ACS Sustainable Chemistry & Engineering

Research Article

pubs.acs.org/journal/ascecg

Molecular Extraction of Peptides in Ionic Liquid Systems

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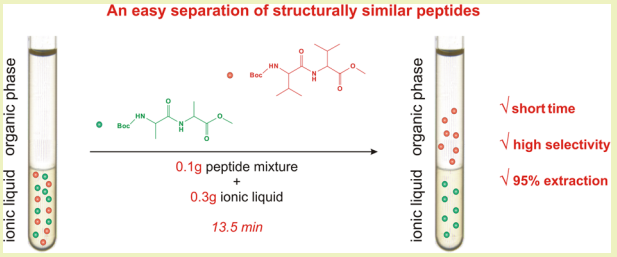
ABSTRACT: The extraction of peptides was studied in a two-phase ionic liquid (IL)/organic solvent system, which displayed outstanding chain length sensitivity (dipeptide vs tripeptide) and separation ability, even for structurally similar peptides (divaline vs dialanine). The extraction process could be performed under substoichiometric conditions; an IL-to-peptide ratio as low as 3:1 led to a high extraction selectivity of divaline/dialanine = 6. For practical applications, two systems were developed for the extraction of peptides from ILs under heterogeneous and homogeneous conditions, with selectivities of 6 and 3.5, respectively. The developed system has shown excellent recycling properties and was reused several times without any visible changes in the selectivity and extraction efficiency. A nuclear magnetic resonance (NMR) experiment with molecular-level spatial resolution was successfully performed to study the mechanism of the extraction process and to visualize the two-phase system.



KEYWORDS: *Extraction, Ionic liquids, Selectivity, Peptides, NMR spectroscopy*

INTRODUCTION

The use of solvents is necessary for performing chemical transformations in the field of organic synthesis. Solvents are required in the vast majority of industrial and laboratory synthetic processes involving biologically active molecules and functional molecules across the fields of pharmaceutical science, materials science, organic electronics, and life science. The key problems that arise from solvent use are the extraction/purification of synthesized products and the recovery/recycling of the employed solvent. In fact, the treatment of solvent systems contributes up to 50% of the energy consumption and up to 75% of the mass utilization during chemical transformations. Moreover, the global solvents market is expected to increase by 4% per annum until 2021.[1] Therefore, the development of sustainable solvent systems with advanced extraction/purification and recovery/reuse characteristics is unquestionably a high-priority goal of modern research and development.

A promising solution to this issue involves task-specific optimization, in which significant success has been achieved using ionic liquids (ILs).[2−4] ILs are nonflammable and nonvolatile reaction media that are being successfully employed in various fields, such as catalysis,[5−7] electrochemistry,[8,9] organic synthesis,[10,11] biomass conversion,[12,13] biological research,[14−16] and extractions.[17,18] Among the most important applications of ILs are extractions, particularly liquid- and solid-phase extractions and separations,[18−20] as well as those involved in the nuclear fuel cycle.[17] ILs have also been used in the modification of silica and polymeric materials,[18] in the mobile and stationary phases of liquid chromatography,[18,20] as the stationary phase in gas chromatography,[18,19] and for capillary surface modification in capillary electrophoresis and electrochromatography.[18,21,22]

Due to the unique effects of ILs on biological molecules, particularly amino acids, peptides, and proteins,[23,24] the possibility of applying ILs to the extraction of these molecules has attracted significant attention. The separation of amino acids has been studied in several aqueous biphasic systems, and the importance of hydrophobic interactions between amino acids and ILs has been demonstrated.[25−30] Protein extraction has also been actively investigated, with ILs having been employed to extract proteins from body fluids[31] and *Chlorella pyrenoidosa* cells;[32] to extract cytochrome C,[33,34] horseradish peroxidase,[35] lipase A,[36] and lactoferrin[37] from aqueous solutions; and to extract keratin from feathers.[38,39] The processes underlying protein partitioning in IL systems have been studied, and models have been proposed that involve the electrostatic interactions between surface amino acid residues and charged IL cations,[40] as well as hydrophobic interactions and the salting-out effect.[41] In recent years, several IL/aqueous two-phase systems for protein extraction and separation have been established.[42−44]

