H-NMR, while the recycle process was repeated up to three times.

Biodegradability test

The biodegradability of four ILs has been assessed by measuring the Biochemical Oxygen Demand (BOD).[18,48] Biodegradation tests were carried out according to the manometric respirometric method to determine the oxygen demand for the biochemical degradation of each organic substance after five days. A detailed description of the method is described on the Supplementary data.

Results and Discussion

Effect of hydroxyl ammonium ILs on the activity of various metalloproteins.

In the present study, the effect of four hydroxyl ammonium-based ILs (HEAF, HMEAF, HDMEAF and BHEAF) on the activity of various metalloproteins such as cytochrome c from horse heart (cyt c), horse radish peroxidase (HRP), mushroom tyrosinase, laccase from Trametes versicolor and alcohol dehydrogenase (ADH) from baker's yeast was investigated (Table 1). In most cases studied, the presence of 5% (v/v) hydroxyl ammonium ILs in the reaction medium affected the activity of metalloproteins. The effect of ILs used on the enzymatic activity depended on the biocatalyst used, as well as on the nature of the cation of IL used. Particularly in the case of HRP and ADH, the activity remained unchanged or decreased, depending on the IL used. The oxidation activity of laccase was significantly reduced in all ILs tested, which is in accordance to that reported for imidazolium-based water miscible ILs.[49] However, it must be pointed out that, in the case of cyt c, the presence of hydroxyl ammonium ILs in the reaction medium significantly enhanced (up to 3.4 fold) its peroxidation activity. An enhanced activity was also observed for tyrosinase in the presence of HMEAF and BHEAF IL.

Effect of ILs on the peroxidative activity of cyt c

In order to further investigate the effect of hydroxyl ammonium-based ILs on the catalytic behaviour of metalloproteins, cyt c was chosen as a model protein. The effect of the concentration of various hydroxyl ammonium ILs on the peroxidase activity of cyt c using guaiacol as a substrate is shown in Fig. 2. As it can be seen, the peroxidase activity of cyt c strongly depends on the nature of cations and the concentration of ILs used, which is in accordance to that observed previously for imidazolium, alkyl ammonium and choline-based ILs[42, 50-52]. In most cases, the increase of the ILs concentration significantly increases the peroxidase activity of cyt c. A 9-fold and 20-fold activity enhancement was observed respectively in BHEAF and HEAF at a concentration of ILs equal to 60% (v/v), compared to buffer. Similar catalytic activation of cyt c in the presence of these ILs was observed for ABTS oxidation (data not shown), indicating that this activation effect is independent of the substrate used. Although cyt c activity has been studied in other biocompatible ILs, such as alkyl ammonium and choline-based ILs, in those cases no activation was observed, in contrast to the results obtained in our study.[50,52] It is interesting to note that, when equimolar amounts of individual components of these ionic liquids (hydroxyl amines and formic acid) were both added in buffer solution (the amounts of the individual components corresponded to the ones present in ILs and were adjusted according to the desired concentration of ILs in buffer), no or low catalytic activity of cyt c was observed (Fig.S.17. Supplementary). This clearly indicates that, the beneficial effect of ILs on the catalytic activity of cyt c is associated to the formed salt and not to their individual components.

In order to gain a deeper insight into the influence of the hydroxyl ammonium-based ILs on the peroxidase activity of cyt c, the effect of the ILs on the apparent kinetic constants V_{max}^{app} and K_m^{app} of cyt c for the oxidation of guaiacol with H_2O_2 were determined. The effect of the nature and concentration of ionic liquids on the catalytic efficiency, expressed by the ratio V_{max}^{app}/K_m^{app} (in all cases studied the cyt c concentration was the same), is presented in Fig. 3. As it can be seen, the presence of ILs enhances, in most cases, the catalytic efficiency of cyt c compared to buffer solution. The highest catalytic efficiency was observed when HEAF was used as co-solvent, causing a more than 20-fold increase in catalytic efficiency at a concentration of this IL higher than 60% (v/v). The increased catalytic efficiency observed at high concentrations of HEAF and BHEAF was the result of a simultaneous increase of V_{max}^{app} and decrease of K_m^{app} compared to that observed in buffer (Table S1). The low apparent K_m^{app} values observed at high concentrations of HEAF and BHEAF indicate that the affinity of cyt c towards the substrate was increased, which may be correlated with structural changes in the active site of cyt c and therefore, changes in the microenvironment of heme.[38,53] The increased catalytic efficiency observed here is in accordance to that reported for various enzymes in other ionic liquid-based systems.[53-56] It was suggested that, the presence of ILs in the reaction medium can increase the affinity of the enzyme to the substrate, resulting in a higher catalytic efficiency compared to

other media, such as organic solvents and aqueous solutions.[55,56]

It has been proposed by several researchers that the enzyme performance in hydrophilic ILs could be affected by the kosmotropicity/chaotropicity properties of the ions of ILs.[57-59] Ions that are considered as kosmotropes promote water structure, while chaotrope ones can suppress it.[60] In the present study, formic acid, used as the anion for the formation of all ILs tested, is considered as a kosmotropic anion.[61] On the other hand, hydroxyl alkyl ammonium cations, which are more hydrophilic than the chaotropic choline cation, could be assumed to be highly chaotropic.[43] This indicates that, the presence of a kosmotropic anion and a chaotropic cation improved the catalytic efficiency of cyt c which is in accordance to that proposed for other enzymes in different ionic liquids.[57-59] It seems that, there is a correlation between the chaotropicity of the cation and the catalytic efficiency of cyt c in these media. More specifically, in the case of more hydrophilic and thus more chaotropic BHEAF and HEAF cations, the catalytic efficiency of cyt c was higher compared to that observed for less chaotropic HMEAF and HDMEAF.

In order to further investigate the effect of these ILs on the peroxidase catalytic behavior of cyt c, the activation energy (E_a) of cyt c for the oxidation of guaiacol in buffer, as well as in the presence of 30% (v/v) aqueous solutions of hydroxyl ammonium Ils, was determined over a temperature range of 20 to 60 ^oC. The activation energies in various media, calculated from the slope of the Arrhenius plots, are presented in Table 2. As it can be seen, the presence of ILs in the reaction mixture decreases the activation energy compared to that in buffer solution and this decrease is more pronounced when HMEAF and HEAF are used. This decrease in E_a value observed here may be correlated to the effect of ILs on the structure of the protein molecule and the formation of enzyme-substrate complex, as it was proposed for imidazolium-based ionic liquids.[56]

Stability of cyt c against H_2O_2 in ILs

It is well known that, heme-containing enzymes, such as peroxidases and cyt c, are inactivated by H_2O_2 in the absence of reducing substrate in aqueous media.[43,62] This inactivation may be correlated to the modification of heme resulting in the formation of a verdohemoprotein, an inactive form of heme, as a final product, as well as in the formation of radical species that could react and inactivate the heme center.[60-65]

In order to investigate the stability of cyt c against H_2O_2 in hydroxyl ammonium-based ILs, cyt c was incubated in the presence of 1 mM H_2O_2 for 15 min at 30 ^oC in buffer containing various ILs (30-60% v/v) and the remaining peroxidase activity was determined using guaiacol as a reducing substrate (Fig. 4).

As seen in Fig. 4, the peroxidase activity of cyt c in buffer was reduced by 40% after incubation with H_2O_2. The tolerance of cyt c, in the presence of all ILs used, strongly depends on the nature of ILs cations, while the effect of their concentration is not so obvious. Namely, in the presence of HMEAF and especially HDMEAF, the remaining activity of cyt c in most cases was higher compared to that observed in buffer, indicating that these ILs protect the protein from H_2O_2 deactivation. However, in the presence of HEAF or BHEAF, the remaining peroxidase activity of cyt c after incubation with H_2O_2 was significantly decreased compared to that observed in buffer, for all concentrations tested.

It was reported that the stability of cyt c in the presence of various hydrophilic ILs was strongly influenced by the kosmotropicity/chaotropicity of the ions of ILs.[50] However, in our study, the effect of ILs on stability of cyt c does not follow the Hofmeister series and therefore, the chaotropicity of their cations. It seems that kosmotropicity/chaotropicity is not the only key in determining the cyt c behavior in ILs.

It is interesting to note that, all the ILs used in the present work are formed by hydroxyl ammonium cations. As it has been proposed, these cations can mimic the molecular structure of water with H-bond accepting/donating functionalities forming hydrogen bonds with the polypeptide backbone of protein and thus affecting its structure and function.[59] It is worth noting that, these interactions should not be too strong in order to avoid the dissociation of the hydrogen bonds between the amino acids which could lead to the disruption of the protein structure.[66] Based on the basicity of nitrogen of the amine residues of the ILs studied, the H-bonding capability should increase by the following order: HDMEAF <HMEAF < HEAF <BHEAF.[67] As it can be seen in Fig. 3, cyt c is more stable in aqueous solutions of HDMEAF and HMEAF ILs, which could be possibly explained by the

decreased H-bond ability and thus reduced interaction of these ILs with the protein molecule.

Moreover, the increased stability of cyt c against H_2O_2 in HDMEAF could be attributed to the less hydrophilic environment surrounding the protein created by this IL. The less hydrophilic environment created by HDMEAF could limit the diffusion of hydrophilic H_2O_2 towards the protein microenvironment, thus reducing its denaturing effect. The possible limited diffusion of H_2O_2 to the microenvironment and thus to the active center of cyt c could probably also explain the decreased activity of cyt c observed in HDMEAF-based reaction medium (Fig. 3).

Structural characterization of cyt c using spectroscopic techniques.

The effect of the hydroxyl ammonium ILs on the conformation of cyt c was investigated through ATR-FTIR, UV-Vis and circular dichroism (CD) spectroscopy. The conformational changes of cyt c in the presence of hydroxyl ammonium ILs-based media compared to its structure in buffer (50 mM sodium phosphate, pH 7.0) were investigated by ATR-FTIR spectroscopy. The analysis of the Amide I band at approximately 1600–1700 cm^{-1} (mainly due to the C=O stretching vibration) makes it possible to obtain information on the effect of ILs on the secondary structure of the protein.[68-71]

Correlation coefficients (r) between the Amide I spectra of cyt c dissolved in buffer and 30% (v/v) aqueous solutions of ILs were evaluated according to previous studies.[71] In particular, the correlation coefficient was calculated using the formula

$$r = \Sigma x_i y_i / \sqrt{\Sigma x_i^2 y_i^2},$$ [54]

where x and y are the absorbance values of the cyt c spectrum dissolved in buffer and 30% (v/v) of ILs respectively, at the ith frequency position for the range 1600-1700 cm^{-1} (Amide I). For identical spectra, a value of 1.0 will be returned.[71] Table 3 shows the correlation coefficients and the differences on α-helix content of cyt c in the presence of various ILs-based media compared to buffer, as a result of ATR-FTIR analysis. As it can be seen in Table 3, the relative structure of cyt c in the presence of all ILs used is close to that in buffer. Similar retention of the secondary structure of cyt c in various media composed by other hydrophilic ILs has also been previously reported.[50,51]

Since the most abundant element (about 40%) of the secondary structure of cyt c is α-helix,[72] we also determined the effect of ILs on α-helix content (Table 3). The α-helix content was identified from the second-derivative ATR-FTIR spectra of the protein in various media, taking into consideration that the bands at 1650–1660 cm^{-1} were assigned to α-helix.[73,74] As it can be seen, a slight increase in α-helix content was observed in aqueous solutions of HMEAF, HDMEAF and HEAF that can be correlated to a more rigid structure of the protein.[75] On the other hand, the decrease in α-helix content observed in BHEAF-based media could be attributed to a less rigid structure of cyt c that could be correlated to the substantially low stability of the protein observed in this IL (Fig. 4).

