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Article

Isopropylammonium Formate as a Mobile Phase Modifier for Liquid Chromatography

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Isopropylammonium formate (IPAF), a new alkylammonium formate (AAF) room temperature ionic liquid, has been synthesized from isopropylamine and formic acid and characterized as an organic solvent mobile phase replacement for reversed-phase liquid chromatography (LC). Characterization of IPAF solvent properties in water such as pH, conductivity, and viscosity, as well as its synthesis, is described. The LC polarity (P′) and the solvent strength (S) parameters are determined to be 6.0 and 2.4, respectively, similar to those same parameters for methanol and acetonitrile. Application of this RTIL is demonstrated as an organic solvent replacement for reversed-phase LC to separate a test mixture of niacinamide, acetophenone and *p*-nitroaniline. The van Deemter plot profile for several columns of different dimensions, particle size, pore size and stationary phase are compared using an IPAF–water mobile phase. At flow rates above 2 mL/min, on-line mixing of the viscous IPAF with water appears not to be uniform. A flattening of the van Deemter profile is noted for particularly short (50 mm) wide bore (4.6 mm) columns packed with larger particles (10 μm). Small particle longer columns likely facilitated mixing at the beginning of the column generating typical linearly increasing van Deemeter curves. IPAF has been further shown as a function of temperature to be a non-denaturing modifier solvent for the separation of the protein cytochrome c from tryptophan compared to methanol. This is important to show, because the semi-preparative separation of native proteins using AAF mobile phases is the long-term goal of this research program.

Introduction

Room temperature ionic liquids (RTILs) are a class of solvents composed of a co-existing organic cation and organic anion. The cation is commonly a 1,3-dialkylimidazolium ion or an alkylammonium species. The anion is commonly a dissociated inorganic or organic Bronsted-Lowry acid, such as nitrate or formate ion. The most common studied class of ionic liquids are the 1,3-dialkylimidazolium series. They have been used as solvents for organic chemistry reactions (1) and for bio-fuel applications such as dissolution of cellulose using 1-butyl-3-methylimidazolium chloride (2). Their versatility is attributable to their unique properties such as thermal stability, non-flammability, non-volatility and extended shelf life.

Several reviews are available describing the use of ionic liquids for advancing research in gas chromatography (GC), liquid chromatography (LC), and capillary electrophoresis (CE) (3, 4, 5). They have been used widely as stationary phases in GC due to their thermal stability and selectivity, such as for chiral compounds (6). Stationary phases with covalently bound imidazolium groups with various counter-anions have been characterized for mixed-mode reversed-phase (RP) LC (7). Ionic liquids have been developed as micelle forming surfactants for chiral and achiral CE separations (8). Recently, LC of cellulose using 1-ethyl-3-methyl imidazolium methyl phosphonate as the mobile phase with refractive index detection was reported; however, the flow rate was only 0.01 mL/min, even with the mobile phase heated to 55°C (9).

The most common application of RTILs for LC is as an additive in mM concentrations in the mobile phase for the liquid chromatography of amines such as catecholamines (10) and pharmaceuticals (11) to suppress silanol interactions by silica based columns. However, two classes of RTILs, alkylammonium nitrates and alkylammonium formates (AAF), have been used as organic solvent replacements for traditional RP-high-performance liquid chromatography (HPLC) solvents (12–16). Nitrate, however, absorbs sharply in the ultraviolet-visible (UV-VIS) region and suffers from a high UV cutoff wavelength. Alkylammonium nitrates have been particularly useful for separating nitroaromatic compounds with an absorbance in the visible region (12). Methylammonium formate (MAF) has been shown to have a lower plate height than methanol over the entire mobile phase velocity range using a silica based column. This effect was likely due to suppression of silanol interactions (13). Ethylammonium formate (EAF) has been shown to better resolve *o*- and *p*-nitrophenol when it is used as a mobile phase replacement for methanol, using a polymeric column (14). Limitations of MAF include difficulty in preparation of high volumes, availability of the amine as a dilute solution in water or alcohol, and potential instability due to amide formation (17). EAF is more stable and has been characterized quite completely as a mobile phase modifier for LC (15, 16), but is also difficult to prepare in high volumes, again due to availability as the amine dissolved in solution. Longer straight chain AAFs such as *n*-propyl- and hexyl-ammonium formate are not stable at room temperature for several months. The instability is evidenced by formation of a yellow color and an unacceptable UV-VIS cutoff (A = 1.0) of 380 nm. Recently, the tertiary amine AAFs diisopropylmethylammonium formate and diisopropylethylammonium formate have been synthesized and reported to have viscosities around 20 cP (18). However, these RTILs are too expensive to make in quantities necessary as mobile phase modifier solvents for analytical scale LC. We have made diethylamine formate; however, it is yellow with an unacceptable high UV cutoff. Triethylammonium formate forms two immiscible layers and triethylammonium actetate also has a high UV cutoff at 285 nm.

RTILs are becoming more widely used by biochemists in fields such as biocatalytic reactions (19) and protein stabilization (20). Fujita *et al.* were able to maintain the secondary structure of cytochrome c while it was dissolved in the RTIL choline dihydrogen phosphate (21). It is well known that unfolded proteins such as cytochrome c fluoresce due to the

presence of exposed tryptophan residues. The fluorescence of tryptophan in cytochrome c in its native conformation is quenched by the heme iron group. Previous studies have indicated that organic solvents such as methanol denature cytochrome c. The unfolding of the protein begins at approximately 60% methanol–40% water volume fraction, versus not until 90% ionic liquid–10% water (22). Recently, fluorescence energy transfer studies with tetramethylrhodamine labeling have been used to further determine the structure of proteins dissolved in 1,3-dimethylimidazolium RTILs (23).