However, few studies have demonstrated the satisfactory performance of ILs in the transformation and separation of peptides. ILs are used as reaction media for peptide synthesis,[45] and there have been several studies of the behavior of peptide−IL−water systems.[46−48] Investigations of the solvation of glycine peptides in ammonium-based ILs have revealed stabilizing effects of ILs on the peptide structure.[49] Recently, we investigated the interactions between imidazolium-based ILs and peptides using ^{13}C nuclear magnetic resonance (NMR) spectroscopy and demonstrated that the ILs were very sensitive to the nature of the peptides.[50] For example, a small change in

Received: November 29, 2014
Revised: December 22, 2014
Published: December 26, 2014

DOI: 10.1021/sc500770v
ACS Sustainable Chem. Eng. 2015, 3, 357−364

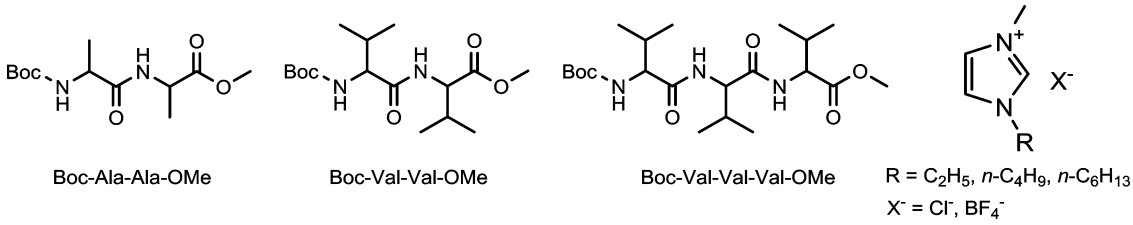


Figure 1. Structures of the peptides and [RMIM][X] ionic liquids used in this study.