Conformational changes of the heme prosthetic group of cyt c in aqueous solutions of hydroxyl ammonium ILs-based media were further investigated using UV-Vis spectroscopy. As it can be seen in Fig. 5 and Fig. 6, the oxidized (Fe(III)) form of cyt c in buffer has a characteristic UV-vis spectrum consisting of a sharp Soret band at 409 nm, a weaker, broad Q-band at 530nm and a very weak charge transfer band from the sulfur atom of Met80 (the axial ligand) with heme Fe(III) observed at 695 nm, which is in accordance to that reported by other researchers.[76-77]

The incubation of cyt c in 60% (v/v) aqueous solutions of HDMEAF, BEHAF and HEAF did not affect the UV-Vis spectrum of the protein (Fig.5) suggesting that the polypeptide environment around the heme has been kept intact.[78] However, the UV-vis spectrum of cyt c in 60% (v/v) aqueous solution of HMEAF was significantly changed (Fig. 5). More specifically, the Soret band of the protein was moved at 413 nm, while at Q-band region a sharp α-band at 550 nm and a sharp β-band at 520 nm appeared, indicating cyt c in reduced state.[79]

Moreover, in HMEAF-based media, the charge transfer band at 695 nm was significantly reduced compared to that observed for the native protein (Fig. 6). This spectral change indicates the partial perturbation or cleavage of the coordination bond of Met80 with the heme iron.[80] The loss of the heme's axial sulfur-coordinated ligand has been correlated with the progressive breaking of hydrogen bonds in the protein interior and gradual exposure of amino acid residues and the

porphyrin ring and hence loss of iron and the catalytic activity of the protein.[78,81]

The structural changes of cyt c observed in HMEAF-based media were further investigated using CD spectroscopy. Unfortunately, due to high absorbance of the IL in far and near UV region, only the Soret region of the CD spectrum (350-450 nm) of cyt c could be investigated, which can provide further insight on structural changes of the heme crevice.[38,82,83] Fig. 7 shows the CD spectra of cyt c in 0.5 mM sodium phosphate buffer pH 7.0 and in the presence of HMEAF (60% v/v). The spectrum of cyt c, in its native conformation in buffer, exhibits a negative peak at 416 nm and a positive peak at 402 nm, due to a split Cotton effect.[83] As it can be seen in Fig. 7, the addition of HMEAF increases the positive band at 402 nm and significantly vanishes the negative band at 416 nm. Similar spectral changes have been previously reported due to interactions of cyt c with other ILs and organic solvents,[33,51,52,84,85] as well as with denaturants, such as guanidine, HCl or urea.[86]

The increase of the positive peak at 416 nm and the disappearance of the negative peak at 402 nm observed here could be attributed to the disordered orientation and disturbance of the distance between the heme on the Met80 side and the aromatic residues Try-59 and Phe-82 in the polypeptide backbone of the heme crevice.[87] Similar results have also been observed when cyt c was dissolved in hydrated pyridinium-based and neat imidazolium based ILs.[51,85] The findings from circular dichroism studies correlate well with the UV-vis data described before, as well as with the reduced peroxidase activity of cyt c in the presence of HMEAF compared to the other ILs used in the present work (Fig. 3, 5, 6).

Decolorization of pinacyanol chloride by cyt c in ILs-based media

The accumulation of industrial dyes in wastewater has a profound environmental and health impact and, therefore, their removal is a substantial challenge for the scientific and industrial community.[88] Several oxidative biocatalysts have been used for the enzymatic elimination of dyes including cyt c, laccases, peroxidases, etc.[44,89-92] In order to investigate the effect of the hydroxyl-ammonium ILs on the decolorization activity of cyt c, we used pinacyanol chloride as a model substrate, which is a symmetric trimethinecyanine dye with industrial use.[93] In this case, the initial concentration of H_2O_2 was kept low (0.3mM) in order to reduce its denaturing effect. As it can be seen in Fig. 8 and Table 4, the decolorization activity of cyt c, in the presence of all ILs studied, strongly depends on the nature of the cation used, as well as the concentration of the ILs in the reaction media. In the case of HMEAF, the decolorization efficiency decreased, while the use of BHEAF and HEAF enhanced the ability of cyt c to decolorize the dye compared to that observed in buffer. More specifically, the decolorization rate in the presence of HEAF and BHEAF in the reaction mixture was up to 4-fold and 5-fold higher, respectively, compared to that in buffer. It is worth noting that, the decolorization yield was about 90% after 20 min of incubation in media containing various concentrations of HEAF and BHEAF (15-60% v/v), while in buffer the decolorization yield was less than 40%. The positive effect of these two ILs increases with the increase of their concentration in the reaction mixture. The high decolorization activity of cyt c in reaction media containing BHEAF or HEAF could be attributed to the enhanced peroxidation activity of cyt c observed in these media (Fig.3). In order to further demonstrate the green properties of these novel ILs, their recyclability and reusability were also investigated, using the catalyzed by cyt c decolorization of pinacyanol chloride as a model reaction (see Experimental section). All ILs were reused in the same decolorization reaction up to three times with comparable decolorization yields to those observed for the initial reaction. The beneficial effect of hydroxyl ammonium-based ILs on the decolorization activity of cyt c together with their reusability indicate that these ILs could be considered as promising environmentally friendly media for biocatalytic decolorization of dyes.

Biodegradability assessment of ILs

In order to determine the biodegradability level of the synthesized ILs, the biological oxygen demand (BOD) for the biochemical degradation of each IL after five days was determined. During the BOD test, the carbonaceous demand (which refers to the conversion of organic carbon to carbon dioxide) was taken into account and the results are reported as carbonaceous BOD (CBOD).[18.] The results of the biodegradation of the ILs are presented in Table 5.

The experimental results indicated that all ILs studied present remarkable biodegradability potential, since a percentage of more than 50% of the organic carbon was biodegraded within five days. The relatively high biodegradability of these ILs could be attributed to the presence of hydroxyl groups on the ILs cations. It has been reported that the biodegradability level of similar ethanolamine-based ILs depends mainly on the cation of the IL molecule and on the groups that provide possible sites for enzymatic hydrolysis, especially oxygen atoms (e.g. in the form of hydroxyls) that present high degradation potential.[14,94-96]

Conclusions

Herein, neoteric, low cost and biodegradable ILs, based on the combination of a hydroxyl ammonium cation and formic acid, have been prepared and used as media for biocatalytic oxidation catalyzed by metalloproteins. Kinetic and structural studies of cyt c indicate that the presence of these ILs in the reaction mixture has a considerable beneficial effect on the catalytic efficiency and tolerance against hydrogen peroxide, while the protein structure is slightly affected. The effect of these ILs on the catalytic behaviour of cyt c strongly depends on the structure of the hydroxyl ammonium cations used for their formation. Moreover, the beneficial effect of hydroxyl ammonium-based ILs on the biocatalyzed degradation of an industrial dye, together with their efficient recyclability and reusability, indicate the potential application of these novel ILs as green media for biotransformations of industrial interest. The use of immobilized enzymes[97,98] or the use of ILs as a support for enzyme immobilization[99,100] is expected to facilitate the recovery and reuse of both the biocatalyst and the ILs, enhancing therefore the green character of such biocatalytic processes. Further investigation on the effect of the nature and physicochemical properties of cations and anions used for the formation of such environmentally friendly ILs on the catalytic behaviour of various industrial enzymes is in progress in our lab.

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Figure Captions

Fig. 1. Structure of ILs used in the present study

Fig. 2. Relative peroxidase activity of cyt c for the oxidation of guaiacol in the presence of various amounts of hydroxyl ammonium-based ILs. As 1.0 is indicated the peroxidase activity of cyt c in 50 mM phosphate buffer pH 7.0. Initial reaction rate of the activity of cyt c in buffer is 10 μM/min.

Fig. 3. Effect of the concentrations of hydroxyl ammonium-based ILs on the catalytic efficiency of cytochrome c for the oxidation of guaiacol with H_2O_2. The black line represents the ratio of V_{max}^{app}/K_m^{app} of cyt c in buffer aqueous solution.

Fig. 4. Stability of cyt c in buffer and 30, 45 and 60 % v/v aqueous solutions of hydroxyl ammonium-based ILs, after incubation for 15 min with 1 mM H_2O_2 at 30 ^oC. As 100% is indicated the activity at t = 0 min.

Fig. 5. UV-visible spectra (300-600 nm) of cyt c in 50 mM phosphate buffer, pH 7.0 and in the presence of 60% (v/v) ILs. The insets show the absorption spectra of the media (60% v/v ILs).

Fig. 6. Absorption spectra of cyt c at the charge transfer band (695 nm) in phosphate buffer 50 mM, pH 7.0 and in the presence of 60% (v/v) ILs.

Fig. 7. Soret region CD spectrum of cyt c in 50 mM phosphate buffer, pH 7.0 and in the presence of 60% (v/v) aqueous solution of HMEAF.

Fig. 8. Cyt c-catalyzed decolorization of pinacyanol chloride with H_2O_2 in phosphate buffer 50 mM, pH 7.0 and in the presence of various amounts (15-75 % v/v) of ILs a) HMEAF, b) HDMEAF, c) BHEAF and d) HEAF.