The primary objectives for this research study are to synthesize a stable, inexpensive RTIL, provide a fundamental chromatographic study of this mobile phase modifier in terms of polarity and efficiency, and demonstrate its potential for the separation of proteins. Isopropylammonium formate (IPAF), a new AAF ionic liquid, has been synthesized from isopropylamine and formic acid and characterized as an organic solvent mobile phase replacement for reversed-phase LC. The availability of the high purity (99.5%) liquid amine instead of a dissolved amine solution allows for a streamlined purification and reduced cost. Both high purity acetic acid and formic acid are commercially available; however, isopropylammonium acetate is not an RTIL, turning to solid when vacuum is used to take off the methanol reaction solvent. The branched character of the isopropyl ammonium cation leads to an AAF less prone to amide formation (17, 24). Longer chain branched AAFs such as 2-methylpropylammonium formate (2MPAF) have been shown to have a low surface tension but a viscosity over 200 cp, apparently because viscosity increases with chain length (17). The low surface tension predicts that this AAF will be a relatively strong reversed-phase LC solvent, based on the solvophobic retention mechanism (25). However, the high viscosity could be problematic due to high column backpressure. High viscosity should, however, provide the advantage of increased protein stability, particularly at increased temperatures. IPAF, with its branched character, but only three carbons instead of four as in 2MPAF, should have a low surface tension but also a lower viscosity, making it an effective reversed-phase LC modifier. IPAF, with a primary amine cation in conjunction with the formate anion, should also be an amphoprotic buffer, as demonstrated for MAF and EAF (13, 14).

Characterization of the solvent properties of IPAF and its synthesis are described. Application of this RTIL is demonstrated as an organic solvent replacement for RPLC to separate a test mixture of niacinamide, acetophenone and *p*-nitroaniline. Various van Deemter plots using columns of different pore size and dimensions, as well as stationary phases, are compared to understand previous studies using AAF mobile phases that showed van Deemter plots with a slightly non-linear trend for the C term (13, 14). IPAF has been further shown as a non-denaturing modifier solvent as a function of temperature for the separation of cytochrome c from tryptophan, compared to methanol. This is important to demonstrate because the semi-preparative separation of native proteins using AAF mobile phases is the long-term goal of this research program.

Experimental

A Dionex (Sunnyvale, CA) Ultimate 3000 Rapid Separation ultrahigh-performance liquid chromatography instrument equipped with both multi-wavelength UV-VIS and fluorescence detection was used for all chromatography measurements. It was interfaced to a computer using Chromeleon software. Height equivalent of a theoretical plate (HETP) was calculated using the software plate count (N) feature and dividing each value by the column length. The software was configured to use $N = 5.54(t_r/w_{0.5})$ where t_r = retention time and $w_{0.5}$ = peak width at half height to find the plate count. Standard UV-VIS measurements were made using an Agilent (Santa Clara, CA) 8453 diode array spectrophotometer. Fluorescence spectra were obtained using a Perkin Elmer (Waltham, MA) LS55 luminescence spectrometer; slit widths were set to 4 and 8 nm, respectively. Density measurements were made using a Denver Instruments (Bohemia, NY) analytical balance and an Eppendorf (Hauppauge, NY) Repeater Plus pipet. Conductivity measurements were taken with an Oakton Con 6 meter purchased from Cole-Parmer (Vernon Hills, IL). Viscosity measurements were taken at 25°C using a size 300 446Z Cannon-Fenske routine type viscometer. The viscometer constant was 0.2571 mm^2/s^2. A Thermo Scientific Orion 2 Star pH meter with an automatic temperature compensation (ATC) epoxy probe from Fisher Scientific, Pittsburgh, PA, was used for all pH measurements.

Reagents

Isopropylamine of ≥99.5% purity (bp = 33–34°C, d = 0.69) was obtained from Aldrich (Milwaukee, WI). Formic acid was purchased as 98% v/v grade from Fluka (St. Gallen, Switzerland). Methanol and acetonitrile were obtained as HPLC grade from Fisher Scientific. Water was purified using a Millipore MilliQ filtration system to a purity of 18.2 MΩ resistivity. Test analytes, including caffeine, niacinamide, *p*-nitroaniline, acetophenone and tryptophan, were obtained from Sigma–Aldrich (St. Louis, MO). Cytochrome c (≥95%, MW = 12,327) from bovine heart was also purchased from Sigma–Aldrich.

Synthesis

The preparation of this class of ionic liquids involves two steps, synthesis and solvent medium evaporation, similar to that previously reported (13, 14). Isopropylammonium formate was synthesized as shown in the following reaction:

$$(CH_3)_2CHNH_{2(liq)} + HCOOH_{(liq)} \rightarrow \left[(CH_3)_2CHNH_3^+\right][HCOO^-]$$

The isopropylamine was diluted by 50% with HPLC-grade methanol for the reaction. It was chilled in a three neck round bottom flask using a combination of dry ice and ice bath. The formic acid was chilled with ice water using a jacketed glass addition funnel suspended above the amine. The formic acid was added to the amine drop-wise to initiate the reaction. Bubbling nitrogen in the round bottom flask during the entire reaction time ensured no possible degradation due to oxygen. Equimolar amounts of isopropylamine and formic acid were used. A batch of 500 mL was able to be produced in 2.5 h. The reagent methanol was evaporated at room temperature under vacuum for 48 h and trapped in liquid nitrogen. Freezing drying was also done to reduce further any residual solvent content.

