ABSTRACT

WEISNER, NATHANIEL HALL. Fluoroalcohol Mediated Aqueous Two-Phase Systems: Characterization and Applications in Organic Synthesis and Extraction. (Under the direction of Dr. Edmond Bowden).

Solvatochromic and small molecule partitioning studies were used to characterize two-phase solutions formed by the addition of a third miscible solvent to aqueous solutions of 1,1,1,3,3,3-hexafluoroisopropanol (HFIP). These aqueous HFIP-Organic (Aq/H-O) two-phase solutions are composed of an Aqueous(Aq) phase and HFIP-Organic (H-O) phase. Solvatochromic studies were used to determine the dipolarity, hydrogen bond basicity and hydrogen bond acidity in both phases of Aq/H-O systems formed under different conditions. Small molecule partitioning allowed for methylene and functional group selectivity of the two-phase systems to be determined. In addition ternary phase diagrams were prepared and phase compositional analysis was done to determine the amounts of each solvents in both Aq and H-O phases for selected systems.

Applications for these type of two-phase systems for organic synthesis were then demonstrated. The primary reaction investigated was a Friedel-Crafts alkylation which benefits from the unique properties of HFIP. The presence of the Aq phase and two-phase nature of the solution was shown to benefit the reaction and allow for easy product recovery. Diels Alder reactions were also shown to proceed in these solutions offering a new largely aqueous solution for organic synthesis.

The use of these systems for extractions was then demonstrated on both small molecules and biological molecules for proteomics applications. Two-phase solutions formed using small amounts of solvents were used for microextraction of amines and polycyclic aromatic hydrocarbons from water and subsequent GC-MS analysis. The

inclusion of an acid anhydride derivatizing agent in the HFIP-Solvent mixture allowed for simultaneous derivatization and extraction of amines increasing their extraction and improving GC analysis.

Application of Aq/H-O systems for proteomics applications were demonstrated by extracting proteins from mouse red bloods cells (RBCs). Proteomics analysis was then done on the proteins in both phases to identify the types of proteins that partition into each phase. The H-O phase was shown to extract more hydrophobic proteins based on GRAVY values and a higher % of the proteins identified in the H-O phase were integral membrane proteins compared to the Aq phase and control.

Fluoroalcohol Mediated Aqueous Two-Phase Systems: Characterization and Applications in Organic Synthesis and Extraction

by
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DEDICATION

This dissertation is dedicated to my parents, Brad and Elaine Weisner, who have helped get me to this point by always supporting me. Also to my beautiful girlfriend Lindsay whose patience and support during these past few years has made things much easier on me. Your love and support is greatly appreciated.

BIOGRAPHY

Nathaniel Hall Weisner was born in Rocky Mount, North Carolina on July 27, 1987. He graduated from North Carolina State University in 2010 with a B.S. in Polymer and Color Chemistry from the College of Textiles. In 2010 he enrolled in Graduate School in the Department of Chemistry at NC State University where he studied under Dr. Morteza Khaledi. His final year and a half of research for his PhD was completed at the University of Texas Arlington while still remaining a NC State student.

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Chapter 1 - Introduction

Aqueous- Based Two-Phase systems in chemical synthesis and analysis

The miscibility of solvents is primarily determined by their relative polarity/hydrophobicity. Water is a polar solvent with a strong hydrogen bonding network and is immiscible with non-polar solvents due to the hydrophobic effect. The hydrophobic effect arises when nonpolar solutes are introduced into a solution of water causing changes in the water hydrogen bonding strucuture.[1] The hydrophobic effect is cited as the reason nonpolar molecules cluster in water and has been attributed to rate accelerations observed for organic reactions in water[2] as well as protein folding in aqueous solutions[3]. The size of the nonpolar solute plays a large role in determining how the hydrogen bonding structure of water is affected and whether or not an interface is formed. For a small non polar solute, water restructures its hydrogen bonding network around the solute with only a small thermodynamic cost, maintaining four hydrogen bonds per water molecule.[4] When many nonpolar solutes are present water is not able to maintain its hydrogen bonding network around the solutes and it becomes more favorable for an interface between water and the nonpolar molecules to be formed, leading to a two-phase solution. The formation of an interface comes at a high free energy cost which must be compensated for by the forces driving phase separation. The driving forces behind interface formation come from the fact that the interaction energy between water molecules and solute molecules are much lower than those of water-water and solute-solute.[5] The forces contributing to the formation of the interface increase linearly with the volume of the two phases while the forces against it increase linearly with the surface area of contact. So as the amount of nonpolar solute

increases, the forces contributing to interface formation are increasing faster relative to the forces against it due to a decreasing surface area / volume ratio.[4] Two-phase solutions can also be formed in aqueous solutions containing either two polymers or surfactants which are known as aqueous two-phase systems (ATPS). The solutions separate into two-phases, each containing one of the polymer or surfactant molecules. The driving force behind separation in ATPS is again that the interaction between individual polymers or surfactants is greater than the mixed interaction at certain concentrations. ATPS have been used extensively for the separation of biomaterials due to their aqueous nature leading to lower interfacial tension and causing less denaturation of molecules such as protiens.[6] Nonionic surfactants undergo phase separation with increasing temperature which is known as cloud point separation. The resulting ATPS has one phase that is enriched in surfactant and another largely aqueous phase.[7]

In addition to traditional solvents and polymer/surfactant ATPS, room temperature ionic liquids (IL's) are a relatively new liquid medium that can be used to create two phase solutions with water. IL's are liquids composed entirely of ions that are known for their low volatility, low flammability and high boiling points and are considered as greener alternatives for traditional organic solvents in many areas.[8] Two-phase solutions can be created by using a hydrophobic IL in water similar to an organic solvent or using two hydrophilic IL's that will form an ATPS. Deep eutectic solvents are a related type of ionic solvent and are formed from mixtures of quaternary ammonium salts and hydrogen bond donors such as amines or carboxylic acids and are typically cheaper and easier to form than IL's.[9] Similar to IL's deep eutectic solvents have been used to form two-phase solutions with water for extraction purposes.[10]

Applications involving two-phase systems take advantage of their two-phase nature typically either to separate or extract molecules or allow molecules to interact that have different solubility's. Liquid-liquid extraction (LLE) is a very well established extraction method traditionally involving the use of an organic solvent to extract molecules from aqueous solutions although extractions in ATPS would also be classified as LLE. LLE has been applied to a wide range of molecules from studying pesticides in water [11,12] to purifying proteins [13]. With LLE being such a well-established technique, current research focuses on improving LLE methods by improving their performance or their greenness by using less toxic solvents for extraction or using smaller amounts of solvents while maintaining performance.

Another application of two-phase systems is for synthesis reactions involving phase transfer catalysis (PTC). PTC allows for a reaction to occur between two substances present in two immiscible solvents. Typically an anionic reactant is present in an aqueous phase while the other reactant is in the solvent phase. The addition of tetraalkylammonium or phosphonium salt greatly accelerates the reaction rate by forming a complex with the anion in the aqueous phase allowing it to partition into the organic phase for the reaction to occur although the exact mechanism is still debated.[14] Biphasic water-organic solutions have been used for biocatalyst reactions where the reactant has low water solubility such as steroids or lipids. The biocatalyst, such as an enzyme, is present in the aqueous phase or at the interface while the organic phase serves a reservoir for the substrate. With agitation some of the substrate is transferred to the aqueous phase where the biotransformation occurs and the products can return to the organic phase. PTC and biphasic biocatalyst both take advantage of the two-phase nature by allowing substrates with different solubility's to react.[15,16]

This work will investigate an unexplored biphasic medium for both extraction and biphasic synthesis applications. The nature of phase separation is based on the presence of hexafluoroisopropanol (HFIP) in aqueous solutions which can selectively solvate both water miscible and immiscible solvents leading to two-phase solutions where one phase is largely aqueous and the other contains HFIP and the other solvent. Previous research in our group has explored coacervates induced by the addition of HFIP to surfactant or polymer solutions.[17] The properties of the HFIP-mediated two-phase systems will be investigated through solvatochromic and partitioning probe studies. Additionally applications for these two-phase systems for synthesis and extraction of both small and biological molecules are demonstrated.

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Chapter 2

Analysis of Phase Properties for Aqueous HFIP-Solvent Two-Phase solutions

Abstract

The addition of a third solvent to aqueous solutions of hexafluoroisopropanol (HFIP) that is miscible with both water and HFIP has been shown to cause phase separation in certain cases. One of the phases is largely aqueous while the other is composed primarily of HFIP and the other solvent. Systems formed with the solvents acetone, N,N-dimethylformamide (DMF), tetrahydrofuran (THF) and butanone were investigated for this work. The THF and butanone systems were shown to generate larger two-phase regions than DMF and acetone through the production of ternary phase diagrams. Solvatochromic studies were used to characterize the phases based on their dipolarity as well as hydrogen bonding acidity and basicity. Dipolarity and basicity could be influenced by changing either the solvent used for phase formation or changing the ratio of HFIP:solvent used for phase formation. All of the HFIP-solvent phases showed strong hydrogen bond acidity based on the solvatochromic results. The partitioning of small molecules in the two-phase solutions was also studied. The phases formed with THF and butanone showed higher partition coefficients for all compounds due to their more hydrophobic nature which was confirmed by determining methylene selectivity values for the different two-phase solutions. Functional group selectivity was also investigated and could be influenced by the solvent used as well as the HFIP:solvent ratio.

Introduction

Takamuku and co-workers first reported phase separation in aqueous solutions with two other miscible solvents of hexafluoroisopropanol (HFIP) and dimethylformamide[1] (DMF) and subsequently in mixtures of water – HFIP and a miscible analog of DMF [2]. In this study, three component systems composed of water, HFIP, and one other solvent that is miscible with both HFIP and water (ketones, ethers) are studied. Takamuku et al. developed ternary phase diagrams for several of these systems formed with amides providing valuable information on conditions where phase separation occurs and helps in determination of the underlying mechanism. They found that increasing hydrophobicity of the third miscible solvent would facilitate the phase separation and leads to larger organic-rich phase.

The physio-chemical properties of solvents determine how they interact with solutes through various types of intermolecular interactions and are important to know when choosing solvents for a specific application. Developing an understanding of the properties of two-phase solutions formed by mixing three miscible solvents is important in gaining further understanding of the mechanism of phase separation as well as potential applications. Solvent properties such as dielectric constant (ε_r) and dipole moment (μ) are often used to define solvent polarity but cannot by themselves take into account all of the different kinds of interactions that are involved in the solvation of solutes such as hydrogen bonding, dipolarity and dispersion.[3,4] The combination of all of these solvent-solute interactions influence a wide range of chemical processes such as solubility[5], retention in chromatography[6] and partitioning[7]. In this study, a combination of solvatochromic measurements and partitioning probe studies are used in order to gain an understanding of the general solvent properties exhibited in these Aqueous / HFIP-solvent two phase systems. In addition, ternary phase

diagrams are prepared to establish the conditions under which these two-phase solutions form.

Solvatochromism is exhibited by molecules that show changes in their spectroscopic properties in different solvent environments. Substances that exhibit solvatochromism have been used to develop scales to quantify solvent polarity and the specific interactions such as hydrogen bonding and dipolarity that constitute polarity. One of the earliest solvatochromic scales was developed by Kosower in 1958 and was known as the Z scale.[8] The scale utilized the hypsochromic shift of 1-ethyl-4-(methoxycarbonyl)pyridinium iodode with increasing solvent polarity. One benefit of this compound was that its charge transfer band occurs in the visible region avoiding interference by solvent peaks which do not often absorb in that region.

Eventually a new set of pyridinium betaine dyes was introduced by Drimroth and Reichardt that had much larger λ_{max} shifts allowing for the creation of more sensitive scales.[9] In particular, compound #30 (2,6-diphenyl-4-(2,4,6-triphenyl-N-pyridinio)phenolate) which became known as Reichardt's Dye, exhibited the largest shift ($\Delta\lambda$=357nm) when changing the solvent from water to diphenyl ether. This dye was used to develop the $E_T(30)$ scale with E_T standing for "energy of transition". The structure of $E_T(30)$ and a related dye known as $E_T(33)$ are shown in figure 2-1. The $E_T(30)$ scale is calculated by the equation:

$$E_T(30)\left(\frac{kcal}{mole}\right) = (N_A \cdot c \cdot h)/(4.184 \times 10^6 \cdot \lambda_{max}) = 28592/\lambda_{max} \qquad \text{Eq. 1}$$

Where N_A(Avagodro's number) = 6.022×10^{23} /mole, c (speed of light) = 2.998×10^8 m/s, and h (Planck's constant) = 6.626×10^{-34} J·s and 4.184×10^6 is the conversion factor for Joules to calories. A normalized version of the scale was also developed, E_T^N, which is unit less and varies between 0 and 1 with the 0.000 value being represented by tetramethylsilane and 1.000

corresponding to water. This scale has been used to measure the polarity of many solvents and solvent mixtures.[10-12]

While E_T scales give good indications of solvent polarities they do not provide the contributions of the various types of interactions between solvents and solutes such as hydrogen bonding and dipolarity. By using various solvatochromic probes to measure the contributions from these different interactions Kamlet and Taft quantified the hydrogen bond donor strength (α), hydrogen bond acceptor strength (β) and dipolarity (π^*) for hundreds of solvents.[13-15] The ability to determine the contribution of these different interactions led to the development of the Linear Solvation Energy Relationship (LSER) equation which describes solvation effects on physio-chemical processes as follows:

$$XYZ = XYZ_0 + s\pi^* + a\alpha + b\beta \qquad \text{Eq 2-2}$$

Where XYZ can represent various properties such as a reaction rate, equilibrium constant or spectroscopic properties for a compound.[16] The constants a and b represent how much changing the hydrogen bond donor (HBD) acidity (α) or hydrogen bond acceptor (HBA) basicity (β) of a solvent impact XYZ. The $s\pi^*$ term represents the combined effects of solvent dipolarity/polarizability.

The values of the different solvatochromic parameters were determined by measuring the λ_{max} of appropriate pairs of probes in different types of solvents: 1) solvents with no hydrogen bonding capabilities; 2) HBA solvents and 3) solvents that are both hydrogen bond donors and acceptors. 4-nitroaniline (4NA) and N,N-diethyl-4-nitroaniline (DENA) were used as one probe pair because of their similar structures giving them similar polarity and HBA abilities while DENA is incapable of being a HBD. β values for solvents were determined from the difference in Δν between the two probes. A normalized equation was

developed with a β value of 1.00 being represented by hexamethylphosphoramide and 0.00 is cyclohexane. The probe pair 4-nitrophenol and 4-nitroanisole can also be used in a similar manner.

In order to measure π* values for solvents the contribution from hydrogen bonding had to be taken into account. For non-hydrogen bonding solvents this was straight forward since α=β=0. By measuring probes with no HBD ability in HBA solvents they were able to measure π^* for HBA solvents since no hydrogen bond interactions could take place between the probe and solvent. Determining π* for solvents with both HBA and HBD properties was more difficult as it requires probes without any hydrogen bonding capabilities whose spectroscopic properties were sensitive to changes in π*. These types of probes proved more difficult and they used compounds such as 4-nitroanisole for which they determined the spectroscopic properties were not influenced by HBD solvents. This allowed them to determine π* for water and alcohols. [14] Normalized scales were developed for DENA and 4-nitroanisole with cyclohexane as 0.00 and dimethylsulfoxide as 1.00.

The α scale was formed using Et(30) and 4-nitroanisole as the probe pair and following the same method used for creating the β scale. ET(30) was found to act as a hydrogen bond acceptor so if the π^* value for the solvent was known α could be determined since it has no hydrogen bond donor ability making bβ ~0.[13] The scale was normalized with methanol as 1.00 and later applied to two other similar probes, ET(33) and Michler's ketone (MK)[17] which are used in this work to determine α values. In addition to solvatochromic studies partition coefficients of test solutes can be used to gain insight into the interactive nature of two-phase systems. Current research in these areas focuses on defining the

Kamlett-Taft parameters of new types of liquids such as ionic liquids[18,19] as well as developing new solvatochromic probes that give yield further insight on solution properties.[20]

Partitioning in these two-phase systems will be primarily driven by hydrophobicity with smaller contributions from other types of interactions measured by the solvatochromic probes. Partitioning in octanol-water biphasic systems is traditionally the standard system used for determination of solute hydrophobicity.[21] Since the partition coefficient is an equilibrium value it only depends on the nature of solutes and the phases involved and not on the relative phase volumes. The effects of changing paramteters such as the ratio of HFIP:solvent, % solvent and solvent type on compound partitioning was studied. Partitioning of a homologous series allows for the determination of methylene selectivity (α_{CH2}) which is the slope of linear relationship between Log(K) and the number of methylene groups on the compound as shown in the following equation:

$$\log K = (\log \alpha_{CH2}) * n_{CH2} + b$$

Methylene selectivity has been used as a measure of hydrophobicity in liquid-liquid extraction[22,23] and chromatography[23,24]. In addition to methylene selectivity, functional group selectivity can be determined from partition coefficients. Functional group selectivity is a measure of the partition coefficient of a functional group from one environment to another which in this case is from the aqueous phase to the organic phase.[25] Functional group selectivity was determined for mono-substituted benzenes using the following equation:

$$\tau = \log (K_{Bz\text{-}R}) - \log (K_{Bz})$$

where τ is functional group selectivity and $K_{Bz\text{-}R}$ and K_{Bz} are the partition coefficients of the substituted benzene and benzene respectively. It is assumed that partition coefficient of benzene (C_6H_6) is the same as C_6H_5 phenyl group.

Combining knowledge gained from both solvatochromic and partitioning studies will give insight into the interactive properties exhibited by the HFIP-solvent phases and how the different variables influence them. In addition to learning about the interactive properties of the phases, ternary phase diagrams for these different systems were determined to gain insight into conditions for formation of these phases.

Experimental

Materials

1,1,1,3,3,3-Hexafluorosiopropanol was purchased from Oakwood chemicals at >99%. N,N-dimethylformamide and acetone were purchased from Fisher Scientific and were ACS grade. THF and butanone were both 99% from Alfa Aesar. N,N-diethyl-4-nitroaniline was purchased form Oakwood chemicals at 99% purity. 4-nitroaniline, 4-nitrophenol and 4-nitroanisole were all purchased from Acros Organics at 99% purity. Michler's ketone was purchased at 98% purity from Sigma Aldrich. The following compounds used for partitioning studies were all purchased from Sigma Aldrich at ≥99% purity: phenol, 4-fluorophenol, 4-iodophenol, acetophenone, propiophenone, butyrophenone, valerophenone, nitrobenzene. Benzene was purchased from Alfa Aesar at 99.8% purity and naphthalene was 99% from Spectrum Chemicals.

Abbreviations

The HFIP-Organic phase will be referred to as the H-O phase while the two-phase solutions will be termed Aq/H-O.

Methods

Preparation of Ternary phase diagrams

Various fractions of water, HFIP, and a third miscible solvent, DMF, acetone, THF and butanone, were added together for a total volume of 1 mL in a clear microcentrifuge tube. The solution was briefly vortexed to mix and allowed to sit overnight at room temperature. The following day the solution was examined to determine whether a phase separation has occurred. The results were recorded and plotted as ternary plots using JMP Pro11 software (SAS).

Measurement of HFIP/solvent phase volume

Two phase solutions of HFIP in water with the four solvents were prepared at 4 different percentages of combined solvent (HFIP + third organic solvent). HFIP and the third solvent were at a 1:1 mole ratio and prepared in triplicate at 9,18,36 and 80% (HFIP + organic solvent) based on a 1 mL total solution volume. The solutions were vortexed and centrifuged. In two-phase systems, the volume of the bottom phase was determined by removing it with a graduated syringe and recording the volume.

Solvatochromic Studies

Solvatochromic probes were used to measure the dipolarity (π^*), hydrogen bond basicity (β), and hydrogen bond acidity (α) for both phases of the Aq/H-O systems. For π^* measurements N,N-diethylnitroaniline (DENA)(structure Fig. 2-2A) was used as a probe and π^* was calculated according to Eq(2) in Table 2-1. 4-Nitroaniline (4NA)(structure Fig. 2-2B) was used as the β probe and β was calculated according to Eq(4) and Eq(5) in Table 2-1. α was determined using Michler's Ketone (MK)(structure Fig. 2-2C) and calculated according to Eq(6) in Table 2-1. The only exception was for the β measurement of pure

HFIP for which the probe pair of 4-nitroanisole(structure Fig. 2-2D) and 4-nitrophenol(Structure Fig. 2-2E) was used due to poor peak shape of 4NA in pure HFIP. β for HFIP using 4-nitroanisole(4NAnis) and 4-nitrophenol(4NP) was calculated with Eqs. 3,6 and 7 in table 2-1. Stock solutions of each probe were made in acetonitrile to be added to the various two-phase solutions for absorbance measurements.

Aqueous /HFIP-Solvent (Aq/H-O) two phase systems were prepared in 10 mL volumes for solvatochromic analysis to obtain large enough HFIP-Solvent phases to fill the cuvette for absorbance measurements. After mixing the appropriate amounts of HFIP, solvent and water the solution was mixed by inversion and centrifuged. 1 mL of the aqueous phase was then removed for analysis and the H-O phase was removed with a syringe. UV-Vis measurements were taken using a Shimadzu UV-1800 spectrophotometer. Measurements were taken over the wavelength range of 250-500 nm with a baseline being taken with the solution of interest prior to the addition of the probe. Once the baseline was taken a small volume (typically 1 μL or less) of the probe solution was added such that 0.1< absorbance at λ_{max} <1.0. λ_{max} could then be converted to ν_{max} and used to calculate the different solvatochromic parameters.

Determination of partition coefficients (K) The partition coefficients in Aq/H-O was determined from the ratio of equilibrium concentrations of an analyte in the two phases. Partition coefficients were determined using HPLC peak areas according to the following equation:

$$K = \frac{(Peak\ area\ in\ HFIP{-}solvent\ phase\ x\ dilution\ factor)}{(Peak\ area\ in\ aqueous\ phase)} \qquad \text{Eq. 9}$$

Aq/H-O solutions were prepared containing the solutes for which log(K) was being determined. The solutions were briefly vortexed and then allowed to rotate for the desired

equilibration time. The solutions were then centrifuged and the aqueous phase was removed to be analyzed without dilution while the H-O phase was diluted 101X in 40% isopropanol in water except for the 36% total solvent H-O phases which were diluted 51X.

Equilibrium time study

A time study was done in order to determine how long compound partitioning takes to reach equilibrium in order to ensure accurate K values. The K values for 4-fluorophenol, nitrobenzene and valerophenone were measured after allowing them to equilibrate for either 0.5, 24 or 48 hours in a 1:1 HFIP:THF system where the total HFIP and THF concentration was 9% v/v. After forming the two phase systems, 1 μL of the analyte was added for the liquid compounds nitrobenzene and valerophenone. Since 4-fluorophenol is a solid, a concentrated solution was prepared in acetonitrile of which 1 μL was added to the two phase system. The solutions were then briefly vortexed to mix and then rotated for the desired equilibration time. Figure 2-3 shows the results of the time study. Log(K) values were seen not to change much with time, suggesting that the solutions reach equilibrium after 30 minutes. Nonetheless, 24 hour equilibration time was chosen for the remainder of the study to ensure equilibration.

Partitioning mixture study

Using HPLC, it would be possible to determine K values for several solutes in test mixtures rather than in pure form, which would significantly reduce the overall analysis time. However, a preliminary study was conducted to ensure that K values of test solutes that were determined in mixtures did not differ significantly from those measured individually. A mixture containing all 10 test compounds was prepared by adding 10 μL of each liquid compound plus 10 μL of saturated solutions of the solid compounds prepared in acetonitrile.

Samples of the test compounds were prepared in the same manner as that described for the time study above and the same 1:1 HFIP:THF 9% solvent system was used as well. The K values for each compound were measured in the solutions containing all 10 compounds as well as for each compound individually and those results are shown Fig. 2-4. These results confirm that the K values can be reliably determined in mixtures.

Partition coefficient HPLC analysis

Based on the mixture and time study, partition coefficients were determined using a mixture of the analytes that were allowed to equilibrate by rotation for 24 hours. The H-O and Aq phases were analyzed by HPLC using an Agilent 1100 HPLC instrument equipped with a photodiode array detector and 214 nm was used for detection. The column was a Zorbax SB-C18 4.6 mm I.D., 150 mm and 3.5 μm particle size. Mobile phase A was HPLC grade water and B was gradient grade acetonitrile. The HPLC method was 20% B to 40% B over 20 minutes then to 50% B over the next 7 minutes followed by a 3 minute equilibration at 20% B. Figure 2-5 depicts a chromatogram of the 10 component mixture.

Phase composition analysis using gas chromatography with a thermal conductivity detector

An Agilent 7890 Gas chromatograph equipped with a thermal conductivity detector was used to characterize both phases from the Aq/H-O solutions. Aq/H-O solutions prepared with THF and acetone were analyzed. Calibration plots were prepared for water, HFIP, acetone and THF. The GC samples were prepared by diluting 100 μL of either the aqueous or H-O phase in 800 μL of DMF and 100 μL of an internal standard (1 mL 1-pentanol diluted with 10 mL DMF) since manual injection was used.

The column was an Agilent DB-624, 30m x 0.32mm x 1.8µm capillary column. The carrier gas was helium at 3.5mL/min constant flow. The sample was injected manually at 0.5 µL with a 50:1 split ratio. The injector and detector temperatures were both held at 250ºC. Temperature programming was used where the initial temperature was held at 110 ºC for 3 minutes and then ramped up to 170 ºC at 30 ºC/min and held for 1 minute for a total time of 6 minutes.

Results and discussion

Ternary phase diagrams

The ternary phase diagrams for 3 component mixtures of HFIP, water and either acetone, THF or butanone are shown in Fig.2-6 A, B and C respectively while figure 2-7 overlays the approximate two-phase regions for each system. The HFIP-water-DMF system is also being studied and its ternary plot has been published in the literature.[1] One way to examine the size of the two phase regions is to compare the mole fraction of water X_{water} at which these ternary mixtures enter and exit the two-phase region with equal volume amounts of each solvent. Table 2-2 shows the X_{water} values for each H-O system where the phase formation begins (X_{lower}) and where the two phase system dissolve into one phase (X_{upper}) with increasing amounts of solvents. These results show that DMF has the smallest two-phase region as it enters the two-phase region last and exits first compared to the other solvents. Acetone is the next smallest with both THF and butanone being larger. This trend seems to be related to solvent hydrophobicity as DMF and acetone are less hydrophobic than both THF and butanone. Based on the results using equal volumes of HFIP and solvent, the THF and butanone systems enter the two-phase regions at similar X_{water} while the THF systems exits at a much lower X_{water}. This also applies to the regions of the plot where one

solvent is in excess to another with acetone being smaller than THF and butanone. All of these solvents are completely miscible with HFIP and water except for butanone, which is not completely miscible with water having a solubility of 27.5g / 100mL. This is the reason why in the butanone ternary plot there is no reentrance into a single-phase solution in the high butanone region like with the other solvents after the $X_{butanone}$ of 0.054, which represents its solubility in water. It is obvious from these plots that acetone has the smallest two-phase region likely due to its lower hydrophobicity than both THF and butanone. The butanone two-phase region is similar in size to THF but this is largely due to the immiscibility of butanone and water at higher concentrations. So increasing the hydrophobicity of the solvent increases the size of the two-phase region, which is consistent with observations reported by Takamuku et al. with amides.[2]

H-O Phase volume

The volume of the H-O phase formed under varying conditions for each solvent was measured to determine differences between the solvents as well as the effect of varying the amounts of the solvents used for two-phase formation. The effect of increasing the total % of solvent is shown in table 1-3 where 1:1 H-O two phase systems were formed with the % of both solvents combined being 9,18, 36 and 80% of the total solution volume meaning the remaining % volume is water. As expected, the volume of the H-O phase increased with increasing amounts of solvents. The THF and butanone systems formed the largest phases until the 80% total solvent solutions. At 80% solvent, the DMF H-O phase is the largest and acetone is tied with butanone for second largest while THF is the smallest. These phases are significantly larger than the % of solvents added indicating that water is now likely making up larger portions of the predominantly organic phases. Since we know from the

ternary plots that DMF and acetone exit the two- phase region at lower HFIP concentrations than phases composed of THF and butanone; it makes sense that they form larger phases at these high solvent regions as they are closer to becoming a single-phase solution. They are likely incorporating more water into their H-O phase, which will ultimately result in transitioning into a single-phase solution, which is supported by phase composition data (Table 2-4).

The ternary plots show that these two-phase systems can be formed under a variety of conditions in terms of % total solvent as well as the ratio of HFIP to the other solvent. The effect of changing the HFIP:solvent ratio on H-O phase volume was investigated and the results are shown in Fig. 2-8. This plot illustrates how the H-O volume changes for 9% total solvent systems as the relative amounts of HFIP and the solvent are varied. Changing the HFIP:Solvent volume ratio from 9:1 to 1:9 represents a change in the HFIP:Solvent mole ratio of approximately 7.0 to 0.08 for all solvents. It is apparent that all of the solvents follow a similar pattern in terms of how the phase volume changes with changing solvent ratios. Starting from the high HFIP end (9:1 H:O the volume of the two phase systems increase until they reach somewhat of a maximum plateau between 7:3 and 5:5 except for DMF which plateaus earlier at 8:2 to 6:4. After this plateau the volumes continue to decrease until a single-phase solutions form for all solvents except THF, which still has a small H-O phase at 1:9 HFIP:THF. For all solvents the phases are much larger in the high HFIP regions (9:1, 8:2) compared to the low HFIP regions (2:8, 1:9) indicating the importance of HFIP and the clustering of its trifluoromethyl groups in causing phase separation. Larger volumes are likely observed around the 6:4 H:S range because there is a good balance between HFIP and the solvent as both are needed to cause phase separation. In the high HFIP region there is

plenty of HFIP to form clusters but not as much of the other solvent for it to cluster around leading to smaller phase volumes. In the low HFIP region there is plenty of the solvent for HFIP to interact with but not enough HFIP to form clusters around all of the available solvent resulting in smaller phases. When either HFIP or the other solvent is in a large excess it is more likely that they will interact with the water in solution as well since both HFIP and the solvent are miscible with water. Water can interact with HFIP as a HBA and with the other solvents as a HBD due to its amphoteric nature.

Phase Composition analysis.

GC-TCD was used to measure the amounts (%v/v) of each solvent in both phases of Aq/H-O systems formed with THF under varying conditions as well as the 1:1 9% acetone system (Table 2-4 A and B). The aqueous phases were observed to consist of greater than 92% (v/v) water in all solutions measured. Changing the HFIP:THF ratio was reflected in the amounts of those solvents in the aqueous phases with the 3:7 HFIP:THF solution having a higher % of THF in the aqueous phase and 7:3 having a higher % of HFIP in the aqueous phase. In comparison to the 9% total solvent HFIP-THF system, the ones formed with 18 and 36% total solvent contained more THF in their aqueous phase. The 1:1 9% HFIP-Acetone system contained a higher % of solvents in its aqueous phase compared to the 1:1 9% HFIP-THF system. A few of the systems show negative values for % HFIP with a small standard error and the total % v/v adds up to less than 100%. This could indicate non ideal mixing in these solutions with the resulting volumes not simply being a combination of the amounts of each solvent.

The effect of changing the HFIP:THF ratio on the amounts of each solvent in the H-O phases of 9% total solvent systems can be seen in Figure 2-9. Similar to what was observed

with the aqueous phases changing the HFIP:THF ratio influences the relative amounts of each solvent in the H-O phase with 3:7 HFIP:THF having more THF and 7:3 having more HFIP. The % of water was also shown to slightly increase in the 7:3 solution compared to 1:1. This is likely a result of their being more HFIP(7:3) that is not involved in clustering compared to the 1:1 system. These molecules are then free to interact with water through hydrogen bonding. Although changing the HFIP:THF ratio changes the relative amounts of the two solvents present in the H-O it does not change as drastically as the 3:7 and 7:3 ratios might indicate as shown in Table 2-4 B. The mole ratio of HFIP:THF in the organic phase is higher than the nominal ratio of 3/7 for the 3:7 system, while for the 7:3 system, the mole ratio of HFIP-THF in the H-O phase is lower than 7/3. This would occur because both molecules are needed for the clustering and hydrogen bonding interactions to occur and why an increase in the excess solvent is observed in the aqueous phase for these systems. In the 1:1 systems the % of HFIP is higher than the other solvent in the H-O phase. This is much more pronounced in the acetone system compared to THF indicating that the higher amount of HFIP compared to the other solvent may be due to HFIP interacting with water through hydrogen bonding more than the other solvent does. Changing the % total solvent used for two-phase formation had less of an impact on the H-O phase composition compared to changing the HFIP:THF mole ratio as shown in Fig. 2-10. The 9% and 18% H-O phases had nearly identical compositions while at 36% composition, the H-O phase had slightly more THF relative to HFIP when compared to the 9 and 18% H-O phases. The acetone H-O phase contained a much higher % of water compared to the THF H-O phase formed under the same conditions. It also contained more HFIP relative to acetone compared to the THF phase

which could be a result of how HFIP is able to cluster around different molecules or more HFIP interacting with the larger amounts of water in the acetone H-O phase.