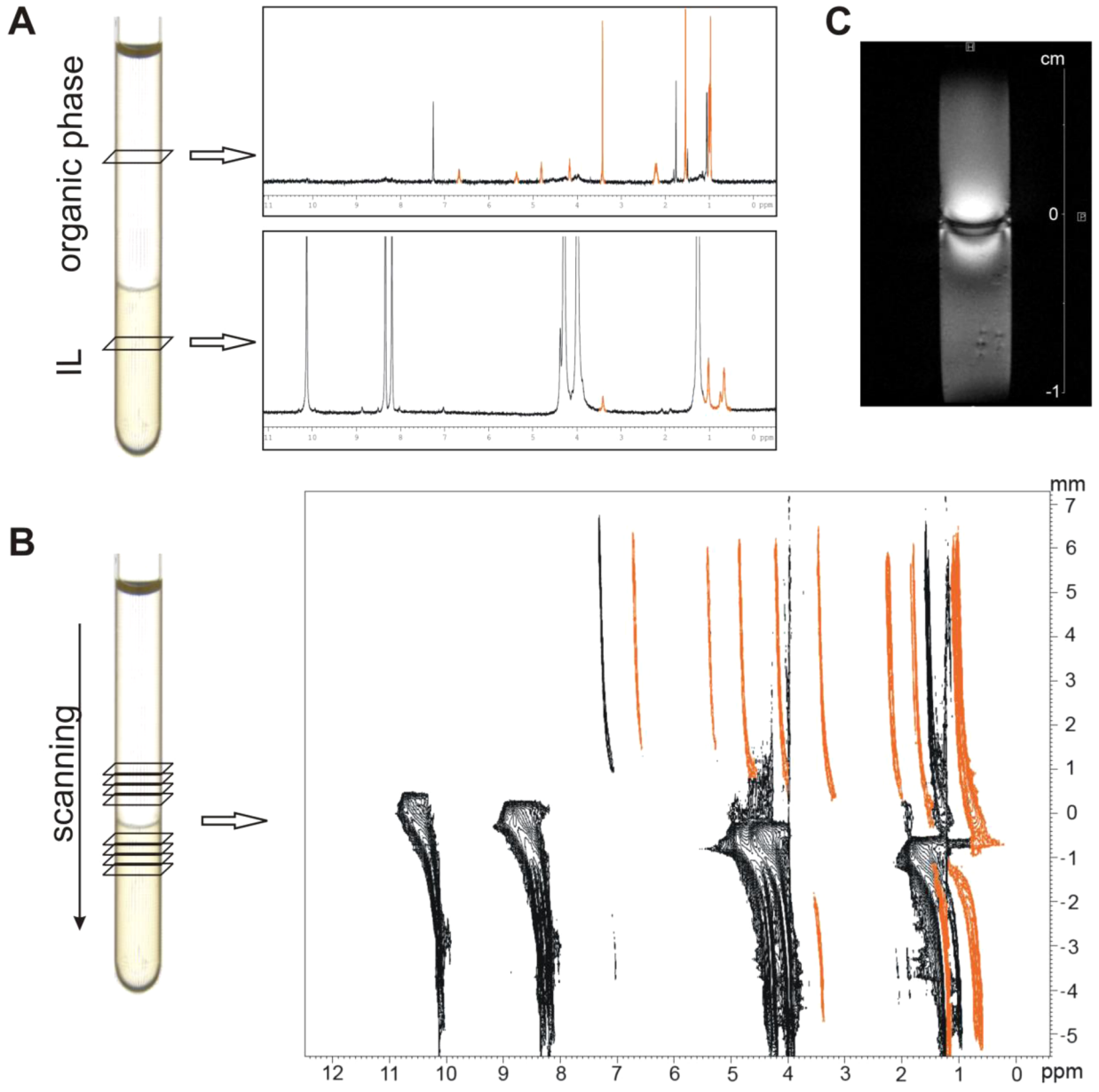


Figure 2. NMR study of the peptide transition from the IL to the organic solvent. (A) Slice-selective spectral study of the IL and organic phases. (B) 2D plot of the vertical NMR scan of the two-phase sample. (C) Magnetic resonance imaging of the NMR tube containing the IL and organic solvent phases. In panels A and B, the peptide signals are plotted in orange and the IL signals in black. Experimental conditions: ^{1}H NMR, 600 MHz, $[C_2MIM][Cl]/C_6D_6$ system.

the amino acid sequence of a peptide resulted in a significant change in that peptide's solubility. Among the peptides tested, those based on L-valine, namely, di-, tri-, and tetravaline, showed poor solubility and exhibited a form of the odd−even effect. A difference in the solubility of the two key peptides studied, Boc-Ala-Ala-OMe and Boc-Val-Val-OMe, in different ILs was also observed.

Despite significant interest, scarce data on the use of IL-based systems for the extraction of peptides are available, and the mechanisms of peptide−IL interactions remain to be established. Amino acids and small peptides represent unique

DOI: 10.1021/sc500770v
ACS Sustainable Chem. Eng. 2015, 3, 357−364

model compounds for understanding these mechanisms at the molecular level. To this end, the present study investigated the extraction of peptides in IL systems and studied the nature of the extraction process using NMR spectroscopy with spatial resolution at the molecular level.

EXPERIMENTAL SECTION

General. The peptides (Boc-Ala-Ala-OMe and Boc-Val-Val-OMe) were prepared as described previously.[50] The ILs (1-ethyl-3-methylimidazolium chloride, 1-hexyl-3-methylimidazolium chloride, and 1-butyl-3-methylimidazolium tetrafluoroborate) and all of the organic and deuterated solvents were purchased from commercial sources.

Slice-Selective NMR and Imaging. The samples were placed in a 5 mm NMR tube, and the NMR spectra were measured at 343 K on a Bruker Avance II 600 MHz instrument equipped with a BBI probe. The slice-selective experiments were performed using a 270° self-refocusing excitation shaped pulse with a 300 μs duration, and the pulse power was calibrated for every sample. The Z-gradient strength varied according to the required slice thickness; for 5.1 G/cm (10% of the gradient amplitude), a thickness of 0.1 mm was achieved. The slice position was determined from the shaped pulse frequency offset (ranging from −25,000 to +25,000 Hz in increments of 500 Hz), and the 100 spectra measured at different increments were packed into a pseudo-2D ser file, with 16 scans acquired for each spectrum. High-quality NMR spectra were measured in the range of 2−7 mm from the sample center in both directions, while lower-quality spectra were recorded at ±1 mm from the phase boundary (i.e., in an overall range of −1 to +1 mm) due to the significant magnetic susceptibility difference between the IL and the organic solvent, which caused local magnetic field inhomogeneity and line broadening. The spectra were processed in automatic mode using exponential multiplication on f2 with LB = 2 and individual phase correction. The 2D FLASH MR images were obtained on an Avance III 400 WB spectrometer with a Micro5 microimaging probe and standard parameters: FOV = 2 cm × 2 cm, matrix = 256 voxel × 256 voxel.

Sample Preparation for Slice-Selective NMR and Imaging. A mixture of 20 mg of divaline and 200 mg of 1-ethyl-3-methyl imidazolium chloride ([C_2MIM][Cl]) was stirred in an NMR tube for 1 h at 80 °C, after which 400 mL of benzene-d_6 was added. The system was stirred for additional 20 min at 70 °C and was then transferred to an NMR spectrometer.