Chromatographic conditions

The IPAF ionic liquid was used as an LC mobile phase with several column types and stationary phases including: a Basic 10 μm, 10 nm C18 silica (150 × 4.6 mm) from YMC (Allentown, PA), a Gemini 3 μm, 11 nm C6 phenyl silica (50 × 2 mm) and a Jupiter 5 μm, 30 nm C5 silica (50 × 4.6 mm), both from Phenomenex (Torrance, CA); an Econosphere 5 μm, 30 nm C4 silica (250 × 4.6 mm) and an RSil 10 μm, 10 nm C18 silica (250 × 4.6 mm), both from Alltech (Deerfield, IL); and an PRP-3 10 μm, 30 nm polystyrene-divinylbenzene (150 × 4.6mm) from Hamilton Co. (Reno, NV). Chromatograms were obtained at 25°C and at a flow rate of 0.5 mL/min unless otherwise specified. Mobile phases were degassed using vacuum while in an ultrasonic bath and also degassed online by the instrument. The column was conditioned and equilibrated for at least 10 min before injections. UV-VIS absorbance measurements were taken at 220, 254, 270 and 409 nm unless otherwise noted; the units are all in absorbance units (AU). Fluorescence measurements are in units of detector counts. Excitation and emission wavelengths for tryptophan and cytochrome c were 270 and 350 nm, respectively. Multiple ($n = 5$) measurements were taken at selected flow rates (less and greater than 1 mL/min) for the van Deemter plots to ensure reasonable precision. The retention factor k' was calculated using [$k' = (t_r - t_0)/t_0$]; t_0 = retention time of solvent front and t_r = retention time of the solute. Separate standards were used to identify all chromatographic peaks.

Results and Discussion

Solvent characterization

Replicate density measurements were made with the average of three trials using three different batches of IPAF ($n = 9$). The average density was 1.095 g/mL with a standard deviation of 0.006 g/mL. This is consistent with other RTILs, which can have densities typically greater than water (1.0–1.6 g/mL) (13, 14). IPAF is slightly denser than other branched alkylammonium formates. 2MPAF and 2-methylbutylammonium formate have previously reported densities of 0.978 and 0.965 g/mL, respectively (17).

The apparent pH of 100% IPAF was measured to be approximately 7.3; this is close to the predicted pH of 7.2 for an amphiprotic buffer calculated from the average of the pKa values for the protonated amine and the carboxylic acid. As water was added, the pH of the solution was found to decrease, as shown in Figure 1, due to the hydrolysis of the isopropylammonium ion with water (pKa = 3.4), which is significantly more favored than the hydrolysis of formate ion (pKb = 10.3). Recently, it has been reported, using small- and wide-angle X-ray scattering, that protic ionic liquids, including alkylammonium nitrates and AAFs, form micellar type aggregates with the alkyl groups in the interior and the protonated amine group on the exterior, ion pairing with the anion (26). Infrared analysis indicated that bulk water, primarily associated with itself, is evident. This is in contrast to just the amine dissolved in water, which has water in the interior of a ring of amines arranged head-to-tail. At approximately pH 5, where the pH appears to level out in Figure 1, the concentration of protonated amine is still low, calculated to be approximately 2.5×10^{-7} M.

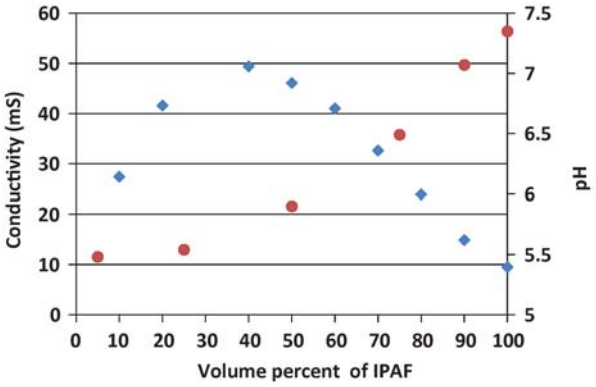

Figure 1. Conductivity in milliSiemans (mS) (left scale) and pH (right scale) as a function of percent (v/v) IPAF in water. Conductivity: diamonds; pH: circles.

The conductivity profile as a function of %IPAF in water is also shown in Figure 1. The conductivity increases as expected with % IPAF due to the increase in ionic species to a maximum of approximately 40% v/v or 0.10 molar IPAF, before decreasing fairly linearly to 100% IPAF. This drop is attributable to the reduction of ion mobility due to increased viscosity and likely also increased ion-pair formation. The conductivity profile is similar to that previously reported for other short chain AAFs (14). In addition, maximum conductivities of alkylammonium nitrates have been reported to be in the range of 0.07 to 0.14 molar fraction (26).

Absorbance measurements were taken from 190 to 800 nm to determine the UV cutoff for the newly developed ionic liquid. The wavelength at which the solvent is opaque with absorbance of greater than 1.0 is 252 nm, but there is essentially no background absorbance above 260 nm. This is acceptable because detection wavelengths at 254 nm for aromatic molecules and 280 nm for proteins have been used often for reversed-phase LC. Detection below this wavelength is possible due to zeroing of the signal at the start of an LC run, but was difficult due to the low signal-to-noise ratio. Cytochrome c is a colored protein that is easily detectable at 409 nm. All efficiency calculations and retention times were taken using wavelengths of 254 nm and above.