Solvatochromic analysis

Dipolarity

The π* values measured for individual solvents as well as various HFIP solvent two-phase systems are shown in table 2-5A and B respectively. As expected the π* of the aqueous phases from the two-phase systems is similar to that of water. Figure 2-11 illustrates how the π* value changes when the solvent used for two-phase formation is changed as well as how the π* values for the H-O phase compares with the solvent used for formation. The HFIP:THF phase has the lowest π* value and HFIP:DMF has the highest π* value which corresponds with the π* values measured for the individual solvents in which THF was the lowest and DMF was highest. A similar trend is observed when changing the mole ratio of the two solvents as shown in Fig 2-12. The π* value shifts toward whichever solvent is present in excess. With ideal mixing the observed π* value should be a weighted sum of the π* values for the solvents in the H-O phase according to the following equation:

$$\pi^*(\text{H-O}) = \pi^*(\text{water})X(\text{water}) + \pi^*(\text{HFIP})X(\text{HFIP}) + \pi^*(\text{HBA solvent})X(\text{HBA solvent})$$

where X is the mole fraction or volume % of each solvent.[26,27] Figure 2-13. compares the theoretical π* values calculated using the previous equation to the values measured using the solvatochromic probe. The measured π* values for the 1:1 HFIP:THF systems with 9 and 18% total solvent were both very close to the calculated values while the 3:7 and 7:3 systems showed deviations from the theoretical values. The measured value for 3:7 HFIP:THF systems was significantly lower than the calculated value while the measured value for the 7:3 systems was significantly higher than calculated. This is likely due to the probe

interacting with whichever solvent is in excess since the π* value for THF is low that is why the 3:7 system measures much lower than calculated with the same thing occurring in the 7:3 system which measures higher than expected since HFIP has a much higher π* value than THF. The 1:1 9% acetone system measured lower than the calculated value. From the GC-TCD data this phase is composed of approximately 12% water, 53% HFIP and 30% acetone v/v. Based on the trend observed with THF π* would be expected to measure higher than the calculated value because of the excess HFIP but instead it measured lower. This could be due to differences in how HFIP clusters around different molecules.

These results show that π* is mainly dependent on the amounts of each solvent in the H-O phase and is likely not influenced by any interactions occurring in the phase. Deviations from theoretical π* values calculated using a weighted sum equation are observed when either HFIP or the HBA solvent are present in large excess to one another.

Hydrogen bond basicity

The β values were also measured for the individual solvents as well H-O two-phase solutions shown in Table 2-6A and B respectively. Again, the β value for the aqueous phases measured was similar to that of water with some being slightly higher. The small amounts of the solvent other than HFIP in the aqueous phase could cause an increase in β from pure water as all of those solvents recorded higher β values. With HFIP being such a poor hydrogen bond acceptor, it is not surprising that its β value was measured at -0.02 so its presence in the H-O phases is likely to have little influence on the β value due to its hydroxyl group but may influence β through interactions with the hydrogen bond accepting group of the other solvent. Figure 2-14 illustrates how the β value changes when the solvent used for two-phase formation is changed as well as how the β values for the H-O phase compares the

solvent used for formation. The β values for H-O phases do not show the same trend as the π* since the H-O phase formed with the solvent having the highest β value (DMF) has the lowest β value of the four 1:1 9% H-O phases. If the β values were a weighted average of HFIP and the other solvent, the HFIP:DMF system should show the highest β value. This could indicate that the interactions between HFIP and the HBA solvent in terms of both hydrogen bonding and clustering influence β. This type of influence was not observed with π*, possibly due to how HFIP clusters around the different solvents. It can influence β by forming hydrogen bonds with the acceptor group of the solvent preventing the solvatochromic probe from interacting with it or due to clustering that sterically inhibits the probes from interacting with the HBA group. Differences in solvent structure and type of acceptor group could have more influence on β than with π*. β values showed a similar trend to π* in terms of their dependence on the HFIP: solvent ratio. Figure 2-15 compares the measured β values to the theoretical values calculated using the weighted sum equation and GC-TCD composition data. Similar to what was seen with the π* values for the THF systems the 3:7 and 7:3 H-O phases show a larger deviation from the calculated value than the 1:1 systems formed with either 9 or 18% total solvent. As expected when THF is in excess the β value for the H-O phase is higher and when HFIP is in excess it is lower. The probe may be able to interact more easily with whichever solvent is in excess leading to greater changes than expected using the weighted sum equation. Again changing the % of solvent used to form the phase showed little influence on β when comparing 1:1 HFIP:THF phases with 9 and 18% total solvent which is what was also observed for π*.

Hydrogen bond acidity

All of the solvents used besides HFIP are very poor hydrogen bond donors and thus have α values close to 0 while HFIP is known as a very strong hydrogen bond donor has the largest α value (0.93) followed by water at 0.4.Table 2-7A. The α values for the aqueous phases are again similar to that of water. The H-O phases prepared under the same conditions but with different solvents all show similar α values ranging from 0.63-0.70 so they are all around 68-75% of pure HFIP (Table 2-7B). This makes sense from the fact that all of these solvents have very low α values themselves with HFIP making the main contribution to the α value of the H-O phase. Unlike both π* and β, the α value did not really change with changing HFIP:THF mole ratio in the 9% total solvent systems with the value staying around 0.68-0.75 and actually showing a slight increase with less HFIP relative to THF. This could be due to how the H-O phase forms. If the trifluoromethyl groups are clustering around the solvents that means the HFIP hydroxyl groups are free to interact with other compounds such as the solvatochromic probe. While the HFIP hydroxyl groups may form H-bonds with each other due to the weak hydrogen bond accepting nature of HFIP it is more likely to H-bond with stronger H-bond acceptors when they are present in solution. This indicates that the mechanism of phase separation does not involve hydrogen bonding for all HFIP molecules with clustering from their trifluormethyl groups playing a larger role as reported by Takamuku[2]. It is also possible that the probe is not accurately measuring the α character of these phases due to whatever microenvironment it is present in. GC-TCD results showed that at higher HFIP:THF ratios more HFIP is present in the H-O phase which would seem to indicate stronger α character. Again increasing the total amount of solvent used

while maintaining the same solvent ratio had little effect on the α value which is what was also observed for π* and β

Summary of Solvatochromic results and discussion on mechanism of phase separation

In summary, the solvatochromic studies of the H-O phases indicate that both π* and β are influenced by the type of HBA solvent used for formation of the two-phase system as well as the relative mole fractions of HFIP and the HBA solvent. π*and β are both influenced by the HFIP:solvent ratio while α was fairly constant in all H-O phases. When HFIP is in excess relative to the HBA solvent, clustering likely prevents the HBA site from interacting with the probe molecule or all of the HBA sites are involved in hydrogen bonds with HFIP. α is dominated by HFIP and does not seem to change with varying solvent conditions. The aqueous phase values for these three properties were all fairly close to that of water. Thus, all H-O phases will likely exhibit strong α while π* and β can be influenced by changing HBA solvents and HFIP:solvent ratios.

These measurements also seem to give some insight on the mechanism of phase separation and how the molecules interact in the H-O phase. The previously reported mechanism by Takamuku and coworkers [2] emphasizes that phase separation occurs due to HFIP clustering around the hydrophobic regions of the other solvent. This clustering of trifluoromethyl groups eventually leads to the exclusion of water molecules causing phase separation. It is also possible that hydrogen bonding between HFIP and the HBA solvent could play a role in phase separation. HFIP is a strong HBD and should preferably interact with the HBA solvents than with water since the HBA solvents are stronger hydrogen bond acceptors than water. The role of hydrogen bonding between HFIP and the HBA solvent was largely discounted in the Takamuku paper, which focused exclusively on clustering, by

HFIP. It has been demonstrated with water immiscible solvents that contain HBA groups such as anisole that HFIP will solvate that solvent and form an H-O phase in mixtures with water (Fig. 2-16A) while for a solvent such as mesitylene that does not contain HBA groups this solvation by HFIP does not occur (Fig. 2-16B). If another water immiscible solvent containing a HBA group is added to the mesitylene solution such diethyl ether then H-O phase formation occurs (Fig 2-16C). When the solvent is not solvated by HFIP it floats on top of the aqueous phase due to its lower density while bottom phases result from HFIP solvation due to its much higher density than water. Since HFIP is miscible with both anisole and mesitylene if clustering was solely responsible, it would form H-O phase with mesitylene even though it lacks a HBA group. These results indicate that the ability of the solvent to interact with HFIP through hydrogen bonding is likely to play a role in at least initiating phase separation by forming a stronger hydrogen bond with the solvent than water. This allows other HFIP molecules to then cluster around their trifluormethyl groups as well as the hydrophobic portions of the HBA solvent creating a hydrophobic environment and ultimately excluding water resulting in phase separation. The fact that all H-O phases demonstrate the same relatively high α values indicates that many HFIP molecules in the phase are free to donate hydrogen bonds so they cannot all be interacting with the HBA solvents through hydrogen bonding. In conclusion, both hydrogen bonding and HFIP clustering play a synergistic role in phase separation.

Partitioning probe studies.

The interactive nature of the H-O phases, relative to the aqueous media, was probed through determination of partition coefficients of 10 test compounds in various Aq/H-O two-phase systems. Partition coefficient is a thermodynamic quantity that is directly related to

the change in free energy of transfer of a solute from the aqueous phase to the organic phase. In general, hydrophobic interaction is the predominant driving force foe solute partitioning between an aqueous and an organic phase. Hydrogen bonding and dipolar interactions play a secondary, but important role in this process. The test compounds were carefully selected based from different classes with specific functional groups with hydrogen bond donor, hydrogen bond acceptor, and dipolar functional groups. Two test compounds, benzene and naphthalene could be classified as non-hydrogen bond compounds, albeit they have weak hydrogen bond acceptor property due to the π electrons imparted by aromatic ring. The list of compounds along with their octanol-water (OW) partition coefficients if available are shown in table 2-8 along with their HPLC capacity factors (k') in the method used for analysis. The goal of these experiments was to determine how the partitioning of these compounds is influenced by the many variables involved in these two-phase systems such as the % volume v/v of solvent (HFIP + HBA solvent), HFIP:solvent ratio, and the type of HBA solvent used.

To test the effect of solvent type and composition on partitioning into the H-O phase, two-phase solutions were formed using 9% total solvent with a 1:1 mole ratio of HFIP:Solvent and the partition coefficients for the 10 compounds were determined (Fig. 2-17). The test solutes had the highest affinity (as evident by largest Log(K) values) toward the HFIP-THF system followed by HFIP-butanone with the HFIP-DMF and HFIP-acetone systems typically having the lowest Log(K) values. This can be explained by THF and butanone being more hydrophobic than DMF and acetone leading to better extraction of these organic molecules. Another aspect to consider is that with DMF and acetone forming smaller H-O phases at 9% total solvent, there is more HFIP and DMF in the aqueous phase as compared to the aqueous phases of THF and acetone systems. This could lead to an increase

in the concentration of organic solvents in the aqueous phase relative to THF and butanone where more of the solvents are incorporated into the H-O phase and the water content of the H-O phases is also lower. It should be noted that the volume of the phases should not influence partition coefficients. In other words, the observe effects are due to differences in phase composition and not the phase volume, which should not have any effect on partition coefficients.

Next, the effect of increasing the total % of solvent was investigated by comparing the partition coefficients for the 10 compounds in 1:1 HFIP:THF two-phase systems prepared with 9, 18 and 36% total solvent v/v (Fig. 2-18). For several compounds, the effect of total solvent concentration their partition coefficient is small and there is a lack of any recognizable trend. The solvatochromic results showed that changing the % total solvent had little effect on the π^*, α and β properties of the H-O phase so changes in phase hydrophobicity would likely account for any changes in partitioning. One very noticeable trend is for the three phenol molecules whose partitioning increases with increasing % total solvent. The phenol compounds are hydrogen bond donors and are thus likely to interact with hydrogen bond acceptors in the H-O phase. The GC-TCD results showed a slight increase in the % volume of THF in the H-O phase as the % total solvent was increased which could explain the increase in partitioning for the phenol compounds. Since K is a measure of the relative affinity of the solute between the Aqueous and H-O phase it is also possible changes in partitioning with either changing HFIP:THF ratios or % total solvent are the results of increasing differences in properties, π^*, β, α, between the aqueous and H-O phases in these systems.

Next, the effect of changing the HFIP:THF mole ratio used to form the two-phase solution on partitioning was investigated (Fig. 2-19). While changing the solvent ratio had little impact on the partitioning of compounds like benzene and nitrobenzene, it had a noticeable effect on others. Log(K) of all of the phenols decreases with increasing HFIP relative to THF. Phenols should be able to interact with both HFIP and THF through hydrogen bonding as it can accept H-bonds from HFIP (stronger HBD than phenols) and donate H-bonds to THF (stronger HBA). Solvatochromic results, indicated that α of the H-O phase remains constant at all solvent ratios while β is highest at 3:7 HFIP:THF. So the increased ability of the H-O phase to accept hydrogen bonds from phenol compounds at 3:7 HFIP:THF ratios increases their partitioning into the H-O phase. Phenone compounds show the opposite trend and their partitioning increases at in the higher HFIP relative to THF phase. Although solvatochromic studies showed a constant α value at varying HFIP:THF ratios, the GC-TCD results did show that the % HFIP of the H-O phase increases with higher HFIP:THF ratios. Since phenones can only act as hydrogen bond acceptors the presence of more HFIP in the H-O for them to interact with could play a role in their increased partitioning. Also the difference in the α value between the H-O and aqueous phases will influence K. All of the 1:1 9% solvent systems have similar α values for their H-O phases but THF and butanone have larger Δα ($\alpha_{H\text{-}O} - \alpha_{Aq}$) which likely plays a role in the higher Log(K) values for the aryl phenones in these systems.

Methylene Selectivity (α_{CH2})

Methylene selectivity is a measure of the change in free energy of solvation for a methylene group in the H-O phase as compared to the aqueous phased and is thus used as a measure of the hydrophobicity of the H-O phase relative to the top aqueous phase. Aryl

phenones differing in their number of methylene groups were used to determine α_{CH2}, which is the slope of the linear relationship between Log(K) and the number of methylene groups. Fig. 2-20 shows the linear function for a 1:1 HFIP:DMF 9% total solvent system from which the slope represents α_{CH2}. The α_{CH2} values for several H-O two-phase systems are shown in Table 2-9. When comparing the two-phase systems prepared with different solvents it is clear that the THF and butanone systems exhibit much higher α_{CH2} than both DMF and acetone, which is not surprising as these are the more hydrophobic solvents, having lower π^* values, and based on the GC-TCD results contain less water in their H-O phase. This would explain why the THF and butanone systems generally had higher Log(K) values for all compounds in systems made under the same conditions (Fig. 2-17). Although the properties measured through solvatochromic studies play a role in compound partitioning the hydrophobicity of the phase is likely the dominant factor. Increasing the total % of solvent leads to a decrease in α_{CH2} as shown in Table 1-10 for the 1:1 HFIP:THF systems formed with 9, 18 and 36% total solvent. The reason that α_{CH2} decreases with increasing solvent % could be due to small increases in the % of the HBA solvent in the aqueous phase since it is a more hydrophobic solvent which was indicated from the GC-TCD results. Changing the HFIP:THF mole ratio for 9% solvent systems also influenced the α_{CH2}. The system with excess THF relative to HFIP (3:7) showed higher α_{CH2} than 1:1 and 7:3, which showed the lowest α_{CH2}. So similar to trends in π^* and β values, Thus α_{CH2} is influenced by changing the conditions under which the Aq/H-O systems are formed.

Functional Group Selectivity (FGS)

The functional group selectivity (FGS) is basically a measure of the Log(K) of a functional group and is directly related to the free energy of transfer from the aqueous phase to the organic phase, in our case this is the H-O phase. FGS can be used to probe the microenvironment of the two phases with a negative FGS value indicating higher affinity for the aqueous phase and a positive value showing a higher affinity for the H-O phase. The FGS of various HFIP-two phase systems for five functional groups are shown in Fig. 2-21. As expected the addition of a hydroxyl functional group decreases selectivity in all systems. The hydroxyl group increases the polarity of the molecule increasing its affinity for the aqueous phase. The acetone and DMF H-O phases were shown to have a higher affinity for hydroxyl groups compared to THF and butanone. This is likely due to increased amounts of water in their H-O phases decreasing the hydrophobicity of the phase and allowing for more hydrogen bonding interactions since water and hydroxyl groups are both HBA and HBD. Increasing the % of total solvent (9, 18 and 36% 1:1 HFIP:THF) was shown to increase partitioning for the hydroxyl group into the H-O phase. Methylene selectivity was also shown to decrease in this same direction which essentially the opposite ability to extract a hydrophobic CH_2 so it makes sense that the opposite trend would be observed for a hydrophilic OH group. This could also be due to the slight increase in % THF of the H-O phase with increasing % total solvent, which hydroxyl groups can interact with through hydrogen bonding.. When changing HFIP:THF mole ratio hydroxyl selectivity was higher in the 3:7 HFIP:THF system than either 1:1 or 7:3.This increased selectivity likely results from the increased β character of the phase allowing the hydroxyl group to interact more through hydrogen bonding. Nitro group selectivity remained fairly constant throughout all systems

with only slight changes observed. The nitro group imparts a negative charge on the molecule, which should make it more water-soluble and also imparts a strong dipole. This is possibly why an increase in nitro FGS is observed in the 7:3 HFIP:THF system compared to 1:1 and 3:7 since it had a higher π^* value. The selectivity of the carbonyl group was observed to go from positive to negative when changing the HFIP:THF ratio from 7:3 to 3:7. The increased dipolarity and potentially stronger α character at higher HFIP ratios may allow for more interactions with the carbonyl functional group that can only act as a HBA. Although α values remained fairly constant in solvatochromic studies the GC-TCD results showed that the 7:3 HFIP:THF system contained a higher % of HFIP. Slight decreases in carbonyl FGS were observed with increasing % total solvent which is likely due to the small decrease in HFIP relative to THF at higher % solvent. An increase in hydrophobicity of the aqueous phase at higher % solvents due to the an increased % of THF could also play a role. Fluoro FGS remained consistent throughout all systems and conditions with a slightly positive value. The iodo group did show differing selectivity among the systems showing higher selectivity in 3:7 HFIP:THF compared to 7:3. The iodo functional group is large and polarizable and due to its size hydrophobicity plays a role in its partitioning. This is likely why the 3:7 HFIP-THF system has increased iodo selectivity compared to 1:1 and 7:3 as it showed the highest α_{CH2} of the three HFIP:THF mole ratios. The same reasoning would explain why iodo FGS decreased with increasing % total solvent and was higher in the butanone and THF systems compared to acetone and DMF. Overall each of the variables investigated, % total solvent, HBA solvent used and HFIP:solvent mole ratio, were shown to influence FGS altering the properties of both the aqueous and H-O phases.

Correlation between Log(K) and Log(P)

Table 2-10 shows the correlation matrix(R^2) of the linear fit between Log(K) values of the various Aq/H-O systems and Log(P) values from O-W partitioning with Fig 2-22 A-D showing four examples of the plots used to determine correlation.. In relation to Log(P) values from O-W Correlation was shown to increase in the 9% HFIP-THF system in the order 7:3 > 1:1 > 3:7 of HFIP:THF (Fig. 2-22 A and B). This is likely a result of the increasing hydrogen bonding basicity as the amount of THF is increased relative to HFIP since octanol is a much better HBA than HFIP as is THF. When looking at the aryl phenones in the in Figures 2-22 A and B the less hydrophobic aryl phenones, acetophenone and propiophenone, are closer to the regression line in the 3:7 system compared to 7:3 indicating higher correlation. The more hydrophobic aryl phenones, butyrophenone and valerophenone, are right on the line for both systems compared to O-W likely due to hydrophobicity playing a larger role in their partitioning than hydrogen bonding. THF and butanone showed higher correlations to Log(P) than acetone and DMF in the 1:1 9% systems. This is a result of THF and butanone demonstrating higher α_{CH2} making them closer to the O-W system. The Aq/H-O systems all showed fairly high correlation to each other (Fig. 2-22 C). The smallest correlation between Aq/H-O systems was observed between 3:7 and 7:3 9% HFIP-THF systems (Fig. 2-22D). In figure 2-22 D the phenols lie below the regression line indicating greater affinity for the 3:7 system. The first three aryl phenones; aceto, propio and butyoro, lie above the regression line indicating greater affinity for the 7:3 system. Valerophenone is right on the regression line, again likely due to its hydrophobicity playing a larger role than hydrogen bonding compared to the less hydrophobic phenones. Altering the HFIP:solvent

ratio seems to affect selectivity more than the other variables investigated; % total solvent and HBA solvent.

Conclusion

In conclusion, Aq/H-O systems form over a wide range of conditions in terms of the relative volume (or mole) fractions of the three constituent solvents with THF and butanone giving larger two-phase regions than DMF and acetone likely due to their higher hydrophobicity. Acetone phases formed under the same conditions as THF phases were shown to contain larger amounts of water in their H-O phases. The mechanism of phase separation seems to stem from the combination of HFIP interaction with the other solvent through hydrogen bonding and clustering of HFIP molecules around the hydrophobic moiety of the solvent, which forms a hydrophobic environment that excludes water.

Solvatochromic studies showed that all H-O two-phase systems exhibit high HBD character) α) while dipolar (π^*) and HBA (β) properties are more dependent on the conditions under which the phase is formed particularly the HFIP:solvent ratio. Changing the total amount of solvent while maintaining the same HFIP:solvent ratio had little effect on any of these properties. These solvatochromic properties can be used to explain several of the trends observed in the small molecule partitioning experiments. Methylene selectivity (α_{CH2}) is higher in HFIP-THF and -butanone phases, which suggest these solvents form more hydrophobic H-O phases than DMF and acetone . These phases are interesting in that all of their properties except for α can be influenced by changing the variables involved in forming them such as; HBA solvent, amount of solvent and HFIP:solvent ratio.

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Tables

Table 2-1. Equations used for calculation of solvatochromic parameters π*, β, and α

Parameter	Probe	Equation	Eq.
π*	DENA	$\pi^*(DENA) = (\nu_{max} - 27.52) / 3.13$	Eq(2)
π*	4NAnis	$\pi^*(4NAnis) = (\nu_{max} - 34.12) / 2.343$	Eq(3)
β	DENA and 4NA	$\nu_{max}(4NA)_{calc} = 1.035 \cdot \nu_{max}(DENA)_{obsd} + 2.64$ $\beta(4NA) = \frac{-\Delta\Delta\nu max}{2.80} = \frac{-(\nu \max(4NA)calc - \nu \max(4NA)obsd)}{2.80}$	Eq(4) Eq(5)
β	4NAnis and 4NP	$\nu_{max}(4NP)_{calc} = 0.901 \cdot \nu_{max}(4NP)_{obsd} + 4.16$ $\nu_{max}(4NP)_{calc} = \frac{-\Delta\Delta\nu max}{2.31} = \frac{-(\nu \max(4NP)calc - \nu \max(PNP)obsd)}{2.31}$	Eq(6) Eq(7)
α	DENA and MK	$\alpha(MK) = \frac{30.059 - \nu \max - 2.241 \cdot \pi*}{1.807}$	Eq(8)

Table 2-2. Mole fraction of water (X_{water}) and total % solvent (%HFIP +%HBA) at which each system enters (X_{lower}) and exits (X_{upper}) the two phase region with equal volume amounts of HFIP and the HBA solvent.

	X_{water}		% Total solvent(v/v)	
Solvent	**X_{lower}**	**X_{upper}**	**lower**	**Upper**
DMF	0.987	0.485	6.0	84.0
Acetone	0.985	0.358	7.0	89.6
THF	0.99	0.245	5.0	94.0
butanone	0.989	0.306	5.0	92.4

Table 2-3. Effect of increasing % total solvent on HFIP-solvent phase volume.

% Total Solvent (v/v)	% volume bottom phase (std. error)			
	DMF	Acetone	THF	Butanone
9	3.03(0.12)	3.70(0.06)	5.93(0.07)	5.97(0.03)
18	12.63(0.03)	14.60(0.23)	16.57(0.23)	16.63(0.09)
36	35.93(0.09)	41.17(0.09)	42.60(0.31)	42.53(0.31)
80	88.50(0.58)	87.07(0.03)	85.07(0.58)	87.07(0.32)

Table 2-4. Phase compositional data for A) aqueous and B) H-O phases determined with GC-TCD analysis.

A.

Solvent	HFIP:Solvent Ratio	% total solvent	% Water (std. error)	% HFIP (std. error)	% Third solvent (std. error)
THF	1:1	9	93.94 (0.80)	0.10 (0.02)	2.56 (0.03)
THF	3:7	9	93.56 (0.89)	-0.62 (0.04)	4.83 (0.01)
THF	7:3	9	96.45 (0.53)	2.21 (0.07)	1.22 (0.00)
THF	1:1	18	92.76 (0.03)	-0.20 (0.04)	3.20 (0.12)
THF	1:1	36	93.10 (0.14)	-0.67 (0.01)	4.74 (0.01)
Acetone	1:1	9	93.69 (0.49)	1.20 (0.04)	2.55 (0.06)

B.

Solvent	HFIP:Solvent Ratio	% total solvent	% Water (std. error)	% HFIP (std. error)	% Third solvent (std. error)	HFIP:THF ratio in H-O phase
THF	1:1	9	5.53 (0.03)	47.20 (0.16)	40.82 (0.58)	0.89
THF	3:7	9	6.07 (0.55)	41.60 (0.55)	46.45 (0.39)	0.69
THF	7:3	9	7.53 (0.15)	52.73 (0.13)	33.82 (0.44)	1.20
THF	1:1	18	5.67 (0.15)	47.45 (1.28)	41.73 (0.79)	0.88
THF	1:1	36	5.59 (0.12)	44.61 (0.60)	43.54 (1.03)	0.79
Acetone	1:1	9	11.97 (0.33)	53.17 (1.48)	30.00 (0.32)	1.23

Table 2-5. π* values for A) individual solvents and B) HFIP-Solvent two phase systems

A.

Solutions	π*
THF	0.62
Butanone	0.70
Acetone	0.74
DMF	0.92
H_2O	1.22
HFIP	1.38

B.

Solvent	% total solvent v/v	HFIP:solvent ratio	π* Aq	π* H-O	Δ (π*)
THF	9	1:1	1.38	0.96	-0.42
THF	9	7:3	1.35	1.13	-0.22
THF	9	3:7	1.00	0.84	-0.16
THF	18	1:1	1.06	0.94	-0.12
DMF	9	1:1	1.38	1.13	-0.25
Acetone	9	1:1	1.31	1.08	-0.23
Butanone	9	1:1	1.35	1.02	-0.33

Table 2-6. β for A) individual solvents and B) HFIP-solvent two phase systems,

A.

Solutions	β
HFIP	-0.02
H_2O	0.12
Acetone	0.52
Butanone	0.54
THF	0.56
DMF	0.72

B.

Solvent	% total solvent v/v	HFIP:solvent ratio	β Aq	β H-O	Δ ($\beta_{H-O} - \beta_{Aq}$)
THF	9	1:1	0.10	0.30	0.20
THF	9	3:7	N/A	0.41	
THF	9	7:3	N/A	0.13	
THF	18	1:1	N/A	0.29	
DMF	9	1:1	0.17	0.18	0.01
Acetone	9	1:1	0.23	0.19	-0.04
Butanone	9	1:1	0.19	0.23	0.04

Table 2-7. α values for A) individual solvent and B) HFIP-solvent two phase systems

A.

Solutions	α
DMF	0*
THF	0*
Butanone	0.06*
Acetone	0.08*
H_2O	0.40
HFIP	0.93

* Literature values

B.

Solvent	% total solvent v/v	HFIP:solvent ratio	α Aq	α H-O	Δ ($\alpha_{H\text{-}O}$-α_{Aq})
THF	9	1:1	0.32	0.68	0.36
THF	9	3:7	N/A	0.75	
THF	9	7:3	N/A	0.71	
THF	18	1:1	N/A	0.71	
DMF	9	1:1	0.40	0.63	0.23
Acetone	9	1:1	0.48	0.70	0.22
Butanone	9	1:1	0.36	0.69	0.33

Table 2-8. Compounds used for partitioning study along with their OW partition coefficient ($Log(P)_{ow}$) and HPLC capacity factor(k').

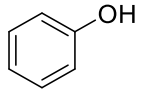
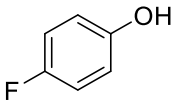
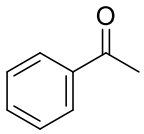
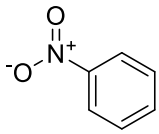
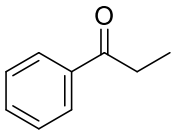
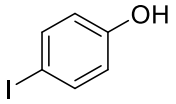
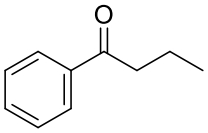
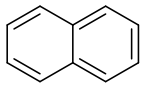
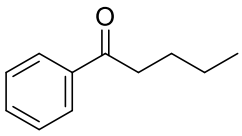
Compound	Structure	$Log(P)_{ow}$	k'
Phenol		1.46	2.48
4-fluorophenol		1.77	3.14

Table 2-8. continued			
Acetophenone		1.58	4.14
Nitrobenzene		1.85	5.72
Benzene		2.13	6.16
Propiophenone		2.19	6.87
4-iodophenol		2.91	7.48
Butyrophenone		2.77	9.90
Naphthalene		3.30	12.37
valerophenone		3.15	12.51

a. [28]

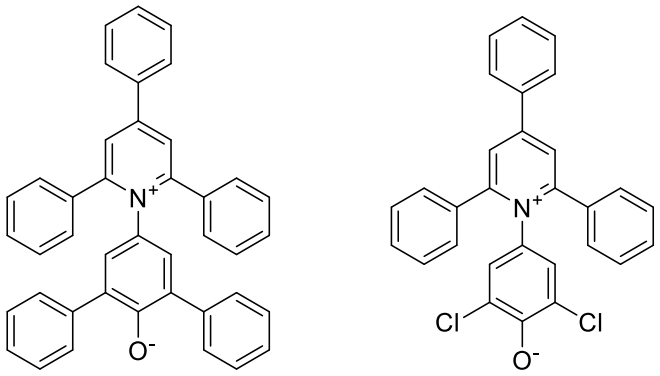
Table 2-9. Methylene selectivity of various HFIP-solvent two phase systems.

Solvent	% Total Solvent	H:T ratio	α_{CH2}	std. dev. of α_{CH2}
THF	9	1:1	0.485	0.013
THF	18	1:1	0.473	0.008
THF	36	1:1	0.448	0.045
THF	9	3:7	0.503	0.018
THF	9	7:3	0.476	0.020
DMF	9	1:1	0.41	0.008
Acetone	9	1:1	0.408	0.013
Butanone	9	1:1	0.491	0.030

Table 2-10. Correlation matrix between Log(K) values for all Aq/H-O systems as well as octanol-water Log(P) values.