Extraction Studies. A mixture of the two peptides containing 10 mg of each was vigorously stirred with IL (350 mg) for 1 h at 80 °C in PTFE screw-capped tubes equipped with a magnetic stir bar. An organic solvent was then added to the IL−peptide mixtures, and the systems were stirred for a specified time (10 s, 20 s, 30 s, 1 min, or 2 min) at 70 °C before being left undisturbed for two separate phases to establish. The probe was subsequently removed from the top organic layer, and ^{1}H NMR spectra were recorded. All NMR measurements were performed with Bruker Avance 600 and DRX 500 spectrometers operating at 600.1 and 500.1 MHz for ^{1}H, respectively. The ^{1}H NMR chemical shifts are reported relative to TMS as internal standard. The spectra were processed with the Bruker Topspin 2.1 software package.

Quantitative Assessment of Extraction of Peptides. A mixture of the Boc-Ala-Ala-OMe (or Boc-Val-Val-Val-OMe) and Boc-Val-Val-OMe containing 50 mg of each component (0.1 g in total) was vigorously stirred with IL for 1 h at 80 °C in PTFE screw-capped tubes equipped with a magnetic stirrer bar. An organic solvent was then added to the IL−peptide mixtures, and the systems were stirred for different durations (30 s, 1 min, 2 min, etc.) at 70 °C before being left undisturbed for approximately 3 min for a clear phase separation boundary to establish. The top organic phase was removed, and the organic solvent was evaporated. The overall mass of the extracted peptides was measured, and the ratio of the peptides was determined by ^{1}H NMR.

After the first step of extraction, the extraction selectivity was calculated as the ratio of the masses of the target peptide (divaline) and the second peptide (dialanine or trivaline) in the organic phase extract. Following the subsequent extraction steps, additional portions of organic solvent were added, and the selectivity was calculated as the ratio of the sum of the masses of the target peptide (current step plus all prior steps) to the corresponding sum of the masses of the second peptide. The procedure was performed until >96% extraction of the target peptide.

RESULTS AND DISCUSSION

The extractions were performed in a native two-phase system containing an IL and an organic phase, without any additives for spectral studies. It is important to avoid the typical additives used for spectral studies (such as deuterium solvents and internal standards) because even small amounts of such compounds may noticeably perturb the IL system, making mechanistic studies unreliable.[4]

The 1:1 mixture of peptides was dissolved in the IL, and the extraction process was mediated by the addition of an organic solvent. Figure 1 displays the ILs and peptides employed in this study. It should be noted that a direct experimental study of extraction is challenging because none of the available standard analytical tools allow the visualization of the phase transition process.

To study the extraction process and visualize the boundary between the phases, a special NMR experiment was designed (Figure 2) in which NMR spectra were recorded from small sections of the sample, i.e., slices along the *Z*-axis of the sample. The position and thickness of the slice were controlled by the NMR pulse sequence parameters (see Experimental Section). Using this method, spectra from the IL and organic phases were recorded for the same sample. The high quality of the measured NMR data allowed us to resolve the signals of the IL and peptides in the bottom phase as well as those of the organic solvent and peptides in the upper phase (Figure 2A). The transition of the peptide from the IL phase to the organic phase was observed by NMR analysis. Thus, this type of spectral study can reveal molecular level information in a spatially resolved part of a sample volume.

Recording a series of 100 NMR spectra provided an in-depth vertical scan of the sample (Figure 2B), and representing the data using a 2D plot afforded molecular-level information along one axis (NMR chemical shifts; unit: ppm) and the geometrical position in the two-phase system along the other axis (distance from the phase boundary; unit: mm). In addition, magnetic resonance imaging was performed to map the two-phase IL−organic solvent samples (Figure 2C).

At the molecular level, the transition of the peptide molecules from the IL to the organic solvent can be accomplished in different ways (Figure 3). For example, the peptide molecule can pass the IL as a supramolecular associate surrounded by IL molecules (Figure 3A). Accordingly, significant deviations in the NMR chemical shifts along the vertical axis would be observed in the organic phase due to changes in the conformational behavior, solvation effects, and shielding environment upon decomplexation of IL solvent.[51] The presence of IL signals should also be detected in the organic phase. In the studied samples, we did not observe significant changes in the chemical shifts of the target molecule in benzene-d_6; moreover, similar values were measured compared with those in [C_2MIM][Cl] (Figure 2A and 2B). Specifically, the chemical shift (δCH_3) of divaline was 1.0 ppm in benzene-d_6 and 0.7 ppm in the IL (a difference of 0.3 ppm). For the other protons, no significant changes were observed, that is, the deviations of the chemical shifts did not exceed ±0.1