Viscosity of the mobile phase will have an effect on the overall HETP for a mobile phase. It is a dominant factor in the diffusion coefficient of the solute in the mobile phase, D_m, present in the numerator of the B term and the denominator of the C_m term. Viscosity measurements were taken for different percentages of IPAF in water and plotted as ln viscosity, a function of volume fraction of IPAF in water, as shown in Figure 2. The viscosity diminishes markedly as the water content is raised. The viscosity of undiluted (volume fraction 1.0) IPAF was found to be 61 cP; however, even the presence of trace solvents can markedly affect the viscosity of AAFs, which are quite hygroscopic. This viscosity trend in Figure 2 is similar to those found for other short chained AAFs (13, 14). This curve can be fitted as a third order polynomial $y = A_1 + A_2x + A_3x^2 + A_4x^3$, where y = ln viscosity and x = volume fraction of IPAF in water; the equation is given in Figure 2. The inverse ln of A_1 should be similar to the viscosity of water, as previously described (12); this value is 1.08, which reasonably compares to 0.89 cP for water at 25°C. Greaves *et al.* reported the

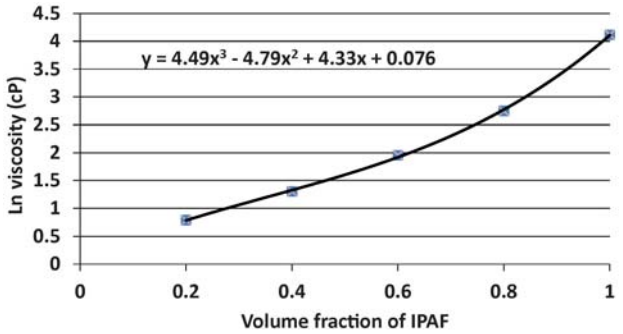

Figure 2. Plot of ln viscosity as a function of volume fraction of IPAF in water. Line fitted to third-order polynomial.

viscosities of BAF and 2MPAF as 70 and 225 cP (17). Considering the shorter branched nature of the IPAF cation, a viscosity in the range of 60–70 cP seems reasonable.

The apparent polarity index (P′) was calculated using experimental data fit into the equation $k'_2/k'_1 = 10^{(P'_2-P'_1)/2}$, where $P'_2-P'_1$ is solved and set equal to $[P'_{IPAF}\Phi_{IPAF(2)} + P'_W\Phi_{W(2)}] - [P'_{IPAF}\Phi_{IPAF(1)} + P'_W\Phi_{W(1)}]$, where P'_W is the polarity index of water (10.2) and the unknown of interest P'_{IPAF}, the polarity index of IPAF, and Φ = fraction of the corresponding solvent, either IPAF or water (w). Three pairs of k' values were used for two different analytes, caffeine and *p*-nitroaniline, to obtain the average apparent P′ of 6.0 ± 0.3 ($n = 6$) for IPAF. This value is in line with traditional RPLC solvents. For comparison, it has been previously reported that EAF, propylammonium formate (PAF) and BAF have polarity values of 6.4 ± 0.1, 5.8 ± 0.1 and 5.2 ± 0.1, respectively (14). Methanol has a P′ value of 5.1 and acetonitrile has a P′ value of 5.8, indicating that IPAF is a slightly weaker modifier solvent, as expected, but not nearly as nonpolar as tetrahydrofuran (THF), with a P′ of 4.0, or hexane, with a P′ value of 0.1. The P′ for IPAF is also similar to analogous short straight chain AAFs. That the polarity of AAFs is similar to that of polar organic solvents is likely due to the micelle type ion pair structures that are formed, as previously described in the discussion about pH. The stability of these micelle structures are thought to be stable over a wide range of water content (26), and therefore should act like an organic solvent in competing for the hydrophobic sites of a reversed-phase column packing, reducing solute retention.

Solvent strength (S) parameters were compared for methanol, acetonitrile and IPAF using both caffeine and *p*-nitroaniline, as predicted by the literature (27) equation $\log k' = \log k'_w - S\Phi$, where k' = retention factor k', k'_w = retention factor for 100% water, and Φ = volume fraction for the modifier solvent (Figures 3A and 3B). This equation is considered to be linear over a limited range of 20 to 75% modifier solvent. The solvent strength parameter was shown experimentally to be 2.41 for IPAF, similar but slightly less than those for methanol (2.74) and acetonitrile (2.72), all from the slopes of the linear least squares regression equations of Figure 3A (14). The similarity of the S values for methanol and acetonitrile is comparable to that reported previously (28); THF would be expected to have a S value approximately 1.5 times higher. Caffeine, possibly because of the polar structure, shows a curved relationship for IPAF as well as methanol (Figure 3B). These data can be fitted