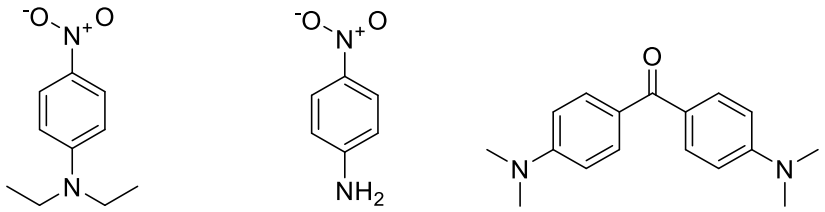
	Log(P) O-W	1:1 9% HT	1:1 18% HT	1:1 36% HT	3:7 9% HT	7:3 9% HT	1:1 9% HD	1:1 9% HA	1:1 9% HB
Log(P) O-W	1.000	0.578	0.639	0.644	0.729	0.486	0.513	0.464	0.564
1:1 9% HT		1.000	0.993	0.991	0.967	0.990	0.993	0.985	0.996
1:1 18% HT			1.000	0.994	0.987	0.970	0.978	0.961	0.989
1:1 36% HT				1.000	0.980	0.967	0.977	0.960	0.986
3:7 9% HT					1.000	0.929	0.943	0.917	0.965
7:3 9% HT						1.000	0.996	0.999	0.992
1:1 9% HD							1.000	0.996	0.995
1:1 9% HA								1.000	0.988
1:1 9% HB									1.000

Figures

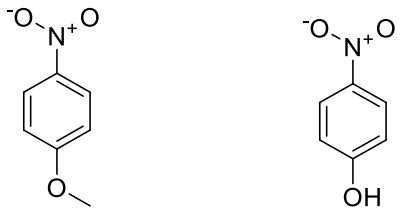


A B

Figure 2-1. Structure of A) Betaine 30 (Reichardt's Dye) and related B) Betaine 33



A B C



D E

Figure 2-2. Solvatochromic probes used for determination of π*, β, and α. A) N,N-diethyl-4-nitroaniline B) 4-nitroaniline C) Michler's ketone D) 4-nitroanisole E) 4-nitropehnol

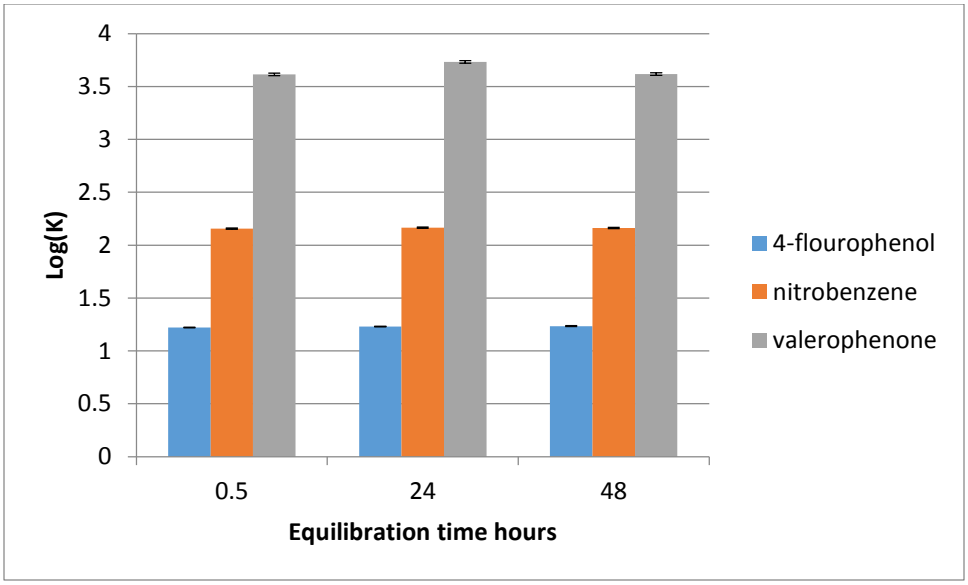


Figure 2-3. Partition coefficient equilibration time study. Measuring the effect of time on the measured partition coefficient of 4-fluorophenol, nitrobenzene and valerophenone in a 1:1 HFIP:THF 9% solvent two phase system

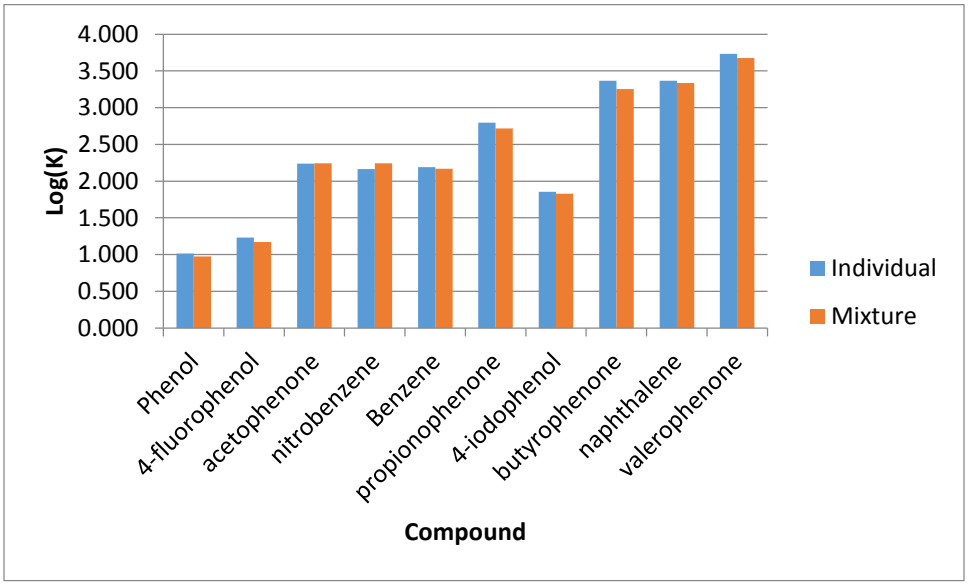


Figure 2-4. Comparison of Log(K) values measured for compounds in 1:1 HFIP:THF 9% two phase system when compounds are measured individually versus all at once.

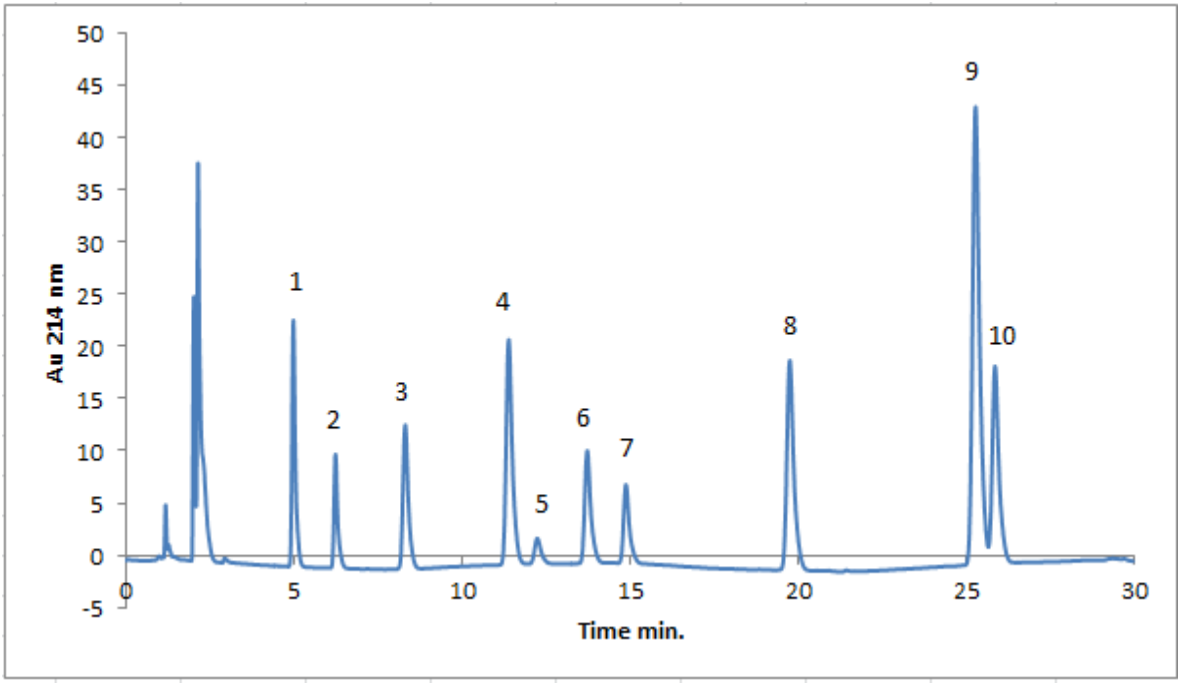
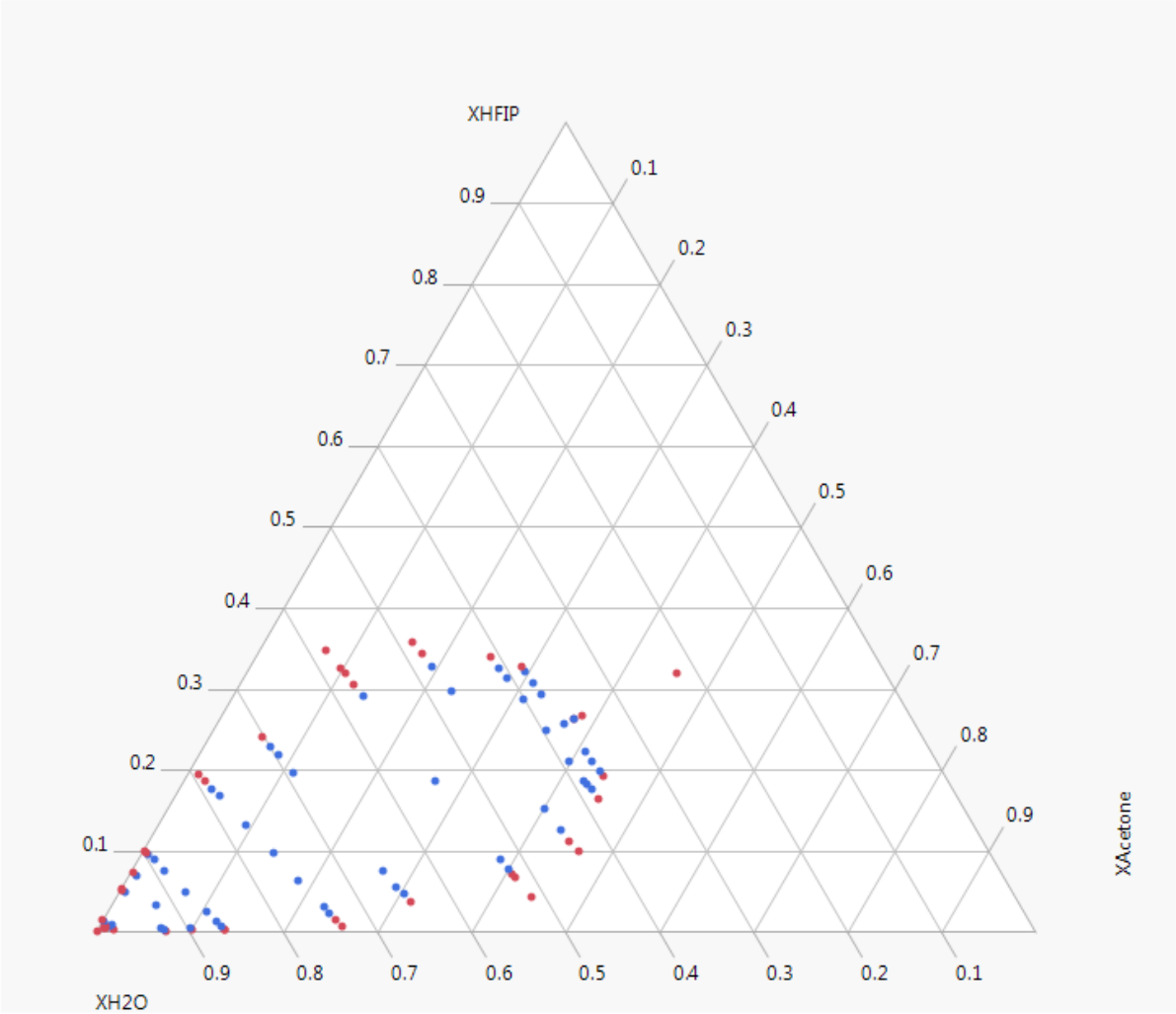
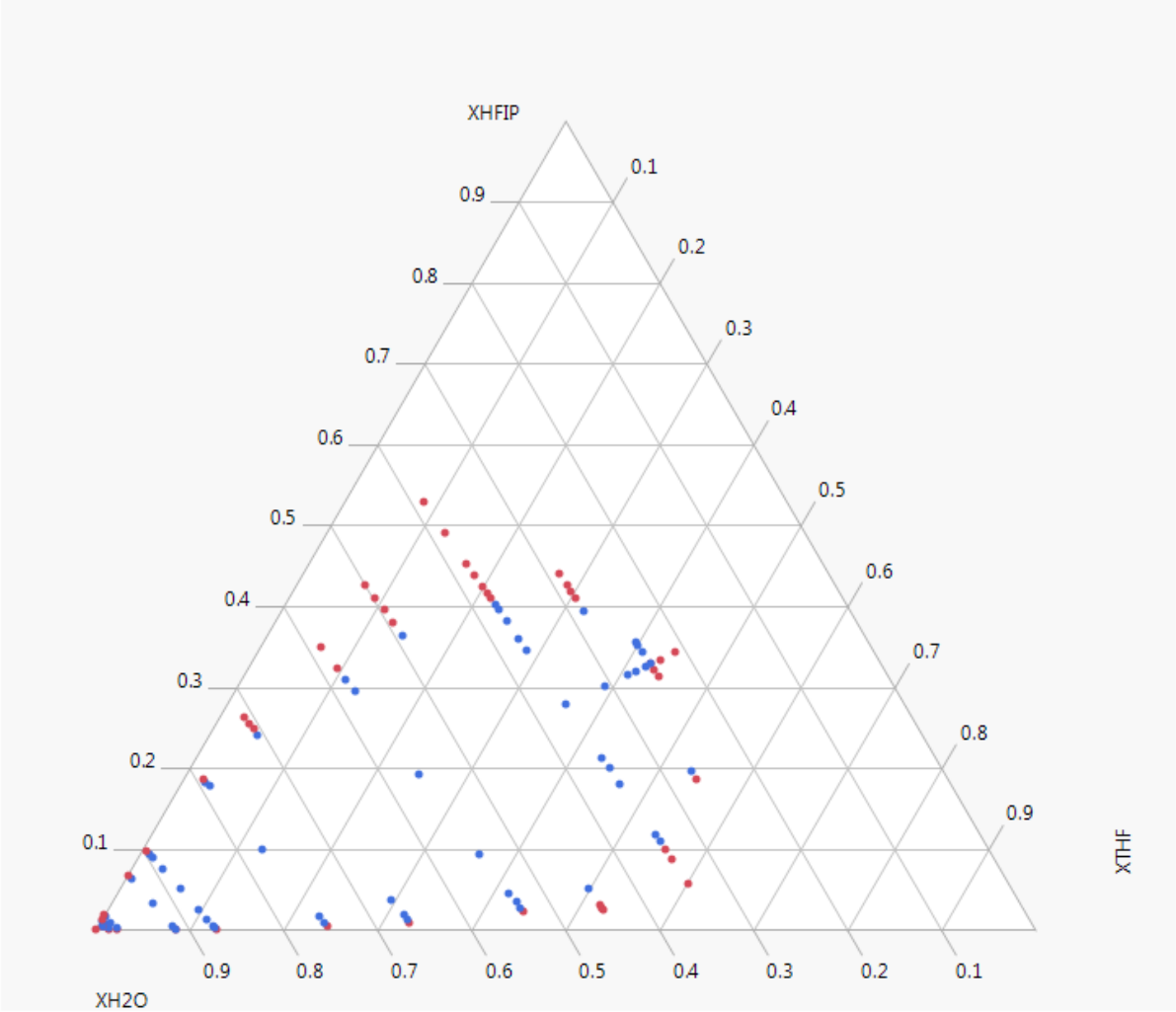


Figure 2-5. Chromatogram of 10 component mixture used for partitioning studies. 1) phenol, 2) 4-fluorophenol, 3) Acetophenone, 4) nitrobenzene, 5) benzene, 6) propiophenone, 7) 4-iodophenol, 8) butyrophenone, 9) naphthalene, 10) valerophenone. Mobile phases are A water and acetonitrile, C18 stationary phase.

Figure 2-6. Ternary phase diagrams for HFIP-water 3 component mixtures with A) Acetone B) THF and C) Butanone. Blue = two-phases, Red = single phase


XHFIP
0.1
0.9
0.2
0.8
0.3
0.7
0.4
0.6
0.5
0.5
0.6
0.4
0.7
0.3
0.8
0.2
0.9
0.1
XAcetone
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
XH2O

A


XHFIP
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0.1
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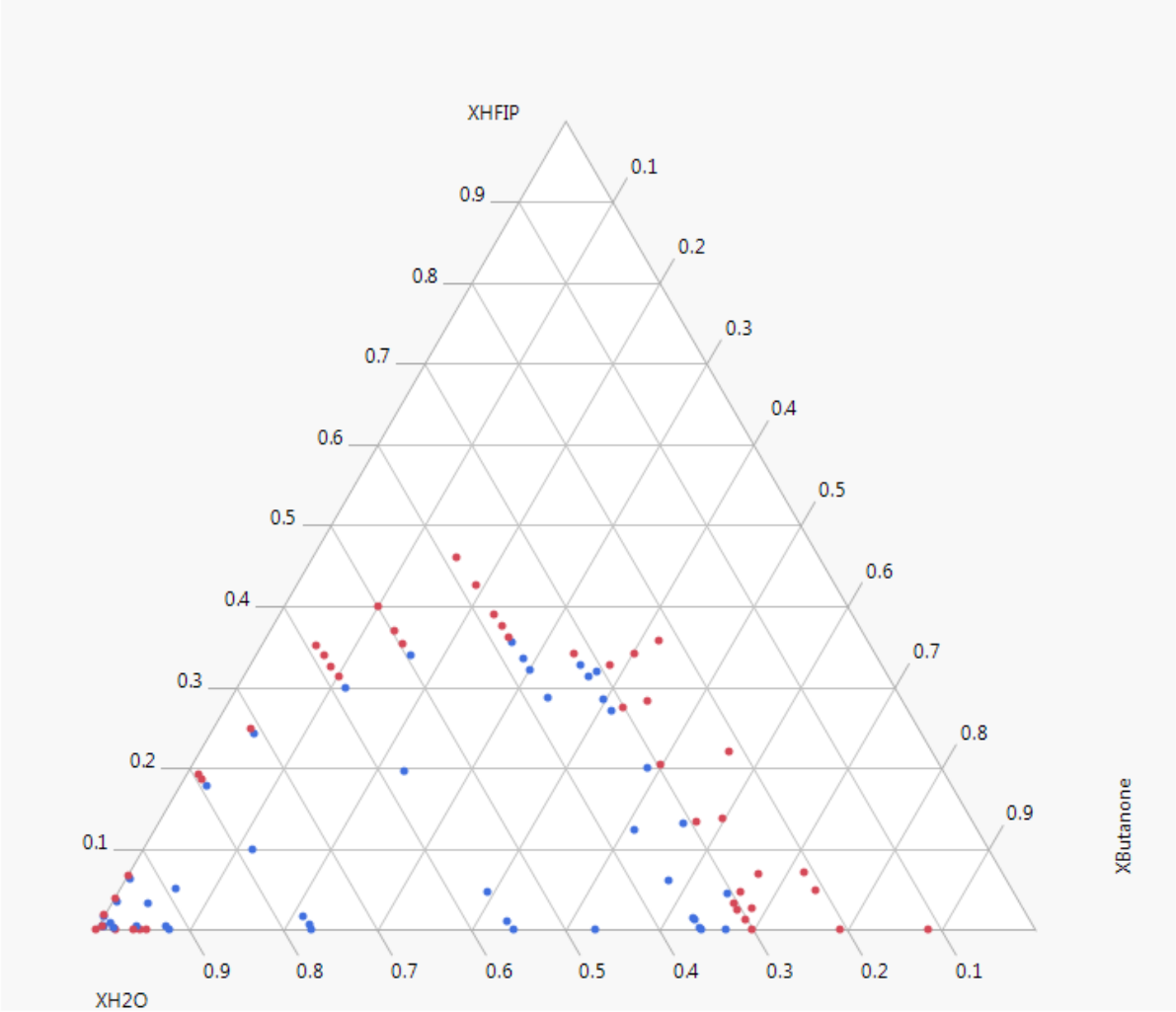
0.2
0.1
XH2O
XTHF

B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0.1
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0.8
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XButanone
0.9
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0.5
0.4
0.3
0.2
0.1
XH2O

C

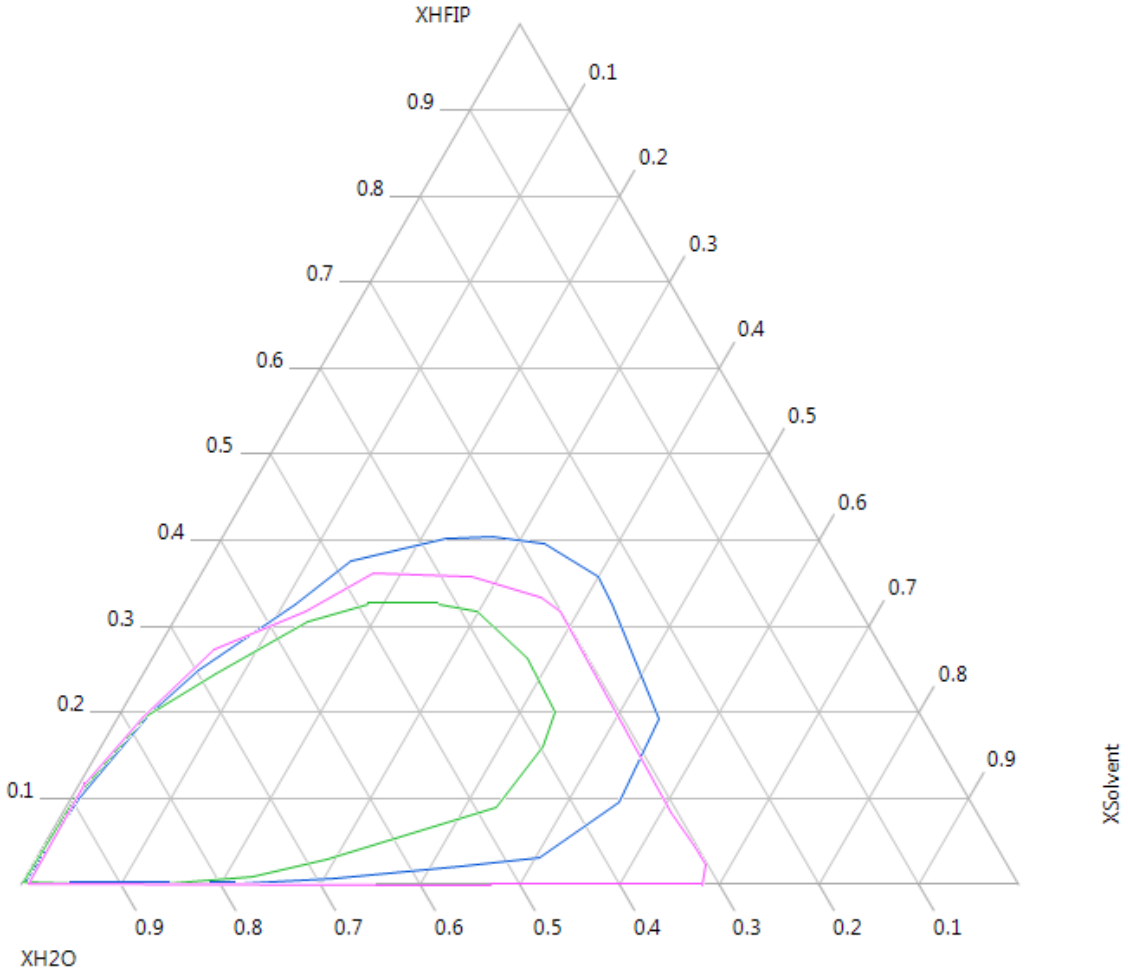


Figure 2-7. Overlay of approximate two-phase regions from ternary plots of HFIP and water with acetone (green), THF (blue) and butanone (purple).

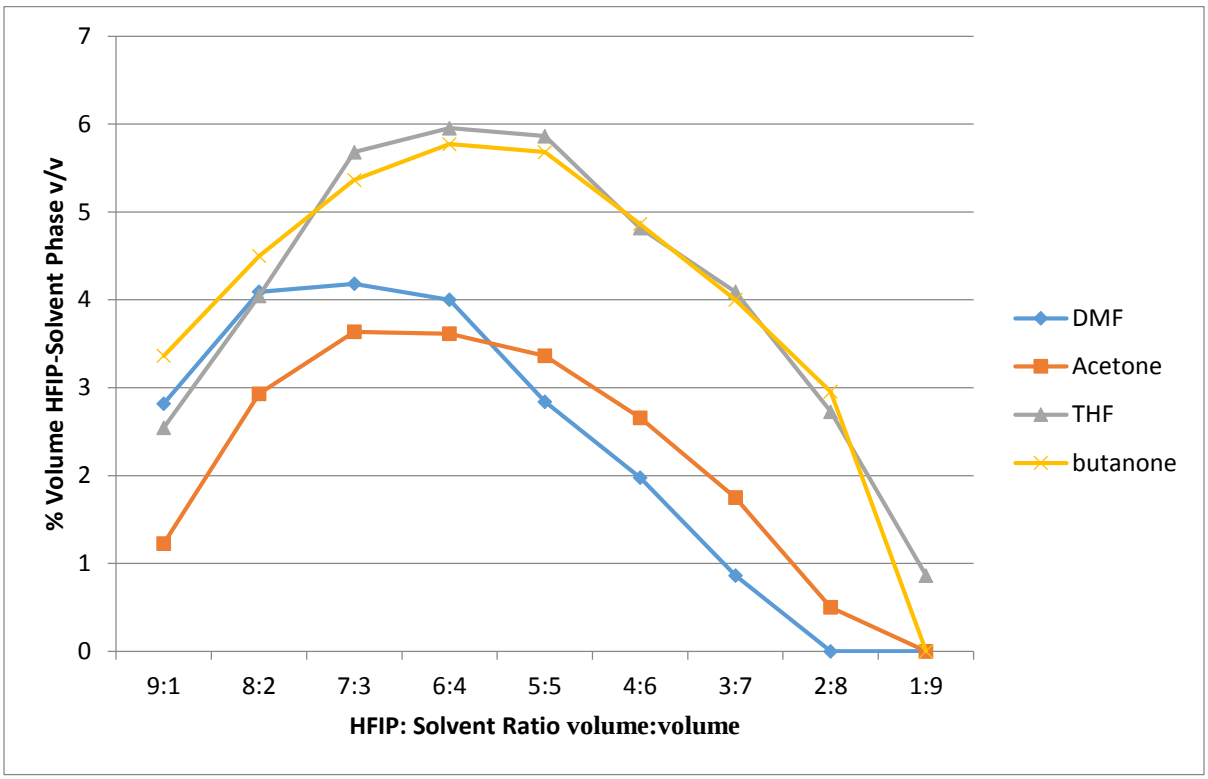


Figure 2-8. Effect of changing HFIP:solvent ratio on HFIP-solvent phase volume. All systems contain 9% total solvent v/v (HFIP + solvent combined)

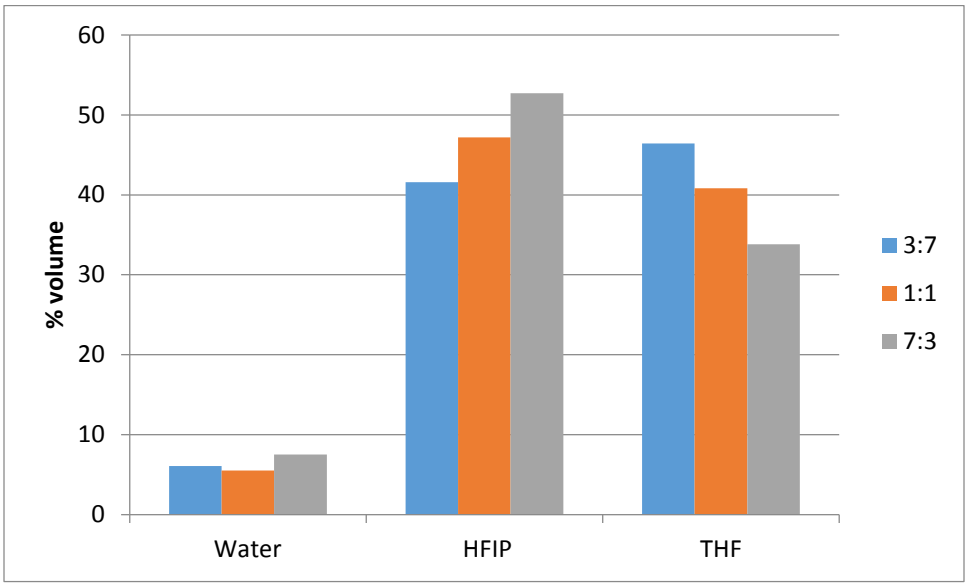


Figure 2-9. Effect of HFIP:THF ratio on composition of H-O phases from 9% total solvent HFIP-THF two-phase systems

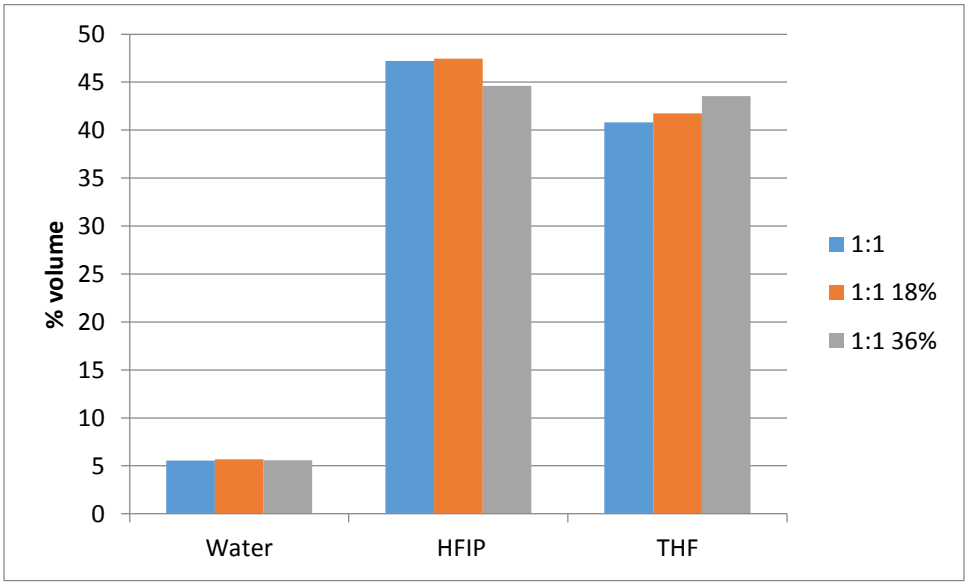


Figure 2-10. Effect of % (v/v) total solvent on H-O composition of 1:1 HFIP-THF two-phase systems

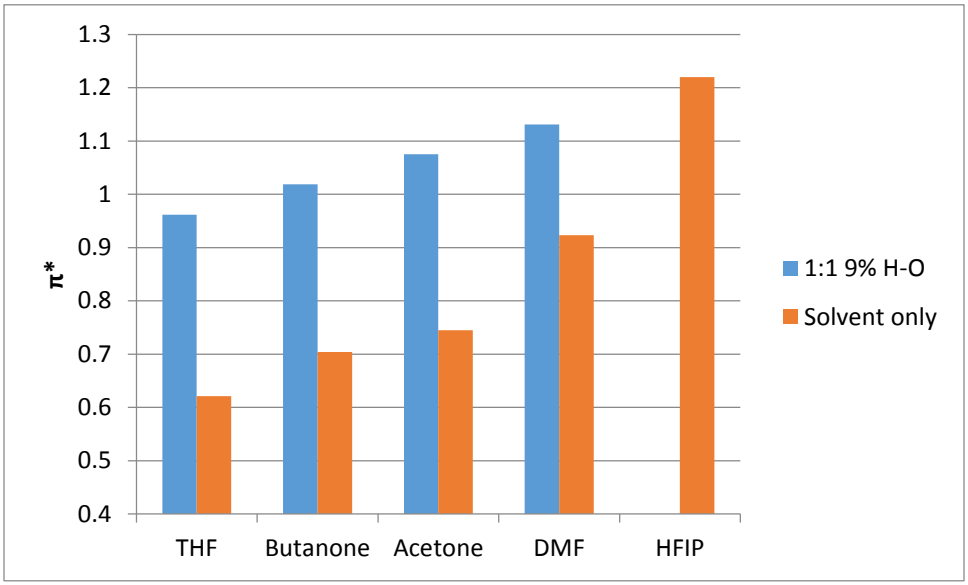


Figure 2-11. Comparison of π* values for 1:1 HFIP:solvent 9% total solvent two phase system solvent phases.

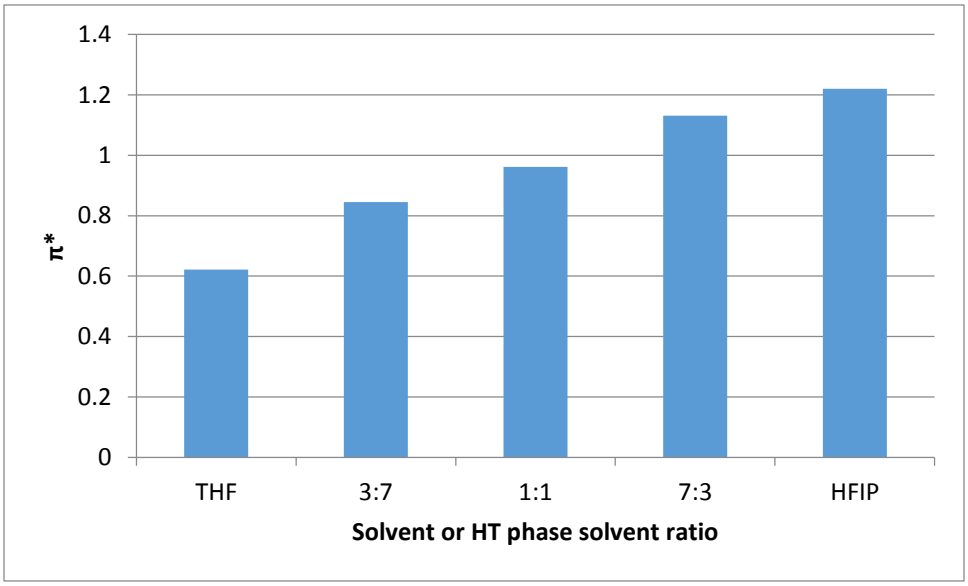


Figure 2-12. Effect of changing HFIP-THF mole ratio on the π* value observed for the HFIP-THF phase formed using 9% total solvent.

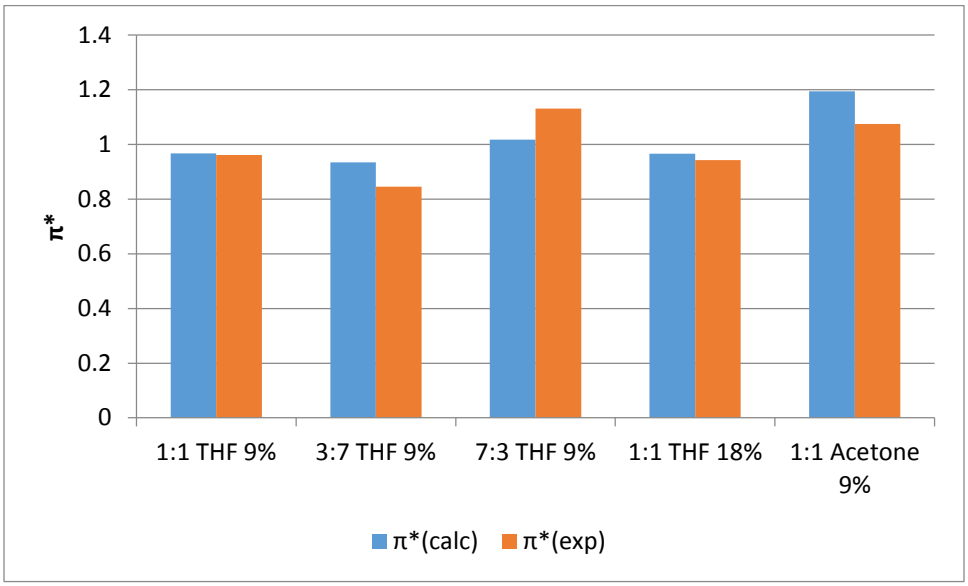


Figure 2-13. Comparison of measured (exp) π* values for H-O phases to the theoretical (calc) values calculated using weighted sum equation and GC-TCD data.