DOI: 10.1021/sc500770v
ACS Sustainable Chem. Eng. 2015, 3, 357−364

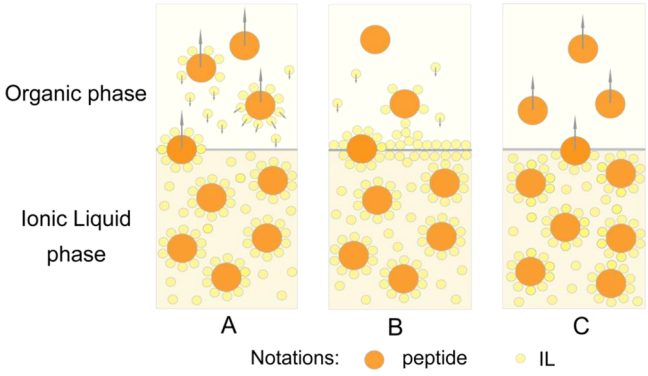


Figure 3. Possible mechanisms of the peptide transition from the IL to the organic phase. (A) Peptide molecules pass the boundary as supramolecular associates. (B) Partial mixing of the phases with/without a warped phase boundary. (C) Peptide molecules pass individually by molecular diffusion.

ppm. Signals corresponding to a complex between the peptide and the IL were not detected in the organic phase. Therefore, the measured spectral data did not correspond to an extraction process involving stable NMR-detectable supramolecular associates.

In the second case, partial mixing of the solvents near the phase boundary may occur with a plausible nonuniform (or warped) phase boundary (Figure 3B). In such a case, noticeable changes in the chemical shifts along the vertical axis would be observed, and a warped phase boundary should be visible in the magnetic resonance image data. Neither of these was observed in the measured NMR data or magnetic resonance imaging of the NMR tube (Figure 2), respectively.

In the case of pure molecular extraction, the solute molecules pass the phase boundary without an associate, and a clear phase boundary should be observed (Figure 3C). The transition would not be accompanied by specific changes in the chemical shifts inside one phase, and the signals of the IL should not be detected in the organic phase in noticeable amounts. Indeed, the case of pure molecular extraction is supported most strongly by the measured experimental data (Figure 2), as neither significant changes in the NMR chemical shifts along the vertical axis nor significant deviations near the phase boundary were experimentally observed (Figure 2).

Of course, it should be noted that the phase transition might involve varying contributions of different mechanisms, with each process occurring on a different time scale. Therefore, the present data should not be considered as an ultimate proof of a single possible mechanism; rather, they indicate that in the studied system, the extraction process preferably occurs by the molecular diffusion of the peptide molecules.

A clear understanding of the molecular extraction process and a well-defined phase boundary are of great importance for extraction/separation applications. Several ILs (Figure 1) and organic solvents (benzene, toluene, cyclohexane, ethyl acetate, and hexanes) were used in the present study to develop an efficient system for practical applications. A combination of $[C_2MIM][Cl]$ (IL phase) and a mixture of ethyl acetate/hexanes (organic phase) was found to be a cost-effective solution with high selectivity. Two structurally similar peptides, Boc-Ala-Ala-OMe and Boc-Val-Val-OMe, were added to this model mixture to demonstrate its ability to distinguish between even small changes in the peptide structure.

A 1:9 solvent ratio of ethyl acetate:hexanes was found to provide the best selectivity for divaline extraction. After stirring and settling, the top organic phase was removed, and the selectivity was calculated as the ratio of the masses of divaline and dialanine extracted from the organic phase. We did not observe significant selectivity changes when varying the temperature in the range of 70−90 °C. In the case of $[C_2MIM][Cl]$ and ethyl acetate, the optimal temperature was found to be 70 °C, which is above the melting point of the IL but below the evaporation point of the organic solvent.