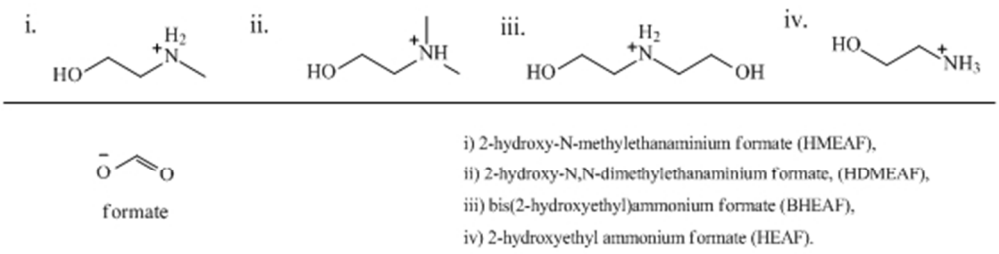


Fig. 1. Structure of ILs used in the present study

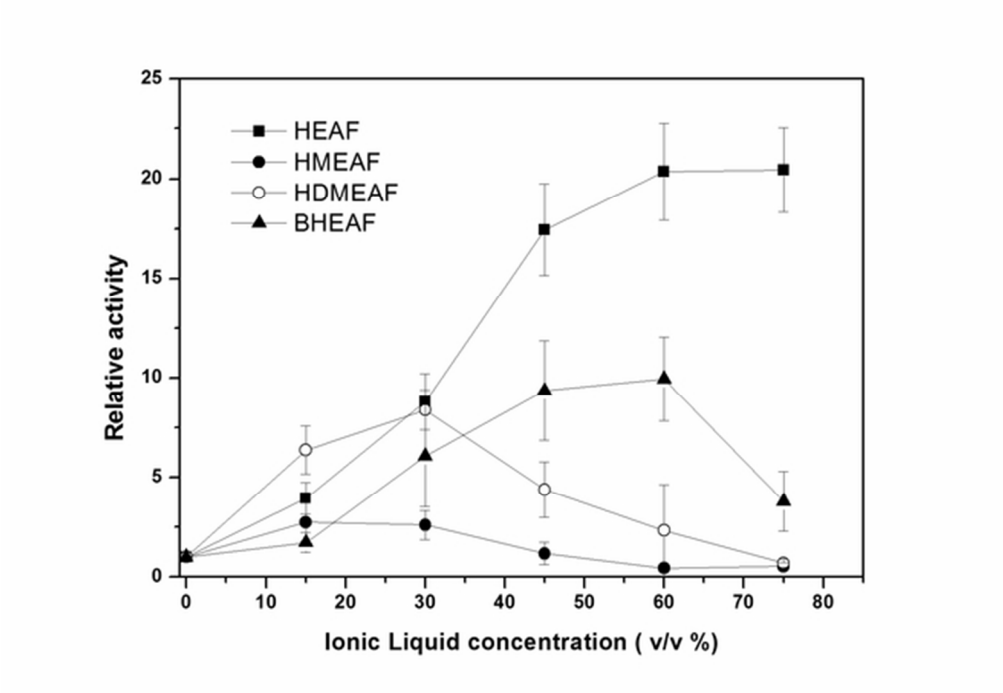
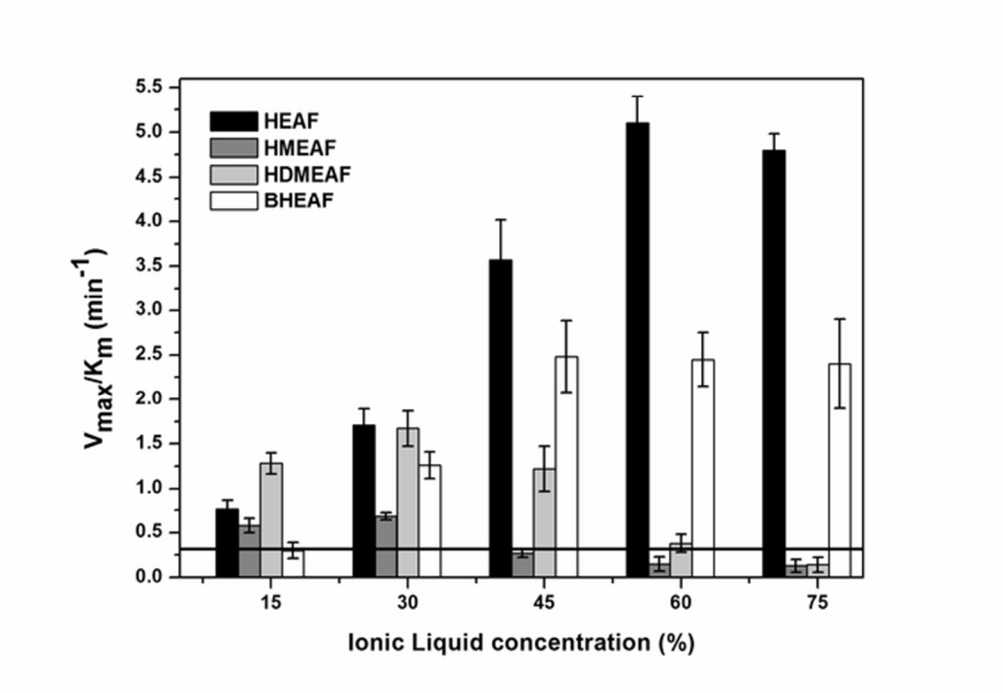
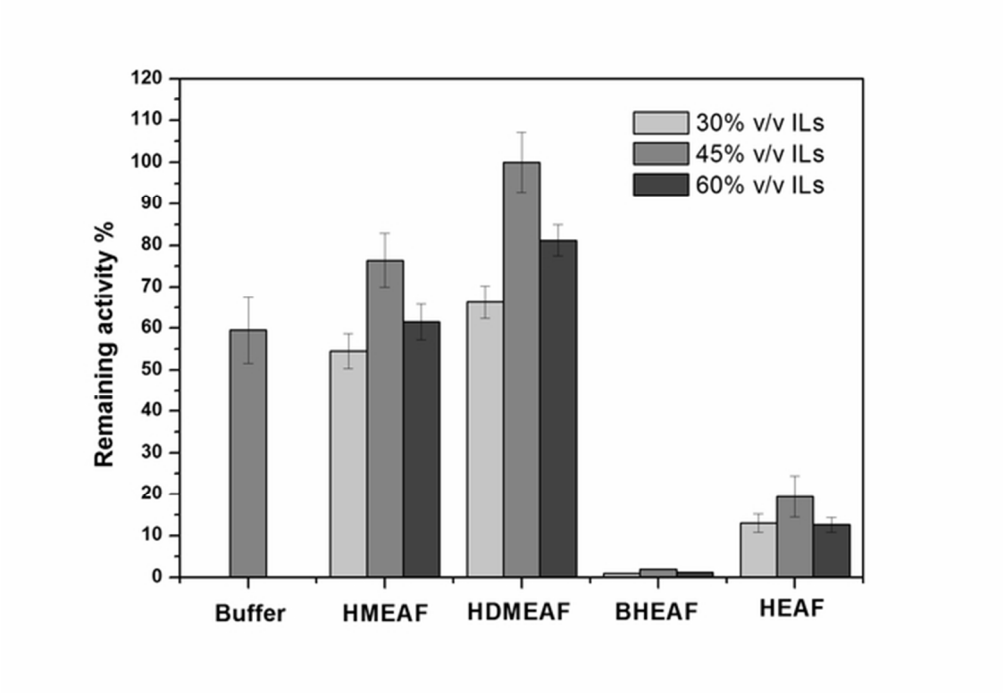


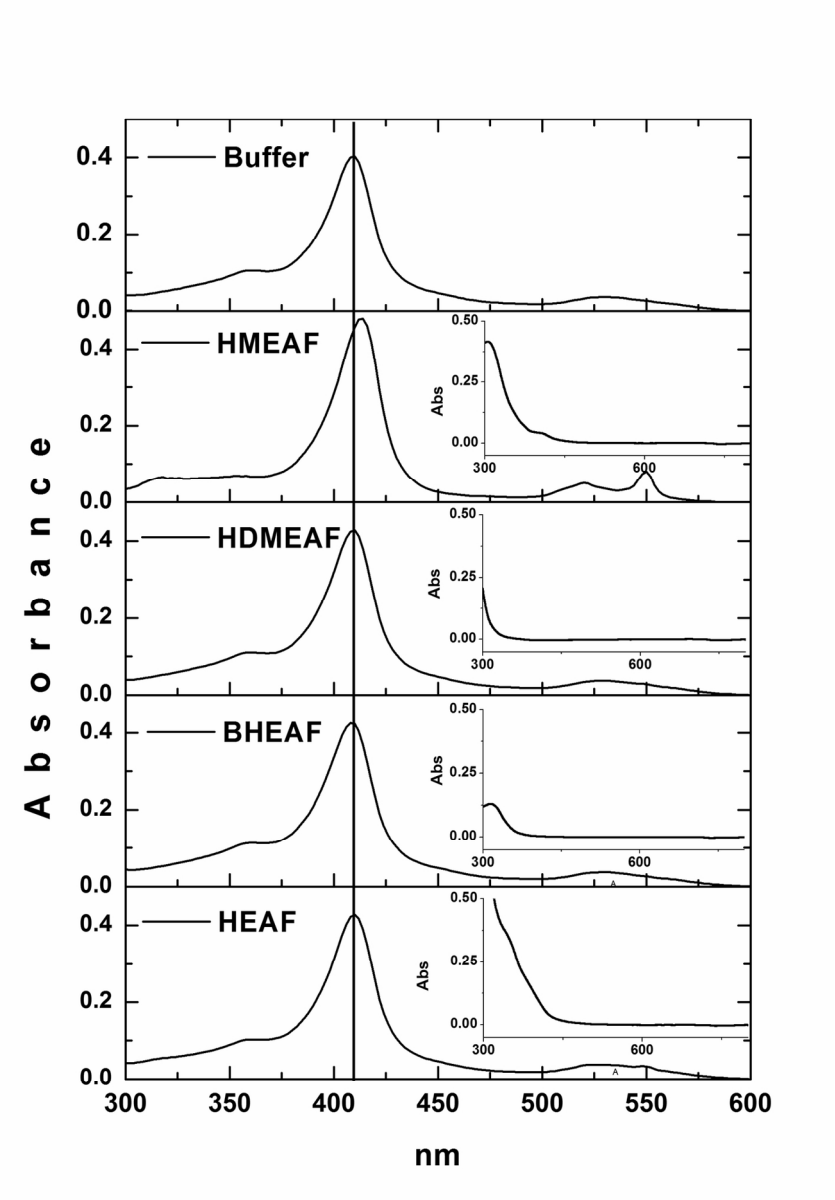
Fig. 2. Relative peroxidase activity of cyt c for the oxidation of guaiacol in the presence of various amounts of hydroxyl ammonium-based ILs. As 1.0 is indicated the peroxidase activity of cyt c in 50 mM phosphate buffer pH 7.0. Initial reaction rate of the activity of cyt c in buffer is 10 μM/min
55x38mm (300 x 300 DPI)



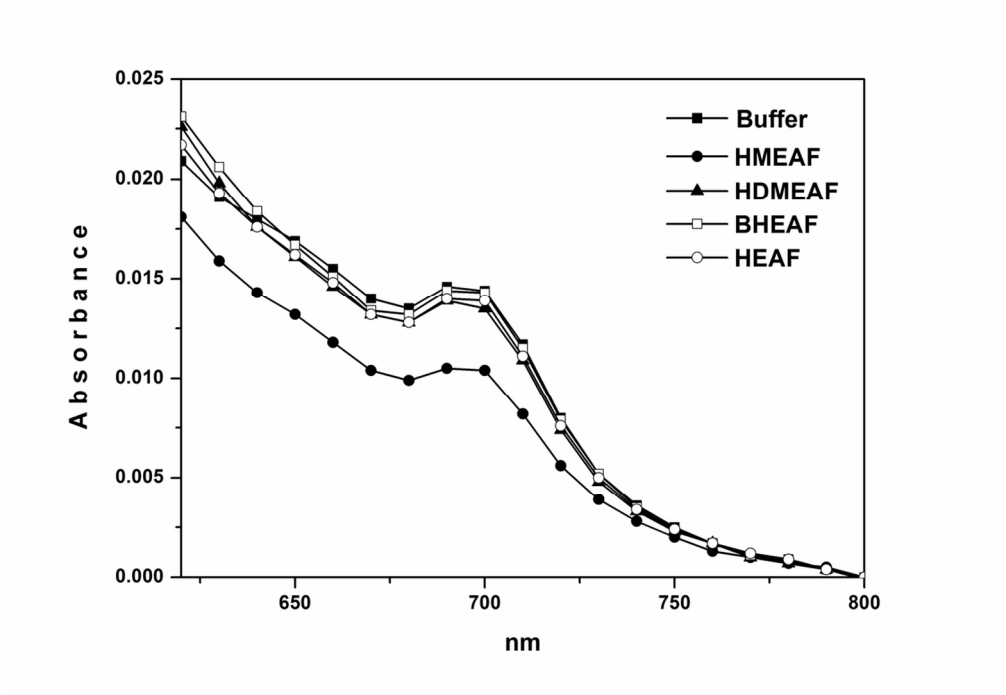
Effect of the concentrations of hydroxyl ammonium-based ILs on the catalytic efficiency of cytochrome c for the oxidation of guaiacol with H_2O_2. The black line represents the ratio of $Vmax^{app}/Km^{app}$ of cyt c in buffer aqueous solution
57x39mm (300 x 300 DPI)



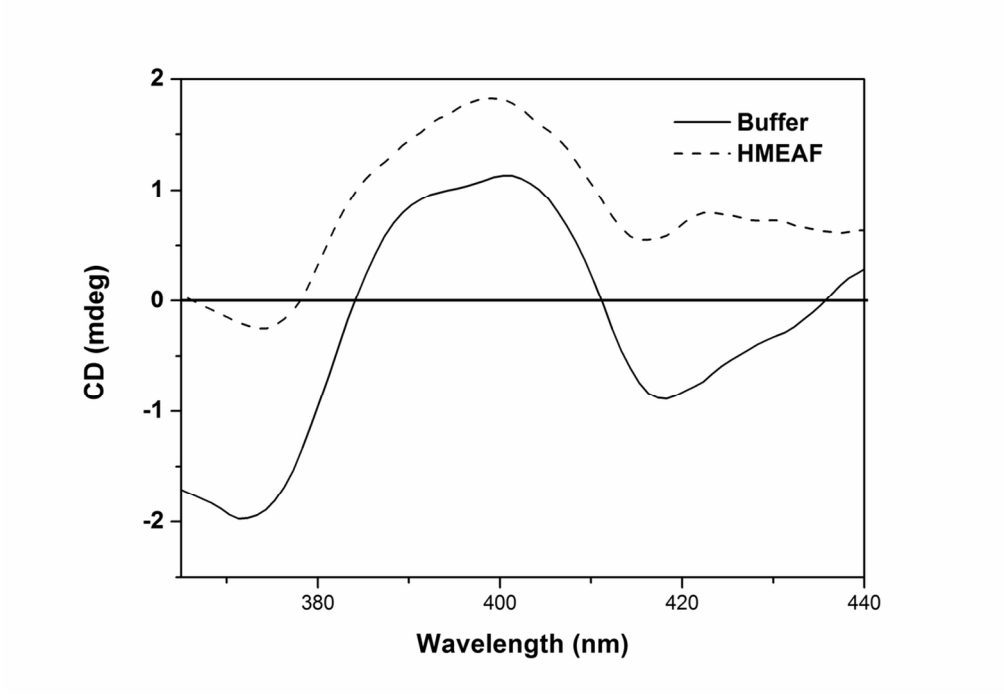
Stability of cyt c in buffer and 30, 45 and 60 % v/v aqueous solutions of hydroxyl ammonium-based ILs, after incubation for 15 min with 1 mM H_2O_2 at 30 ^{o}C. As 100% is indicated the activity at t = 0 min.
57x39mm (300 x 300 DPI)