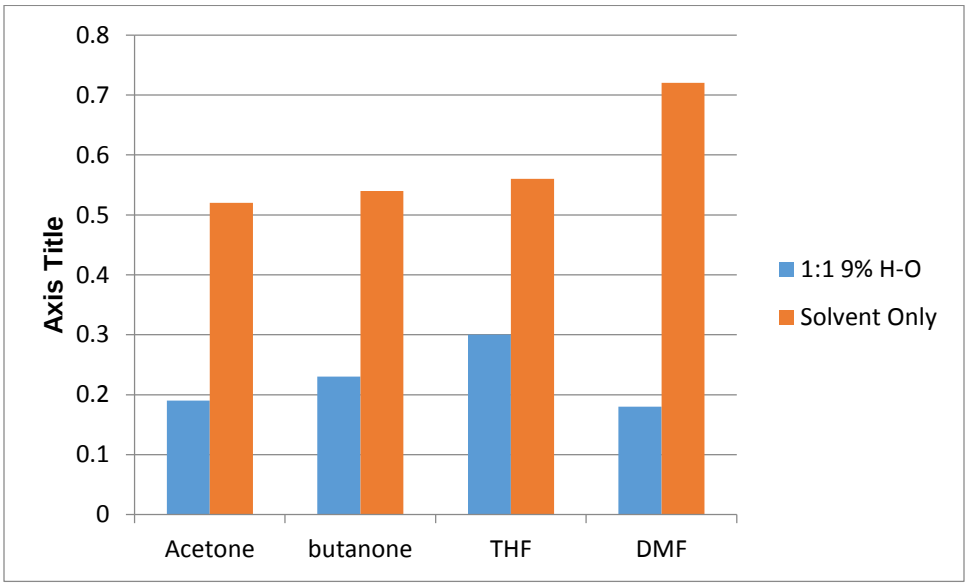


Figure 2-14. Effect of HBA solvent used for two-phase formation on measured β values for 1:1 9% H-O phases and comparison to the β values for the individual solvents.

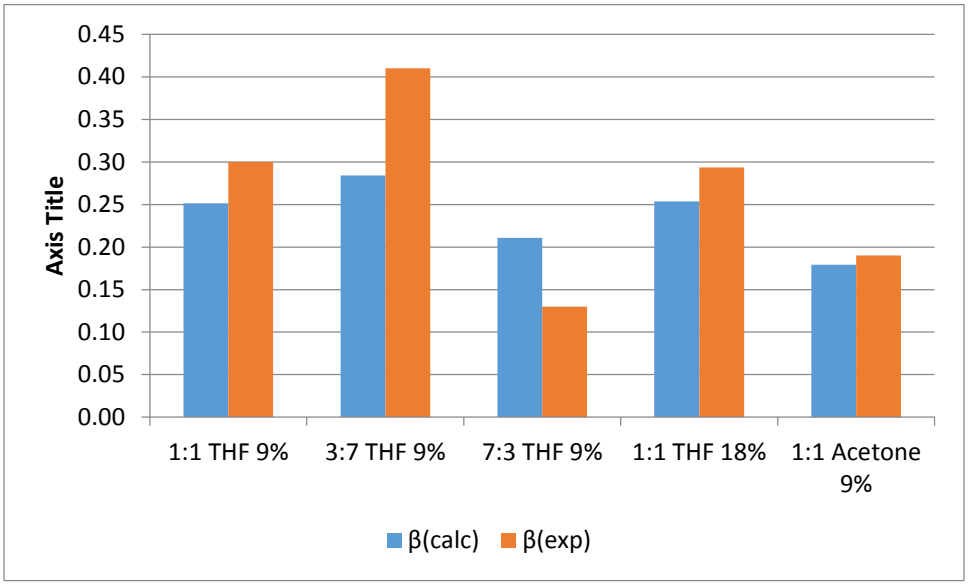


Figure 2-15. Comparison of measured (exp) β values for H-O phases to the theoretical (calc) values calculated using weighted sum equation and GC-TCD data.

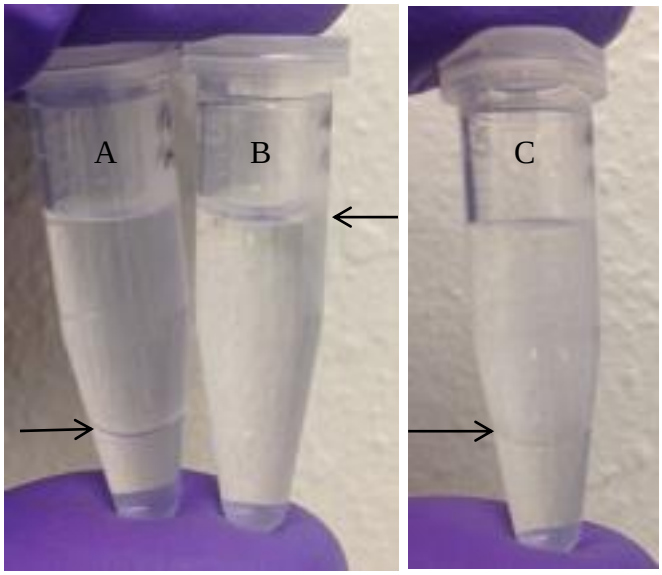


Figure 2-16. 10% HFIP solutions in water with the addition of 100 µL of A) anisole B) mesitylene and C) 100 µL mesitylene + 40 µL diethyl ether. Arrows indicate the interface between the two-phases.

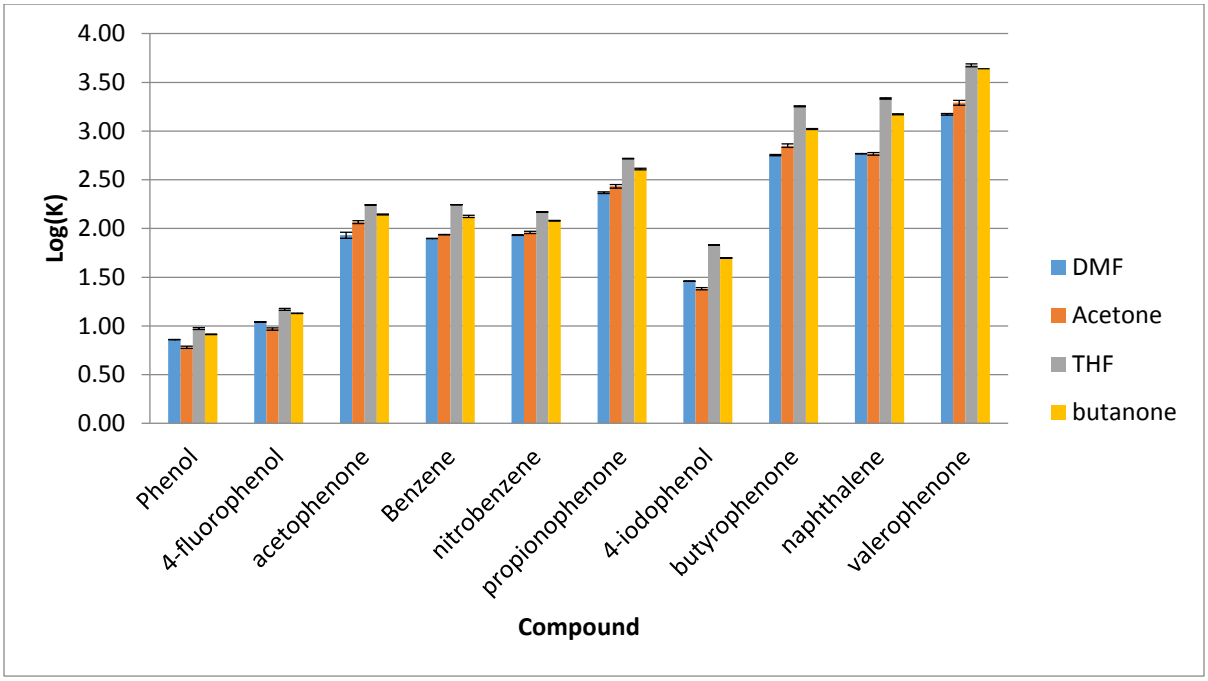


Figure 2-17. Effect of solvent on small molecule partitioning in HFIP-solvent 1:1 9% two-phase systems.

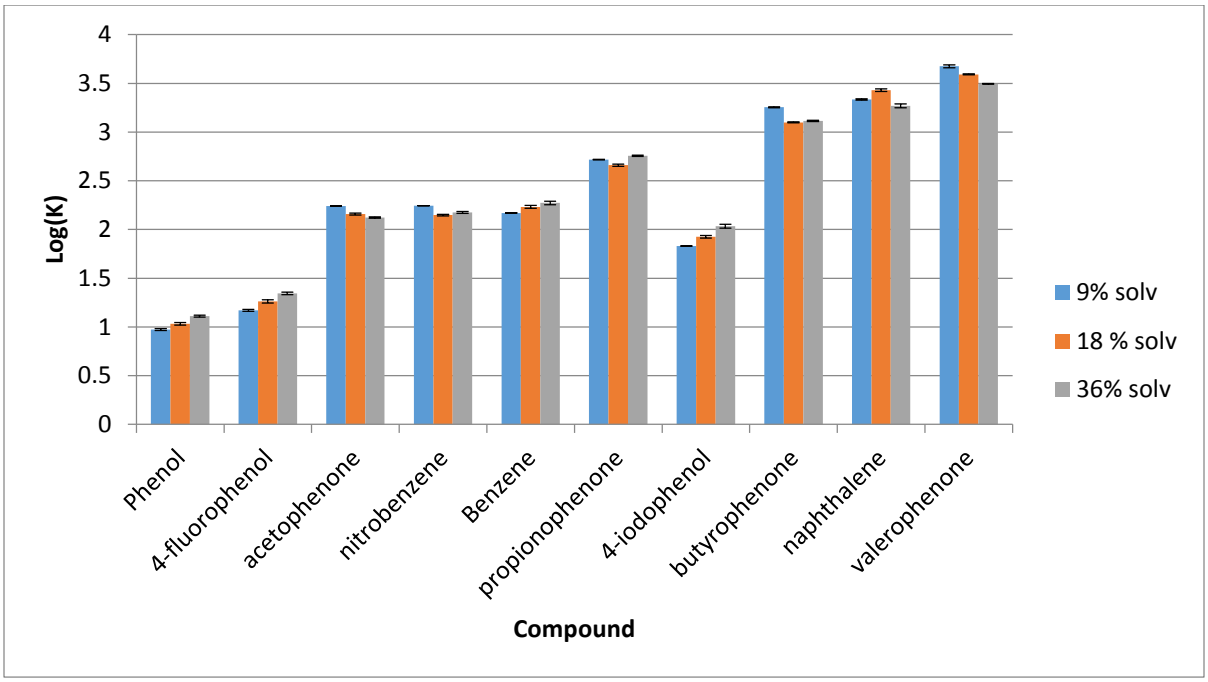


Figure 2-18. Effect of total % (v/v) of solvent used to form 1:1 HFIP:THF two-phase solutions on the partitioning of small molecules.

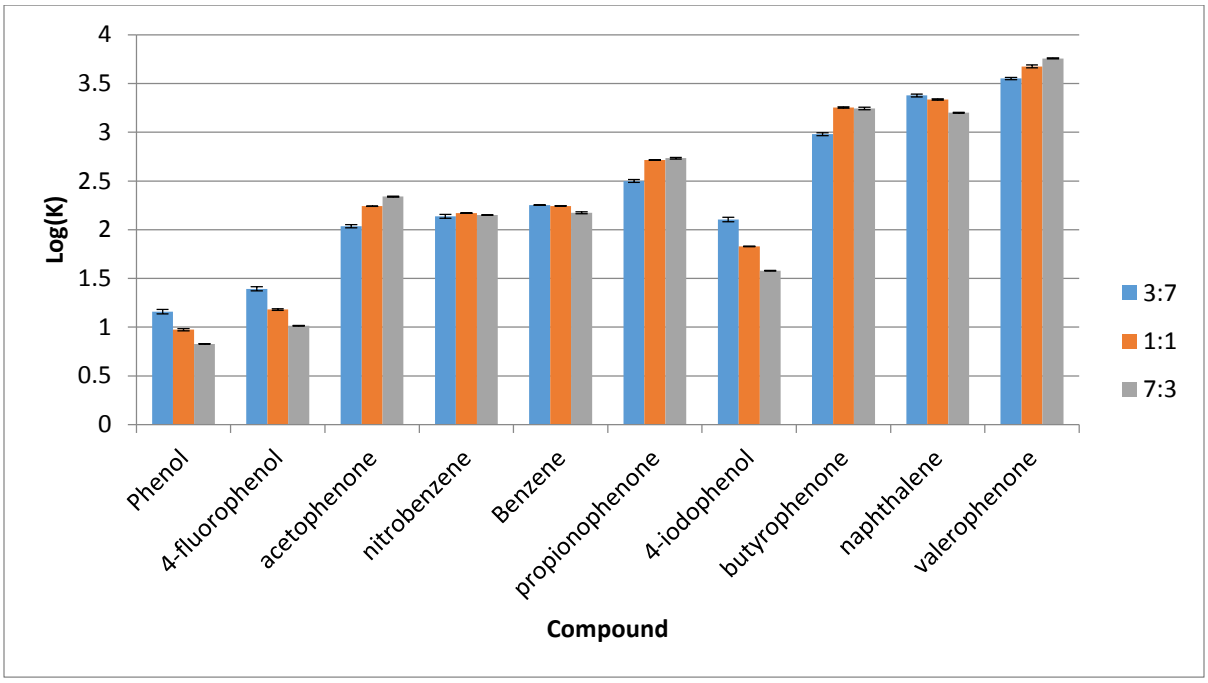


Figure 2-19. Effect of HFIP:THF ratio used to form 9% total solvent systems on small molecule partitioning in HFIP-solvent two phase systems.

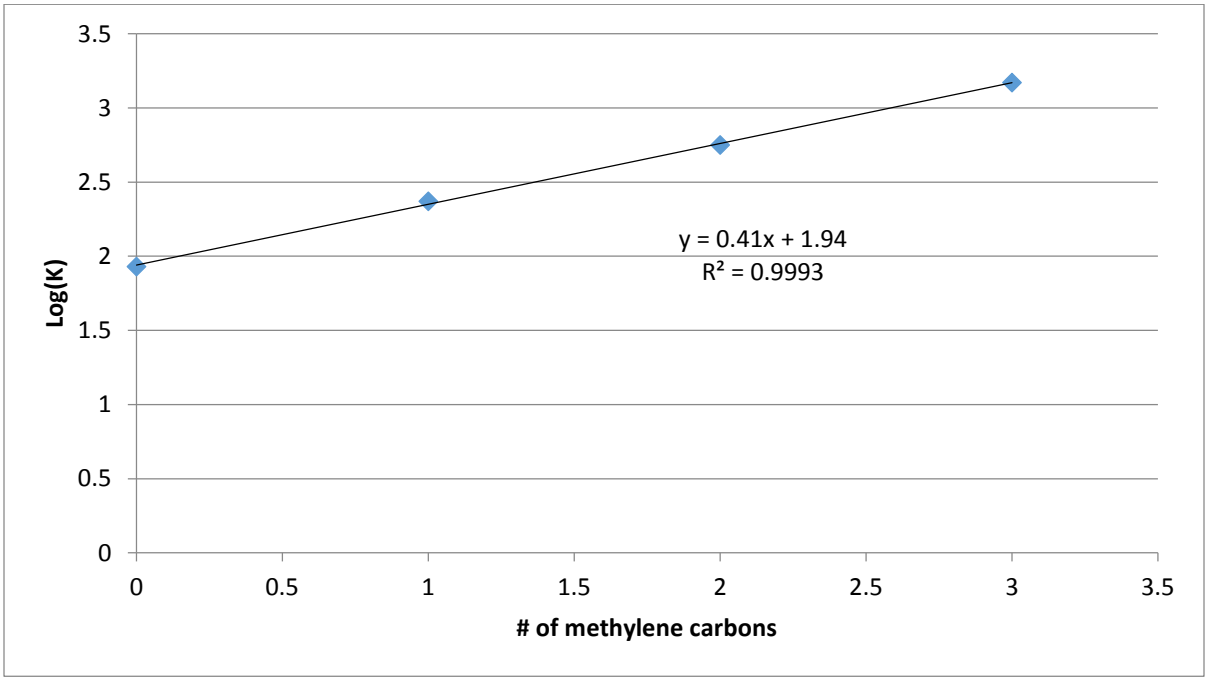
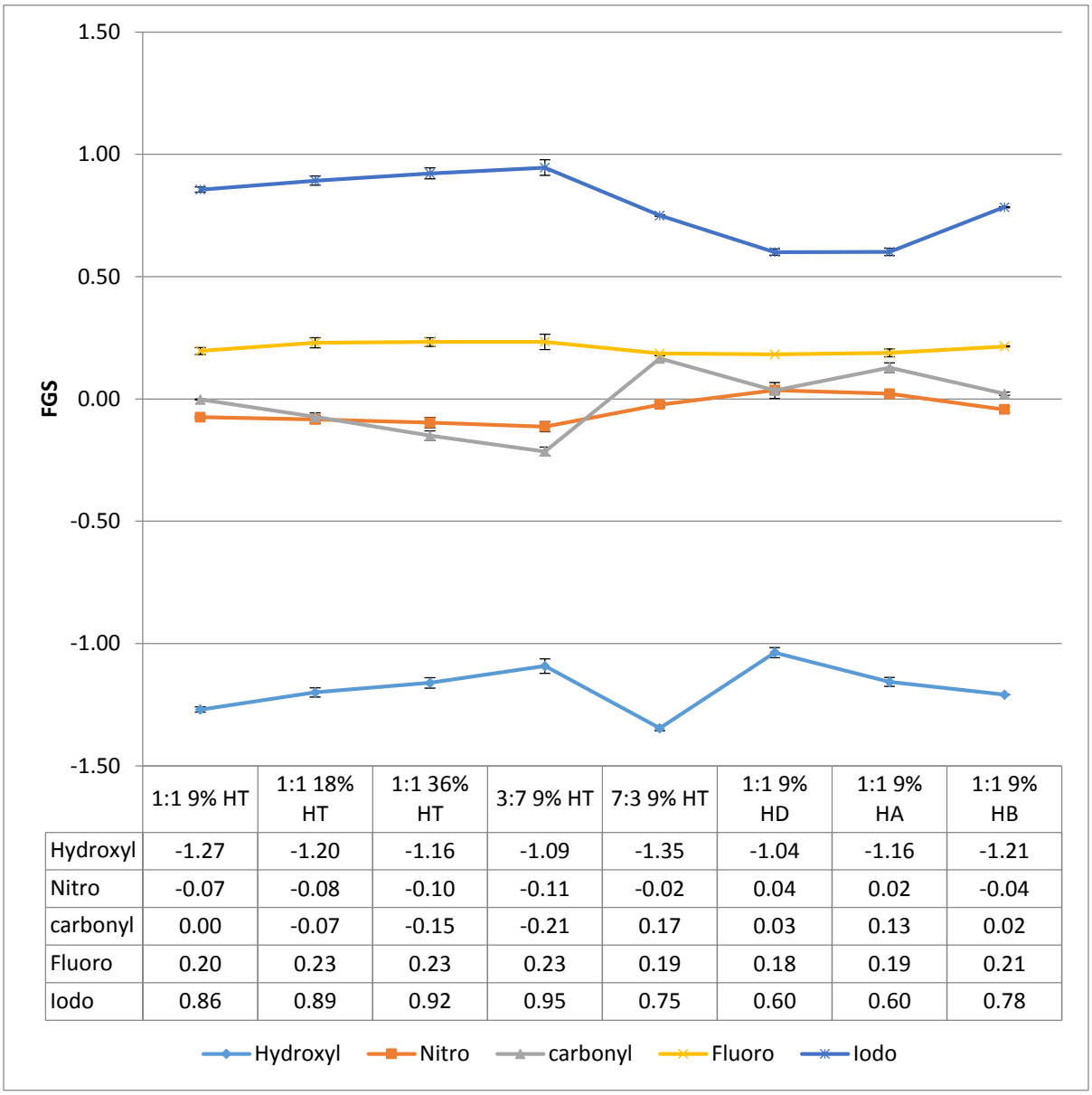


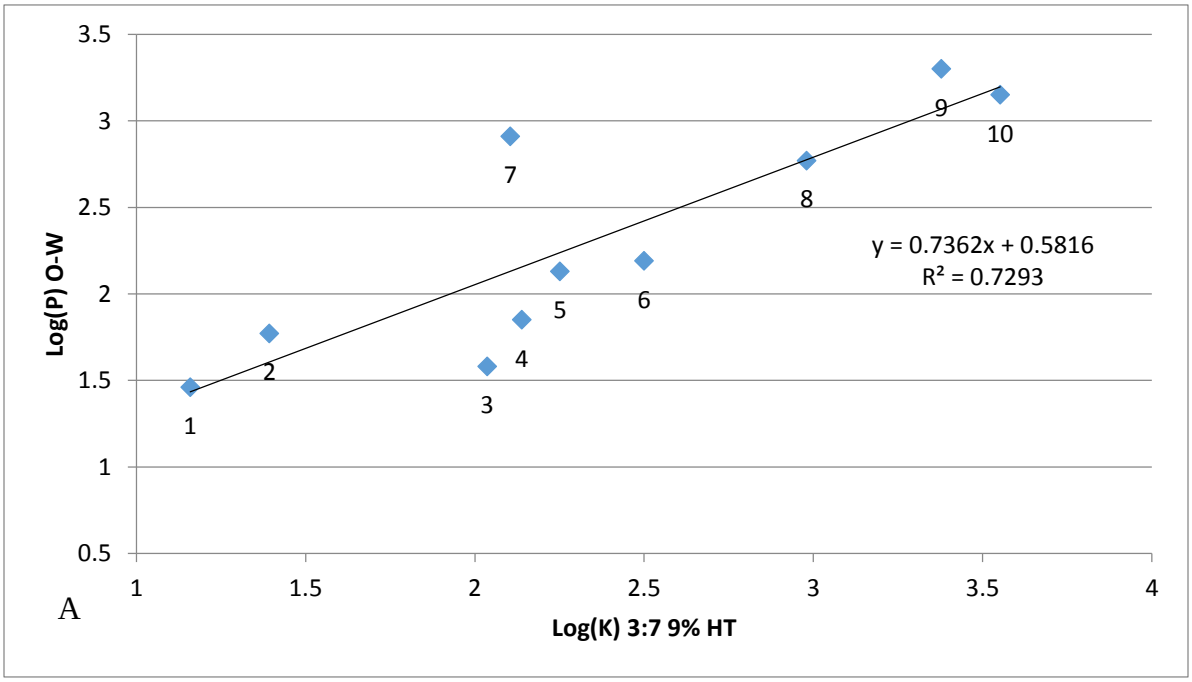
Figure 2-20. Plot used for determining methylene selectivity of 1:1 HFIP:DMF 9% total solvent two phase system.



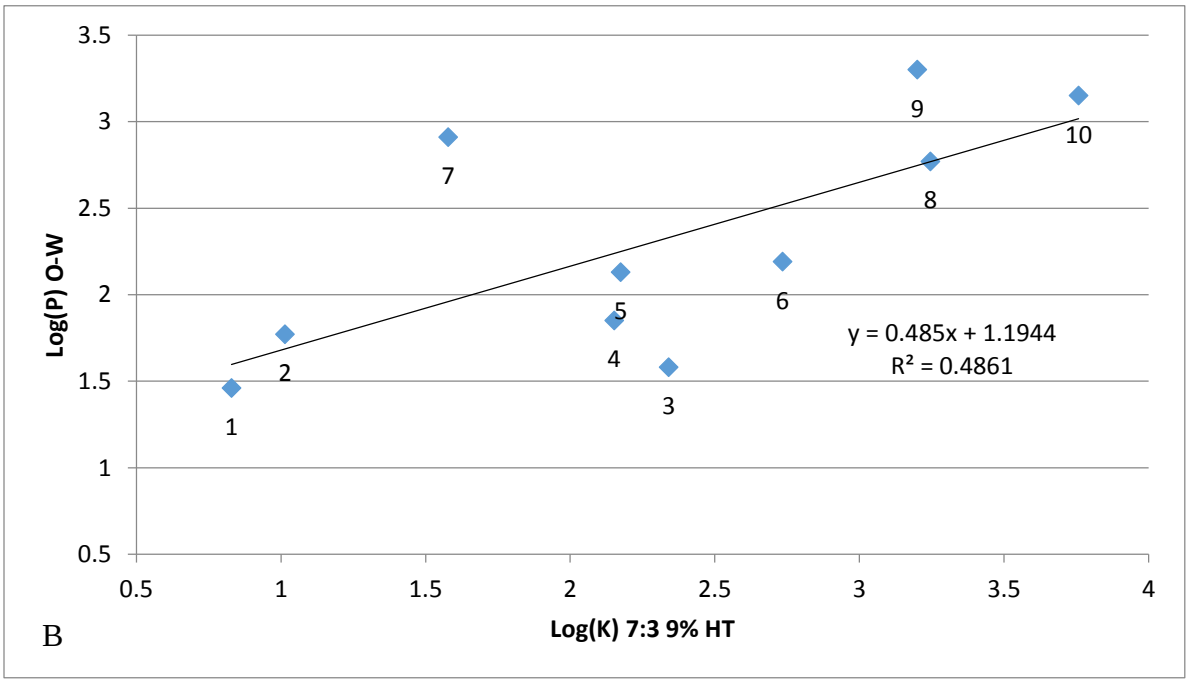
	1:1 9% HT	1:1 18% HT	1:1 36% HT	3:7 9% HT	7:3 9% HT	1:1 9% HD	1:1 9% HA	1:1 9% HB
Hydroxyl	-1.27	-1.20	-1.16	-1.09	-1.35	-1.04	-1.16	-1.21
Nitro	-0.07	-0.08	-0.10	-0.11	-0.02	0.04	0.02	-0.04
carbonyl	0.00	-0.07	-0.15	-0.21	0.17	0.03	0.13	0.02
Fluoro	0.20	0.23	0.23	0.23	0.19	0.18	0.19	0.21
Iodo	0.86	0.89	0.92	0.95	0.75	0.60	0.60	0.78

Figure 2-21. Functional group selectivity in various Aq/H-O two-phase systems. HT = HFIP-THF, HD = HFIP-DMF, HA = HFIP-Acetone, HB = HFIP-Butanone

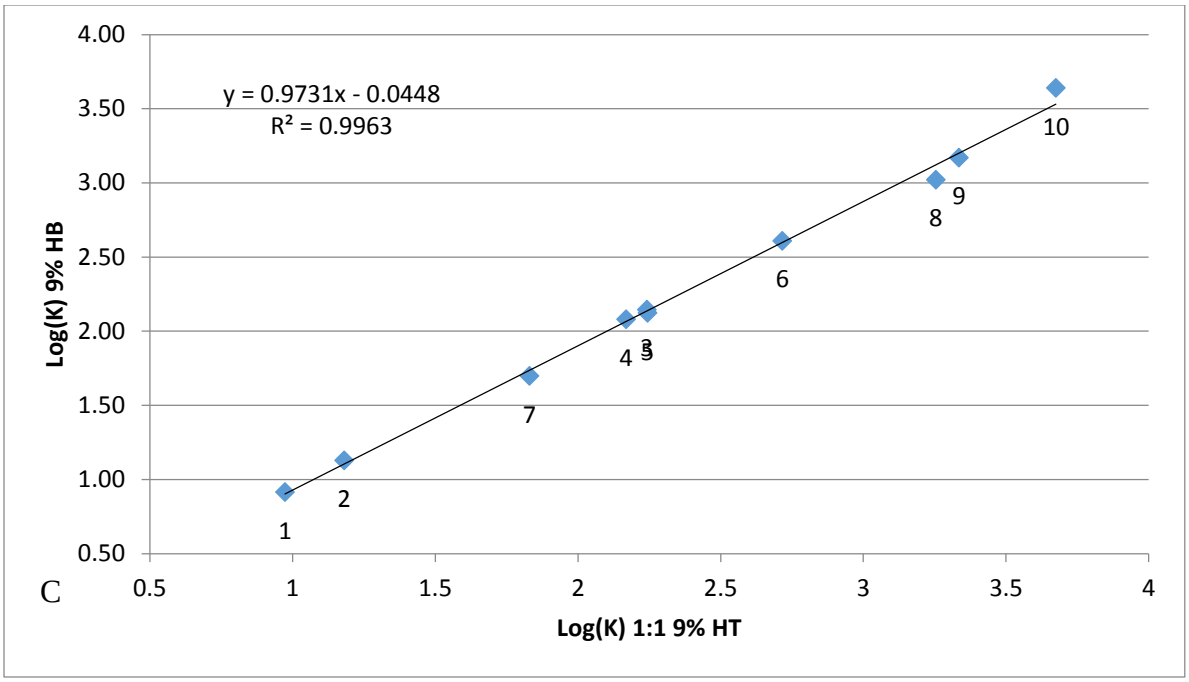
Figure 2-22. Linear correlation between Log(P) from Octanol-Water and Log(K) values for HFIP-Solvent two-phase systems. A) 3:7 9% HFIP:THF vs. O-W B) 7:3 9% HFIP:THF vs. O-W C) 3:7 9% HFIP:THF vs. 7:3 9% HFIP:THF D) 1:1 9% HFIP:Acetone. Data labels: 1) Phenol, 2) 4-fluorophenol, 3) acetophenone, 4) nitrobenzene, 5) benzene, 6) propiophenone, 7) 4-iodophenol, 8) butyrophenone, 9) naphthalene, 10) valerophenone


3.5
3
2.5
2
1.5
1
0.5
1
1.5
2
2.5
3
3.5
4
Log(P) O-W
Log(K) 3:7 9% HT
y = 0.7362x + 0.5816
R² = 0.7293
1
2
3
4
5
6
7
8
9
10

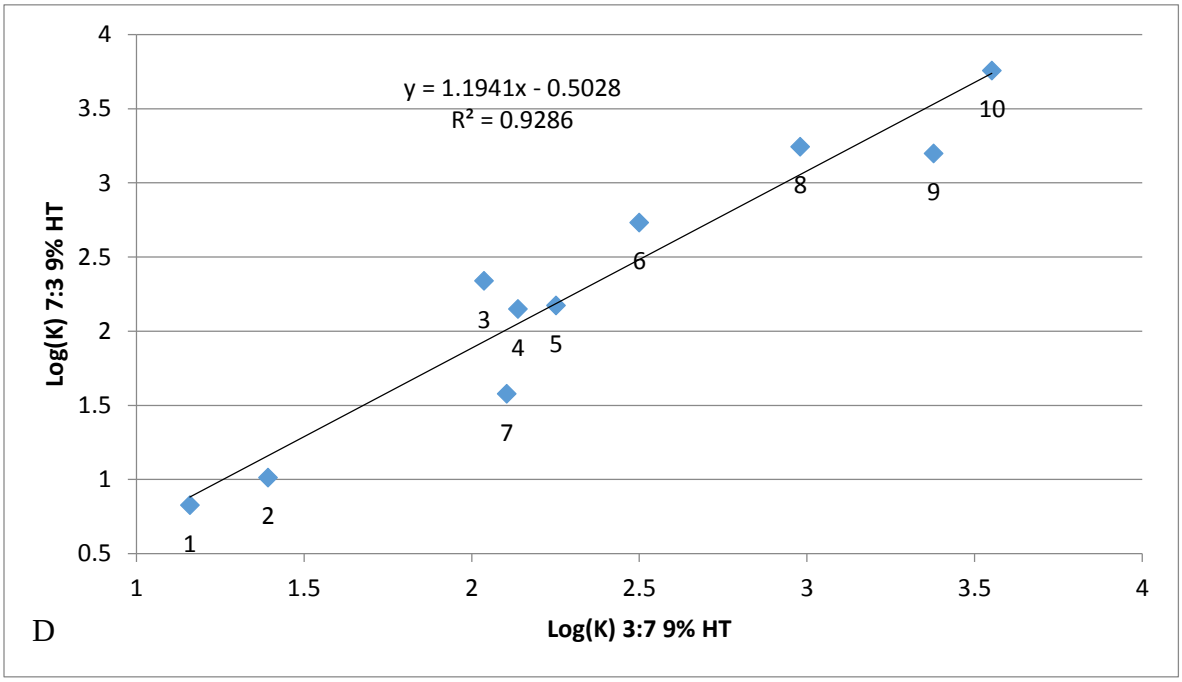
A


3.5
3
2.5
2
1.5
1
0.5
0.5
1
1.5
2
2.5
3
3.5
4
Log(P) O-W
Log(K) 7:3 9% HT
y = 0.485x + 1.1944
R² = 0.4861
1
2
3
4
5
6
7
8
9
10

B


y = 0.9731x - 0.0448
R² = 0.9963
Log(K) 9% HB
Log(K) 1:1 9% HT
4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.5
1
1.5
2
2.5
3
3.5
4
1
2
3
4
5
6
7
8
9
10

C


y = 1.1941x - 0.5028
R² = 0.9286
Log(K) 7:3 9% HT
Log(K) 3:7 9% HT
4
3.5
3
2.5
2
1.5
1
0.5
1
1.5
2
2.5
3
3.5
4
1
2
3
4
5
6
7
8
9
10

D

Chapter 3

Organic Synthesis in Aqueous HFIP-Organic Two-Phase solutions

- **Part of this chapter has been published in:**

  Weisner, N.; Khaledi, M. G. Organic synthesis in fluoroalcohol-water two-phase systems. *Green Chem.* **2016**, *18*, 681-685.