With an initial dipeptide amount of 50 mg (1:1 ratio of divaline to dialanine) mixed with 0.32 g of $[C_2MIM][Cl]$, the first divaline extraction step exhibited a high selectivity of m(divaline)/m(dialanine) = 6 (Figure 4A). The separation of the organic phase was performed after contact times as short as 30 s. Although the remaining amount of divaline gradually decreased after the first extraction step, it was still possible to achieve good selectivity. For example, a combined selectivity of m(divaline)/m(dialanine) = 5 was obtained after two extraction steps. The divaline was almost completely extracted after five steps with a corresponding good combined selectivity of m(divaline)/m(dialanine) = 2.6, whereas not extracted dialanine remained in the IL phase (Figure 4A). The total contact time between the organic phase and IL was 13.5 min (combined for all steps), and the total amount of the extracted peptide reached 95% after five steps.

Increasing the amount of IL and lowering the peptide concentration led to lower extraction selectivity (Figure 4B and C). Specifically, 0.5 g of the IL led to selectivity in the range of 4.6−1.9, whereas 0.65 g of the IL caused it to decrease to 3.1−1.5. Therefore, the extraction efficiency apparently increased with increasing concentrations of the dipeptides in the IL phase. Using molar concentrations of the dipeptides in the range of 0.5−0.7 mol/L resulted in high extraction efficiencies.

The change in selectivity upon increasing the number of steps was particularly noteworthy. With the initial 1:1 ratio of the peptides, the highest observed selectivity was 6 (Figure 4A), and the amount of target peptide extracted during this step was 30%. Although the overall selectivity decreased as the number of steps increased, it should be emphasized that the separation was performed on gradually decreasing amounts of the extracted component. Prior to step 5, although the peptide ratio in the IL was m(divaline):m(dialanine) = 0.12:1.00, the selective extraction of divaline was still possible. The overall shape of the curve reveals the outstanding performance of the studied system for the separation process, which produced a 95% extraction of the target peptide after five steps.

Upon increasing the amount of IL to 1.3 g, divaline was extracted with a nearly constant selectivity that ranged from 3.5 to 3.0 and changed only slightly during the extraction process (Figure 5). After six extraction steps, the total amount of the extracted peptide was 96%, for which the total time of contact between the organic and IL phases was 16.5 min.

When the IL mass was further increased to 2.6 g, the selectivity remained stable, but quantitative separation did not occur in six steps (extraction efficiency reached 80%). Thus, the optimal amounts of the IL for the studied systems are 1.3 g (Figure 5) and 0.32g (Figure 4A).

It is of interest to compare the dependence of the separation selectivity on the number of steps for the two different cases (Figure 4 vs Figure 5). At the lower amount of the IL (Figure 4), the bulk of the divaline remained as a precipitate and initially passed into the organic solvent, whereas the