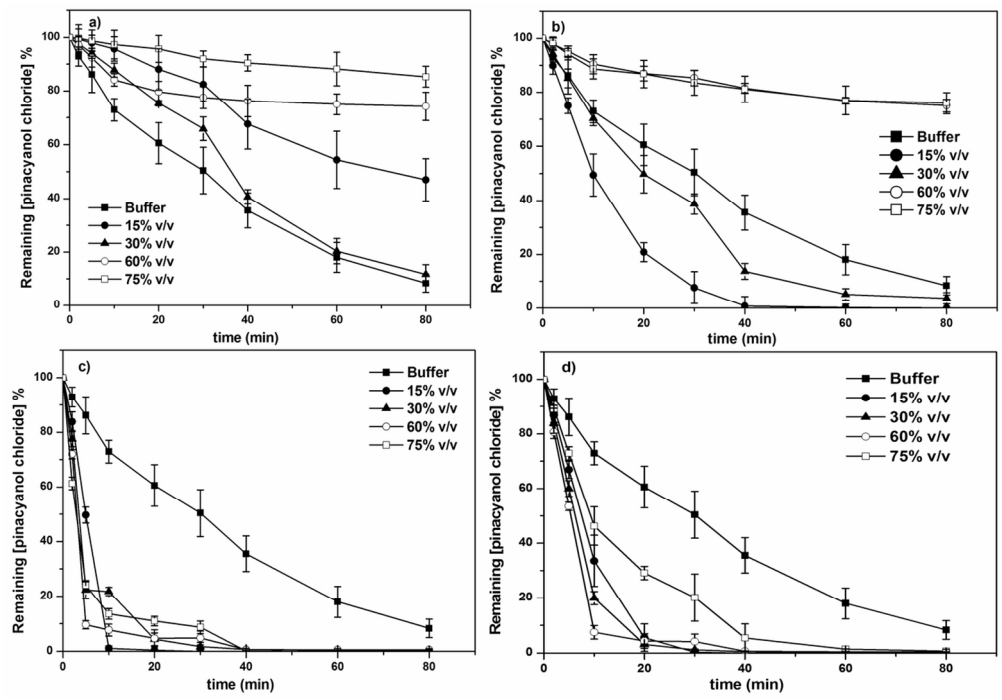
UV-visible spectra (300-600 nm) of cyt c in 50 mM phosphate buffer, pH 7.0 and in the presence of 60% (v/v) ILs. The insets show the absorption spectra of the media (60% v/v ILs).
119x172mm (300 x 300 DPI)



Absorption spectra of cyt c at the charge transfer band (695 nm) in phosphate buffer 50 mM, pH 7.0 and in the presence of 60% (v/v) ILs.
57x39mm (600 x 600 DPI)



Soret region CD spectrum of cyt c in 50 mM phosphate buffer, pH 7.0 and in the presence of 60% (v/v) aqueous solution of HMEAF
57x39mm (600 x 600 DPI)



Cyt c-catalyzed decolorization of pinacyanol chloride with H_2O_2 in phosphate buffer 50 mM, pH 7.0 and in the presence of various amounts (15-75 % v/v) of ILs a) HMEAF, b) HDMEAF, c) BHEAF and d) HEAF
118x82mm (300 x 300 DPI)

Table 2. Activation energy E_a for the oxidation of guaiacol catalyzed by cyt c in various ILs (30% v/v).

Activation Energy E_a (kcal/mol)				
Buffer	**BHEAF**	**HDMEAF**	**HMEAF**	**HEAF**
1.28	1.17	1.19	0.67	0.58

Table 3. Correlation coefficient (r) between the ATR-FTIR spectra of cyt c dissolved in 50 mM sodium phosphate buffer, pH 7.0 and in 30% (v/v) aqueous solutions of ILs. Δα-helix estimation (%) is the difference between the percentages of α-helix content of cyt c in 30% (v/v) ILs compared to that in buffer calculated by ATR-FTIR analysis in Amide I region.

Ionic Liquid	r	Δα-helix (%)
HMEAF	0.97	+0.80
HDMEAF	0.93	+2.50
BHEAF	0.98	-0.68
HEAF	0.94	+1.72

Table 4. Reaction rates of the cyt c-catalyzed decolorization of pinacyanol chloride with H_2O_2 in phosphate buffer 50 mM, pH 7.0 and in the presence of various amounts of hydroxyl-ammonium ILs.

% v/v ILs	Decolorization rate (μM min^{-1})			
	HMEAF	HDMEAF	BHEAF	HEAF
0	3.9	3.9	3.9	3.9
15	0.9	7.8	11.3	9.9
30	1.8	4.0	14.0	12.5
60	2.5	1.5	17.4	14.5
75	0.4	1.3	18.6	8.0

Table 5. (%) Biodegradability assessment of the hydroxyl ammonium ILs.

Ionic Liquid	(%) Biodegradation *
2-HEAF	58.9
HMEAF	55.0
HDMEAF	57.4
BHEAF	52.2

* The percentage biodegradation is calculated by dividing the specific carbonaceous BOD (CBOD) by the ultimate carbonaceous BOD (UCBOD). The **CBOD** value expresses the oxygen demand by microorganisms for degradation of the ILs within five days (only carbonaceous stage). CBOD is the BOD value (mg O_2/L) reading affected due to the drop of pressure in the water sample bottle, minus the BOD value reading affected due to the drop of pressure in the dilution water bottle (blank). The **UCBOD** value (mg O_2/L), expresses the oxygen demand by microorganisms for the ultimate degradation of the organic compound referring only to the conversion of organic carbon to carbon dioxide, water, and new microbial cellular constituents.

Hydroxyl ammonium ionic liquids are a biodegradable, non-toxic family of third generation ionic liquids with a beneficial effect on the catalytic efficiency of metalloproteins such as cytochrome c.

Electronic Supplementary Information

Hydroxyl ammonium ionic liquids as media for biocatalytic oxidations

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Biodegradability test

Biodegradation is the natural process for the removal of organic substances from the environment. The determination of the biodegradability level of organic substances such as ILs is essential in order to estimate their environmental impact. Biodegradability assessment of ILs have been examined by measuring the Biochemical Oxygen Demand (BOD) [1],[2].

In this work, biodegradation tests were carried out according to a manometric method so as to determine the oxygen demand for the biochemical degradation of each organic substance after five days. VELP BOD manometric apparatus was used to measure the BOD of the IL inoculated samples. This method is based on the steady decrease of the pressure in a closed system as a result of oxygen consumption. The carbon dioxide which is produced is bounded by a strongly alkaline medium (KOH pellets above the solution) so as not to interfere with the final measurements. The nutrients prepared are:

- Ferric chloride hexahydrate: 0.25 g $FeCl_3 \cdot 6H_2O$ to a final volume of 1 L with distilled water.
- Calcium chloride anhydrous: 27.5 g $CaCl_2$ to a final volume of 1 L with distilled water.
- Magnesium sulfate heptahydrate: 22.5 g $MgSO_4 \cdot 7H_2O$ to a final volume of 1 L with distilled water.
- Phosphate salts solution (buffer): 8.5 g KH_2PO_4, 21.7 g K_2HPO4, 33.4 g $Na_2HPO_4 \cdot 7H_2O$ and 1.7 g NH_4Cl to a final volume of 1 L with distilled water.

This method consists of filling each BOD flask with specific amount of IL, 135 mL aqueous solution of nutrients and 15 mL microorganisms. The seed source of microorganisms was mixed liquor which was taken from a secondary sedimentation tank of urban waste water of Psyttaleia sewage treatment plant in Greece. A blank solution was also prepared, containing only nutrients and mixed liquor.

In general, two stages of degradation take place during the BOD test, carbonaceous and nitrogenous but in this work only the carbonaceous demand taken into account and the BOD values will be reported as CBOD (degradation of the organic carbon). Inhibition of nitrogenous bacteria was achieved by a thiourea solution (2 g thiourea to a final volume of 1 L with distilled water) which was also added to BOD samples (0.5 mL of the solution in each flask). The samples were kept at 20 ± 1°C in darkness in tightly closed bottles for an incubation period of 5 days.

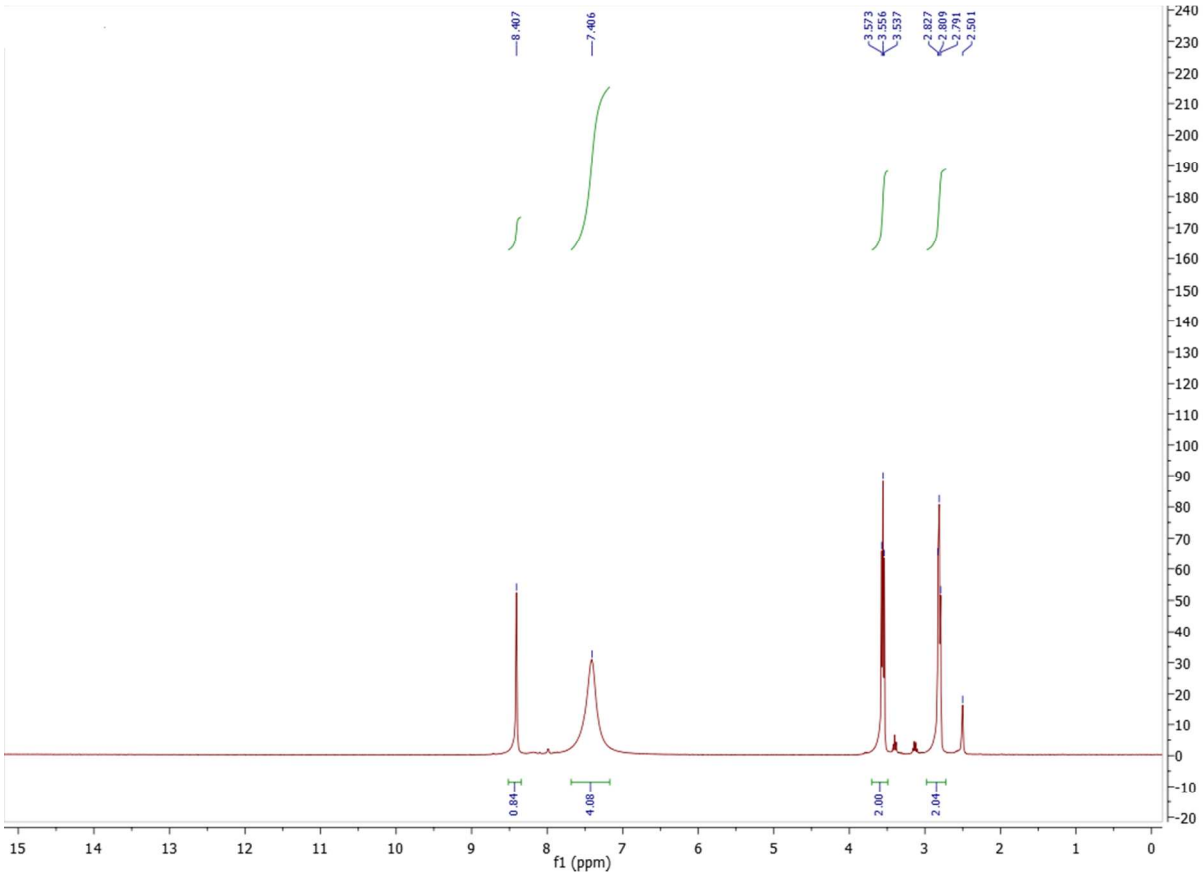


Fig. S1. ^{1}H NMR spectrum of *2-hydroxylethylammonium formate* (HEAF).