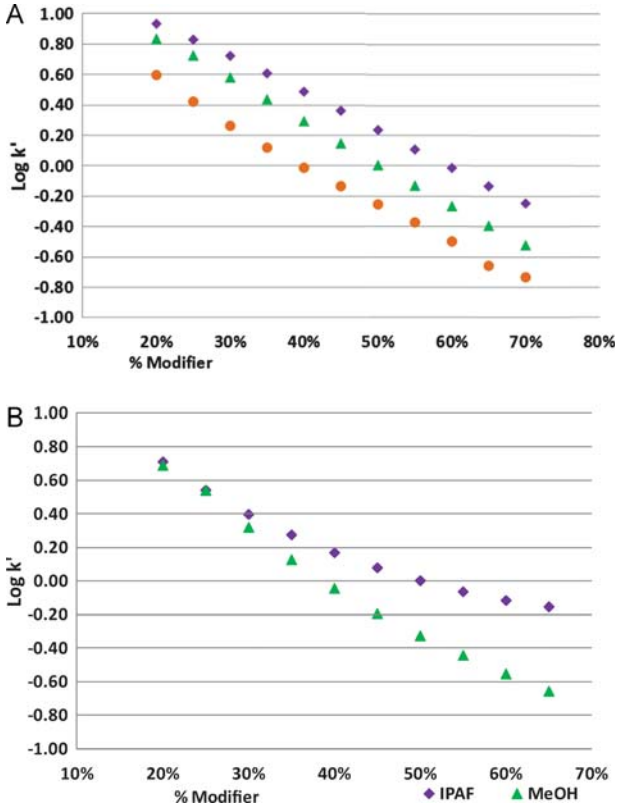

Figure 3. Retention factors for *p*-nitroaniline in methanol, acetonitrile, and IPAF using the YMC-Basic 10 μm C18 column. Linear fit equations (log k′ versus volume fraction of modifier solvent) were used to identify the solvent strength (slope) terms. For methanol, $y = -2.74 + 1.39$ with $R^2 = 0.9993$; for acetonitrile, $y = -2.72 + 1.12$ with $R^2 = 0.9941$; for IPAF, $y = -2.41 + 1.44$ with $R^2 = 0.9991$. IPAF is shown with diamonds, methanol is shown with triangles and acetonitrile is shown with circles (A). Retention factors for caffeine in methanol and IPAF using the YMC-Basic 10 μm C18 column fitted to quadratic equations using log k′ and volume fraction of the modifier solvent. For IPAF, $y = 3.08x^2 - 4.51x + 1.48$ with $R^2 = 0.9997$; for methanol, $y = 3.06x^2 - 5.71x + 1.76$ with $R^2 = 0.9996$. IPAF is shown with diamonds; methanol is shown with triangles (B).

to a quadratic relationship, as discussed by Poole (27). For comparison, MAF showed a solvent strength parameter for *p*-nitroaniline of 2.05 using a Polaris C18 column, and that for methanol was found to be 2.8 (13). Previously reported S values are 1.90, 2.20, 2.50 and 2.55, respectively, for EAF, PAF, BAF and methanol of *p*-nitroaniline using a PRP-1 column (14). Considering the PRP aromatic stationary phase, these values are comparable to ours using a C18 silica phase.

During the course of the previous solvent strength parameter determinations (Figures 3A and 3B), the column backpressure was recorded. Traditional analytical LC columns have a normal operating pressure of approximately 2,000 psi, and a maximum operating pressure of approximately 6,000 psi. The pump operating backpressure for a YMC Basic column with IPAF as the organic solvent was determined from 20 to 60% IPAF in water at 0.5 mL/min. At 20% IPAF–80% water, a pressure of 500 psi was recorded, similar to the backpressure for standard organic solvents in the 10–30% range. The start of an exponential increase occurred at 40% IPAF in water, and at 50% IPAF–50% water, a pressure of 1,250 psi was reached. At

70% IPAF–30% water, a pressure of 3,500 psi was noted and the study was discontinued. EAF, PAF and BAF all showed a more linear relationship proportional to the increasing RTIL concentration (14). The pressure dependence on mobile phase composition is different from traditional RPLC solvents by not gradually increasing to a maximum and then decreasing as 100% methanol, or acetonitrile, is reached (25, 27).

van Deemter plot studies

Figure 4 shows the separation of the test solutes used as potential indicators for the van Deemter studies. The retention of nicotinamide was too low, making accurate determination of the peak width difficult. The other two solutes showed good peak shape and could be used as van Deemter plot test compounds. Figure 5A compares the van Deemter plots for IPAF with those of methanol and ethylene glycol for acetophenone as the test solute. Ethylene glycol was chosen for comparison because it has a higher viscosity, for a standard organic solvent, 16.5 cP at 25°C (25). Online mixing was done in which pure water was used in the "A" solvent reservoir and a mixture of 80% IPAF (or ethylene glycol or methanol)–20% water was used in the "B" solvent reservoir. For example, to obtain the 25% IPAF–75% water mixture, the chromatography software was programmed to draw 31.3% of the B solvent. This achieved the desired net solvent composition. Replicates of $n = 4$ were done at flow rates of 1.0 mL/min. Reproducibilites, as measured by relative standard deviation (RSD) in the acetophenone plot, were 0.2%, 0.7% and 1.5%, respectively for methanol, ethylene glycol and IPAF. RSD for IPAF at 1.6 mL/min increased to 7.5%. Peak symmetry values ranged from 0.98 to 1.02, close to 1, as measured by the ratio of peak widths on either side of the vertical at peak maximum.

Comparing the van Deemter plots in Figure 5A, IPAF shows slightly better separation efficiency at the lowest flow rates studied than both methanol and ethylene glycol. In this region

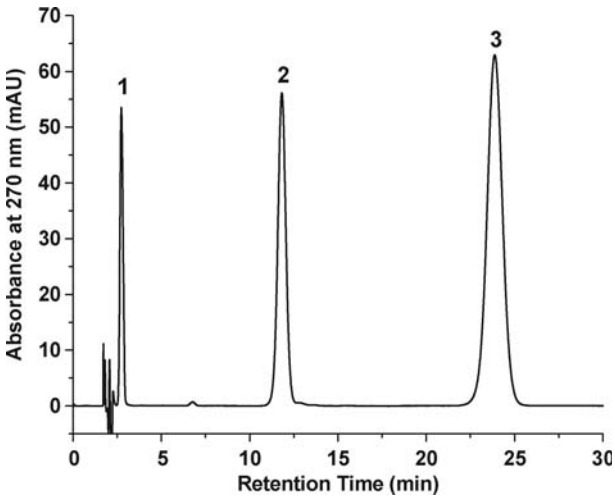

Figure 4. Chromatogram of the van Deemter plot test mixture separation on the YMC C18 column using 25% IPAF–75% water at 1.0 mL/min. Solvent reservoir A was pure water and reservoir B was a mixture of 80% IPAF–20% water. The instrument was programmed to draw 31.3% of solvent B for a net mixture of 25% IPAF–75% water. Absorbance detection at 270 nm; 25 μL injection of 200 mg/L solutes; peaks: 1, nicotinamide; 2, *p*-nitroaniline; 3, acetophenone.