DOI: 10.1021/sc500770v
ACS Sustainable Chem. Eng. 2015, 3, 357−364

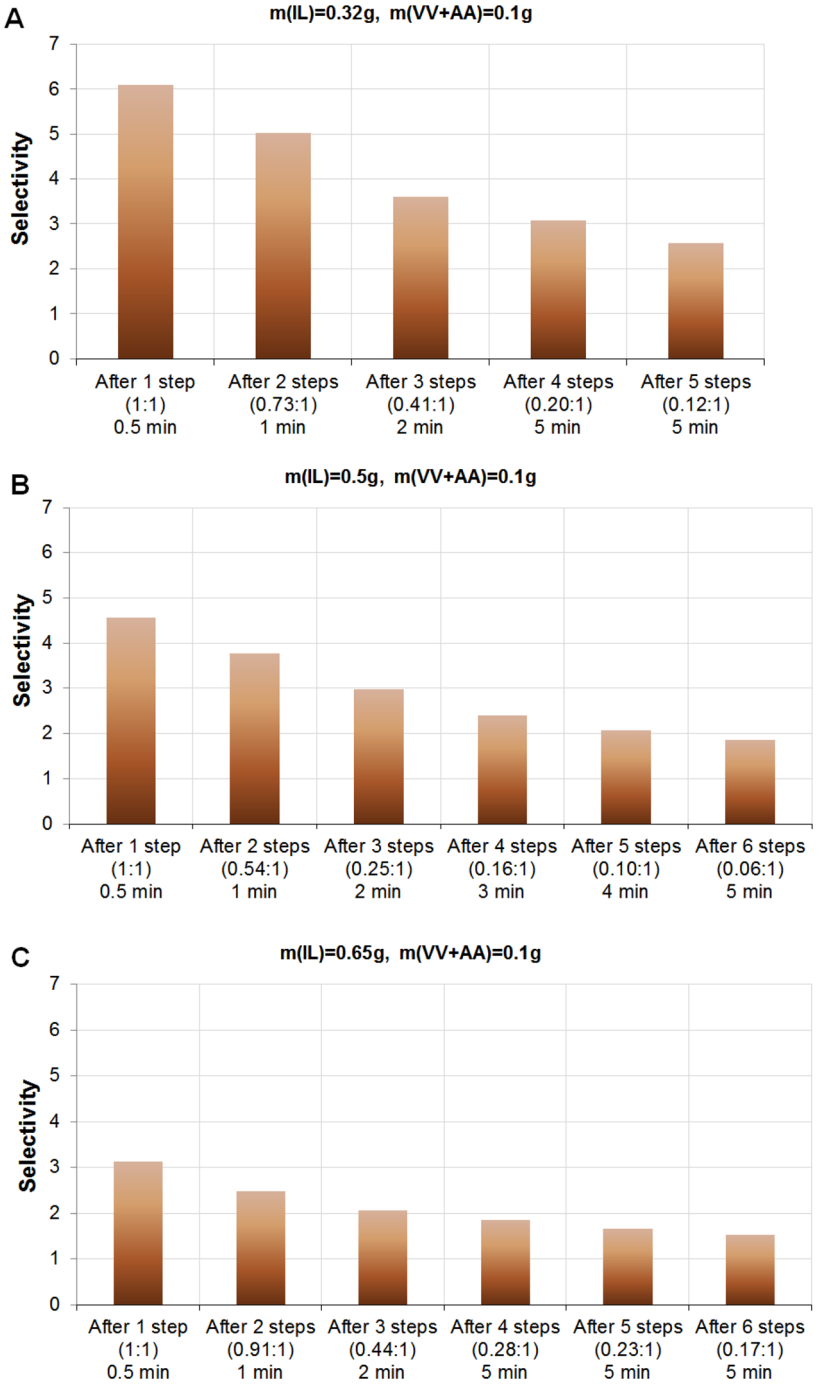


Figure 4. Extraction of divaline from divaline(VV)/dialanine(AA) mixtures using [C_2MIM][Cl] (IL phase) and a mixture of ethyl acetate/hexanes (organic phase) at 70 °C. The initial amount of the two peptides (0.1 g total of divaline and dialanine) and the amount of IL are indicated in each case. The extraction was performed as a stepwise process, and the combined selectivities for all of the preceding steps are shown. The ratios between the amounts of divaline and dialanine present in the IL phase before each step are shown in parentheses (normalized to the amount of dialanine), and the contact time between the organic phase and the IL is indicated for each step.

significantly more soluble dialanine remained in the IL. Therefore, in this case, the extraction occurred from the heterogeneous IL system. The high selectivity of m(divaline)/m(dialanine) = 6 after 30 s of contact time achieved during the first step was due to the immediate extraction of the insoluble divaline component of the mixture (Figure 4A). The overall selectivity was lower because with the lower amount of IL the separation efficiency was noticeably affected by the removal of the precipitate. When a higher amount of IL was used (Figure 5), both peptides were almost completely dissolved, and the separation was performed under homogeneous (i.e., precipitate-free) conditions. Therefore, the extraction efficiency was governed by the interaction between the solubilized peptides and the IL, and the dialanine was more strongly retained by the IL phase. This separation resulted in a somewhat lower selectivity for the initial steps, whereas a higher selectivity was observed for the subsequent steps performed until the >96% extraction of the divaline was achieved (Figure 5).

Next, it was important to evaluate the influence of the nature of the IL on the extraction process. In the case of 1-butyl-3-

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ACS Sustainable Chem. Eng. 2015, 3, 357−364

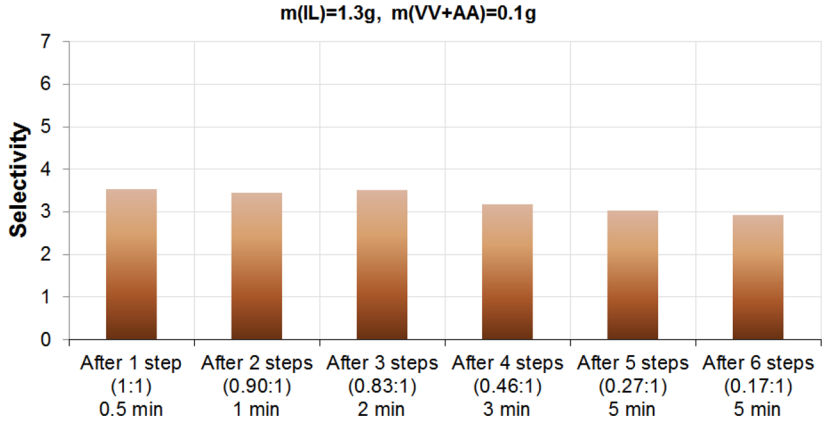

Figure 5. Extraction of divaline from the indicated divaline(VV)/dialanine(AA) mixture with constant selectivity. The extraction was performed as a stepwise process, and the selectivity displayed is that combined for all preceding steps. The ratios of the amounts of divaline and dialanine present in the IL phase prior to each step (normalized to the amount of dialanine) are shown in parentheses, and the contact time between the organic phase and IL is indicated for each step.