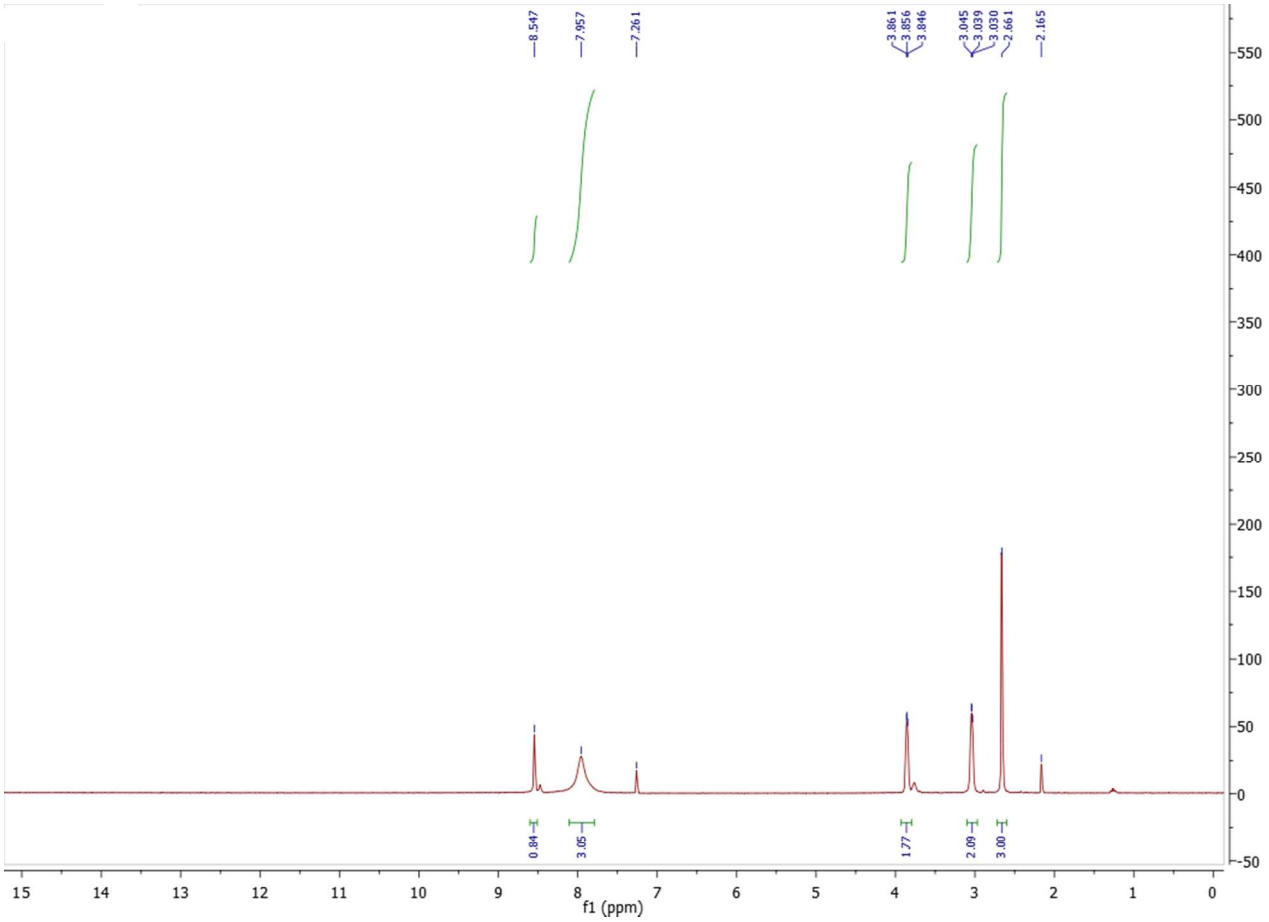


Fig. S2. ^{1}H NMR spectrum of *2-hydroxy-N-methylethanaminium formate* (HMEAF).

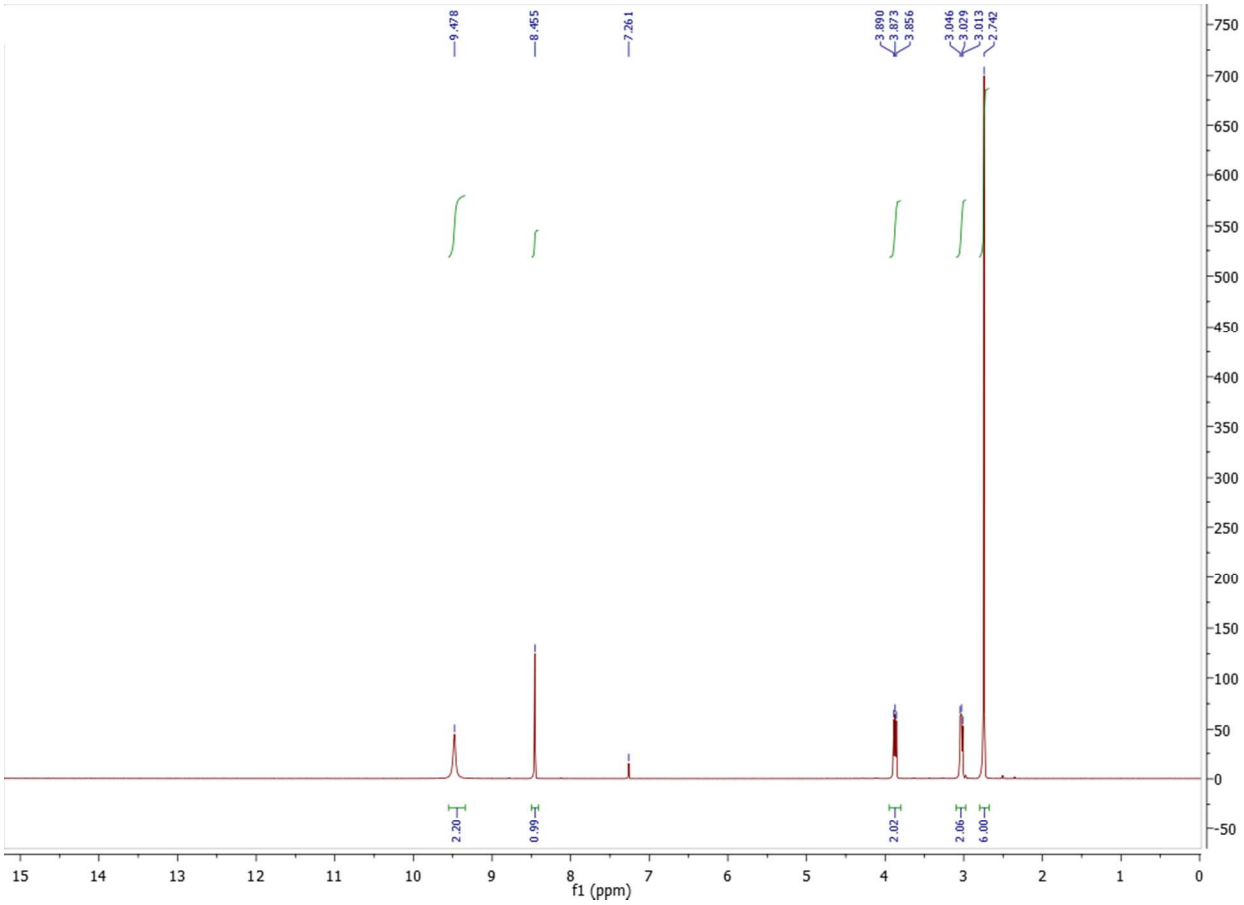


Fig. S3. ^{1}H NMR spectrum of *2-hydroxy-N,N-dimethylethanaminium formate* (HDMEAF).

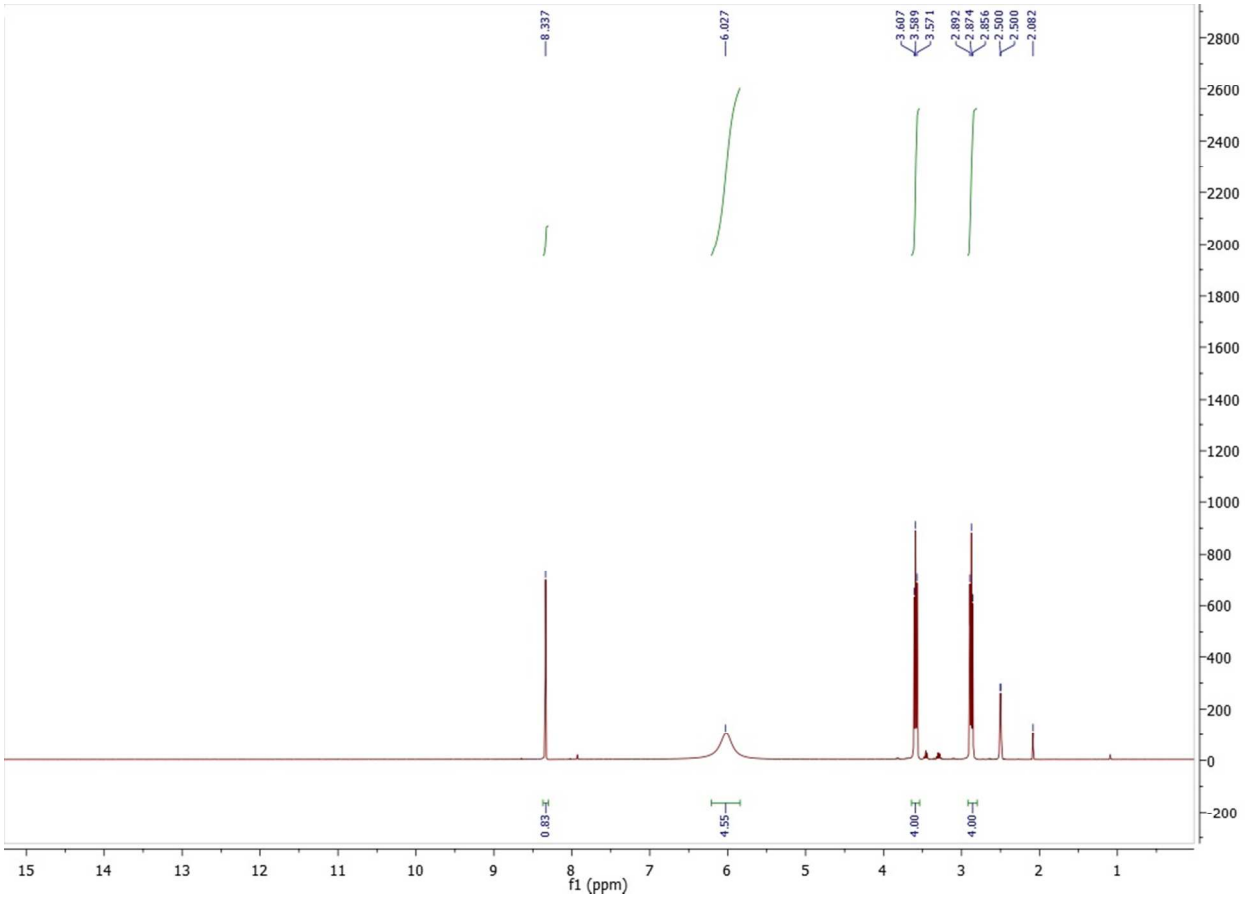


Fig. S4. ^{1}H NMR spectrum of *bis(2-hydroxyethyl)ammonium formate* (BHEAF).