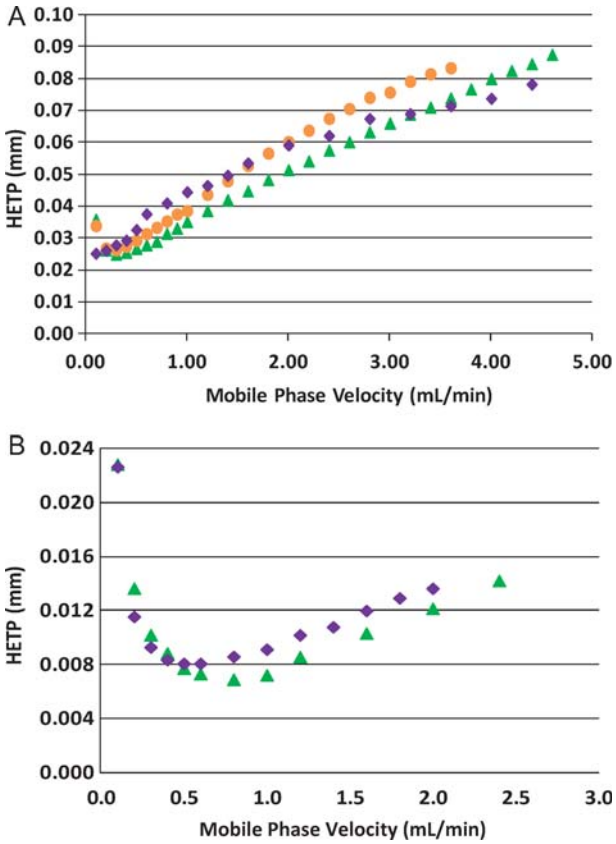

Figure 5. van Deemter plots for separation of acetophenone in 25% of three modifier solvents (ethylene glycol, methanol and IPAF) using the YMC C18 column. Absorbance detection at 254 nm. IPAF is shown with diamonds, methanol is shown with triangles and ethylene glycol is shown with circles (A). van Deemter plot for the Phenomenex phenyl column using *p*-nitroaniline as the test solute. Mobile phases: 25% IPAF–75% water or 25% methanol–75% water. IPAF is shown with diamonds and methanol is shown with triangles (B).

of the plot, the longitudinal diffusion B/u term dominates. This is due to the higher viscosity of IPAF, resulting in a small diffusion coefficient for each analyte. It has been previously reported, but not researched, that the C term in van Deemter plots made using the AAF class of ionic liquids can appear to not increase linearly at high flow rates (13, 14). The flow rate was investigated out far enough in Figure 5A that the C term for IPAF actually crosses over the C term for methanol at 3.2 mL/min for both analytes. This trend results in a lower theoretical plate height when at elevated mobile phase flow rates than traditional reversed phase separation solvents. This unusual C term profile was repeated using a second new 10 μm C18 YMC column and also the RSil 10 μm C18 column several months later by a different researcher.

In Figure 5A, both methanol and ethylene glycol HETP plots are linearly increasing in the C term, as expected. The high viscosity of IPAF, compared to ethylene glycol or methanol, seems to be causing a mixing problem that is not facile or complete enough at high pressure or flow rate. A plot of pressure versus flow rate also shows a discontinuity with a change in slope at the same flow rate for the IPAF van Deemter plot. The Dionex instrument does use a 350 μL online mixer located after the solvents are mixed and before the samples are injected. A

comparison of van Deemter plots using different volume fractions of IPAF to water was made. The C term plateau break point for 15% IPAF in water occurs at 2.0 mL/min, while those for 25% IPAF and 35% IPAF were noted at 1.4 and 0.8 mL/min, respectively. A plot of column backpressure versus flow rate also shows a discontinuity at the same point in the HETP plot. When using a premixed IPAF–water mobile phase, the C term does become linear and follows a more traditional pattern.

However, when doing on-line mixing of the mobile phase similar to that in Figure 5A, the van Deemter plots for a 3 μm phenyl silica column seem linear in the C term for both methanol and IPAF (Figure 5B). The maximum mobile phase velocity able to be reached is 2.0 mL/min, which resulted in a backpressure of slightly over 5,000 psi. As expected, IPAF shows a smaller HETP than methanol at flow rates up to 0.5 mL/min. It seems that the short length and wide ID of the YMC column coupled with the relatively large 10 μm particles may also be significant factors in the flattening of the van Deemter profile at high flow rates.

However, two 250 × 4.1 mm i.d. 10 μm particle columns were tested and found to generate a typical linearly rising van Demeter plot in the C term, similar to that in Figure 6A for the Jupiter C5 5 μm, 30 nm column. The C term trend in Figure 6B using a 10 μm particle size 30 nm pore size polystyrene-divinylbenzene column is fairly linear, but there appears to be some fluctuation of the points at high flow rate. Although pressure constraints limited the flow rate to just above 2 mL/min, only a slight change in slope was noted. Fortunately, longer columns preferably packed with 5 μm particles with 30 nm pores are considered ideal for semi-preparative protein separations (our primary aim) and it appears that these types of columns give typical van Deemter profiles over a wide flow rate range.