Abstract

The addition of organic reactants to aqueous solutions of HFIP results in a two-phase solution where one phase is largely aqueous and the other is enriched in HFIP and the organic reactant. These types of solutions were investigated as a new medium for organic synthesis. The initial reactions investigated were Friedal-Crafts benzylations to produce 1,1-diarylalkanes using a benzyl chloride alkylating agent. Good to excellent yields were observed for arene and heteroarene nucleophiles of varying nucleophelicities in aqueous solutions containing 10-50% HFIP v/v with 30 minute reaction times. Base additives were not needed for removal of the HCl by product since it partitions into the aqueous phase after it is created driving the reaction forward. Reactions using benzyl alcohol alkylating agents were also successful requiring the use of heat and Bronsted acid catalysis. The diarylalkane products were able to be purified from the HFIP-solvent phase using flash chromatography without the need for liquid-liquid extraction. Diels-Alder reactions were also shown to proceed in these two-phase solutions with the strong hydrogen bond donation ability of HFIP influencing the selectivity of the reaction between isoprene and methyl vinyl ketone.

Introduction

The use of water as a solvent for organic synthesis reactions was ignored for a long time due to the fact that most organic reactants are not soluble in water and because of the labile nature of many organic functionalities including organometallic compounds in water which are often used as catalyst.[1,2] Interest increased when Breslow and Rideout reported that the Diels-Alder reaction was greatly accelerated when carried out in water compared to those carried out in traditional volatile organic compounds (VOC's).[3] This acceleration was attributed to the "hydrophobic effect that can accelerate reactions by driving hydrophobic reactants closer together in order to decrease their surface area of contact with water. The same idea applies to many enzymatic reactions where the substrate molecules may be introduced to the active site of the enzyme through hydrophobic interactions.[4] This observation of rate acceleration led to further research of using water as a solvent for organic reactions and it was found that hydrogen bond stabilization of the Diels-Alder transition state also contributed to rate acceleration along with the hydrophobic effect.[5] These results and others showed that water is a unique medium for organic synthesis reactions and offer properties not available in traditional solvents, Volatile Organic Compounds (VOC). In addition, water has the added advantages of being a safer and environmentally benign solvent than traditional hazardous VOC. The problem was that most of these results were done in very dilute aqueous solutions due to the low solubility of the compounds in water which limits the practical uses of water as a solvent for laboratory or industrial synthesis. It was then shown that reactions could be accelerated by mixing the reactants with water to form aqueous suspensions. These reactions were termed as "on-water" and allowed for the use of larger amounts of reactants as they did not have to be soluble in the water and formed

separate phases when not being mixed.[6,7] "On-water" reactions were often shown to give better results than "in-water" or solvent free conditions in terms of yield and reaction rates.[7-9]. Several explanations for the enhanced reactivity of on-water reactions were initially offered such as reactions occurring through small amounts of reactants dissolved in water or enhanced hydrogen bonding.[7] Currently the most widely accepted explanation is that the enhanced reactivity is due to acid base chemistry at the water organic interface. Hydroxide ions are strongly adsorbed to the water interface allowing protons to catalyze reactions in the organic phase.[10,11] "On-water" conditions have been applied to several types of reactions including Friedel–Crafts benzylations (FCB)[9] although only reactive heteroarenes could be used due to the high nucleophilicity of water, which traps the electrophiles, thus preventing less nucleophilic reactants from reacting in the presence of large amounts of water.[12] Although water is considered a green solvent one must be careful when claiming a method that uses water is a green method. Often times VOC's are still used in the workup steps of the protocol since organic products are often insoluble in water. [13] This is often unavoidable but must be taken into account when claiming the greenness of a method and any reduction in solvents used is still a benefit form a green chemistry perspective.

In addition to using "On water" conditions the limitation of reactant solubility in water can be overcome through the use of surfactants either to form aqueous micellar solutions or emulsions in which to carry out organic synthesis reactions. In 1997 Shu Kobayashi's group, one of the key contributors in this area, reported Lewis Acid (LA) catalysis of Aldol additions in water by using a mixture of triflates salts of scandium or lanthanide ions serving as a LA in aqueous solution of SDS.[14] The LA's used were scandium and lanthanide triflates which were found to be water stable and a surfactant such as SDS

was used to solubilize the reactants. They called this a Lewis Acid Surfactant Combined (LASC) catalyst by mixing a LA such as scandium ion with SDS to make $Sc(DS)_3$ which acts as both a catalyst for the reaction and a surfactant to solubilize the reactants.[15] They proposed that by combining the LA counter ion with the surfactant that the reactants will be concentrated near the LA in solution allowing the LA to more easily catalyze the reaction. The use of LASC's was shown to be applicable to several reactions including multicomponent reactions such as the Mannich reaction and the Diels-Alder reaction.[15-17] Similar studies were done with Bronsted acid catalysis in water using surfactants to solubilize the reactants. Dodecylbenzenesulfonic acid (DBSA) was shown to act as a combined Bronsted acid surfactant catalyst (BASC) to catalyze three component Mannich reactions in water.[18] Using the combined surfactant catalyst was shown to work better than using a solution with a separate surfactant and acid such as SDS and HCl. It was later shown that DBSA catalyzed other reactions including esterification. The by-product of the esterification reaction is a water molecule. Dehydration reactions such as this are typically difficult to carry out in the presence of large amounts of water since the reverse reaction, hydrolysis, is typically favored.[19,20] As the water was produced during the esterification reaction it was expelled from the emulsion droplets created by the BASC and reactants as a result of which the reaction shifted favorably to yield more of the product. Even without any kind of acid catalysis surfactants can increase reaction rates through what is known as micellar catalysis or microemulsion catalysis as they can concentrate reactants near micelles and increase rates even though they are not involved in the reaction mechanism like a traditional catalyst.[21] The type of surfactants used for micellar-catalyzed reactions as well as in emulsion solutions can impact the reaction. The use of ionic surfactants often leads to

either acceleration or inhibition depending on the reaction and the charge of the reactants or intermediate species.[21,22] Micellar and emulsion systems can also have a preorientation effect on the reactants and affect the regioselectivity of the reaction.[21,23] The molecules orient themselves to engage in hydrophobic/electrostatic interactions within the micelle or emulsion droplets which effect what region of the molecule is open to react or in what orientation it will be when it reacts.[24,25]

In addition to water, other alternative solvent systems for organic synthesis have been investigated. One example is the use of Ionic Liquids (IL's) as a medium for organic reactions. IL's are organic salts in the liquid state and typically defined as having melting points below 100°C. The usefulness of ionic liquids in organic synthesis stems from their versatility as a solvent due to the ability to change the cation and anion to tailor the solvent properties for a particular reaction.[26] IL's have also been viewed as a green alternative to VOC's due to their low volatility and flammability.[26] The development of "Task Specific Ionic Liquids" which can include functionalities such as Bronsted acids increased their applicability as media for synthesis reactions.[27,28] Most IL's have polarities similar to short chain alcohols such as ethanol and thus may not be able to solubilize very hydrophobic compounds well and in order to make them more nonpolar longer alkyl chains on the cation must be used which can increase the toxicity of the IL's.[26,29] Nevertheless IL's have been applied to a large variety of organic reactions and may find more widespread use in research and industry as advances continue.[28,30,31] Supercritical Fluids specifically supercritical CO_2 (SCCO2) are another alternative solvent system being investigated. The solvation properties of SCCO2 like other supercritical fluids can be adjusted by altering the temperature and pressure near the critical values making it more tunable than traditional VOC similar to ionic

liquids.[32] SCCO2 and other supercritical fluids have been shown to be useful for synthesis in several carbon-carbon bond formation reactions and support the use of metal based catalysts.[33-35] SCCO2 is typically considered to be a weak solvent although all gases are miscible in it.[36] Solubility of organic compounds can be improved by adding a cosolvent such as methanol but this reduces the greenness of SCCO2 which is one of the main appeals of using it as a solvent.[32] Solvent free or solventless chemistry has also been applied to organic synthesis with certain amounts of success. Although the claim of being solvent free can be disputed [37] these methods do use smaller amounts of VOC than traditional methods. Certain problems arise when a solvent is not used such as high solution viscosity and production of solids which are not ideal for industrial applications.[38] These have been overcome to some degree through the use of techniques such as ball-milling and microwave heating.[39-41] Highly exothermic reactions are hard to carry out with solvent free methods as they can become explosive or more dangerous with no solvent to absorb the heat. All of these alternative methods have shown uses as media or methods for organic synthesis which in some cases can give unique results not obtained in traditional VOC. Each also has its own limitations that prevent widespread use in terms of replacing VOC whether it is lower solubility of hydrophobic reactants or difficulties in terms of use in industrial applications.

The initial goal of this research was to investigate coacervate systems as alternative media for organic reactions. A coacervate is a colloidal mixture in which the colloidal assemblies form a separate phase from the aqueous medium, where the two immiscible phases contain an appreciable amount of water.[42] Coacervates are often formed in solutions containing amphiphilic molecules which phase separate under certain conditions to form one phase that is rich in amphiphile called the coacervate phase and another aqueous-rich phase

that is lean in amphiphile. Coacervates can be categorized as either simple or complex with simple coacervates being composed of a single amphiphilic molecule and complex coacervates containing two amphiphilic molecules of opposite charge. Phase separation can occur with the addition of a salt or other additive such as an alcohol or with a change in pH or temperature.[42,43] In particular, this research has focused on a class of coacervate system that was first discovered in our laboratory. These coacervates are induced in the presence of perfluorinated alcohols or perfluorinated acids in aqueous solutions of a wide range of amphiphilic molecules. These coacervates are formed by the addition of a fluoroalcohol, primarily hexafluoroisopropanol (HFIP), trifluoroethanol (TFE), or a fluoroacid, such as trifluoroacetic acid (TFA) and heptafluorobutyric acid (HFBA), to a mixture of two oppositely charged surfactants or a single amphiphile. Without the addition of the fluoroalcohol, the two oppositely charged surfactants would typically form a precipitate. Systems composed of only one amphiphilic molecule have also been discovered under certain pH conditions such as SDS with HCl and HFIP or CTAB with Tris buffer and HFIP. As compared to previously discovered coacervates [42,44,45] these Perfluoro-Aclohol/Acids Induced Coacervates (PFAIC) can be formed over a much wider range of conditions potentially enabling the environment to be tailored to a specific application.

Phase separation in aqueous HFIP solutions upon the addition of amides were first reported by Takamuku et. al.[46,47] In these solutions all three components were individually miscible with another component but the combination of all three at certain concentrations led to a two phase solution. This phase separation was attributed to weakening of the hydrogen bond interaction between water and the carbonyl group of the amide due to the formation of a hydrophobic shell of trifluoromethyl groups from HFIP around the

hydrophobic moieties of the amide as the alcohol concentration increases. This preferential solvation by HFIP eventually leads to a hydrophobic environment from which water molecules are excluded leading to phase separation. This newly formed phase is highly concentrated in HFIP and the amide with low water concertation. During the course of this research it was found that similar selective solvation was observed when hydrophobic compounds were added to aqueous solutions of HFIP. Compounds such as anisole are not miscible and will float on top of water. When added to an aqueous solution of HFIP the HFIP will selectively solvate anisole and form a two phase solution in which anisole and HFIP are concentrated in the bottom phase due to the higher density of HFIP. In order to be solvated by HFIP it is necessary that the compound is able to interact with HFIP in hydrogen bonding and contain a hydrogen bond accepting group such as the oxygen in anisole. Upon this discovery these types of solutions were investigated further as a new medium for organic synthesis. The absence of surfactants in these types of two phase solutions offers an advantage over the coacervate solutions as the surfactants must be separated from the products in order to isolate the product. Additionally, the amounts of reactants used are much larger for the Aq/H-O two phase systems since in the coacervate solutions the reactant concentration is based on the volume of the coacervate phase which is usually a small fraction of the total volume, which will result in larger actual yields even if the % yields are comparable.

Fluoroalcohols have also been shown to be beneficial as solvents or additives for homogeneous catalysis of organic synthesis reactions; often improving yield and reaction time while preserving enantioselectivity.[48] In fluorinated alcohols the inductive properties of the fluorine substituents increase the acidity of the hydroxyl group making FA's good

hydrogen bond donors. For this same reason FA's are poor hydrogen bond acceptors and have very low nucleophelicities when compared to their analogue hydrocarbon-based alcohols.[48,49] These properties along with their high ionizing power, make FA's, particularly TFE and HFIP, useful solvents for several reactions. Ionizing power measures how well a solvent can stabilize carbocations and is important for reactions that form cationic intermediates. TFE and HFIP were shown to be good solvents for Friedal-Crafts alkylation's that could promote the reaction in a regio- and stereo-controlled manner without the presence of a LA due to their acidic properties along with low nucleophelicities.[50] TFE and HFIP have also been used as solvents for cleavage of t-butyl esters and carbonates into their corresponding carboxylic acids, decarboxylated product and alcohols giving a simple method for t-butyl cleavage which is a popular protecting group used in organic synthesis.[49] The usefulness of FA's is further illustrated in that after allowing for t-butyl cleavage the product can be obtained by solvent evaporation which is simple as both TFE and HFIP have fairly low boiling points. TFE was shown to be a good solvent for the promotion of one pot three component reactions between aldehydes or ketones, amines and trimethyl cyanide or trimethyl phosphite to produce α-amino nitriles or α-amino phosphinates in high yields without the presence of any additional catalyst.[51]

The focus of this chapter will be on the use of both coacervate and Aqueous HFIP-Organic (Aq/H-O) two phase systems for organic synthesis reactions and the potential advantages over traditional solvents. Since FA's have already been demonstrated to be good solvents for Friedel-Crafts type reactions [52], the Friedel-Crafts benzylations of arenes and heteroarenes were chosen as the first reaction to investigate in these systems. Since these reactions proceed through a cationic intermediate they are ideally suited to the properties of

FA's. These reactions produce 1,1-diarylalkanes which are important building blocks for many biologically active compounds.[12,53] Traditional synthesis of 1,1-diarylalkanes involves the use of benzyl halides and stoichiometric amounts of a Lewis acid. Current research has focused on developing more environmentally friendly processes or reagents such as the use of catalytic amounts of a catalyst or alkylating agents such as benzyl alcohols which generate small amounts of water rather than acids. Additionally, Diels-Alder reactions were investigated in the Aq/H-O two phase solutions as FA's have also been investigated as solvents for these type of reactions showing the ability to influence reaction rate, regioselectivity and other properties.[54]

Experimental

Materials

Surfactants cetyltrimethylammonium bromide $CH_3(CH_2)_{15}N^+(CH_3)_3Br^-$ (CTAB), and sodium dodecyl sulfate $C_{12}H_{25}OSO_3^-\ Na^+$ (SDS) were purchased from USB Corporation as "ultrapure" and used without further purification. Dodecyltrimethylammonium bromide $CH_3(CH_2)_{11}N^+(CH_3)_3Br^-$ (DTAB) and zwitterionic surfactant 3-(N,N-dimethylmyristylammonio) propane sulfonate (DMMAPS) were both purchased from Sigma Aldrich. Structures are shown in Fig. 3-1.

1,1,1,3,3,3-hexafluoropropanol (HFIP), 99%+ was purchased from Oakwood Chemicals (Estill, SC) and 2,2,2-Trifluoroethanol (TFE), extra pure, was purchased from Acros Organics. 2,6-lutidine, ≥99% was purchased from Sigma Aldrich and ammonium bicarbonate was 99.0% from Alfa Aesar. Π-bond nucleophiles mesitylene 98%, 2-methylfuran ≥98% and 1-methylpyrrole ≥99% were purchased form Sigma Aldrich while anisole ≥99% was purchased from Fisher Scientific. Structures for these 4 reactants are

shown in fig. 3-2. 4-methoxybenzyl chloride (4mbc), 98% was obtained from Acros Organics while 4-methoxybenzyl alcohol (4mba), 98% was purchased from Sigma Aldrich. Hydrochloric acid solutions were prepared from 37% HCl solutions from Sigma Aldrich. Perfluorooctanoic acid (PFOA), 96% was purchased form Sigma Aldrich as well. Buffers were prepared using Tris-HCl 99%. Methyl vinyl ketone (MVK) 90%, isoprene 99% and acrylonitrile 99+% were purchased from Alfa Aesar. 2-propanol (IPA) was ACS and purchased from VWR. HPLC grade acetonitrile and water were both obtained from Fisher and used without further purification for HPLC analysis. Deionized water was used for reactions.

Methods

Friedel-Crafts benzylations in Coacervate Solutions

Complex coacervate solutions were prepared by first mixing the appropriate amounts of anionic (SDS) and cationic (CTAB or DTAB) solutions prepared in DI water resulting in the formation of a precipitate. DI water was then added to dilute the surfactants to the desired concentration followed by the addition of HFIP at the desired % v/v. After the addition of HFIP, the solution was vortexed and the two phase coacervate solution was formed resulting in the disappearance of the precipitate. Two types of simple coacervates were investigated. The first type was the combination of a cationic surfactant, CTAB or DTAB, with HFIP in solutions with a pH higher than 7.0 due to the presence of Tris buffer. When appropriate amounts of HFIP were added to solutions of cationic surfactants in Tris buffer at a pH > 7.0 two phase coacervate solutions were formed. The second kind of simple coacervate resulted from the addition of HFIP to aqueous solutions of the zwitterionic surfactant DMMAPS.

Example Preparation of a 3mL, 1:1 SDS:DTAB (50 mM), 10% HFIP coacervate:
600 μL of both SDS and DTAB 250 mM stock solutions were combined with 1.5 mL of deionized water and 300 μL of HFIP. *The solution is then vortexed for mixing resulting in the two phase coacervate solution.*

In order to keep the concentration of the reactants relatively the same in all of the different coacervate solutions the volume of the coacervate phase under all conditions investigated had to be determined. Approximate coacervate volumes were determined by preparing 5 mL solutions of each coacervate composition investigated followed by vortexing and centrifugation. After centrifugation the volume of the coacervate phase was approximated by comparing to volume standards prepared by adding known volumes of water to the same type of centrifuge vials used to prepare the coacervate solutions.

To carry out the Friedel-Crafts reactions in the coacervate solutions the reactants were added to the coacervate solution such that the π-bond nucleophile was 1 M relative to the volume of the coacervate not the entire solution. The electrophilic reactant, 4mbc or 4mba, was 0.2 M and if a base additive such as 2,6-lutidine was used it was 0.4 M relative to the coacervate volume. Since the reaction is taking place in the coacervate phase the volume of the coacervate phase was used to determine reactant amounts rather than the volume of the entire solution. These ratios and concentrations were based on the work done by Mayr on the same type of reactions in aqueous solvent mixtures.[52] After addition of the reactants the solution was placed on a stir plate and stirred for 30 minutes using a magnetic stir bar. After 30 minutes the reaction was stopped using isopropanol which stops the reaction and reforms a single phase solution. Isopropanol was confirmed to stop the reaction by analyzing reaction solutions by HPLC immediately after stopping the reaction and a day later to

confirm that the product peak area remained the same. In order to determine the reaction yield, HPLC calibrations of the products were prepared and the theoretical value of the product concentration for a 100% yield was calculated from the concentration of the electrophilic reactant which is the limiting reagent. The actual concentration of product was determined by HPLC and used to determine percent yield base on dividing the actual value by the theoretical 100% value. Yield values presented are based on triplicate preps.

Example reaction procedure for a 1:1 SDS:DTAB (50mM), 15% HFIP coacervate:

For a 3 mL total solution volume, the volume of the coacervate produced was 129 *µL. Once the solution was formed the reactants were added: 129 µmoles of 1mp + 51.6 µmoles of 2,6-lutidine + 25.8 µmoles of 4mbc. Upon addition of 4mbc the reaction clock was started and the solution was stirred for 30 minutes. After 30 minutes 3 mL of IPA was added to the solution to form a single phase solution. The reaction solution was then diluted 5x for HPLC analysis.*

Preparation of benzylation HPLC calibration curves

HPLC calibration curves were prepared for the benzylation products formed between 4mbc and the nucleophiles: mesitylene, anisole, 2-methyl furan (2mf), and 1-methylpyrrole (1mp). Larger scale reactions were done according as in the work by Mayr [52] in single phase solutions. The reaction products were then purified using flash chromatography. The identity of the purified products was then verified using ^{1}H NMR and mass spectrometry. ^{1}H NMR data was obtained using a Varian Mercury Plus 300 MHz spectrometer with $CDCl_3$ as the solvent. GC-MS data was obtained using an Agilent 6890 Gas Chromatograph equipped with an Agilent 5973N mass selective detector and used to measure the m/z values for the standards to make sure they corresponded to the desired product.

Molecular masses were determined using an Agilent 6890 GC with a 5973 Network mass selective detector. The GC column was a HP-5 capillary column with a length of 30 m, I.D. of 0.25mm and film thickness of 0.25 μm. Helium was used as the carrier gas with a flow rate of 1.0 ml/min and a split ratio of 50:1. The inlet was operated at a temperature of 250°C and a 4 minute solvent delay was used. Temperature program: The temperature was initially held at 70 °C for 2 minutes and then ramped to 150 °C at 25 °C/min followed by another ramp to 250 °C at 50 °C/min and held for 12 minutes. Electron impact ionization was used and all molecular ions were visible with splitting patterns matching previously reported results for the same compounds.

The reaction and purification conditions as well as characterization data for each product are described below:

Mesitylene-4mbc:

0.2 mmol of 4mbc and 0.4mmol of 2,6-lutidine were added to a 10 mL solution of TFE containing 10 mmol of mesitylene and stirred for 1 hour at room temperature. After 1 hour 10 mL of deionized water was added to the solution and liquid-liquid extraction was performed using diethyl ether. Extraction phase was then dried using sodium sulfate and ether was evaporated. The remaining reaction mix was the solubilized using dichloromethane to prepare for flash chromatography. Following flash chromatography with 8:1 hexane:ether a white solid was obtained.

GC-MS: m/z = 240.0

^{1}H NMR (300 MHz, $CDCl_3$): δ = 2.20 (s, 6H), 2.29 (s, 3H), 3.76 (s, 3H), 3.95 (s, 2H), 6.76-6.79 (m, 2H), 6.88-6.94 (m, 4H)

Anisole-4mbc:

0.2 mmol 4mbc and 0.4 mmol of 2,6-lutidine were added to a 10 mL solution of TFE containing 10 mmol of anisole and stirred for 1 hour at room temperature. Following the same liquid-liquid extraction procedure used for mesitylene the product was then purified using flash chromatography, 8:1 pentane:ether, and obtained as a mostly white solid with a tinge of yellow.

GC-MS: m/z = 228.0

^{1}H NMR (300 MHz, $CDCl_3$): δ = 3.78 (s, 6H), 3.87 (s, 2H), 6.81-6.84 (m, 4H), 7.07-7.10 (m, 4H)

2mf-4mbc:

0.2 mmol of 4mbc and 0.4 mmol of 2,6-lutidine were added to a 10 mL solution of 90/10 acetonitrile/water containing 10 mmol of 2mf and stirred for one hour at room temperature. Following the same liquid-liquid extraction procedure used for mesitylene the purified product was obtained via flash chromatography, 8:1 pentane:ether, as orange liquid.

GC-MS: m/z = 202.0

^{1}H NMR (300 MHz, $CDCl_3$): δ = 2.24 (s, 3H), 3.79 (s, 3H), 3.85 (s,2H), 5.82-5.85 (m, 2H), 6.83-6.85 (m, 2H), 7.14-7.17 (m, 2H)

1mp-4mbc:

0.2 mmol of 4mbc and 0.4 mmol of 2,6-lutidine were added to a 10 mL solution of 90/10 acetonitrile/water containing 10 mmol of 1mp and stirred for one hour at room temperature. Following the same liquid-liquid extraction procedure used for mesitylene the purified product was obtain via flash chromatography, 8:1 pentane:ether, as a dark red liquid.

GC-MS: m/z = 201.0

^{1}H NMR for 2-isomer (300 MHz, $CDCl_3$): δ = 3.43 (s, 3H), 3.79 (s, 3H), 3.88 (s, 2H), 5.87 (m, 1H) 6.06 (m, 1H), 6.57 (m, 1H), 6.81-6.84 (m, 2H), 7.06-7.09 (m, 2H)

Following purification of the products stock solutions for each were prepared in IPA. These stock solutions were used to prepare HPLC standards used to build the calibration curves.

HPLC analysis

HPLC analysis was performed on an Agilent 1100 instrument equipped with a quaternary pump, autosampler, vacuum degasser and UV-vis detector. The column used was a Zorbax SB-300 C18: 15 cm length, 4.6 mm ID, 3.5 μm particle size. The mobile phase was water and acetonitrile with a gradient of 25% ACN to 75% ACN over 20 minutes followed by 5 minute equilibration at 25% ACN with a 1 mL/min flow. UV detection at 224 nm was used for all compounds.

GC-TCD phase composition analysis of aqueous HFIP two phase solutions

The GC used in this experiment was an Agilent HP7890 Gas Chromatograph equipped with a thermal conductivity detector. The column was an Agilent DB-624, 30m x 0.32mm x 1.8μm capillary column. The carrier gas was helium at 3.5mL/min constant flow. The sample was injected manually at 0.5 μL with a 50:1 split ratio. Because the samples were injected manually, an internal standard was incorporated to correct for variations in the injection volume. The injector and detector temperatures were both held at 250ºC. Temperature program: The temperature was initially held at 100 ºC for 3 minutes and then ramped up to 170 ºC at 30 ºC/min and held for 1 minute for a total time of 6 minutes.

Reactions in Aqueous HFIP two phase solutions

Friedel-Crafts Reactions

Friedel-Crafts benzylations were performed in aqueous HFIP solutions using the same π-bond nucleophiles as in the coacervate solutions. In addition to using 4mbc, 4-methoxybenzyl alcohol (4mba) was used as the electrophile as well. To a 1 mL solution containing the desired % of HFIP, 1 M nucleophile was added forming a two phase solution. When base additive was being used, it was added at 0.4 M concentration and for 4mba reactions when Bronsted acid was used it was added at a catalytic amount of 20 μmoles or 10% of the limiting reactant 4mba. Next 0.2 mmoles of the electrophile was added and weighed to increase the accuracy of the % yield calculation. Following the addition of the electrophile the reaction was stirred for the desired time and stopped by adding 4 mL of IPA to form a single phase solution which was then diluted and analyzed by HPLC in the same manner described for the coacervate reactions and all yields reported are based on triplicate preps.

Diels Alder reactions

% Conversion

To a 1 mL aqueous solution containing the desired % of HFIP, Isoprene was added at a 1.5 M concentration. The dienophile MVK was added at 0.5 M and weighed to ensure accurate % conversion calculations. After stirring for desired reaction times 4 mL of IPA was added to form a single phase solution and the reaction was immediately analyzed by HPLC to determine the remaining amount (in mg) of MVK, which was divided by the original amount of MVK added to determine % conversion.

Para/meta (p/m) product ratio

The p/m product ratio for the reaction between isoprene and methyl vinyl ketone was determined using a GC method from the literature.[54] The GC used was an Agilent 6890 GC with a 5973 Network mass selective detector. The GC column was a HP-5 capillary column with a length of 30 m, I.D. of 0.25mm and film thickness of 0.25 μm. Helium was used as the carrier gas with a flow rate of 1.0 ml/min and a split ratio of 50:1. The inlet was operated at a temperature of 250°C and a 3 minute solvent delay was used. The temperature program was 50 °C (3 min)–25 °C/ min–90 °C (15 min). The products were verified by observing their molecular ion matching their m/z as well as the peaks closely matching the reported retention times of 15.0 for the meta product and 15.3 for the para product. The p/m ratio was measured by integrating both peaks and dividing the para peak area by that of the meta product.

Results and Discussion

Friedel-Crafts alkylations in coacervate systems. Complex and simple coacervate solutions formed by the addition of HFIP to surfactant solutions were investigated as a reaction media for Friedel-Crafts reactions. The volume of the coacervate phase formed is dependent on the concentration of surfactants in solution as well as the amount of HFIP used to induce phase separation. Table 3-1 shows the % volume (v/v) of the coacervate phase for the two-phase solutions used in this work. One clear trend that is noticeable is that by increasing the percentage of HFIP in the solution a larger coacervate phase will be formed. Doubling the amount of HFIP from 10 to 20% results in larger than a two times increase in

the coacervate phase volume. This is seen in both the complex systems, SDS:(C or D)TAB, as well as the DMMAPS simple coacervate system. Since the reactants are likely contained in the coacervate their concentration can be controlled by knowing the volume of coacervate formed under different conditions. Larger amounts of coacervate (% volume) should conceivably solubilize larger amounts of reactants leading to higher yields as long as the resulting change in the coacervate environment is not detrimental to the reaction. The nucleophiles used for these Friedel-Crafts alkylations were arenes and heteroarenes that act as π-bond nucleophiles and undergo nucleophilic aromatic substitution. The structures of these compounds along with their nucleophilicity (N) value are shown in figure 3-2. The work by Mayr [52] illustrated that as long as the nucleophilicity of the nucleophile is greater than that of its environment the reaction should proceed to a high yield. Otherwise, the nucleophile will be prevented from reacting with the electrophile due to the electrophile being surrounded and "trapped" by the more nucleophilic solvent. The N value for HFIP is -2.4 which is lower than all of the nucleophiles other than mesitylene whose reactivity was not investigated in the coacervate solutions. With the coacervate phase being highly concentrated in HFIP it should provide an environment of low nucleophilicity facilitating the reaction of these compounds. Initially, reactions between these nucleophiles and t-butyl chloride were attempted with little to no product formation observed. Thus the benzylic halide, 4-methoxybenzyl chloride was used as the electrophilic reactant making these reactions more specifically Friedel-Crafts benzylations. The result of this reaction is the formation of a diaryl alkane product between the arene and 4mbc as well as HCl as a byproduct shown in figure 3-3. With the coacervate phases being highly concentrated in both HFIP and surfactant they should provide an environment of low nucleophilicity to allow

the reactions to occur. One of the first variables investigated was the effect of the stirring speed on the reaction yield, to see if the surface area of the aqueous H-O phase interface had any effect on the reaction as it can in certain emulsion systems [22]. This was tested for the reactions of 4mbc with both anisole and 1mp in a complex and simple coacervate. In both cases it was observed that the yield dropped when the stirring speed was set to the maximum level as shown in figure 3-4. The stirring speed in the slow stirred solutions is fast enough that the coacervate phase is mixed within the aqueous phase but coacervate droplets are still clearly visible. The effect was much more pronounced in the simple coacervate system when compared to the complex system. At fast stirring rates it is likely that more of an emulsion type solution is formed with either the coacervate phase being broken apart or the coacervate phase droplets being so small that the reactants come into contact with much more water. With water being much more nucleophilic than HFIP and all of the nucleophiles it will interfere with the reaction by interacting with the electrophile 4mbc. Additionally based on these results it appears that the complex systems is more stable towards stirring than the simple system. This could be due to the very hydrophobic nature of the complex formed between the anionic and cationic surfactant making it less likely to break up and interact with the aqueous phase with agitation. In the simple system the lone surfactant present is water soluble and the forces causing phase separation may be overcome by the agitation. No precipitation of the catanionic complex formed between SDS and DTAB is observed during fast stirring indicating the coacervate phase remains intact for it to remain soluble. In the case of the simple coacervate it is harder to determine this as the two main components of the coacervate phase, HFIP and CTAB, are individually miscible in water. This may be the reason yields drop much more significantly at high stir rates in the simple system. The use of

sonication instead of stirring was also investigated showing lower yields than the slow stirred reactions. Sonication provides agitation but real mixing of the two phases indicating that the presence of the aqueous phase may also play a role in improving reaction yields. Based on these results the slow stirring speed was used for the remainder of the coacervate yields reported.

The results of the Friedel-Crafts benzylation reactions in complex coacervate systems are shown in table 3-2. The % yields were shown to increase with increasing nucleophilicity of the π-bond nucleophiles in these complex systems. Anisole showed lower % yields than 2mf which showed lower % yields than 1mp. As stated earlier increasing the % HFIP used to form the solution leads to larger coacervate phase volumes. For anisole the % yield increased with increasing % HFIP (entry's 1-3) while for 2mf the % yield slightly decreased (entry's 6-8). Even with the slightly lower % yield for 2mf in the 20% HFIP system compared to the 10% system the total yield of product will be much greater in the 20% system due to roughly 4x larger coacervate volume allowing for greater amounts of reactants to be used. When using CTAB instead of DTAB as the cationic surfactant (entry's 4, 5, 9 and 10) the % yields were fairly similar to those using DTAB. CTAB coacervates do form larger coacervate volumes compared to DTAB under the same conditions so if similar % yields are observed, CTAB systems will likely provide higher actual yields. Unlike the DTAB systems where 2mf % yield decreased with increasing % HFIP the % yield in the CTAB system increased when going from 10 to 20% HFIP providing the best % yield observed for 2mf in these complex coacervate systems.