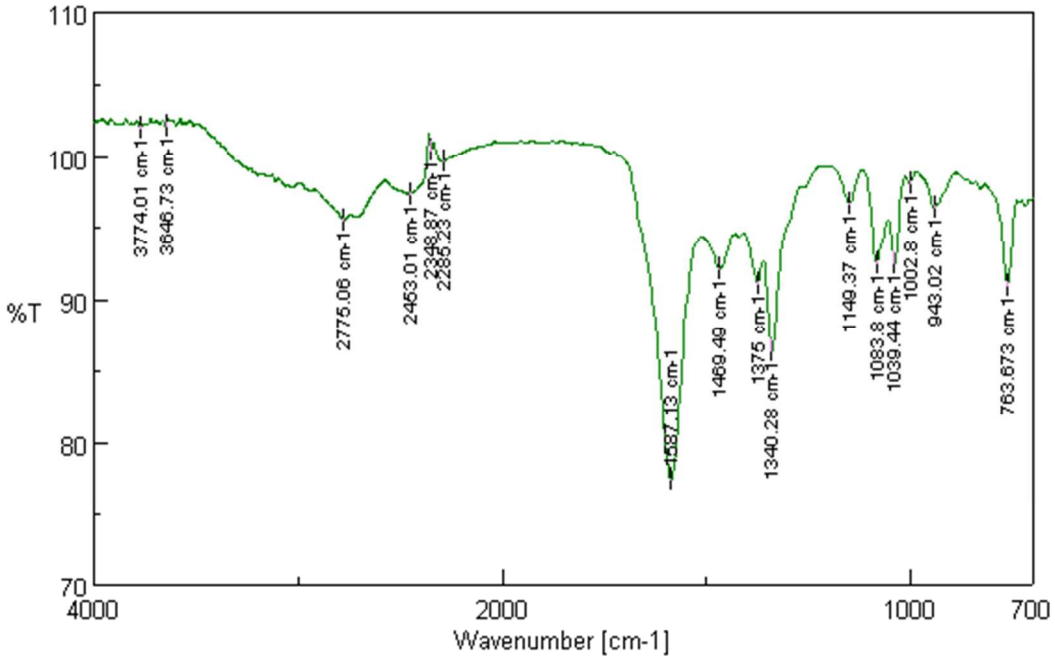


Fig. S5. ATR spectrum of 2-*hydroxylethylammonium formate* (HEAF).

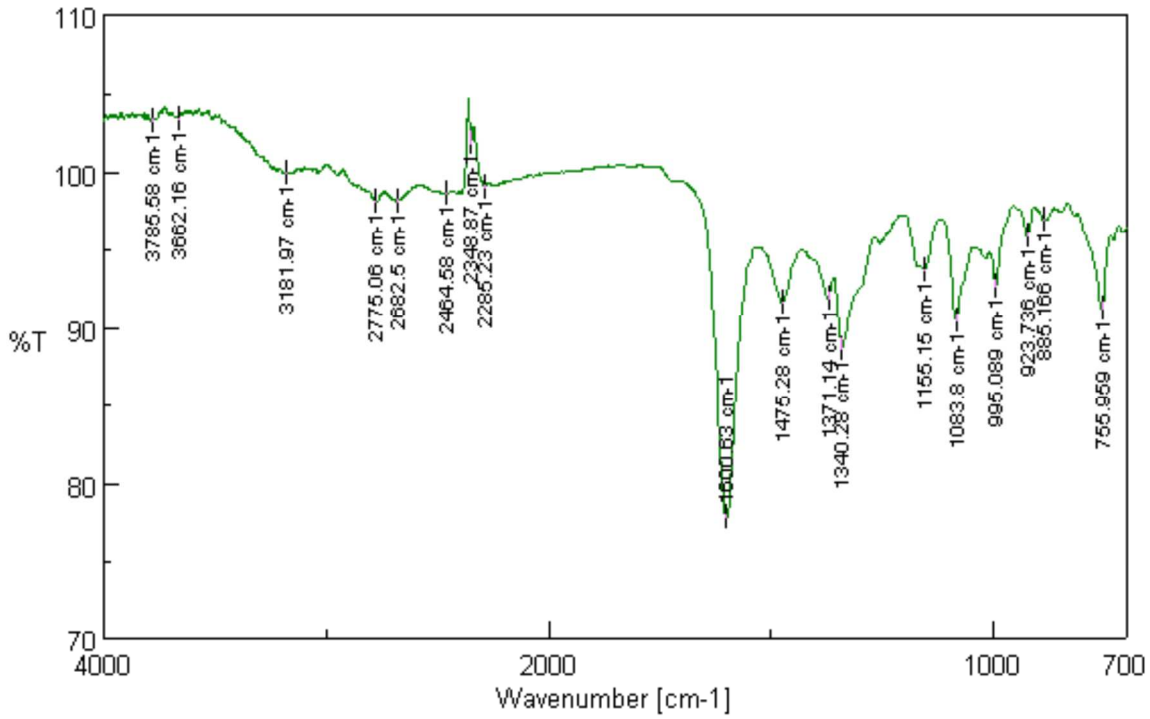


Fig. S6. ATR spectrum of *2-hydroxy-N,N-dimethylethanaminium formate* (HDMEAF).

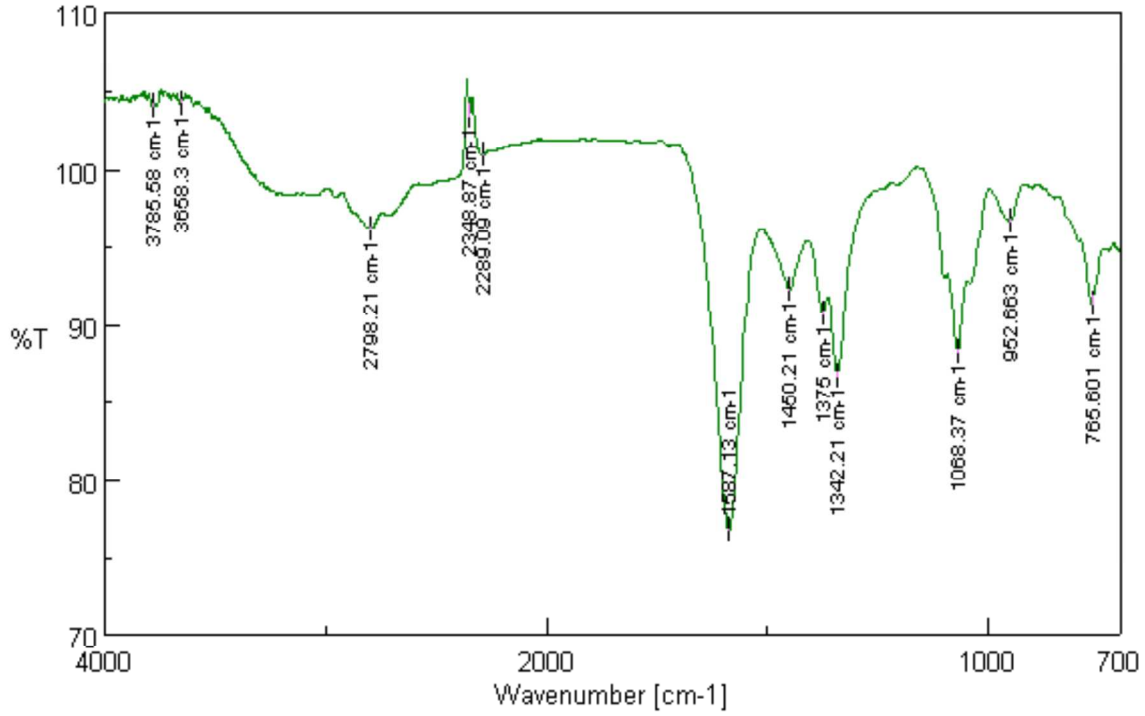


Fig. S7. ATR spectrum of *bis(2-hydroxyethyl)ammonium formate (BHEAF)*

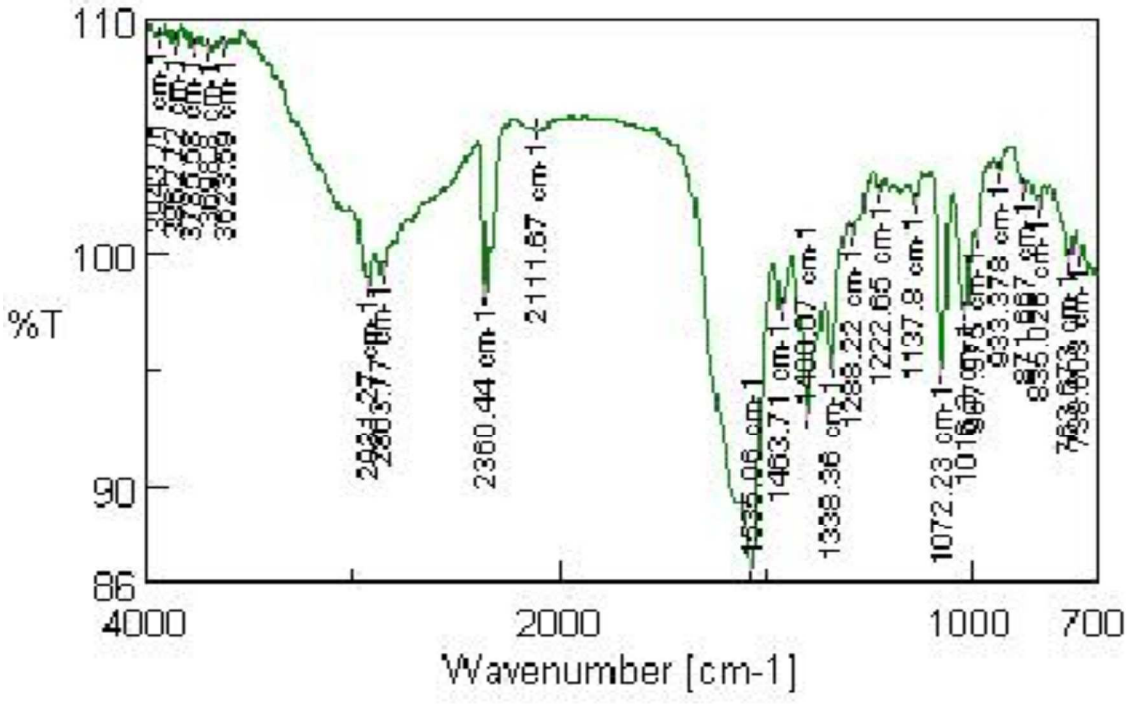


Fig. S8. ATR spectrum of 2-hydroxylethylammonium formate (HEAF):

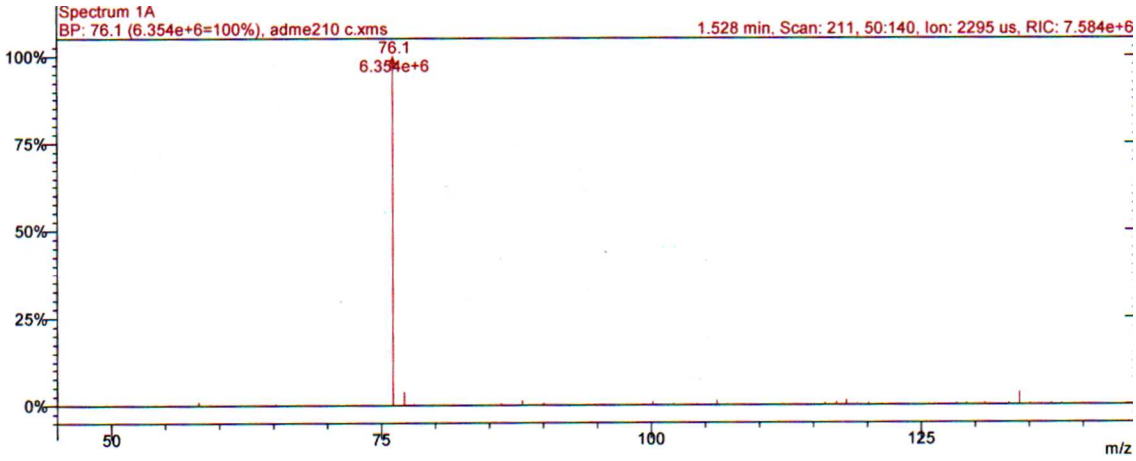


Fig. S9. MS spectrum of *2-hydroxy-N-methylethanaminium formate* (HMEAF).