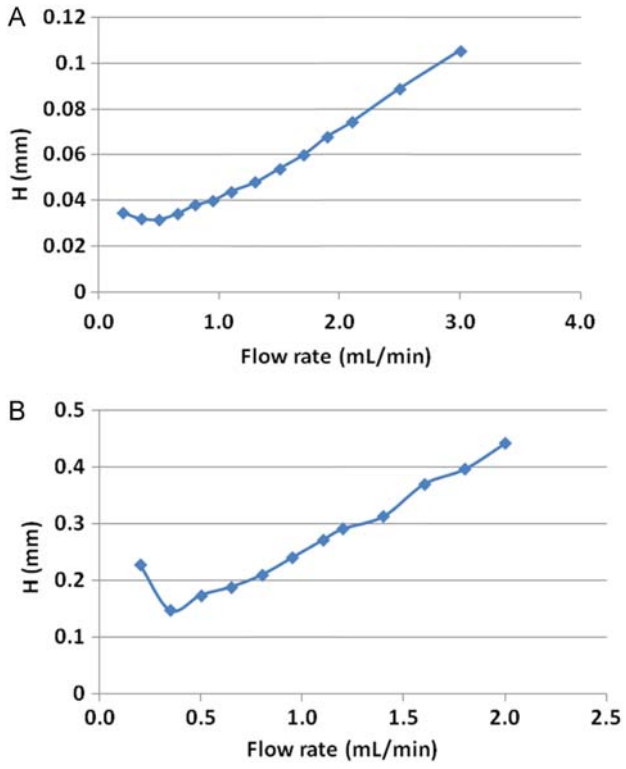

Figure 6. van Deemter plot for the Phenomenex C5 column. Mobile phase: 25% IPAF–75% water. Injection volume: 5 μL of 50 mg/L tryptophan (A). van Deemter plot for the Hamilton PRP-3 column. Mobile phase: 25% IPAF–75% water. Injection volume: 50 μL of 50 mg/L tryptophan (B).

Protein stability in IPAF

RTILs have previously shown to stabilize proteins in solution (19–23); however, this stability has yet to be shown during the course of a chromatography separation. This is important to show because the semi-preparative separation of proteins in their native form using AAF mobile phases is the long-term goal of this research program. The ability of IPAF to stabilize cytochrome c was first verified using fluorescence spectroscopy. Figure 7 shows tryptophan fluorescence of the protein cytochrome c as a function volume percent of IPAF and methanol in water. As the methanol content increases above 40%, fluorescence of cytochrome c increases due to denaturation, as evidenced by exposure of tryptophan and its removal from the heme environment where the fluorescence is quenched. For IPAF, the fluorescence remains low, indicating that the protein remains in native conformation until approximately 70% volume percent.

This type of experiment has been done previously for MAF and EAF (22). Previously, the protein began to unfold at approximately 40% volume percent when dissolved in methanol–water solution, as has again been verified in Figure 7. When MAF or EAF was used as a solvent, the protein began to unfold at 70–80% volume percent.

The fluorescence of cytochrome c was measured using an AAF mobile phase for the first time during a reversed-phase LC separation and compared to that for methanol. Tryptophan is used as an internal standard to follow any changes in fluorescence due to solvent viscosity or polarity. The separation was achieved using a gradient either 5% IPAF to 50% IPAF or 5% methanol to 50% methanol from 0 to 10 min. The peak area ratio for tryptophan using the methanol gradient in Figure 8A to tryptophan with the IPAF gradient in Figure 8B was 2.3 and the corresponding peak area ratio for cytochrome c was 5.0. The

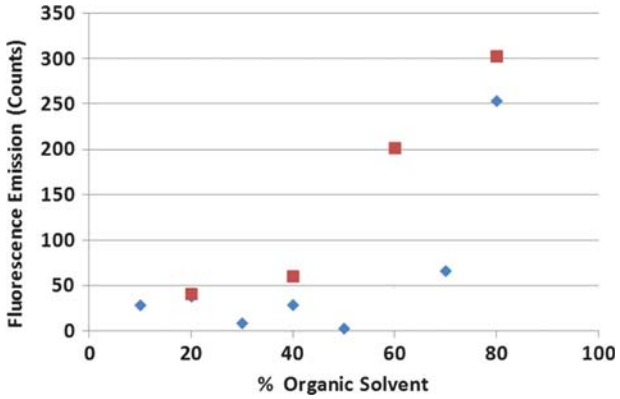

Figure 7. Fluorescence emission at 360 nm of cytochrome c dissolved in varying amounts of methanol and IPAF. Excitation wavelength is 270 nm. The background fluorescence has been subtracted out for both solvents. Methanol is shown with squares and IPAF is shown with diamonds.

higher fluorescence intensity for tryptophan and cytochrome c in methanol as compared to IPAF was verified using a standard fluorescence instrument for an independent sample. This observation is likely due to the background fluorescence of the IPAF, which has a maximum excitation wavelength near 350 nm, somewhat quenching the fluorescence of the tryptophan.

It is well known that, assuming moderate k' values, increased temperature causes better separation efficiencies. However, at a high enough temperature, the protein should denature. For this series of separations from sub-ambient to 50°C, a Hamilton PRP-3 column was used because it is less sensitive to temperature than silica-based columns. These series of chromatograms are shown in waterfall format in Figures 8A and 8B. Cytochrome c elutes first, followed by tryptophan. As expected,