One possible advantage of complex coacervate systems over simple coacervate systems is the ability to form solutions with varying ratios of cationic:anionic surfactants in

solution there by creating a charged environment in the coacervate phase. The surfactant counterions, Na^+ and Br^-, are most likely to remain in the aqueous phase. Having a charged environment could potentially impact reactions that go through ionic intermediates, either hindering or assisting the reaction. These Friedel-Crafts reactions go through a cationic intermediate. Reactions were carried out in complex coacervate solution at ratios of 7:3 and 3:7 SDS:DTAB. The results (entry's 12-15) showed that % yields decreased under both ratios with 7:3 SDS:DTAB having a much more detrimental effect. So for these reactions at least a 1:1 ratio proved to be best in terms of % yield.

Table 3-3 shows the results of Friedel-Crafts benzylations in simple coacervate systems. While the DMMAPS system followed the same trend of increasing % yield with increasing % HFIP as the complex systems the simple system formed with CTAB and Tris did not showing a higher % yield for anisole reactions compared to 2mf although 1mp still gave the highest % yield. So although the trend can be used as a general rule it may not apply in all cases depending on the nature of the specific reactants. Reactions were done in the CTAB Tris system using both 50 mM and 500 mM Tris buffer with anisole and 2mf. The different concentrations of Tris did not seem to have a very significant effect in terms of % yield likely indicating that the coacervate environments formed were fairly similar. In the DMMAPS coacervate both anisole and 2mf showed increased % yields when increasing the % of HFIP from 10 to 20%. The reaction of 1mp in the DMMAPS 10% HFIP system gave the highest % yield observed for any of these reactions in coacervate solutions. The DMMAPS system seems to be suitable for producing larger amounts of products given the good yields and larger coacervate volume at identical surfactant and HFIP concentration when compared to the other systems.

In order to establish the benefits of carrying out reactions in the coacervate environments comparisons were made to single phase solutions composed of an individual component of the coacervate components such as a surfactant or HFIP using the same reactants concentrations. When reactions were carried out in single phase surfactant solutions, composed of 100 mM of one of the surfactants, the % yields dropped significantly. The reactivity pattern was still observed, which depended on Nucleophilicity; but the % yields were much lower than for coacervate solutions due to the absence of HFIP and a much more nucleophilic aqueous environment. For example the % yield of a reaction between 1mp and 4mbc in 100 mM DMMAPS dropped to 69% compared to close to 100% in the 100mM DMMAPS 20% HFIP coacervate solutions.

Synthesis in HFIP-Induced Aqueous-Organic Two Phase Systems.

The next experiment was to examine the reaction yields in the aqueous HFIP solutions and in the absence of surfactants. Interestingly, phase separation occurred in a 10%HFIP in water solution upon the addition of a nucleophile (1mp or anisole) with the nucleophile concentrated in the bottom phase. Since the density of the nucleophile is less than water, the bottom phase must contain large amounts of HFIP, which has a higher density than water. The reactions were carried out in the newly discovered Aqueous HFIP-Organic (Aq/H-O) two phase systems. The reaction yields were equivalent or better than those in the coacervate systems. This discovery provided new opportunities of conducting organic synthesis in aqueous environment and HFIP-enriched phases and in the absence of surfactants.

Although good reaction yields were observed for the Friedel-Crafts alkylation's in coacervates, these systems present some inherent disadvantages as media for synthesis. One problem was that although the % yields were fairly good the actual yields in terms of amount

of product were fairly low based on the entire volume of the solution. This is because the concentration of the reactants had to be based on the volume of the coacervate and not the entire solution. Using larger amounts of reactants would far exceed the volume of the coacervate phase resulting in disruption of the coacervate phase resulting in a two-phase solution due to solvation of the organic reactant by HFIP. Additionally, the presence of surfactants in the coacervate systems would require an additional step of separating the product from the surfactants can cause problems as well. Also, the presence of surfactants would pose challenges in the analysis of reaction mixture using common analytical techniques such as gas or liquid chromatography and mass spectrometry. Although a guard column was used to protect our HPLC columns from the surfactants being injected during coacervate analysis degradation of column performance was observed after many injections. The column could be regenerated using a solvent rinsing procedure but it was only a temporary fix. For these reasons it was decided to focus on the Aqueous HFIP-Organic (Aq/H-O) two-phase systems as a new medium for organic synthesis.

Compositional analysis of (Aq/H-O) systems.

In order to better understand the nature of these types of phase separations and the environments in which the reactions would be taking place in Aq/H-O systems, compositional analysis of the two phases was done. Figure 3-5 shows the solutions resulting from the addition of 1-methylpyrrole (1mp) at a 1 M concentration to aqueous solutions of increasing % HFIP. In the absence and at lower concentrations of HFIP, 1mp floats on top until enough HFIP is present to solvate the 1mp. Due to the higher density of HFIP (1.596 g/mL), the newly formed HFIP-1mp phase (called H-O here on) sinks to the bottom. As the amount of HFIP is increased the volume of the bottom phase increases until at 75% HFIP,

where a single phase solution is formed. Similar trends can be observed with the other π-bond nucleophiles except for mesitylene. Mesitylene does not have any hydrogen bond accepting capabilities like the other compounds and thus is not selectively solvated by HFIP and remains floating on top of the solution. If another compound is added that will selectively solvated and form H-O the mesitylene will be extracted into it as well and will no longer be seen floating on top (fig. 3-6). Gas chromatography with a thermal conductivity detector was then used to determine the composition of both the aqueous and H-O phases formed by the addition of 1M amounts of each π-bond nucleophile in solutions of 10, 20, and 50% (v/v) HFIP in water. Additionally, the same type of analysis was done on 1M solutions of 1-octanol in 10 and 20% (v/v) solutions of HFIP in water. GC-TCD calibrations for all components can be seen in figure 3-7(A-F) The full results in both the aqueous and H-O phases are shown in table 3-4. It should be noted that the H-O results for mesitylene are well below 100% because small amounts of diethyl ether were added to induce phase separation since mesitylene does not contain any hydrogen bond accepting groups. The unaccounted for volume in the mesitylene H-O phase is due to the presence of ether which should partition primarily into the H-O phase rather than the aqueous phase. The more important part of this data was the composition of the H-O phase since that is where the reactions are occurring. Figure 3-8 illustrates the effect of % HFIP on the H-O phase volume for two phase solutions formed by the addition of 1M amounts of the 4 π-bond nucleophiles. H-O phase %volume increases with %HFIP for different types of nucleophiles. All nucleophiles were present at a 1 M concentration relative to the 1mL total solution volume except for mesitylene (0.6 M). With mesitylene, it was found that it did not form a bottom phase with HFIP likely due to lack of interactions with the HFIP compared to the other nucleophiles. In order to form an

HFIP- mesitylene (H-O) phase, a small volume (46 μL, 0.4 M) of ethyl ether was added to the solution (total volume=1.1mL). The volume used corresponds with the volume of 2,6-lutidine base additive used for FCA reaction covered in the next section. Remarkably, the increase in volume is not only due to the extraction of larger amount of HFIP, but also transfer of water molecules into the H-O phase. Figure 3-9 shows the concentration (in %v/v) of water in the H-O phase increases for different nucleophiles as the total amount of HFIP in the solutions increases. This is due to the strong association between HFIP and water molecules through H-bonding; thus as HFIP molecules are extracted into the H-O phase, they would carry the associated water molecules with them to the organic-rich phase. The H-O phases with more hydrophobic nucleophiles (such as mesitylene and anisole) have smaller water concentrations than the other less hydrophobic nucleophiles. Nearly all of the nucleophile remains in the H-O phase as the % HFIP of the total solution is increased but since the volume of the phase is increasing the concentration of the nucleophile in the H-O phase decreases. Figure 3-10 shows the changes in the HFIP concentration in the HFIP-Organic phase as the initial concentration of HFIP increases in the solution. The results show that initially the concentration of HFIP in the H-O phase increases, which along with increasing water content leads to the increase in phase volume. The concentration of HFIP in the H-O phase seems to level out at HFIP concentrations above 20% for all systems. Additionally, based on the GC TCD analysis nearly all of the nucleophile remains in the H-O phase at all HFIP concentrations where the aqueous phases in all systems contained less than 1% v/v nucleophile. This data shows why this type of system provides a good environment for these Friedel-Crafts reactions. The H-O phase provides an environment of low nucleophilicity even in the presence of large amounts of water the reaction to take place.

Friedel-Crafts benzylations of π-bond nucleophiles using 4mbc.

The first reactions carried out in the Aq/H-O two phase systems were the same as in the coacervate solution with the addition of mesitylene as a nucleophile which is less nucleophilic than all of the previous nucleophiles used. Again 2,6-lutidine was used as base additive to trap the HCl generated by the reaction. Table 3-5 shows the results of the reactions carried out between the four nucleophiles with 4mbc in both pure water and HFIP as well as Aq/H-O solutions at different HFIP% that resulted in the formation of two-phase systems. Surprisingly, the yields in the two-phase systems are significantly higher than those in 100% HFIP for nearly all cases. As expected, the reactions did not proceed to any notable extent in "on-water" condition (i.e. 100% water) with the exception of 1-methylpyrrole; the strongest nucleophile where the yield was 34%. The trend is similar for all four reactants in the two-phase systems as the reaction yield reaches a maximum at 20% HFIP. Based on the GC-TCD results it is known that the concentration of water in the H-O phase in the 50% two phase solutions is much higher than at 10 or 20% which is what likely leads to the lower yields in 50% solutions. Surprisingly this seemed to have more of an impact on the more nucleophilic reactants 2mf and 1mp but it can also be noted that the % of water in their H-O phases at 50% is higher than that of both mesitylene and anisole. Another explanation could be based on polarity with 2mf and 1mp being more polar and water soluble than mesitylene and 1mp. During stirring it is possible that the more polar reactants (2mf and 1mp) partition into the aqueous phase where the reaction is less likely to take place while the more nonpolar reactants remain in the H-O phase leading to similar yields to those in the 20% systems. Nevertheless good yields were obtained for all nucleophiles in the 20% two-phase system. Note that the initial concentrations of the reactants were 1M nucleophile and 0.2M 4mbc.

However, in the two-phase systems, the reactants are concentrated in the H-O phase by a factor of 4-5 due to the inverse relationship with the smaller volume of the H-O phase (20%-25% v/v. The other factor is the presence of a small percentage of water (5%-12%) in the H-O phase. Whether the enrichment effect and/or presence of water in the H-O phase play a role leading to higher yields (as compared to 100% HFIP) will be discussed later. Another aspect of the 100% HFIP reactions is that for 3 of the 4 nucleophiles the use of a heterogeneous base, ammonium bicarbonate, instead of the homogeneous base 2,6-lutidine improved the reaction yield dramatically.

Interestingly, good yields were observed in the two-phase systems even in the absence of a base additive. The role of the base additive for these reactions is to trap the HCl that is generated as a byproduct of the reaction. Table 3-6 shows yields obtained from reactions performed without any base additive. It was hypothesized that in the two-phase systems the HCl is removed from the reaction media as it partitions into the aqueous phase; driving the reaction to completion. The pH of the aqueous phase was measured before and after 20% HFIP reactions between anisole and 1mp and was observed to decrease significantly from 5.64 and 6.20 to 0.20 and 0.41 respectively. These results support the hypothesis that the HCl is transferred into the aqueous phase. Thus, the aqueous phase can essentially perform the same role as a base additive such as 2,6-lutidine. The only exception was for the reaction between 2-methylfuran and 4mbc in the two-phase systems without a base additive. This is surprising considering the high nucleophilicity of 2-methylfuran. In 20% HFIP system with 2,6-lutidine, a much better yield of 85% was obtained for the 2-methylfuran - 4mbc reaction. This means that whether or not a base additive is necessary could be dependent on the nucleophile.

In order to demonstrate the advantages of the Aq/H-O system similar experiments were carried out using other solvents. As shown in Table 3-7 the results in the 20% HFIP two-phase system are significantly better than those in the other solvents. Toluene and dichloromethane are not miscible with water and form a separate phase with the nucleophile from the aqueous phase; similar to the aqueous HFIP two-phase systems. The difference is that these solvents like most other organic solvents do not combine the properties of strong H-bond donation and low nucleophilicity like HFIP. Isopropanol, the aliphatic analog of HFIP with hydrogen bonding properties, is completely miscible with water just like HFIP. Unlike HFIP isopropanol lacks the ability to selectively solvate the reactants leading to phase separation and thus the reaction takes place in the presence of much larger amounts of water. Only a modest yield was observed with 1mp in the 20% isopropanol solution which is the strongest nucleophile. These results demonstrate how the unique properties of HFIP combine together to form an environment that can not only promote a reaction through strong H-bond donation but enable the reaction to occur due to its low nucleophilicity.

Effect of two-phase nature on reaction.

It was observed for several reactions that the yields in the Aq/H-O two phase solutions was much higher than that of the same reactions carried out in 100% HFIP single phase solutions. Several attempts were made to determine the role of the two-phase nature by carrying out reactions in solutions that resembled the H-O phase based on the GC-TCD phase composition results. These experiments did not lead to any conclusive answers. The best insight into the benefit of the two phase systems can be gained by looking at the results in table 3-5 particularly the 100% HFIP reactions. It was observed that in most cases the use of the heterogeneous base additive ammonium bicarbonate led to much higher yields in these

reactions. This may mean that the heterogeneous base is much better at trapping the HCl compared to 2,6-lutidine. One reason for this could be that since HFIP is such a strong H-bond donor it interacts with the basic site of 2,6-lutidine preventing it from trapping the protons generated. Since HFIP is a poor solvent for solvating cations it will not interact strongly with these free protons shifting the equilibrium towards the reactants. With the two phase systems the aqueous phase essentially acts as a heterogeneous base as it can remove the protons from the reaction environment which is why good yields can be observed in the two phase systems with or without a base additive.

Friedel-Crafts Benzyltations of π-bond nucleophiles with 4-methoxybenzyl alcohol

In order to widen the scope of electrophilic reactants in FCA, the use of a benzyl alcohol, 4-methoxybenzyl alcohol (4mba), as an electrophilic reactant was investigated. Although the chloride ion is a better leaving group than hydroxide, the use of 4mba is more desirable due to alcohols generally being less toxic than halide reactants as well as producing more benign by products (H_2O vs. HCl). When carrying out reactions with 4mba in the same manner as 4mbc little to no product yield was observed. It was found that by using a Bronsted acid as well as heat and longer reaction times good yields could be observed with 4mba as with 4mbc. Table 3-8 shows the results of benzylations between different nucleophiles and 4mba in 20% two-phase Aq/H-O systems at 50°C with 24 hour reaction time. The reactions were also carried out as "on water" (i.e. using 100% water); due to lack of solubility of reactants in water as well as in 100% HFIP in which all the reactants were soluble. As can be seen 1mp reacts well under "on water" conditions while anisole and mesitylene do not. This is most likely due to the much higher nucleophilicity of 1mp

compared to anisole and mesitylene, and illustrates the usefulness of the Aq/H-O two-phase system in accommodating nucleophiles of varying strengths. As compared to 100% HFIP reaction media, the aqueous HFIP two phase system performed much better for 1mp while the results for anisole were very similar. However, the two-phase system provides the advantage of consuming much smaller amount of HFIP (by a factor of five).

The two Bronsted acid catalysts investigated were HCl and perfluorooctanoic acid (PFOA). For anisole and mesitylene, PFOA gave a higher yield after 24 hours while with 1mp, HCl gave a quantitative yield compared to only 55% for PFOA. It is likely that the HCl remains in the aqueous phase and catalyzes the reaction either at the interface of the two phases or small amounts of HCl may partition into the H-O phase (due to the presence of water in this phase) and catalyze the reaction. Note that the water concentration in the H-O phase of 1mp system is greater than those for anisole and mesitylene; which could lead to the partitioning of larger amounts of HCl in the H-O phase. The long perfluoroalkyl chain of PFOA is likely to interact with the H-O phase and may serve to attract protons to the interface of the two phases as shown in figure 3-11. This hypothesis is supported by yields seen at 2 and 4-hour reaction times for 1mp, anisole and mesitylene with 4mba in 20% HFIP two-phase systems using both acids shown in table 3-9. The results clearly show that a reaction using PFOA as the Bronsted acid has an initial faster rate of reaction than the one with HCl. The effect was most pronounced for mesitylene which also had the lowest water content in its HFIP-Organic phase.

Isolation of Product from HFIP-Organic Phase.

In typical reaction procedures, especially those involving aqueous solutions, liquid-liquid extraction using an organic solvent such as diethyl ether or ethyl acetate is used to

remove the organic components from the reaction solution for purification and isolation of the product. Often large volumes of solvents are used for LLE as multiple extraction steps are done to ensure complete extractions of the product. Due to the two-phase nature of these systems it is possible to simply remove the bottom HFIP-Organic phase containing the product, followed by isolation of the product using chromatography, thus eliminating the liquid-liquid extraction (LLE) step and significantly reducing the amounts of solvents used. Isolated yields were obtained for reactions using both LLE and bottom phase removal. In order to perform LLE, water was added to the reaction solutions following the desired reaction time in order to dilute the HFIP, and ethyl acetate was used to extract the product. The second method takes advantage of the two-phase system where the synthesis and extraction into the organic phase occurs concomitantly. Thus, the reaction solution was centrifuged following the desired reaction time in order to completely separate the phases. The bottom phase (i.e. H-O phase) was then removed using a glass pipette and dried with sodium sulfate to remove any water; then the HFIP was removed by a rotary evaporator leaving behind the product as well as any unreacted starting materials. Following removal of the solvents either after LLE or bottom phase removal the products were purified using flash chromatography and the yields of the product were determined. The results in table 3-10 show that very similar results were obtained using either method. The main takeaway from these results is that LLE and bottom phase removal give similar results because nearly all of the product should be present in the H-O phase. Not having to use LLE is an advantage of these Aq/H-O two phase systems from an economical and environmental standpoint and also simplifies the procedure.

Friedel-Crafts acylations of the same π-bond nucleophiles using acylating reagents of hexanoic anhydride and hexanoyl chloride was also attempted in the Aq/H-O two phase solutions. Very little to no yield was observed for these reactions. This is likely due to the higher electrophilicity of the acylium ion compared to the carbenium ion present in the alkylation reactions. Reaction products were observed in 100% HFIP solutions so the small amounts of water present in the H-O phase of two phase solutions was likely more inhibitive to the acylation reactions. The use of heat and acid or base additives did not show any improvement in reaction yield.

Diels-Alder reactions in Aq/H-O two phase systems.

Diels Alder reactions differ from Friedel-Crafts reactions in that they are pericyclic and do not result in any byproduct formation nor go through a cationic intermediate. Diels-Alder reactions were initially carried out between the diene isoprene with methyl vinyl ketone (MVK) as the dienophile, fig 3-12. The reaction between isoprene and MVK can result in two regio-isomers either the para or meta product. For this reason, the % conversion of MVK was measured for these reactions since it was the limiting reactant and the para/meta ratio can be influenced by the reaction media which is discussed later. The % conversion for MVK was monitored by HPLC at 224nm for Diels Alder reactions with isoprene in Aq/H-O two phase systems as well as 100% water solutions the MVK HPLC calibration curve is shown in fig. 3-13. The % conversion results, table 3-11, show much higher conversions were observed in the two phase systems when compared to 100% water. The reactions were also attempted in 100% HFIP solutions but many interfering peaks were observed in the chromatograms that were not seen in the other solutions possibly due to side reactions other than Diels Alder. These peaks interfered with the MVK peak making it difficult to measure

an accurate peak area and even if it was measured the conversion % may not be solely related to conversion of MVK to the Diels Alder products. The performance of the three different two phase solutions was fairly similar with 20% HFIP giving a slightly higher conversion after 24 hours.

Ratio between the para and meta (p/m ratio) product can be influenced by solvent conditions particularly the hydrogen-bond donation ability of the solvent. Strong H-bond donating solvents like HFIP are able to form H-bonds with the carbonyl of MVK (fig. 3- 14) leading to greater formation of the para product. When the reaction is carried out in an inert (non-directing) solvent the para/meta ratio is 2.21. Table 3-12 shows the p/m ratios of reactions carried out in two-phase solutions as well as 100% water and 100% HFIP. The results show that carrying out the reaction in 100% HFIP gives the highest p/m ratio while using 100% water gives a slightly higher ratio than an inert solvent. The reactions in Aq/H-O two phase systems were in between these two showing a slight trend of increasing p/m ratio as the % of HFIP in the solution increases. The observed p/m ratios were also shown to be fairly independent of the concentration and ratios of the reactants used for the reactions. Table 3-13 shows the results of an isoprene MVK reaction carried out in 10% HFIP two phase systems with varying amounts of the two reactants. Changes to the diene to dienophile ratio gives small changes in the p/m ratio likely due to causing small changes in the H-O phase composition.

Since the p/m ratios in the two phase systems were much lower than that of 100% HFIP the effect of using acid additives for the two phase reactions was investigated to see how the p/m ratio would be affected. Bronsted acids should generate protons which would protonate the carbonyl carbon affecting it in a similar way to H-bonding from HFIP and

Lewis acids provide cationic metal species that can interact with the carbonyl as well and could influence the p/m ratio. The effect of different additives on the p/m ratio in a 10% HFIP two phase systems for the reaction between isoprene and MVK is shown in table 3-13. HCl and $CuCl_2$ both showed very little to no influence on the p/m ratio. Increasing the amount of HCl 8 times gave a small increase in the p/m ratio. This is likely due to the protons from HCl and Cu^{2+} ion existing mainly in the aqueous phase and not interacting with MVK which is primarily in the H-O phase along with isoprene where the reaction is occurring. On the other hand, the addition of PFOA was shown to greatly increase the p/m ratio. This is further evidence for the hypothesis made earlier when using base additives for Friedel-Crafts reactions with benzyl alcohols fig. 3-11. Because PFOA is a strong acid it will completely dissociate in water giving a proton and perfluorooctanoate anion. The perfluoroalkyl chain of the anion will interact with the H-O phase and attract the protons to the interface of the H-O and water phases allowing better interaction with the reactants in the H-O phase. Another possibility could be that PFOA is forming micelles in which the reactions are occurring rather than the H-O phase but the H-O phase is not dispersed by the addition of PFOA and remains intact so it seems more likely that the PFOA is interacting with the H-O phase. Also the concentration of PFOA in the solutions is slightly less than its reported critical micelle concentration of 25mM.[55]

The Diels Alder reaction of isoprene with acrylonitrile was also investigated. These reactions were much slower when compared to MVK. A reaction between isoprene and acrylonitrile showed only a 33% conversion of the acrylonitrile after 10 days.

Conclusion

This is the first report of the use of two phase solutions formed by the selective solvation of organic compounds by HFIP in aqueous solutions as a medium for organic synthesis. HFIP offers unique properties that make it a useful solvent for several types of organic synthesis reactions. Friedel-Crafts and Diels Alder reactions were shown to proceed in these two phase systems which offer several benefits over using HFIP alone such as reducing the amount of HFIP needed, removal of water soluble by products produced by certain reactions by the aqueous phase which drives the reaction to higher yields without the need for other additives, and potential absorption of heat given off by exothermic reactions by the aqueous phase due to its high heat capacity. These systems also allow reactions to be carried out in the presence of large amounts of water which typically hinders these types of reactions. Recovery of the product from the H-O phase without the need for liquid-liquid extraction is another benefit of the two phase nature. Compared to HFIP induced coacervate phases, Aq/H-O two phase solutions offer the advantage of being able to react much larger amounts of reactants in the same volume of solution as well as not using surfactants which can cause more difficulty in product recovery and analysis.

Friedel-Crafts benzylations were carried out on arene and heteroarene nucleophiles to produce diarylalkane products in good yields. Both benzyl chloride and benzyl alcohol electrophiles were shown to work for the reaction with the alcohol requiring heat and Bronsted acid catalysis. For the reactions with the Benzyl chloride the aqueous phase was shown to perform the role typically carried out by a base additive of removing the HCl produced during the reaction from the reaction environment.

The Diels Alder reaction between isoprene and MVK was carried out as well and performed better than when carried out using "on water" conditions. The p/m ratio of the product is largely influenced by H-bonding or the presence of acids. The p/m ratio was fairly constant in the two phase systems formed with different %'s of HFIP. The use of Bronsted acids was shown to increase the p/m ratio in the two phase systems especially for perfluorooctanoic acid whose anion is believed to interact with the H-O phase and attract protons to the H-O aqueous interface facilitating interaction between protons and reactants.

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Tables

Table 3-1. Coacervate phase % volumes (v/v) for the coacervate phase compositions investigated for FCA reactions. Amounts of reactants added were based on the volume of the coacervate phase. (a) Tris concentration 0.5 M (b) Tris concentration 50 mM

Composition	Total surfactant concentration mM	% HFIP	% Volume (v/v) of coacervate phase
DTAB:Tris[a] (8.0)	50	10	2.6
DTAB:Tris[b] (8.0)	50	10	4.3
1:1 SDS:DTAB	100	10	2.6
1:1 SDS:DTAB	100	15	6.0
1:1 SDS:DTAB	100	20	10.7
7:3 SDS:DTAB	100	10	4.6
3:7 SDS:DTAB	100	10	2.4
1:1 SDS:CTAB	100	10	2.8
1:1 SDS:CTAB	100	15	3.2
1:1 SDS:CTAB	100	20	12.5
DMMAPS	100	10	8
DMMAPS	100	15	12.5
DMMAPS	100	20	18

Table 3-2. HPLC yields between π-bond nucleophiles and 4mbc in complex coacervate solutions containing a total surfactant concentration of 100 mM. 2,6-lutidine was used as a base additive

Entry	Composition	Nucleophile	% HFIP	% Yield (% RSD)
1	1:1 SDS:DTAB	Anisole	10	55.3 (5.8)
2	1:1 SDS:DTAB	Anisole	15	61.1 (4.3)
3	1:1 SDS:DTAB	Anisole	20	64.8 (4.9)
4	1:1 SDS:CTAB	Anisole	10	42.4 (8.3)
5	1:1 SDS:CTAB	Anisole	20	61.6 (5.6)
6	1:1 SDS:DTAB	2mf	10	73.5 (3.4)
7	1:1 SDS:DTAB	2mf	15	72.4 (3.6)
8	1:1 SDS:DTAB	2mf	20	66.3 (3.0)
9	1:1 SDS:CTAB	2mf	10	65.4 (4.7)
10	1:1 SDS:CTAB	2mf	20	75.5 (2.3)
11	1:1 SDS:DTAB	1mp	10	86.1 (4.8)
12	7:3 SDS:DTAB	Anisole	10	27.5 (11.5)
13	3:7 SDS:DTAB	Anisole	10	49.7 (8.4)
14	7:3 SDS:DTAB	2mf	10	22.4 (14.5)
15	3:7 SDS:DTAB	2mf	10	58.6 (9.1)

Table 3-3. HPLC yields between π-bond nucleophiles and 4mbc in simple coacervate systems. 2,6-lutidine was used as a base additive

Composition	Nucleophile	Surfactant concentration mM	Buffer Concentration mM	% HFIP v/v	% Yield (% RSD)
CTAB:Tris(8.0)	Anisole	50	500	10	72.2 (4.3)
CTAB:Tris(8.0)	Anisole	50	50	10	68.6 (4.7)
CTAB:Tris(8.0)	2mf	50	500	10	53.6 (3.4)
CTAB:Tris(8.0)	2mf	50	50	10	59.1 (4.4)
CTAB:Tris(8.0)	1mp	50	500	10	84.5 (6.4)
DMMAPS	Anisole	100	n/a	10	44 (8.9)
DMMAPS	Anisole	100	n/a	20	62.4 (6.3)
DMMAPS	2mf	100	n/a	10	67.9 (5.8)
DMMAPS	2mf	100	n/a	20	77.8 (2.4)
DMMAPS	1mp	100	n/a	10	99.3 (1.3)

Table 3-4. GC-TCD results to determine composition of two-phase systems formed in aqueous HFIP-solutions by the 4 π-bond nucleophiles

% HFIP total solution v/v	Phase	% HFIP in Phase v/v (%RSD)	% Water in phase v/v (%RSD)	% Mesitylene in phase v/v (%RSD)
10	Aq	4.8 (0.9)	89.6 (1.5)	N/A
10	HO	31.7 (0.8)	-0.2 (3.7)	39.9 (1.4)
20	Aq	8.9 (2.2)	80.6 (3.9)	N/A
20	HO	47.8 (17)	5.6 (1.2)	25.9 (0.2)
50	Aq	12.2 (3.5)	73.7 (7.2)	N/A
50	HO	55.2 (0.5)	22.1 (1.6)	8.3 (4.7)

% HFIP total solution v/v	Phase	% HFIP in Phase v/v (%RSD)	% Water in phase v/v (%RSD)	% Anisole in phase v/v (%RSD)
10	Aq	5.6 (3.0)	91.2 (1.4)	0.12 (22)
10	HO	29.0 (1.8)	1.6 (3.2)	58.4 (1.8)
20	Aq	7.9 (5.4)	81.3 (2.3)	0.13 (14.6)
20	HO	48.8 (2.3)	8.3 (3.3)	35.3 (3.5)
50	Aq	11.1 (1.0)	78.0 (1.0)	0.30 (2.4)
50	HO	53.4 (4.2)	24.2 (0.7)	11.6 (3.1)

Table 3-4 continued

% HFIP total solution v/v	Phase	% HFIP in Phase v/v (%RSD)	% Water in phase v/v (%RSD)	% 2mf in phase v/v (%RSD)
10	Aq	6.7 (4.2)	90.0 (2.5)	0.30 (4.3)
10	HO	34.7 (13.6)	2.9 (5.5)	46.5 (2.9)
20	Aq	9.2 (6.0)	84.0 (2.7)	0.32 (5.9)
20	HO	50.4 (3.6)	12.9 (2.8)	23.4 (3.6)
50	Aq	13.6 (2.8)	76.8 (0.6)	0.43 (13.7)
50	HO	50.1 (4.8)	14.8 (1.3)	7.9 (2.1)

% HFIP total solution v/v	Phase	% HFIP in Phase v/v (%RSD)	% Water in phase v/v (%RSD)	% 1mp in phase v/v (%RSD)
10	Aq	3.3 (1.1)	95.4 (1.3)	0.8 (1.9)
10	HO	38.3 (2.7)	8.4 (6.3)	43.3 (0.6)
20	Aq	5.9 (1.8)	92.2 (1.9)	0.5 (0.2)
20	HO	51.5 (3.2)	12.7 (1.6)	26.4 (2.0)
50	Aq	9.7 (4.4)	84.5 (1.5)	0.4 (0.3)
50	HO	8.4 (1.4)	29.5 (0.2)	9.6 (1.3)

Table 3-5. Reactions of π-bond nucleophiles with 4mbc with 2,6-lutidine as a base additive in solutions of HFIP and water

Entry	Nucleophile	% HFIP	% Yield[a]
1	Mesitylene	0	0
2	Mesitylene	10	61.2
3	Mesitylene	20	81
4	Mesitylene	50	77.5
5	Mesitylene	100	14.5
6	Mesitylene	100	66.2[b]
7	Anisole	0	0
8	Anisole	10	42
9	Anisole	20	70.5
10	Anisole	50	69.3
11	Anisole	100	38.9
12	Anisole	100	42.8[b]
13	2-methylfuran	0	<5
14	2-methylfuran	10	80
15	2-methylfuran	20	85
16	2-methylfuran	50	69
17	2-methylfuran	100	<5
18	2-methylfuran	100	<5[b]
19	1-methylpyrrole	0	34
20	1-methylpyrrole	10	92.5
21	1-methylpyrrole	20	95
22	1-methylpyrrole	50	75
23	1-methylpyrrole	100	69
24	1-methylpyrrole	100	85.6[b]

[a] Yields determined by HPLC [b] Used NH_4HCO_3 as a heterogeneous base additive instead of 2,6-lutidine. Both base additives were present at a 0.4 M; nucleophile was 1 M while 4mbc was 0.2 M.

Table 3-6. Reactions of π-bond nucleophiles with 4mbc with no base additive

Entry	Nucleophile	% HFIP	% Yield[a]
1	Mesitylene	10	73
2	Mesitylene	20	81
3	Mesitylene	100	41
4	Anisole	10	68
5	Anisole	20	79
6	Anisole	100	30
7	2-methylfuran	10	66
8	2-methylfuran	20	37
9	2-methylfuran	100	no rxn
10	1-methylpyrrole	10	93
11	1-methylpyrrole	20	88
12	1-methylpyrrole	100	79

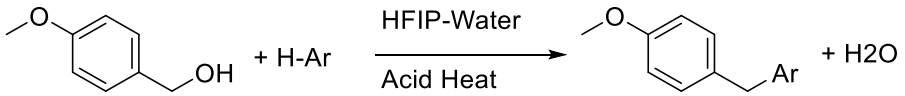
Nucleophile was 1 M while 4mbc was 0.2 M, same as the reactions done with base additives. Mesitylene reactions included diethyl ether to aid in phase separation as previously mentioned. [a] Yields determined by HPLC

Table 3-7. Effect of other solvents on Friedel-Crafts benzylation reactions under the same conditions as HFIP-Water systems.