methylimidazolium tetrafluoroborate ($[C_4MIM][BF_4]$), in which dialanine is highly soluble and divaline is nearly insoluble, the separation process was also performed with excellent selectivity. With 0.65 g of the IL, the observed selectivity was in the range of 6−3 (similar to the results shown in Figure 4A), and the total amount of the extracted peptide reached 93% after an overall contact time of 15.5 min over 6 steps. To address the role of the cation, $[C_6MIM][Cl]$ was also included in the study; however, a much lower selectivity was observed in this case (effective extraction did not occur).

As we recently reported, an outstanding characteristic of ILs is their ability to distinguish slight variations in the peptide structure.[50] The solubility of the studied dipeptides changed in the following order: dialanine ≫ divaline in $[C_2MIM][Cl]$ and $[C_4MIM][BF_4]$ and dialanine ≈ divaline in $[C_6MIM][Cl]$.

Thus, the task-specific optimization of the IL properties was the key to achieving successful extractions. For example, a minor change in the structure of the IL (C_2, C_4 vs C_6 groups) affected the solvent−solute interactions and allowed for selective extractions.

Moreover, even small variations in the structure of the peptides could be distinguished by the IL media, which was reflected by their difference in solubility, for example, trivaline > divaline in $[C_2MIM][Cl]$.[50] This finding may provide an amazing opportunity to extract peptides based on their number of amino acid residues. To investigate this possibility, experiments involving a mixture of the divaline Boc-Val-Val-OMe and trivaline Boc-Val-Val-Val-OMe peptides were performed in the $[C_2MIM][Cl]$:ethyl acetate/hexanes system. Indeed, it was possible to selectively extract the divaline from the mixture. Good selectivity in the range of 4.5−3.5 was observed at peptide molar concentrations of ∼0.1 mol/L, and the total amount of the extracted divaline reached 93% after a total contact time of 20 min over 7 steps.

The developed system has shown excellent recycling properties. After extraction of one portion of the peptides, a new portion was simply added, and the process was repeated. The IL was reused several times without any visible changes in the selectivity and extraction efficiency. An NMR study has confirmed that the IL system stayed intact after carrying out the extraction processes.

CONCLUSIONS

The molecular origin of the transition of peptides from the IL phase to the organic phase during an extraction process was determined using slice-selective NMR experiments and magnetic resonance imaging. The extraction efficiency and selectivity were shown to be easily tuned by adjusting the structure of ILs and optimizing the experimental conditions. The highest value of the divaline/dialanine selectivity, 6, was achieved for the $[C_2MIM][Cl]$:ethyl acetate/hexanes system at 70 °C, for which as little as 0.32 g of the IL was required to mediate the separation of a total of 0.1 g of peptides. This system demonstrated very good performance for the separation of a mixture of a dipeptide and tripeptide, with observed selectivities as high as divaline/trivaline = 4.5.

It should be noted that the extraction process can be performed under substoichiometric conditions with a ∼3:1 ratio of IL (solvent) to peptides (solute). This result is superior to those of regular extraction systems, for which a large excess of the solvent is usually required to achieve the controlled release of the dissolved compounds. In addition, high performance peptide separation was achieved in a completely recyclable IL system.

We anticipate further studies on this subject because the results have potential applications in the separation and analysis of biomolecules and they open new opportunities for the development of sustainable chemical processes.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors thank Dr. Detlef Moskau (Bruker Biospin AG, Switzerland) for helpful discussions in designing slice-selective NMR experiments. The work was supported by the Russian Science Foundation (RSF Project 14-13-00448) and Russian Foundation for Basic Research (Projects 13-03-12231 and 14-03-31478).

DOI: 10.1021/sc500770v
ACS Sustainable Chem. Eng. 2015, 3, 357−364

DEDICATION

The article is dedicated to the memory of Dr. Detlef Moskau (deceased 2014).

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