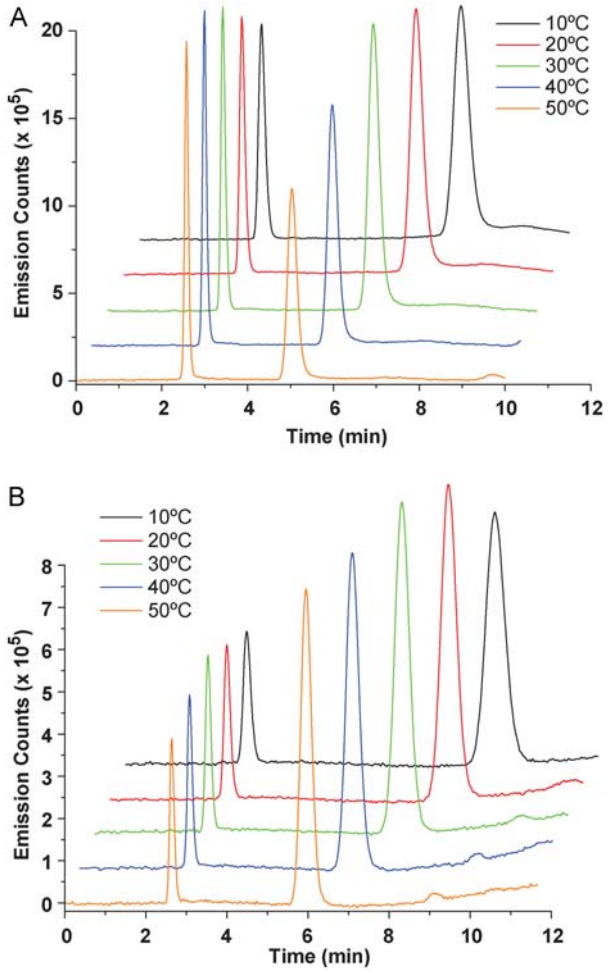

Figure 8. Overlay-style chromatograms of a tryptophan and cytochrome c separation at different temperatures from 10 to 50°C using the PRP-3 column. The gradient was programmed from 5% methanol–95% water to 50% methanol–50% water mobile phase gradient from 0 to 10 min. Fluorescence detection was 270 nm excitation and 350 nm emission. The chromatograms from top to bottom, right to left are: 10, 20, 30, 40 and 50°C. Injection volumes (25 μL) were 50 mg/L tryptophan and 200 mg/L cytochrome c (A). Overlay style of five chromatograms of a tryptophan and cytochrome c separation at different temperatures ranging from 10 to 50°C using the PRP-3 column. The gradient was programmed from 5% IPAF–95% water to 50% IPAF–50% water mobile phase gradient from 0 to 10 min. Fluorescence detection was 270 nm excitation and 350 nm emission. The chromatograms from top to bottom, right to left are: 10, 20, 30, 40 and 50°C. Injection volume was (25 μL) 50 mg/L tryptophan and 200 mg/L cytochrome c (B).

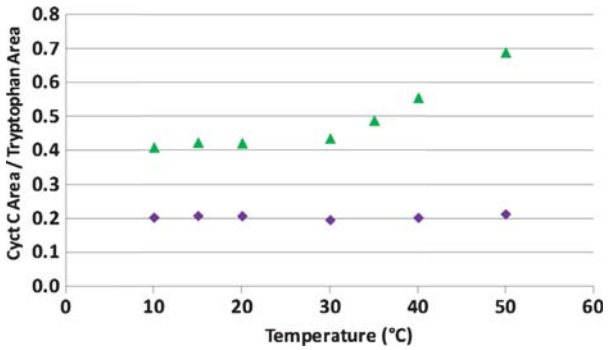

Figure 9. Ratio of cytochrome c to tryptophan fluorescence peak areas obtained during the temperature study separations in Figure 8. Methanol is shown with triangles and IPAF is shown with diamonds.

increased temperature causes decreased retention and faster analysis times for both peaks. Two factors influence the intensity of the cytochrome c fluorescence peak: (i) change in viscosity of the mobile phase and (ii) a possible tendency to denature at higher temperatures. Column compartment temperatures were increased in 10°C increments from 10 to 50°C to show any protein denaturing effects. The samples, dissolved in water, were all kept at 15°C in the autosampler tray for the duration of the experiment to prevent any denaturing effects before injection. The backpressure as a function of temperature for IPAF at the beginning of the gradient decreased from 600 to 400 psi from 10–50°C, while the pressure range for the end of the solvent gradient was approximately 1,800 to 1,200 psi.

Using IPAF, the cytochrome c/tryptophan peak area ratios were consistent at approximately 0.20 over the entire tested temperature range (Figure 9). Using methanol, the cytochrome c/tryptophan peak area ratios rose modestly from 0.41 to 0.44 from 10–30°C and then increased markedly to 0.68 at 50°C. For methanol, at 35°C, the ratio of cytochrome c fluorescence with respect to tryptophan, the internal standard, increases, clearly indicating denaturing of the protein. IPAF did not show this same increase in fluorescence ratio. The ratio remained virtually constant up to a temperature of 50°C. This result for IPAF correlates with standard circular dichroism data previously obtained for EAF (22), in which 20% EAF–80% water was able to maintain protein conformation through 55°C. We feel that IPAF could likely better stabilize proteins at higher temperatures due to its higher viscosity. Temperatures above 50°C were not tried. Potential degradation of IPAF at 60°C under N_2 was monitored by UV-VIS spectrophotometry as a function of time. No significant change in the absorbance at 280 nm or higher wavelengths was noted for the first 4 h; there was a slight increase of 0.08 absorbance units at 5 h. Good stability of IPAF with respect to UV cutoff (no shift to longer wavelength) when stored at room temperature was evident for at least several months.

Conclusion

We have demonstrated that IPAF is a suitable replacement for methanol as an RPLC mobile phase for the separation of small molecules. Its physical properties are consistent with previously reported data for the AAF class of RTILs. IPAF is easily

synthesized and is far less expensive than previously reported AAFs. Flattening of the van Deemter profiles was observed for the separation of *p*-nitroaniline and acetophenone at high flow rates above 2 mL/min using a relatively short, wide diameter, large particle silica-based column. However, typical van Deemter plots were noted for longer 10 micron particle columns or shorter columns packed with smaller particles. It is recommended that IPAF–water mobile phases be mixed on-line using a diluted IPAF stock solution to ensure reproducibility. IPAF has been shown to hold cytochrome c in conformation at both higher solvent volume fractions and temperatures than corresponding methanol solutions. Mobile phase comparisons are underway of standard organic solvent modifiers with IPAF for liquid chromatography with fluorescence detection of other proteins such as lactate dehydrogenase, which has subunits.

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