Solvent	Nucleophile	% Yield
HFIP	Anisole	79
	1-methylpyrrole	88
2-propanol	Anisole	0
	1-methylpyrrole	48
Dichloromethane	Anisole	0
	1-methylpyrrole	<10
Toluene	Anisole	0
	1-methylpyrrole	<10

All reactions were done in 20% solutions of the solvent in water. Nucleophile was present at 1 M and 4mbc was 0.2 M.

Table 3-8. Reactions of π-bond nucleophiles with 4-methoxybenzyl alcohol. Reactions carried out at 55 °C.



Entry	Nucleophile	% HFIP	Acid	% Yield[a]
1	Mesitylene	0	HCl	0
2	Mesitylene	20	HCl	58
3	Mesitylene	20	PFOA	82
4	Anisole	0	HCl	0
5	Anisole	20	HCl	68
6	Anisole	20	PFOA	77
7	Anisole	100	HCl	69
8	Anisole	100	PFOA	81
9	2-methylfuran	20	HCl	49
10	2-methylfuran	20	PFOA	0
11	1-methylpyrrole	0	HCl	98
12	1-methylpyrrole	20	HCl	99
13	1-methylpyrrole	20	PFOA	55
14	1-methylpyrrole	20	None	0
15	1-methylpyrrole	20	HCl	0[b]
16	1-methylpyrrole	100	HCl	44
17	1-methylpyrrole	100	PFOA	43

[a] Yields determined by HPLC [b]Done at room temperature.

Table 3-9. Effect of two different Bronsted acids on 4mba reactions yields

Entry	Nucleophile	Acid	Time (hours)	% Yield[a]
1	Mesitylene	HCl	2	5
2	Mesitylene	PFOA	2	86
3	Anisole	HCl	2	16
4	Anisole	PFOA	2	73
5	Anisole	HCl	4	31
6	Anisole	PFOA	4	81
7	1-methylpyrrole	HCl	2	< 5
8	1-methylpyrrole	PFOA	2	51
9	1-methylpyrrole	HCl	4	11
10	1-methylpyrrole	PFOA	4	57

All reactions were done in 20% HFIP-water two phase systems at 50 °C. [a] Yields determined by HPLC

Table 3-10. Comparison of LLE and bottom phase removal for isolation of reaction products

Nucleophile	With LLE	Without LLE
Mesitylene	86	81
Anisole	70	75
2-methyl furan	73	67
1-methyl pyrrole	N/A[a]	79[b]

Reactions were carried out in 20% HFIP-water two-phase systems using 2,6-lutidine. [a] LLE procedure did not result in clear phase separation. [b] Done in 20% HFIP solution without any base additive.

Table 3-11. % Conversion of MVK in Diels Alder reaction with isoprene in HFIP-water two phase systems and 100% water

% HFIP	Reaction Time (hours)	% conversion of MVK (%RSD)
0	24	20 (22)
10	4	22 (16)
10	24	67 (5.6)
20	4	18 (7.9)
20	24	74 (7.2)
50	4	14 (25)
50	24	65 (7.9)

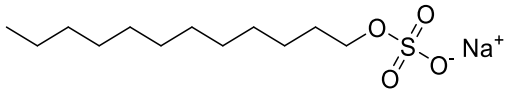
Table 3-12. p/m ratio of reactions between isoprene and MVK carried out in HFIP-water two phase solutions as well as 100% HFIP and water solutions

% HFIP	p/m ratio
0	2.7
10	4.1
20	4.5
50	4.6
100	8.9

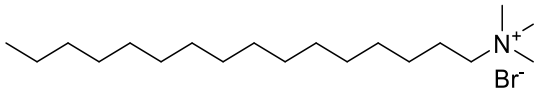
Table 3-13. The effect of reactant amounts and acids on the p/m ratio observed for the Diels Alder reaction between isoprene and MVK in a 10% HFIP two phase system.

Entry	µL Isoprene	µL MVK	Additive	µmoles additive	p/m ratio
1	150	41.6	None	n/a	4.1
2	75	41.6	None	n/a	4.2
3	150	80	None	n/a	3.88
4	100	80	None	n/a	3.83
5	300	80	None	n/a	3.81
6	50	80	None	n/a	3.91
7	150	41.6	HCl	25	4.06
8	150	41.6	HCl	200	4.43
9	150	41.6	$CuCl_2$	60	4.04
10	150	41.6	PFOA	25	7.77

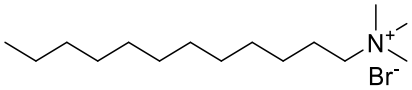
Figures



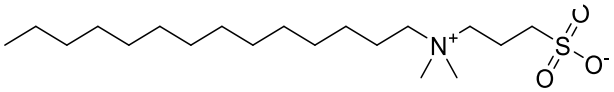
Sodium dodecyl sulfate (SDS)



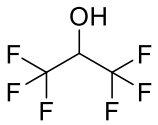
Cetyltrimethylammonium bromide (CTAB)



Dodecyltrimethylammonium bromide (DTAB)



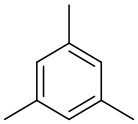
3-(N,N-dimethylmyristylammonio) propane sulfonate (DMMAPS)

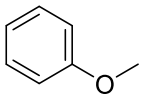


1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP)

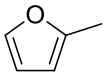
Figure 3-1. Surfactants used for coacervate formation which was induced by the addition of HFIP

Mesitylene = -2.6 anisole = -1.18,





2-methylfuran = 3.8 1-methylpyrrole = 5.85



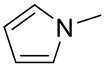


Figure 3-2. π-bond nucleophile structures and their nucleophilicity (N) values

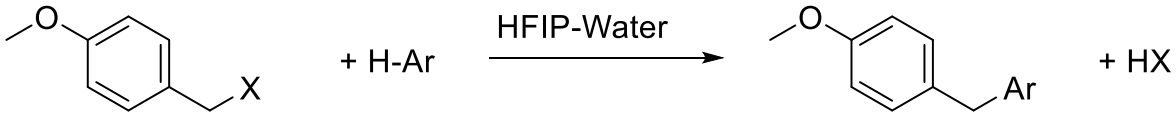


Figure 3-3. Reaction scheme for Friedel-Crafts benzylations. X= halogen or OH

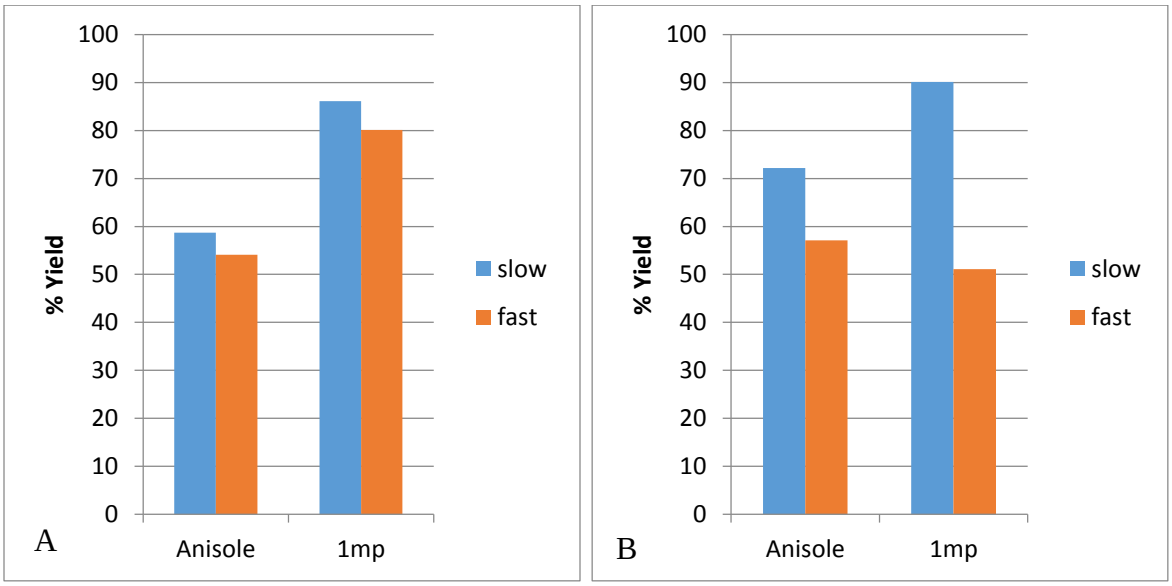


Figure 3-4. Effect of stirring speed on product yield for reactions between 4mbc and both anisole and 1mp in (A) complex coacervate SDS:DTAB (100 mM) 10% HFIP (B) simple coacervate DTAB (50 mM) Tris pH 8.0 (500 mM) 10% HFIP.

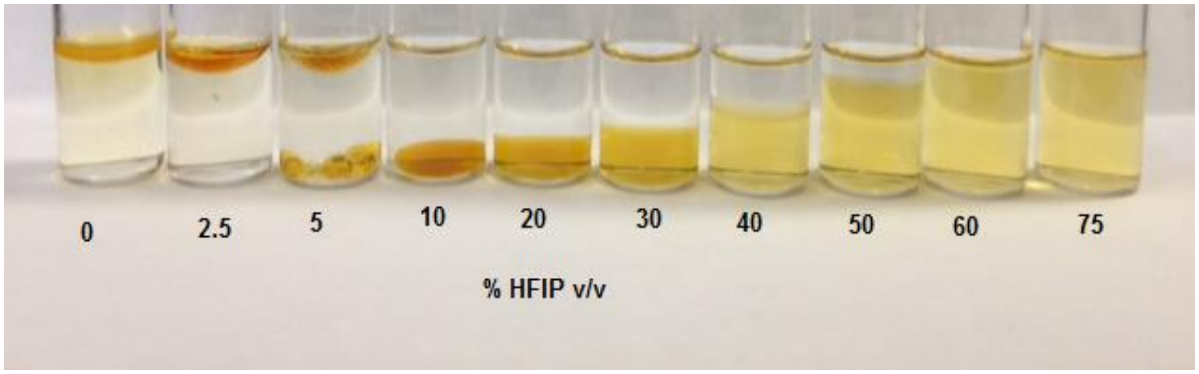


Figure 3-5. Effect of the addition of 1M 1-methylpyrrole to solutions of increasing % HFIP in water (v/v)

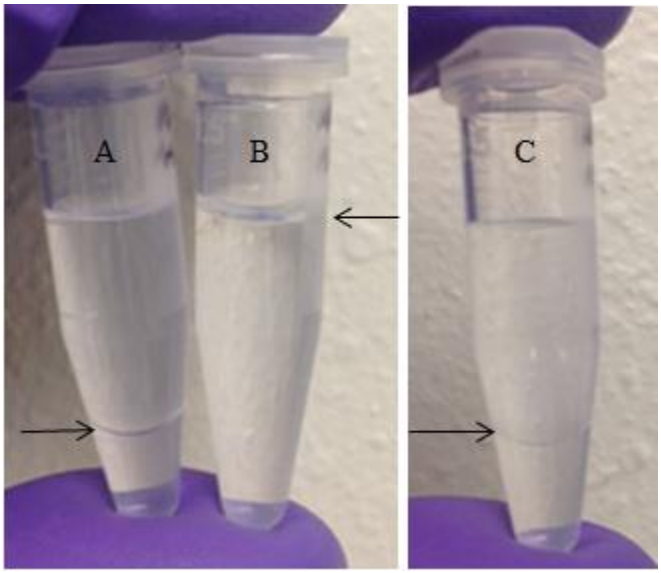


Figure 3-6. 10% HFIP solutions in water with the addition of 100 μL of A) anisole B) mesitylene and C) 100 μL mesitylene + 40 μL diethyl ether. Arrows indicate the interface between the two-phases.

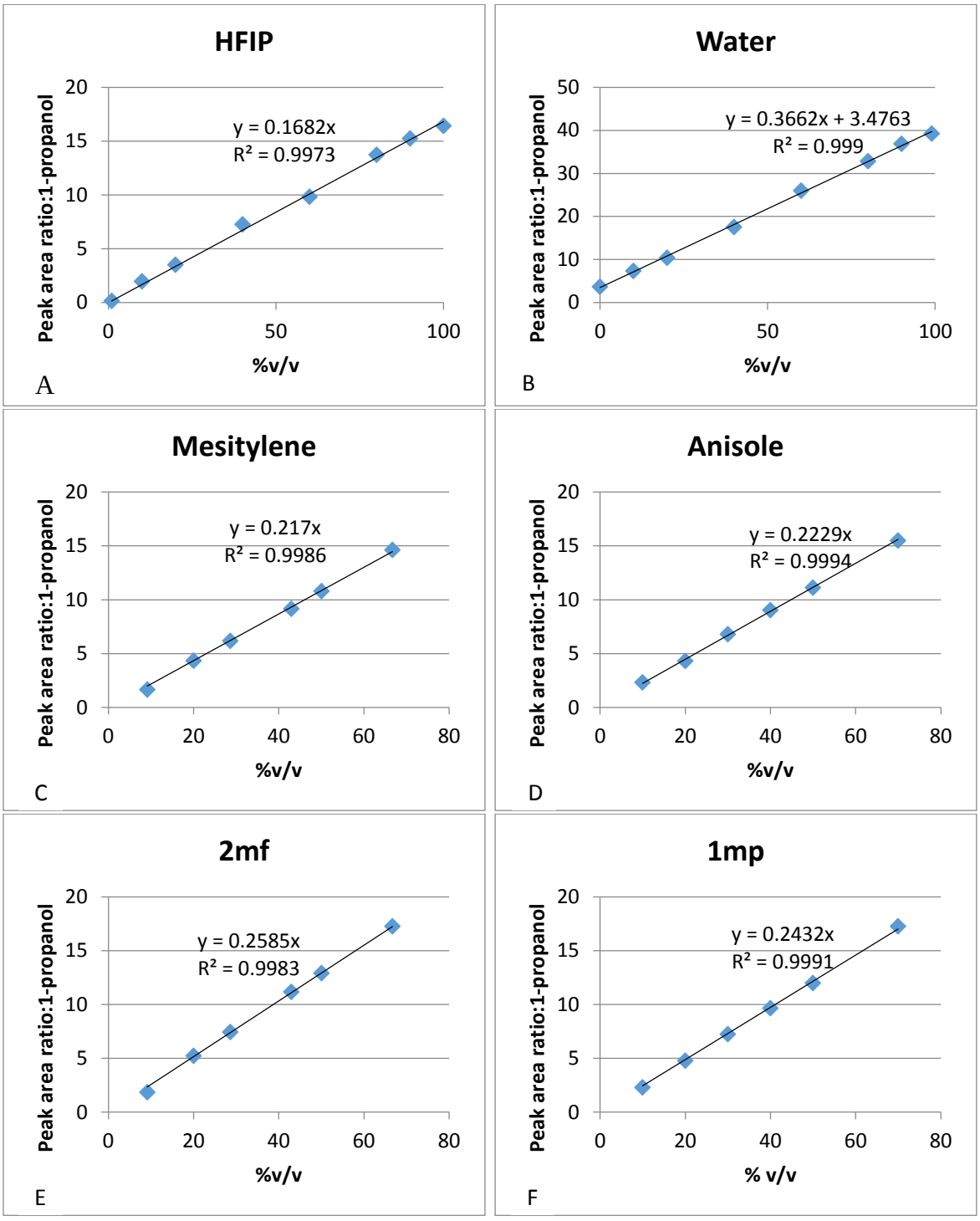


Figure 3-7. GC-TCD calibration curves for (A) HFIP, (B) Water, (C) Mesitylene, (D) Anisole, (E) 2mf and (F) 1mp

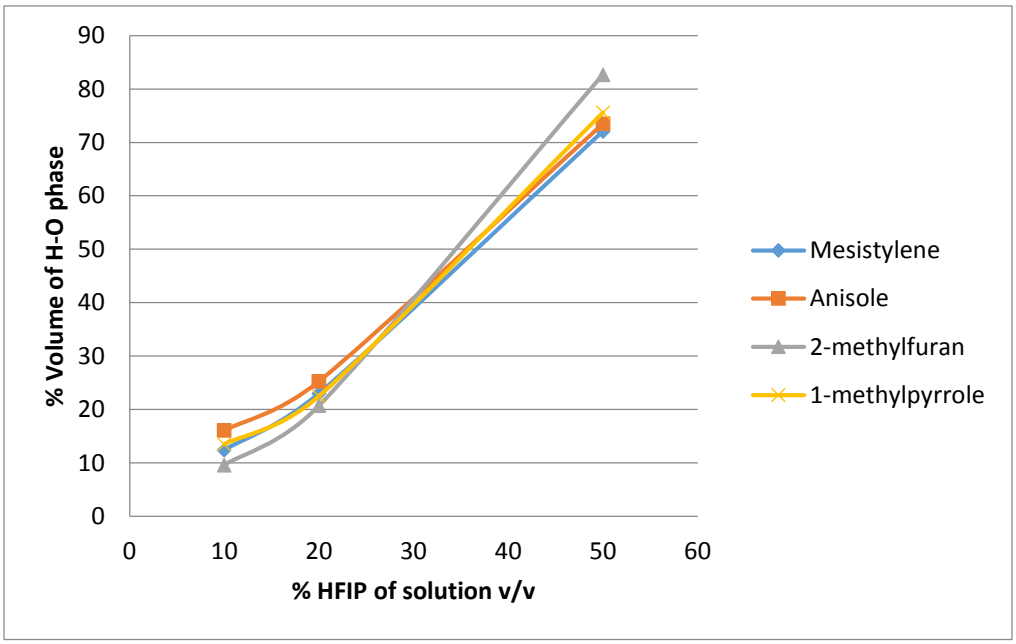


Figure 3-8. Effect of % HFIP on the % volume of H-O phases formed by the addition of 1M amounts of 4 nucleophiles to solutions of HFIP in water

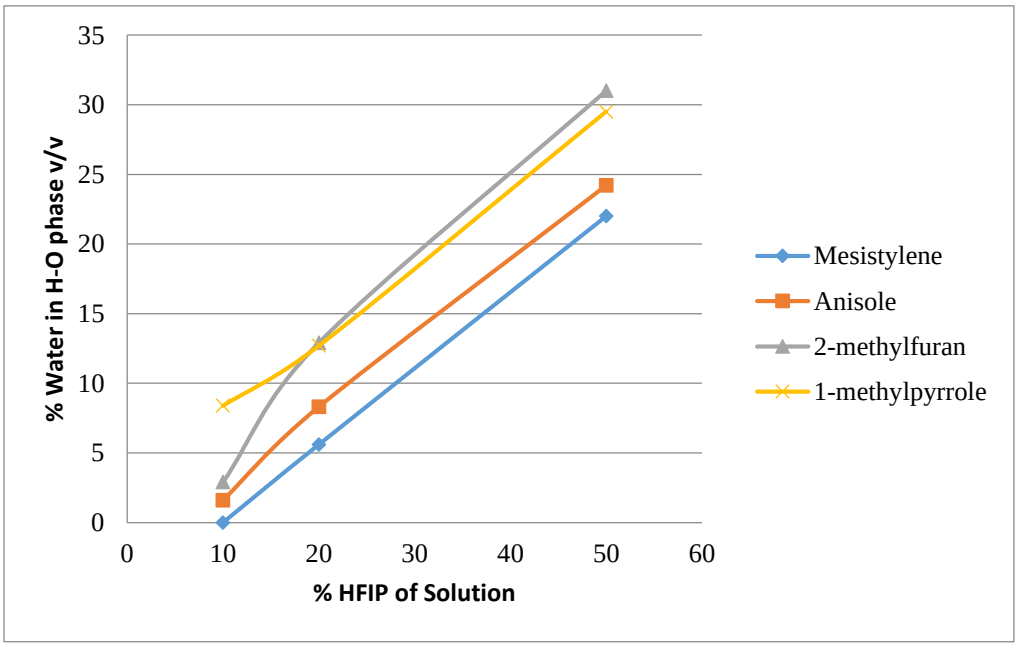


Figure 3-9. % Water in H-O phases formed by different nucleophiles

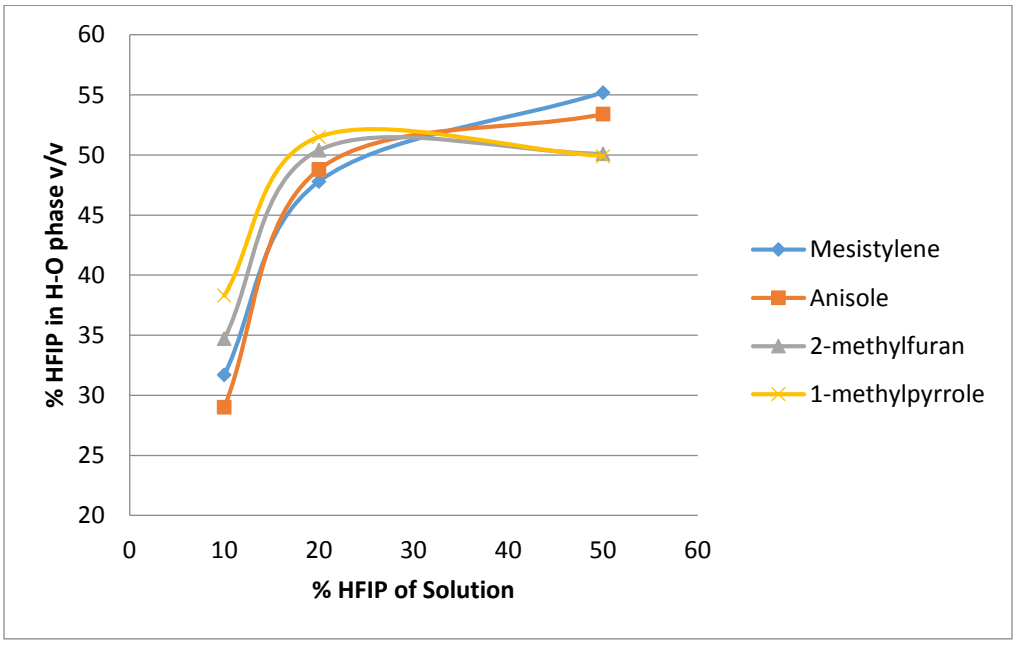


Figure 3-10. % HFIP in H-O phases formed by different nucleophiles

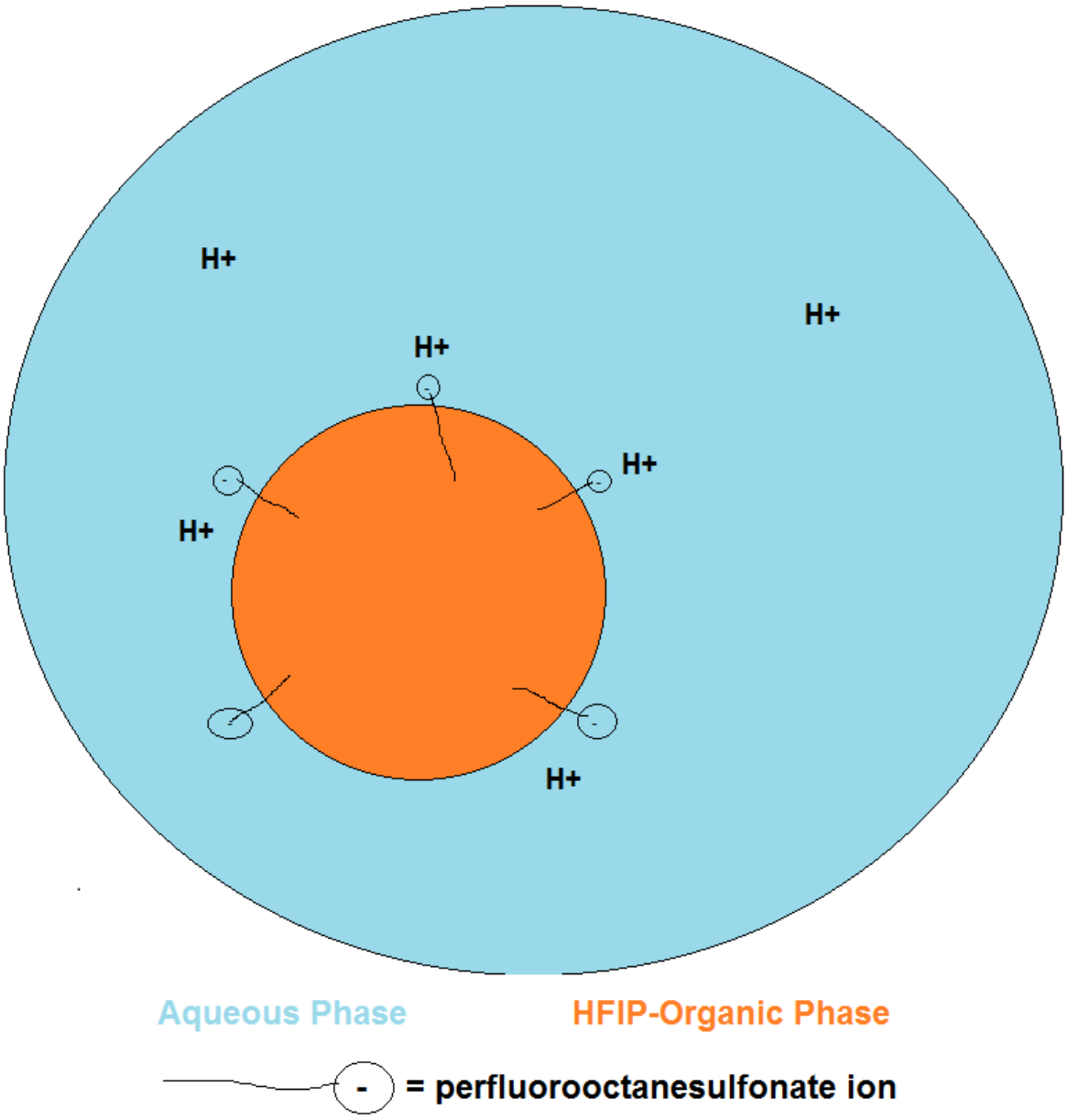


Figure 3-11. Proposed role of PFOA in catalyzing reactions through Bronsted acid catalysis. The PFOA anion attracts protons to the H-O phase where they are able to better interact with the reactants.

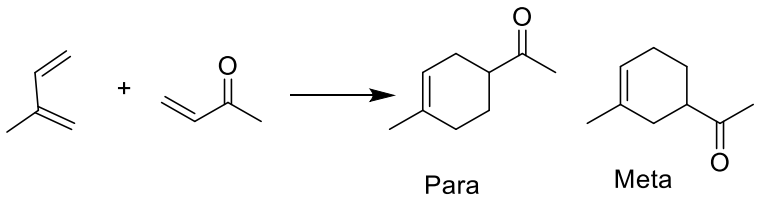


Figure 3-12. Diels Alder reaction between isoprene and methyl vinyl ketone resulting in either the para or meta product

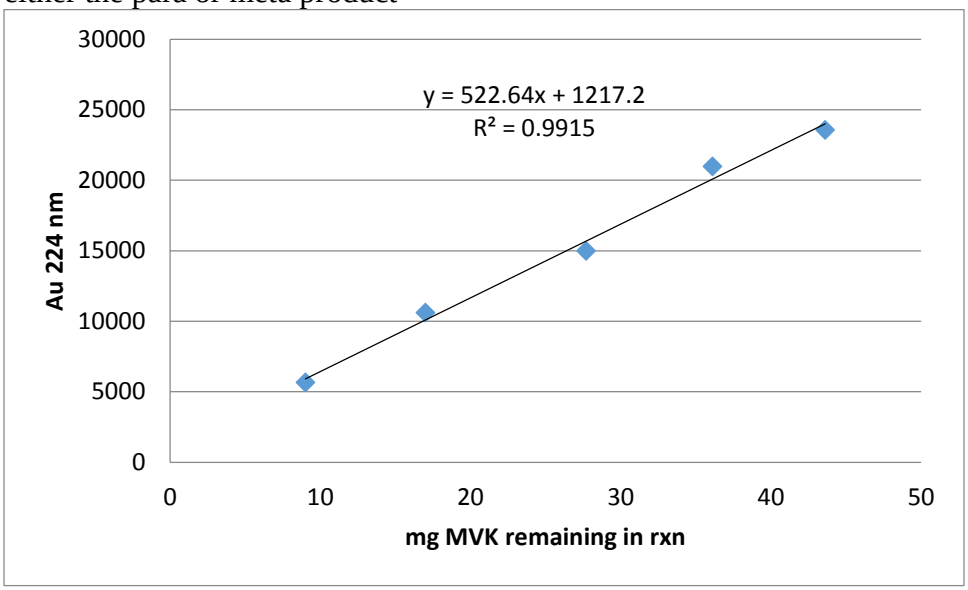


Figure 3-13. HPLC calibration of MVK used for calculating % conversion for Diels Alder reactions.

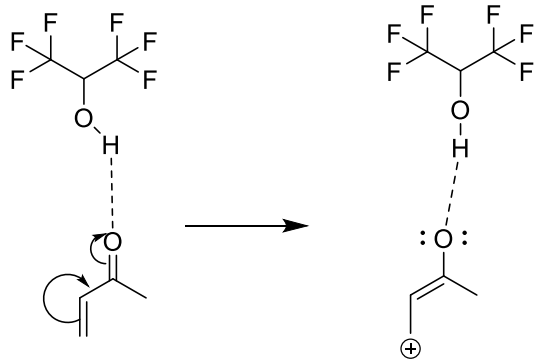


Figure 3-14. HFIP H-bond interaction with the carbonyl group of MVK that results in an increase of the para product formation

Chapter 4

Aqueous / HFIP-Solvent Two-Phase Systems for Liquid-Liquid Microextractions of Small Molecules from Aqueous Solutions

Abstract

The addition of 200 μL of a 1:1 Hexafluoroisopropanol(HFIP):Tetrahydrofuran(THF) mixture to 4 mL of water forms a two-phase solution with a 20 μL HFIP-THF phase. These two-phase solutions were used for liquid microextraction of amines and polycyclic aromatic hydrocarbons (PAH) from water. Analysis was performed by using splitless injection of the HFIP-THF phase into a GC-MS instrument with single ion monitoring. The addition of 0.5% hexanoic anhydride into the HFIP:THF mixture allowed for simultaneous derivatization and extraction of amines leading to improved GC-MS performance as well as increased extraction. The % recovery of the 6 derivatized amines was between 21 and 68% in deionized water representing enrichment factors of 41-135. Recovery of 4 PAH molecules ranged from 56-73% in deionized water with enrichment factors from 112-145. The GC-MS methods gave linear ranges over three orders of magnitudes and limits of detection in the low μg/L range for both derivatized amines and PAH molecules.