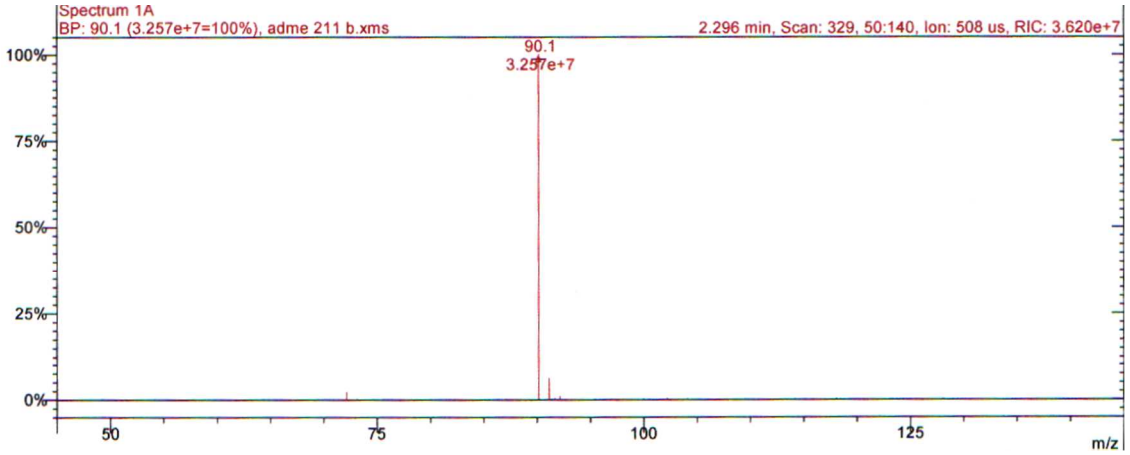


Fig. S10. MS spectrum of *2-hydroxy-N,N-dimethylethanaminium formate* (HDMEAF).

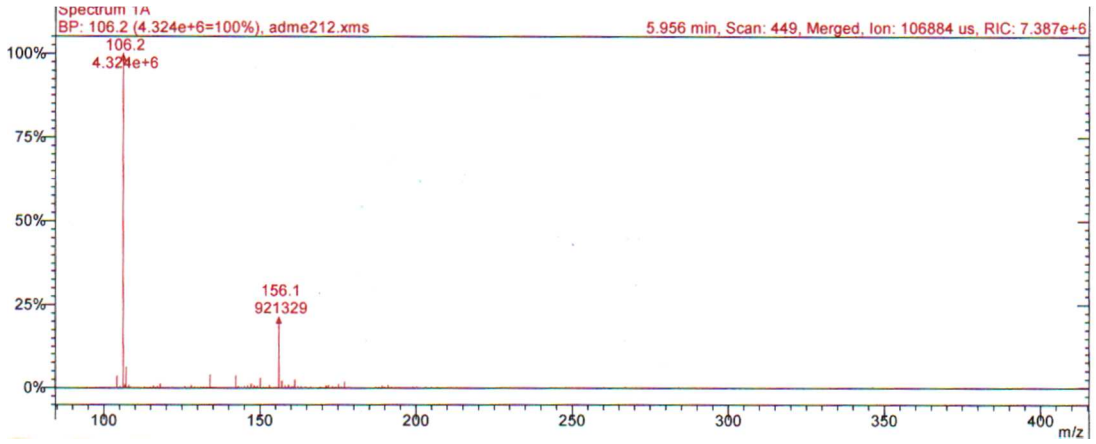


Fig. S11. MS spectrum of *bis(2-hydroxyethyl)ammonium formate* (BHEAF).

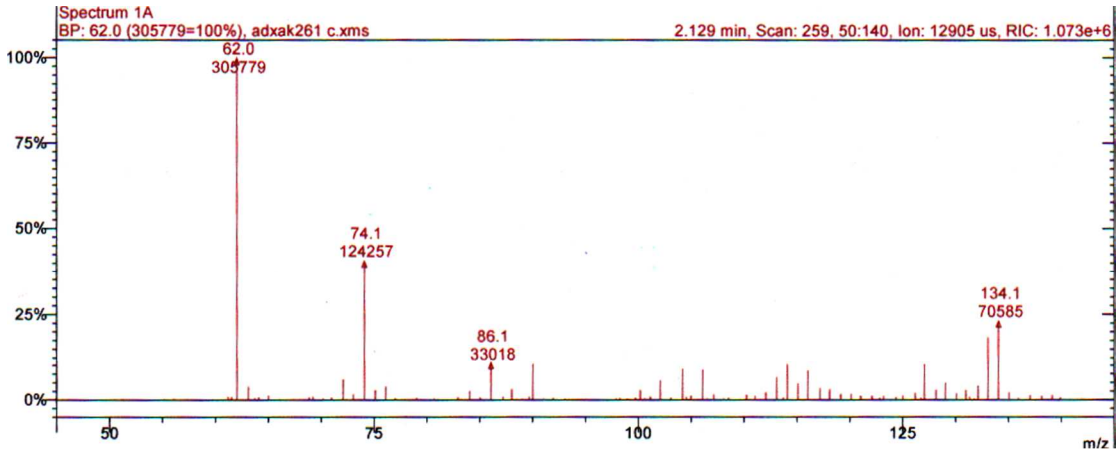


Fig. S12. MS spectrum of *2-hydroxylethylammonium formate* (HEAF).

The UV–Vis spectroscopic measurements were performed on a double-beam UV-vis spectrophotometer (UV-1601 Shimadzu, Tokyo, Japan) in a standard 1 cm path length quartz cuvette.

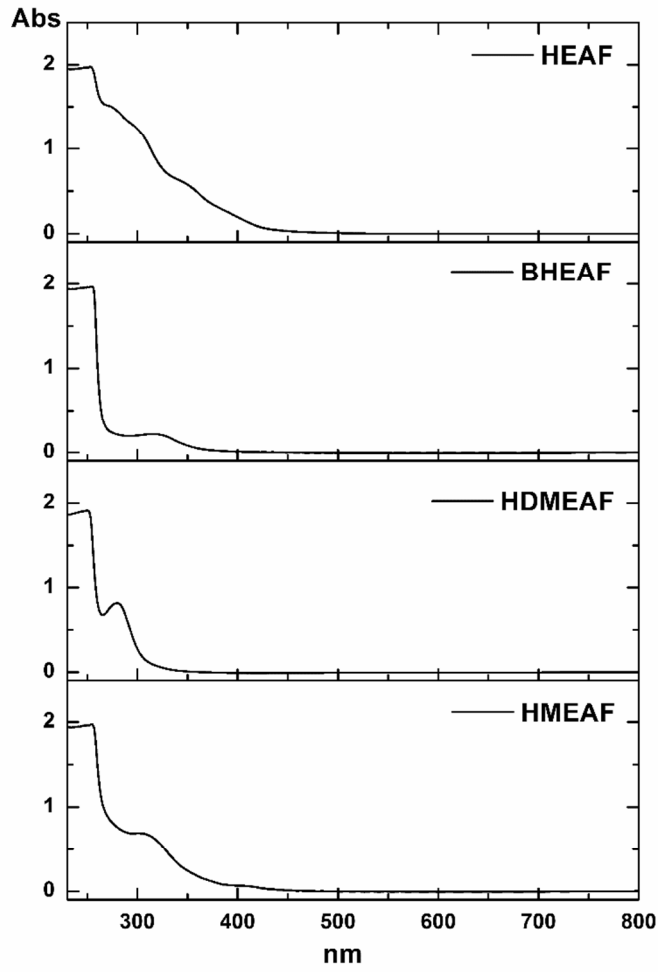
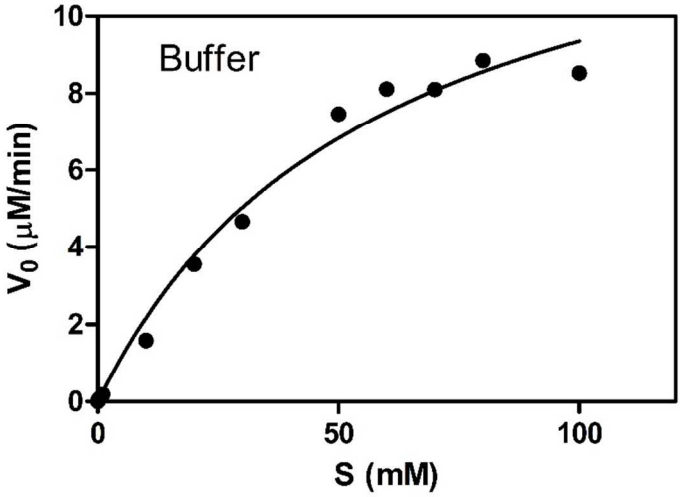
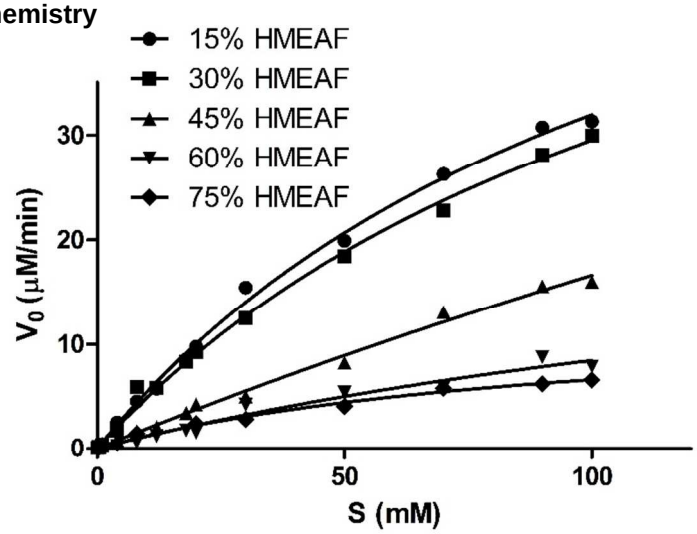
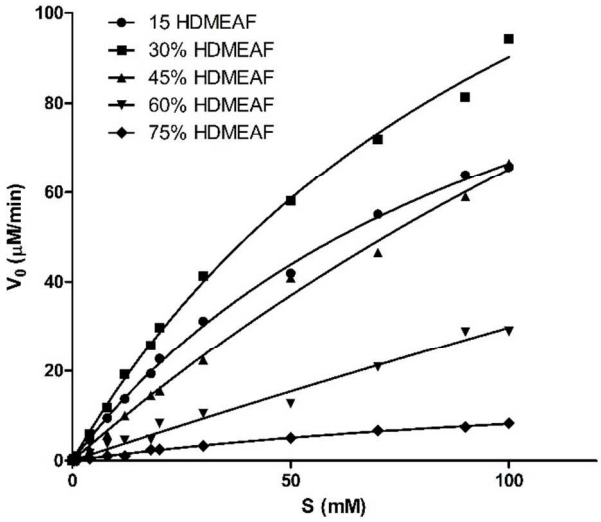
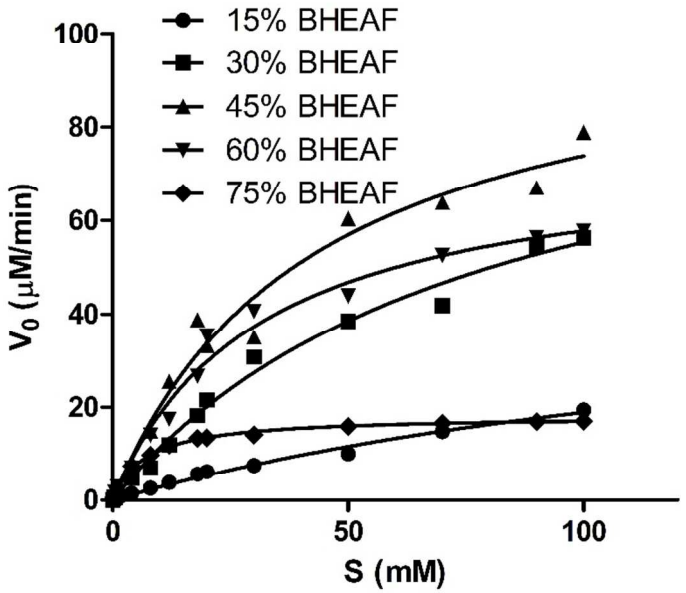


Fig. S13. UV-vis spectra (230-800 nm) of all ILs used in this study.









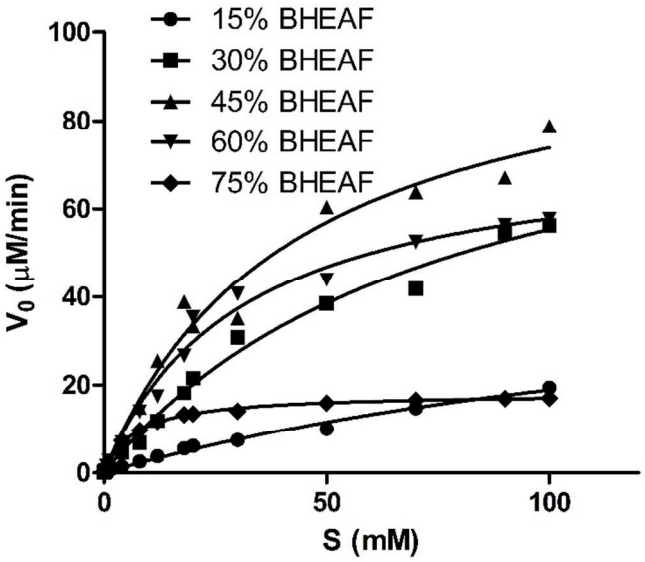


Fig. S14. Michaelis Mented plots for cyt c catalyzed oxidation of guaiacol with H_2O_2 in buffer and in various Ils-based media.

Table S1. Apparent kinetic parameters K_m^{app} (μM) and V_{max}^{app} (μM min^{-1}) of guaiacol oxidation with H_2O_2 by cyt c in the presence of various amounts of ILs (0-75% v/v).

% of IL in reaction medium (v/v)	HEAF		HMEAF		HDMEAF		BHEAF	
	K_m^{app}	V_{max}^{app}	K_m^{app}	V_{max}^{app}	K_m^{app}	V_{max}^{app}	K_m^{app}	V_{max}^{app}
0	58.6 ±1.1	18.8 ±2.5	58.6 ±1.1	18.8 ±2.5	58.6 ±1.1	18.8 ±2.5	58.6 ±1.1	18.8 ±2.5
15	93.3 ±2.2	70.96 ±2.9	120.3 ±5.3	70.3 ±4.2	107.2 ±5.4	137.9 ±5.9	173.2 ±10.9	51.6 ±3.5
30	110 ±1.2	188.7 ±8.5	63.3 ±3.1	43.5 ±3.6	117.2 ±6.8	196.0 ±9.8	77.5 ±5.6	98.2 ±6.8
45	111.1 ±1.4	397.4 ±10.8	118.2 ±6.8	32.1 ±3.9	88.7 ±4.3	108.7 ±7.3	42.2 ±3.6	105.1 ±12.1
60	45.2 ±2.5	231.8 ±12.8	113.5 ±7.2	16.8 ±2.8	281.2 ±8.6	107.8 ±6.4	30.8 ±6.8	75.6 ±9.8
75	34.3 ±1.6	164.4 ±19.3	99.3 ±7.1	13.1 ±1.2	143.2 ±5.9	20.2 ±3.6	7.6 ±4.3	18.4 ±2.3

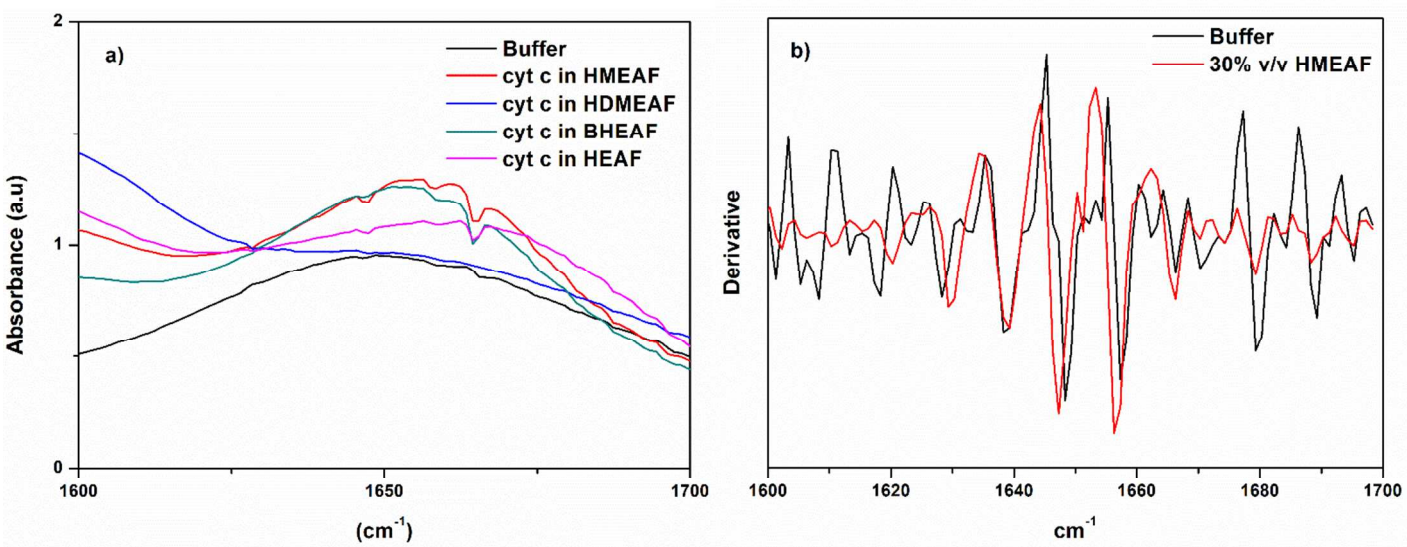


Fig. S15. a) Comparison of ATR spectra of the Amide I region of cyt c in 50 mM phosphate buffer pH 7.0 and 30 % v/v of all ILs studied, b)Comparison of the second derivative spectra in the Amide I region of cyt c in 50mM phosphate buffer pH 7.0 and 30% v/v HMEAF.

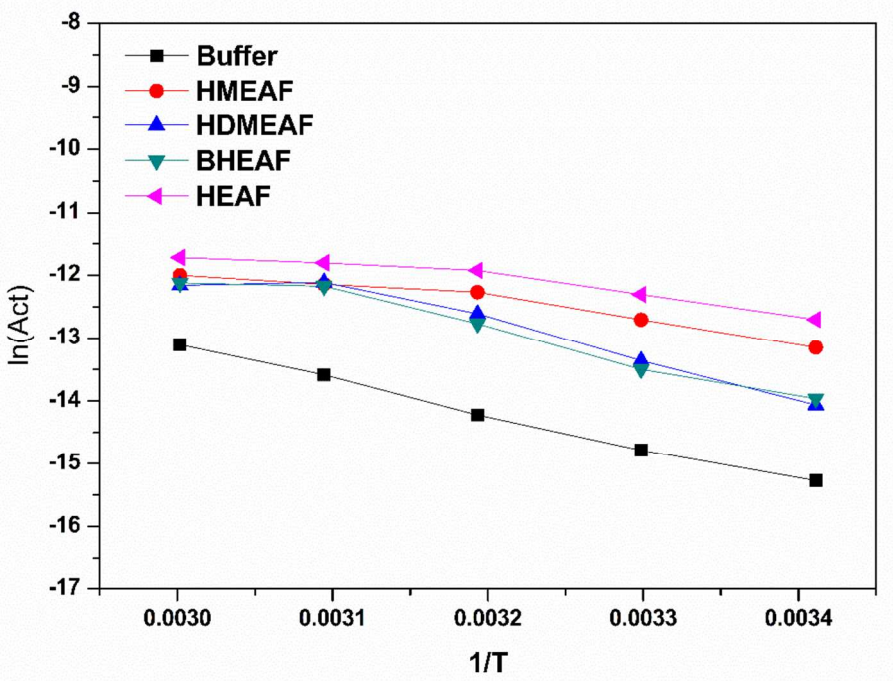


Fig. S16. Arrhenious plots of cyt c activity in buffer and all ILs studied.

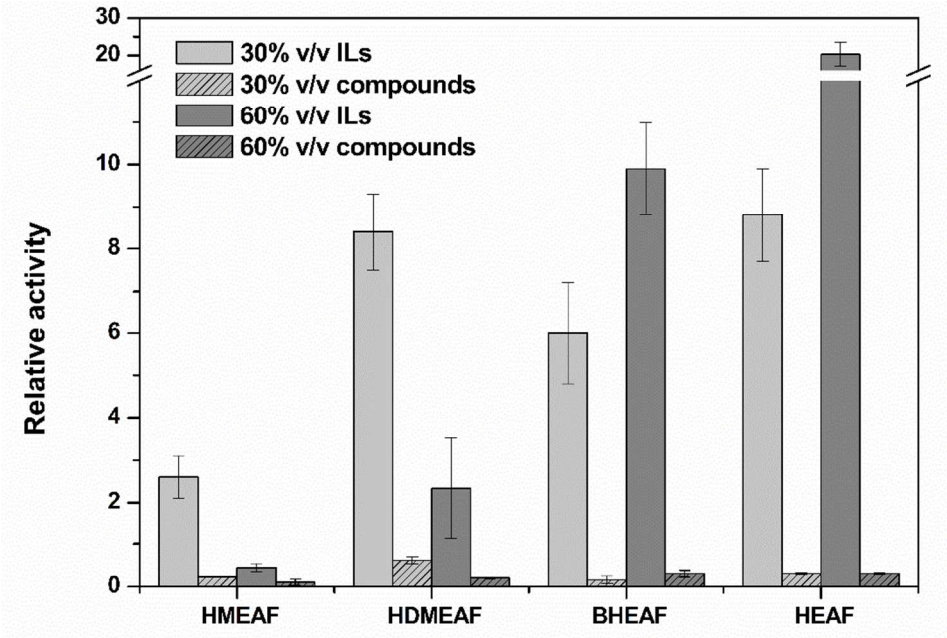


Fig. S17. Relative peroxidase activity of cyt c for the oxidation of guaiacol in the presence of various amounts of hydroxyl ammonium-based ILs and its equimolar amounts of individual components. As 1.0 is indicated the peroxidase activity of cyt c in 50 mM phosphate buffer pH 7.0. Initial reaction rate in buffer: 10 μM/min.

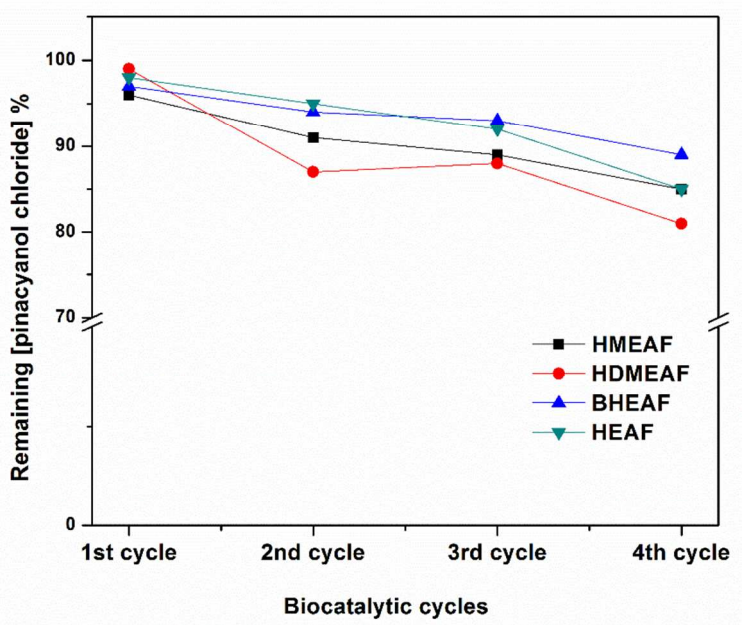


Figure S18. Recycle of ILs used as media in the decolorization of pinacyanol chloride catalyzed by immobilized cyt c.

References

[1] A. Tzani, A. Douka, A. Papadopoulos, E.A. Pavlatou, E. Voutsas, A. Detsi, ACS Sustainable Chem. Eng. 1 (2013) 1180–1185.
[2] A. Romero, A. Santos, J. Tojo, A. Rodríguez, J. Hazard. Mater. 151 (2008) 268–273.