Introduction

Identification and quantitation of various classes of chemicals in water is important for monitoring pollutants in the environment. The analysis is typically done using either gas or liquid chromatography coupled to a variety of detectors. In a typical analytical process for the analysis of compounds in water an extraction step is used for extraction and pre-concentration of analytes prior to analysis of water samples. Organic compounds often have

low solubility in water and are present at low concentrations which can make them difficult to detect. Liquid-Liquid extraction (LLE) is one of the oldest extraction and preconcentration techniques and involves extracting the analytes into a water immiscible solvent by forming an emulsion and then allowing the phases to separate and then remove the organic phase for analysis.[1] Typically large volumes of solvents are used and analyte pre-concentration can be achieved through evaporation of the solvent after extraction. The evaporation method cannot be done in the analysis of volatile analytes that will be lost during solvent evaporation. The performance of an LLE system is often determined by distribution constant (K_d) (Eq. 3-1) which is the concentration of the solute in the organic phase divided by the concentration of the solute in the aqueous phase and is often referred to as the partition coefficient.

$$K_d = C_{org} / C_{Aq} \qquad \text{Eq. 3-1}$$

Throughout its history in analytical chemistry LLE has been used for the extraction and analysis of a wide range of compound classes such as pesticides[2], polycyclic aromatic hydrocarbons[1], metals and various other organic materials [3]. . The development of continuous LLE methods rather than batch methods was one of the first advances for LLE resulting in increased throughput. One of the first continuous LLE setups was reported in 1962 and allowed for 250 mL of an extraction solvent to perform LLE on several liters of seawater.[4] The extractor worked by passing the extraction solvent through flowing seawater to perform extraction and then continuously collecting and evaporating the solvent and passing it back through the seawater again. The extracted analytes were collected when the extraction solvent was evaporated. Similar continuous LLE extractors using internal recovery and recycling of the extraction solvent were later applied to the extraction of

pesticides from water.[5] These early continuous extractors could only be used with solvents that were less dense that water as they relied on the solvents rising through the water and then overflowing into the collection and recycling apparatus. Goldberg and Delong later reported continuous LLE devices that could be used with any water-immiscible solvent independent of whether it was more or less dense than water.[6] They were able to achieve enrichment factors in the range of 10^5 by evaporating large volumes of the extraction solvent after extraction of the solutes from the water. With all of these designs requiring internal evaporation and recycling of the extraction solvent they are difficult to use in the field and require the water sample of interest to be collected beforehand and brought to the apparatus for extraction. Another design for continuous LLE based on mixing and settling was developed and used directly in the field to extract pesticides and polychlorinated biphenyls (PCB) from river water.[RW.ERROR - Unable to find reference:217]

One of the largest areas of focus in the field of liquid extraction over the past couple of decades has been on greatly reducing the total volume of organic solvent used for extraction while still achieving high analyte recovery and enrichment. The reduction of solvents used as well as the use of less toxic solvents are some of the main principles of green chemistry. One of the first examples of a liquid microextraction technique was single drop microextraction (SDME) [7,8] which involves extracting analytes into a droplet of organic solvent suspended from the tip of a syringe and then pulling the droplet back into the syringe for analysis. SDME has been used for the extraction and analysis of a variety of compounds in water such as pesticides[9] and pharmaceuticals[10] and has also been adapted for a continuous extraction procedure in which the water is flowed across the suspended droplet for extraction.[11] SDME can also be applied to headspace analysis of volatile compounds by

suspending the solvent droplet in the air above the sample of interest to allow for extraction.[12,13] Techniques using GC auotsamplers have been developed to automate the SDME process during GC analysis, greatly reducing the amount of work required by the researcher.[14] The benefits of the SDME process are simplicity only requiring a syringe, using very little solvent, and good analyte recovery and enrichment. Difficulties with SDME arise from the stability of the droplet where too much agitation can cause it to fall from the tip. Also, because the solvent is a droplet and not dispersed throughout the solution this limits the surface area available for extraction which slows the kinetics of extraction.[15]

Another microextraction technique termed Hollow Fiber Liquid Phase Microextraction (HF-LMPE) was first reported a couple of years after SDME.[16] In this technique a porous hollow fiber is used in which the pores have been saturated with a water immiscible solvent. The inside of the fiber can then be filled with either more of the extraction solvent or water to back extract the analytes giving either a two or three phase extraction. The solution inside the fiber is termed the acceptor phase while the solution from which the analytes are being extracted is the donor phase. The use of an aqueous acceptor phase is only possible for compounds with acidic or basic groups that are ionizable. The pH of the donor phase should be adjusted such that the compounds are neutral while the pH of the acceptor phase should give the compounds in their ionized form to prevent them from repartitioning into the donor phase due the lower solubility of the ions in the organic solvent filling the pores. Thus HF-LPME is good for the extraction of most hydrophobic compounds particularly those found in the environment whether they are ionizable or not. More polar compounds will not have much affinity for the organic solvent filling the pores allowing these systems to have some selectivity towards more hydrophobic compounds and

eliminating certain interferences from complex matrices.[17] The hollow fiber is attached to the end of a syringe which allows for the collection of the acceptor phase for analysis after extraction. The acceptor phase can then be analyzed directly by GC or HPLC depending on whether the acceptor phase is an organic solvent or aqueous solution. Extraction can either be static or dynamic. Static extraction is when the donor solution is agitated in some way and the acceptor phase remains static during the extraction. In dynamic extraction a syringe pump is used to move the acceptor phase in and out of the hollow fiber in order to extract the analytes from the solvent in the pores.[18] Dynamic extraction was shown to improve extraction speed as well as efficiency but does require some additional instrumentation and optimization compared to static extraction.[19]

Solid Phase Microextraction (SPME) was first reported in 1990 and eliminates the use of solvents for extraction of analytes from an aqueous media. A fiber coated with an extractant, either a liquid polymer or solid sorbent, is used to extract compounds from a liquid or gas phase. After extraction, the fiber can be directly introduced into the injection port of a GC to allow for thermal desorption of the analytes. It is also be possible to remove the analytes from the SPME fiber by using solvents if having them in a liquid is desired for analysis. When the fiber is immersed into a liquid sample an equilibrium is reached for the analytes between the liquid and stationary phase of the fiber.[20] This equilibrium is dependent on the concentration of the analyte in solution was well as the properties of the fiber based on the following equation: $n_1 = KV_1C_2^0$, where n_1 represents the moles of analytes extracted, K is the distribution constant, V_1 is the volume of the stationary phase and C_2^0 is the concentration of the analyte in the aqueous phase. As this equation shows the choice of stationary phase is very important in that distribution coefficient of the analyte plays a large

role in determining the efficiency of the extraction. A variety of stationary phases can be used for extraction allowing for the phase to be picked to best extract the analyte of interest.[21] SPME is very easy to use for in the field analysis of water sources due to only needing the fibers to perform extraction and then analyze them later back at the lab.[22] In addition to coating a fiber to perform SPME several other techniques have been developed such as stir-bar sorptive extraction[23,24], thin-film microextraction[25], and several metal syringe based methods where the sorbent is packed inside of a syringe which provides a little more stability than the traditional coated fibers.[26,27] Most of these new techniques allow for the use of larger volumes of stationary phase which is one of the main variables in improving extraction efficiency. The use of larger volumes of stationary phase does necessitate the use of longer equilibration times. SPME is a well-established technique for the extraction and enrichment for wide ranges of compound classes. Some of the drawbacks associated with SPME are that the stationary phases require a preconditioning step prior to extraction to wet the stationary and improve extraction efficiency. The stability of the stationary phase either during extraction or thermal desorption could cause problems such as decomposition of the stationary phase within the GC inlet. If thermal desorption is not being used then solvents have to be used to remove the analytes removing one of the primary green advantages of SPME.

Another solvent microextraction technique known as dispersive liquid-liquid extraction (DLLE) was first reported in 2006 and has since gained wide spread attention.[28] In this technique two solvents are used to perform the extraction one is water immiscible such as dichloromethane or tetrachloroethylene as the method was originally developed with solvents denser than water. The second solvent, known as the dispersing solvent, is miscible

with both the extraction solvent as well as water like acetone or ethanol. The extraction is performed by using a syringe to inject a solution of mostly dispersing solvent containing a small amount of the extraction solvent into an aqueous solution. For example in the original paper, 1.0 mL of acetone containing 8.0 μL of tetrachloroethylene was injected into 5.0 mL water solution forming a cloudy solution. After gentle mixing and centrifugation a 5.0 μL sedimented phase is present at the bottom. This sedimented phase can then be collected via syringe for analysis, likely through direct injection into a GC instrument. The sedimented phase is composed mostly of the extraction solvent while the dispersing solvent serves to promote mass transfer of the analytes from the aqueous phase into the extraction solvent. Due to the small volume of the sedimented phase relative to the aqueous phase very high enrichment factors are achievable. When the solvents are injected the solution becomes cloudy as the solvent is dispersed as tiny droplets throughout the aqueous solution giving a very large contact area between the acceptor phase and the sample leading to fast extraction kinetics.[15] Due to its simplicity as well as good extraction performance DLLE has been used for the analysis of nearly all types of organic analytes found in various aqueous solutions.[29] Although this technique greatly reduces the amount of the more toxic extracting solvent used it still requires the use of larger amounts dispersing solvent. The presence of this large volume of dispersing solvent in the aqueous phase is also likely to increase the solubility of the analytes in the aqueous phase which could decrease extraction recovery. Methods were then developed that did not require dispersing solvents and compensated by using increased agitation to disperse the extracting solvent through means such as ultrasonication[30], vortexing[31] and repeated pumping using a syringe[32]. New methods were also developed where solvents with lower density than water could be used for extraction. One was based

on the use of special glassware that made collection of the floating extraction phase easier.[33] In another technique 1-dodecanol was used as the extraction solvent. Since it has a freezing point just under room temperature the solution was cooled after extraction to allow for easy collection of the solid droplet for further analysis.[34] Although the small volume of the acceptor phase formed during most of the reported DLLE procedures leads to high enrichment factors it can also cause problems in terms of sampling and analysis. It can be difficult to remove very small volumes of acceptor phase without also collecting some of the aqueous phase. It also makes it more difficult to use autosamplers for analysis. Even with reduced volume inserts for autosampler vials more than a few microliters are needed to make sure the needle can adequately remove the sample for injection. The ability to use autosamplers over manual injection is convenient especially when analyzing large number of samples.

Gas chromatography is often the separation method of choice for small molecules after solvent microextraction due to its high resolving power, high sensitivity and short analysis times. Additionally, direct injection of solvents is much more compatible with GC compared to HPLC where it may cause peak shape problems due to mismatch with the mobile phase in reversed phase HPLC. The GC separation of certain compounds can be difficult if they are very volatile or contain polar functional groups such as primary amines, alcohols or aldehydes. These polar groups tend to be adsorbed onto the column leading to large amounts of peak tailing and small signals. For this reason when analyzing polar compounds such as amines with GC, derivatization reactions are often used to convert polar amine group into more volatile compounds improving the GC separation.[35] One of the most common types of derivatization for primary and secondary amines is an acylation reaction

that converts the amine group into an amide. Acid anhydrides and acyl chlorides are the most common acylation agents for amines and react under mild conditions. The byproducts of these reactions produce either a carboxylic acid or HCl for the acid anhydride and acyl chloride respectively. These often need to be removed from the solution prior to analysis because they may damage the GC column so base additives are often added to the reaction solution which also acts as a catalyst to promote the reaction. In addition to improving chromatographic performance derivatization can be used to improve detection with certain types of detectors. Derivatization can be used to introduce electron withdrawing groups to compounds such as amines in order to make them compatible with electron capture detectors. The most common types of reagents for introducing electron capture properties are fluorinated compounds which introduce groups such as trifluoroacetyl or pentfluorobenzoyl groups.[36,37] The need to derivatize compounds introduces and additional step in the extraction procedure as often many of these reactions need to be carried out in organic solvents rather than the initial aqueous solutions so extraction must be performed prior to derivatization. The ability to perform simultaneous extraction and derivatization is desirous in that not only does it simplify the procedure, it should enhance the extraction of the compounds by converting them to more hydrophobic compounds decreasing their water solubility.

Several examples of simultaneous extraction and derivatization have been demonstrated using various microextraction techniques. One example using SDME incorporated the derivatizing agent, pentafluorobenzoyl chloride, into the extraction droplet and required pH adjustment of the aqueous solution.[38] The use of pentafluorobenzaldehyde as a derivatizing reagent in aqueous solutions was found to easily react with amines to form a

Schiff's base which was simultaneously extracted using HF-LPME although it only works with primary amines.[39] Pentafluorobenzaldehyde and N-succinimidyl benzoate amine derivatization was also combined with headspace extraction using SPME in which the amines were initially derivatized in the aqueous solution and transferred to a headspace vial for extraction.[40,41] The use of DLLE for simultaneous extraction and derivatization has also been studied. Several DLLE simultaneous derivatization procedures utilized extraction solvents that also acted as the derivatization solvent such as butyl chloroformate[42] and pentafluorobenzaldehyde[43]. Methods were also developed using typical DLLE solvents with the derivatizing agent added into the solution.[44,45]

The focus of the research presented here is the application of the Aqueous / HFIP-solvent (Aq/H-O) two phase systems for extraction and enrichment of small molecules from aqueous samples with subsequent GC-MS analysis. The systems were also used for simultaneous extraction and derivatization of amines from water using acid anhydride derivatizing agents.

Experimental

Materials

1,1,1,3,3,3-Hexafluoroisopropanol(HFIP) was purchased from Oakwood Chemical at 99% purity. THF was extra pure anhydrous (99.9%) from Acros organics. Anisole was 99% from Alfa Aesar. Butyryl chloride and hexanoic anhydride were purchased from Acros organics at 99% and 97% purity respectively. Hexanoyl chloride and butyryic anhydride were purchased from TCI America at 98 and 99% purity. The PAH compounds; anthracene, pyrene, chrysene, perylene, 1-chloronaphthalene and 9-bromoanthracene were all purchased

from Sigma Aldrich and were at least technical grade. Hexylamine was 99% from TCI America. o-toludine and o-anisidine were purchased from EMD Millipore at 99% purity. 4-chloroaniline, 4-bromoaniline and 3,5-dichloroaniline were all purchased from Sigma Aldrich at a minimum of 98% purity. River water for spiked extractions was obtained from the Trinity River in Arlington Texas.

Methods

Preparation of amine derivative GC-MS calibration curves

Stock solutions of amines were prepared in acetonitrile at a concentration of 400 mg/L. To prepare the derivative standards 2.5 μL of the appropriate acyl chloride was added to 1mL of the stock solution and stirred for 30 minutes. Since butryric and hexanoic anhydride were used as the derivatizing agents in the extractions, butyryl and hexanoyl chloride were used for preparing the standards. The acyl chlorides were used to prepare the standards because they are much more reactive and guaranteed that complete derivatization of the amines would occur in a short time span. Since the stock solutions were prepared in acetonitrile with no water present, the reactions proceeded smoothly with completion being monitored by GC-MS, the reaction is shown in figure 4-1. Completion of the derivatization reaction was confirmed by the disappearance of the reactant amine peak and appearance of a single derivative peak. The concentration of the amine derivative was then taken to be the same as the initial stock solution for preparing calibration standards at the desired concentration. Calibration standards were prepared in the solvent used to form the two phase solution other than HFIP such as THF or 2-butanone. The calibration curves for 4-bromoaniline and its butyryl and hexanoyl chloride derivatives are shown in figure 4-2.

GC-MS analysis of standards and extractions for amine samples

GC-MS analysis was done on a Shimadzu QP-2010 Ultra gas chromatograph equipped with a single quadrupole mass spectrometer and electron impact ionization source. The column was a Restek rxi-5ms column; 30 m length, 0.25 mm i.d., 25 μm film thickness. The instrument was operated with an inlet temperature of 280°C, ion source temperature of 230°C, interface temperature of 280°C and a solvent cut time of 3 minutes. A 1μL splitless injection was done with a sampling time of 1 minute. The GC temperature program was as follows: 45°C(1 min) - 25°C/min - 260°C(1.5 min) for a total run time of 11.10 minutes. The m/z values for the amines as well as their derivatives along with the SIM parameters are shown in Table 4-1 and 4-2 respectively. The m/z value of the underivatized amine was used for quantitation since it was the largest signal observed for all derivatives and the m/z of the derivative molecular ion was used as a reference ion.

Extraction derivatization process for amines

4 mL aqueous solution was spiked with an amine mixture to a concentration of 20 μg/L in a 15 mL centrifuge vial. The extraction solvent was prepared by mixing equal amounts of HFIP and THF along with 0.5% v/v of the derivatizing acid anhydride. 200 μL of the extraction solvent was quickly injected into the aqueous solution via a syringe and the solution was then rotated for 20 minutes to allow for the derivatization reaction to occur. Following rotation, the solution was centrifuged at 1500 rpm's for 2 minutes to yield a two phase solution with bottom phase being the HFIP-THF with a phase volume around 20 μL. The HFIP-THF phase was then removed using a syringe with care taken not to pull up any of the aqueous phase. The HFIP-THF phase was then transferred to an autosampler vial with a

reduced volume insert for GC-MS analysis. The enrichment factor of the extraction was calculated using the following equation:

$$EF = C_{H\text{-}O} / C_i \quad \text{Eq. 4-1}$$

Where $C_{H\text{-}O}$ is the concentration of analyte measured in the HFIP-solvent phase determined from external calibration curves and C_i was the initial concentration of the analyte spiked into the water. For amine analysis mM was used for concentrations rather than mg/L since derivatization changes the mass of the compound but not its molarity. The % recovery of the extraction was calculated by:

$$\% \text{ Recovery} = (C_{H\text{-}O} / C_{100\%}) \times 100 \quad \text{Eq. 4-2}$$

Where $C_{H\text{-}O}$ is the measured concentration of analyte in the HFIP-solvent phase and $C_{100\%}$ is the theoretical concentration of an extraction with 100% recovery. This value can be determined by knowing the concentration of amine initially spiked into solution as well as the volume of the H-O phase.

GC analysis of PAH molecules with HFIP-THF extraction

GC-MS analysis was done on a Shimadzu QP-2010 Ultra gas chromatograph equipped with a single quadrupole mass spectrometer and electron impact ionization source. The column was a Restek rxi-5ms column; 30 m length, 0.25 mm i.d., 25 μm film thickness. The instrument was operated with an inlet temperature was 340°C, ion source temperature of 230°C, interface temperature of 300°C and a solvent cut time of 3 minutes. The instrument was operated with a constant flow rate of 2.0 mL/min. The GC temperature program was as follows:

45°C(1 min) - 30°C/min - 200°C(0.5 min) - 25°C/min - 300°C(2.5 min)

resulting in a total analysis time of 13.17 minutes. The structure and m/z for the PAH molecules analyzed as well as the SIM program are shown in Tables 4-3 and 4-4 respectively.

The extraction and analysis procedure for PAH's was the same as that described for amines except no derivatization reagent was added to the extraction solvent. Enrichment and recoveries were calculated in the same manner as well.

GC-MS analysis of PAH molecules with HFIP-Anisole extraction

The same column and instrument used for the HFIP-THF extractions were used for HFIP-anisole extraction. The instrument was operated with an inlet temperature was 310°C, ion source temperature of 230°C, interface temperature of 310°C and a solvent cut time of 3 minutes. The instrument was operated with a constant linear velocity of 35 cm/sec and high pressure injection was used for 0.5 μL splitless injection. The GC temperature program was as follows:

135°C (1 min) -25°C/min- 300°C (4 min) for a total run time of 11.6 minutes.

The PAH molecules were detected using SIM monitoring of their molecular ion. External calibrations were prepared in mixtures of HFIP and anisole and used to determine enrichment factors as well as % recovery.

Results and Discussion

Development of extraction derivatization procedure

Acetylation reaction

Both acyl chlorides and acid anhydrides are capable of converting primary amines into amides improving their GC characteristics. In HFIP-solvent two-phase systems acid anhydrides worked much better at converting amines to amides compared to acyl chlorides. Acyl chlorides being much more reactive than acid anhydrides probably immediately reacted with the water present to form a carboxylic acid which is less reactive and not able to derivatize the amines under the conditions used. Aq/H-O extractions were carried out on 4-bromoaniline and o-anisidine with and without the presence of the derivatizing agent butyric anhydride (Fig 4-3). When the derivatizing agent is present the amine peak disappears and a new peak is present which represents the derivative. Although the amine peak is not present in the extractions with butyric anhydride it does not necessarily mean that there was complete conversion to the derivative. The new peak was confirmed to be the expected amide peaks by observing the molecular ion as well as searching the EI-MS spectra and obtaining the predicted structure. Having confirmed that the derivatization procedure works in the HFIP-solvent two phase systems the effect of derivatization on extraction and GC performance was investigated using 4-bromoaniline. Extraction and GC results were compared between using butyric or hexanoic anhydride as well as using no derivatization reagent. Figure 4-4 shows the effect of derivatization on the EF value measured using 200 μL of 1:1 HFIP:THF for extraction from a 4 mL aqueous solution spiked with 20 μg/L 4-bromoaniline. Derivatization greatly enhanced the extraction of the amine by making it less polar and hexanoic anhydride gave a larger EF value than burtyric due to its longer alkyl chain. Table 4-5 shows how the

derivatization affected the GC performance in terms of retention time as well as signal size. Derivatization increased both the retention time and the signal size. Increasing the retention time can be beneficial for analysis of more volatile amines that would elute early under typical GC temperature programs making them difficult to separate or causing them to elute near the injection solvent and not be detected. Increased signal size combined with the higher enrichment factor should improve the detection of amines present at low concentrations. In addition to increasing retention and signal size derivatization also improved the peak shape and reduced tailing as seen in Fig 4-5 A-C which shows the chromatogram of 4-bromoaniline as well as its two derivatives.

Extraction protocol

With the benefits of derivatization being established various methods of performing the extraction derivatization procedure were tested to determine what worked best. Table 4-6 shows the results of some different extraction procedures and how they affected the peak area observed for extractions using hexanoic anhydride as the derivatizing agent. Based on these results it was determined that using 200 μL (per 4mL Aqueous solution) of a 1:1 HFIP:THF solution containing 0.5 % v/v hexanoic anhydride with syringe injection and 20 minutes of rotation, slow constant inversion, would be used for future extractions. Using 200 μL was shown to work better than either 160 or 240 which gave smaller and larger phases respectively. The volume of the HFIP:THF phase produced when using 200 μL of HFIP:THF in 4 mL was about 20 μL which is small enough to allow for high enrichment factors (200 is the theoretical high based on the 4 mL volume) but also large enough to work with easily and use with autosamplers by using small volume inserts in the autosampler vials.

Very little to no difference was observed when changing the % of derivatizing agent from 0.5 to 1.0 % v/v. Increasing the rotation time from 20 to 80 minutes show no benefit on the observed peak area meaning the acetylation reaction reaches its completion within 20 minutes.

Splitless injection of HFIP-THF phase into GC-MS analysis

Splitless injection offers lower limits of detection over split injection because the entire volume of solution injected into the instrument is introduced into the column. More care must be taken when setting up the instrument parameters for splitless injection compared to splitting sample in order to maintain good chromatographic results. When doing splitless injection the split vent is closed for around 1 minute to allow for the sample to enter the capillary column before the split vent is opened. Unlike with split injection the large volume of the injection solvent being introduced into the column can have a profound effect on the separation. If the initial oven temperature is too high the solvent will begin travelling down the column and interact with the analyte molecules preventing them from interacting with the stationary phase. This can lead to poor separation and problems such as broad or split peaks. To overcome this, a technique called solvent focusing is used where the initial oven temperature is at least 15°C below the boiling point of the injection solvent. This causes the injection solvent to condense on the front of the column along with the analytes keeping them in a tight band. After the injection is complete the temperature is increased causing the solvent to elute from the column allowing the analytes to interact with the GC stationary phase without being interfered with by the injection solvent. Thus, for analytes extracted into HFIP-THF phases an initial oven temperature of 38°C was used which is around 19°C below the BP of HFIP and 28°C below THF. For some extractions done using anisole as the other

solvent with HFIP, a much higher initial oven temperature was used of 135°C since the BP of anisole is 154°C. Under these conditions the HFIP should initially be swept down the column upon injection due to the high temperature while the analytes are focused in the remaining anisole. Having to use higher initial oven temperatures can hinder the analysis of more volatile compounds which would not be allowed to interact with the stationary phase. This needs to be taken into consideration when choosing the solvent with HFIP used for two-phase formation.

Derivatization-Extraction of amines

The results of the derivatization extraction procedure on aqueous samples spiked with amines can be seen in table 4-7. Enrichment factors between 41 and 118 were observed corresponding to recoveries between 20.5 and 58.5%. While the % recoveries are not great, the enrichment in combination with the increased signal size due to derivatization allow for easy detection of the amines which were originally spiked in solution at 20μg/L of the amines (Fig. 4-6). The method did not work as well for hexylamine which was the only alkyl amine used. This could possibly be due to the fact that underivatized hexylamine has higher water solubility than most of the other amines or it may have a lower affinity for the HFIP-THF phase. If the derivatization reaction is taking place in the HFIP:THF phase then hexylamine may not partition into the H:T to allow for the reaction to occur as much as the other amines. Therefore, while the method does allow for the use of traditional derivatization agents such as acid anhydrides it may not work as well for more hydrophilic compounds. Even without complete derivatization the reaction appears to be reproducible based on the standard error observed between preps which is most important for quantitation. The extraction

derivatization of amines was also carried out in spiked river water (Table 4-7). These results show enhanced enrichment for early eluting amines while the later eluting amines give results nearly identical to the deionized water extractions. The peak area of the hexylamine derivative was increased to the point where it was outside of the calibration. This is most likely due to what is known as "matrix-induced chromatographic response enhancement effect".[46] This occurs when compounds in the sample matrix elute closely to the analytes of interest while also interacting with the active sites in the GC system which excludes the analytes from those interactions leading to signal enhancement. The results indicate that there are early eluting compounds having this effect particularly near the elution of the hexylamine derivative. This effect can be purposefully induced by incorporating "analyte protectants" into the sample for injection giving larger signals. This effect could make quantitation difficult so it needs to be taken into account when developing extraction methods either by doing some kind of sample cleanup to remove the substances causing the effect or preparing standard solution in the matrix which is free of the analytes. It can be difficult to find matrix which is completely free of the analytes of interest though so the most practical way to account for matrix effects is to incorporate internal standards. Using isotopically labeled versions of the analyte of interest or standards that elute near the analyte of interest will account for any matrix effect occurring. This phenomenon is not unique to the Aq/H-O system and could be problematic for other types of microextractions on the same matrix.

Table 4-8 shows some of the GC-MS analytical figures of merit for this method. All derivatives show linear ranges with at least 3 orders of magnitude and good R^2 values. The limits of detection are based on the mass of the derivatized amine molecule and were

determined by the GC-MS software based on S/N=3 for LOD and S/N=10 for LOQ. Since derivatization increases the molecular weight of all amines by 98 Da so the limits shown actually indicate a lower initial concentration of the amine in terms of μg/L. For example the LOD of the Hexylamine derivative is 19.7 μg/L but since derivatization increases the mass from 101 g/mole to 199 g/mole the LOD corresponds to a hexylamine concentration of 10.0 μg/L. The hexylamine derivative shows significantly higher limits than the other compounds that are all fairly similar. This goes along with it also having lower signal intensities at the same concentration compared to the other amines. These results along with the extractions results indicate the method works better with aromatics amines.

Extraction of polyaromatic hydrocarbons

PAH molecules were extracted with Aq/H-O two phase systems with no derivatization being required. Compared to the amines used PAH's are much more hydrophobic being composed primarily of fused aromatic rings. PAH's were initially extracted using the same 1:1 HFIP:THF solution as was used for amines with none of the derivatization reagent being present. Aqueous solutions were spiked with 0.5 ug/mL of PAH's and extracted using 200 uL of 1:1 HFIP:THF in 4 mL aqueous solution (Fig. 4-7). The results of these extractions in both deionized and river water are shown in table 4-9 A. Enrichments ranged from 111-157 for the 6 PAH molecules in deionized water giving recovery's of 5573%. For non-halogenated PAH's the recovery increases with increasing hydrophobicity with perylene having the highest and anthracene the lowest recoveries. The presence of halogens on the PAH molecule likely increase water solubility lowering extraction of the compounds into the HFIP-THF phase.

The GC-MS analytical figures of merit for extraction of PAH into the 1:1 HFIP:THF are shown in Table 4-10. All molecules showed a linear range with three orders of magnitude and good R^2 values. The limits of detection and quantitation were fairly similar for all molecules in the low μg/L range.

PAH extraction in HFIP-Anisole two-phase system

Although anisole is not miscible with water it is still selectively solvated by HFIP in aqueous HFIP solutions forming two-phase solutions with a HFIP-anisole phase at the bottom. The HFIP-anisole system formed a 23 μL H-O phase in 6 mL of water using 100 μL of total solvent which is half of that used for the HFIP-THF system in a 4 mL of water. This is advantageous from the standpoint of using smaller solvent quantity as well as increasing the potential enrichment. However the measured EF values for the HFIP-anisole system were fairly similar to that of the HFIP-THF extraction indicating lower % recovery (Table 4-9 B). Therefore, unless using less solvent is very important; the HFIP-anisole system did not offer any advantage over the HFIP-THF system for PAH extraction. It also needs to be operated at a higher initial oven temperature because of the higher boiling point of anisole.

Conclusion

In conclusion Aq/H-O two-phase can be used for extraction of small molecules from aqueous solutions for subsequent GC-MS analysis. Simultaneous extraction and derivatization of amines can be performed in these solutions using acid anhydride derivatizing agents to convert the amines to amides which improves their extraction recovery as well as GC-MS performance. The derivatization extraction procedure did not work as well with the alkyl amine hexylamine compared to the aryl amines. The presence of two solvents in the H-O phase can cause difficulties when performing splitless GC injection and

must be taken into account when developing the method particularly optimizing the initial oven temperature to allow for solvent focusing. Other solvent microextraction techniques such as DLLE outperform the HFIP-solvent system in terms of EF and recovery based on the literature. The advantage of the HFIP-solvent system is that it uses less solvent compared to many other techniques which often require larger volumes of dispersing solvents and also allowing for the derivatization with a hydrophobic derivatizing agent such as hexanoic anhydride in an aqueous solution. The amount of derivatizing agent used is also very small compared to most other derivatization procedures and although the reaction does not appear to give a quantitative yield it does seem to be reproducible based on the standard errors measured from triplicate preps of the extractions.

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Tables

Table 4-1. Amines used as test compounds and the m/z values of their hexanoic anhydride derivatives and GC retention times

Amine	m/z for amine	m/z for hexanoic anhydride derivative	Retention time (min.)
Hexylamine	101	199	8.10
o-toluidine	107	205	8.93
o-anisidine	123	221	9.37
4-chloroaniline	127	225	9.61
4-bromoaniline	171	269	10.05
3,5-dichloroaniline	161	259	10.28

Table 4-2. SIM program for GC-MS analysis of amines hexanoic anhydride derivatives

Time (minutes)	m/z values monitored
7.80-8.40	101, 199
8.40-9.15	107, 205
9.15-9.45	123, 221
9.45-9.75	127, 225
9.75-10.15	171, 269
10.15-11.38	161, 259

Table 4-3. PAH molecules analyzed including their structure and m/z value

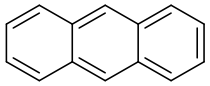
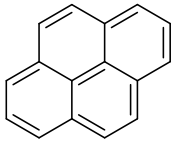
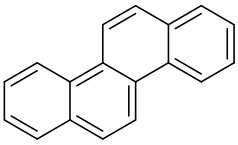
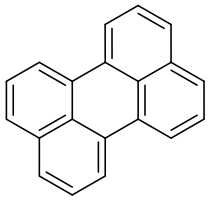
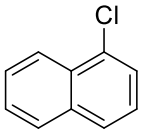
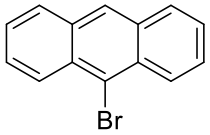
Compound	Structure	m/z used for SIM analysis
Anthracene		178
Pyrene		202
Chrysene		228
Perylene		252
1-chloronaphthalene		162
9-bromoanthracene		256

Table 4-4. GC-MS SIM program used for PAH analysis using HFIP-THF two-phase system for extraction

Time (minutes)	m/z values monitored
3.0-8.5	178
8.5-10.0	202
10.0-11.5	228
11.5-13.0	252

Table 4-5. Effect of derivatization on GC retention time and signal peak area for extractions of 4-bromoaniline in HFIP-THF two-phase system

Derivative	t_R min	Peak area of a 22 µM std
none	6.5	135466
butyric anhydride	7.84	711349
hexanoic anhydride	9.11	820210

Table 4-6. Comparison of different extraction protocols on observed peak area of 4-bromoaniline hexanoic anhydride derivative.

Volume of 1:1 HFIP:THF used for extraction	% v/v Hexanoic anhydride in extraction solvent	Extraction procedure	Peak Area derivative peak
160	0.5	syringe injection 20 minute rotation	983765
200	0.5	syringe injection 20 minute rotation	3281815
240	0.5	syringe injection 20 minute rotation	1810758
200	1	syringe injection 20 minute rotation	3293632
200	0.5	syringe injection 20 minute sonication	2286279
200	0.5	Syringe injection 80 minute rotation	3291624

Table 4-7. Amine derivatization-extraction results with 1:1 HFIP:THF containing 0.05% hexanoic anhydride

Amine	Water	EF (std. error)	% Recovery (std. error)
Hexylamine	DI	41 (2.1)	20.5 (1.0)
	river	N/A	N/A
o-toluidine	DI	118 (2.7)	59.0 (1.3)
	river	153 (7.3)	76.5 (3.7)
o-anisidine	DI	117 (3.4)	58.5 (1.7)
	river	135 (6.2)	67.5 (3.1)
4-chloroaniline	DI	95 (0.8)	47.5 (0.4)
	river	95 (5.5)	47.5 (2.7)
4-bromoaniline	DI	97 (1.9)	48.5 (1.0)
	river	96 (5.1)	48 (2.5)
3,5-dichloroaniline	DI	81 (1.8)	40.5 (0.9)
	river	81 (4.4)	40.5 (2.2)

Table 4-8. Analytical figures of merit for hexanoic anhydride amine derivatives in the derivatization-extraction scheme in Aq/HFIP:THF and GC-MS analysis.

Amine	Linear Range (µg/L)	R^2	LOD(µg/L)	LOQ(µg/L)
Hexylamine	79-78800	0.9983	19.7	78.8
o-toludine	7.7-7660	0.9999	3.8	5.3
o-anisidine	7.2-7188	1.0	4.5	7.2
4-chloroaniline	7.1-7088	0.9998	4.4	7.1
4-bromoaniline	6.3-6292	1.0	3.1	4.7
3,5-dichloroaniline	6.4-6432	1.0	2.4	4.0

Table 4-9. PAH extraction results using A)1:1 HFIP:THF and B) 3:2 HFIP:Anisole

A.

Compound	water	EF	% Recovery
Anthracene	DI	129	65
	River	128	64
Pyrene	DI	149	75
	River	134	67
Chrysene	DI	141	70
	River	145	73
Perylene	DI	147	73
	River	157	79
1-chloronaphthalene	DI	112	56
	River	115	58
9-bromoanthracene	DI	115	57
	River	111	55

B.

Compound	water	EF (std. error)	% Recovery (std. error)
Anthracene	DI	138(6.0)	53(2.3)
	river	143(5.5)	55.1(2.1)
Pyrene	DI	137(6.1)	52.8(2.4)
	river	143(5.9)	55(2.3)
Chrysene	DI	136(5.6)	52.4(2.1)
	river	139(7.0)	53.4(2.7)
Perylene	DI	134(6.3)	51.6(2.4)
	river	137(6.3)	52.8(2.4)

Table 4-10. Analytical figures of merit for PAH extraction in Aq/HFIP:THF followed by analysis by GC-MS analysis.

PAH	Linear Range (µg/L)	R^2	LOD(µg/L)	LOQ(µg/L)
Anthracene	6.3-1250	0.996	3.1	6.3
Pyrene	3.1-1250	0.9998	1.6	3.1
Chrysene	2.5-1250	1.0	1.2	2.5
Perylene	7.4-1250	1.0	3.5	7.4

Figures

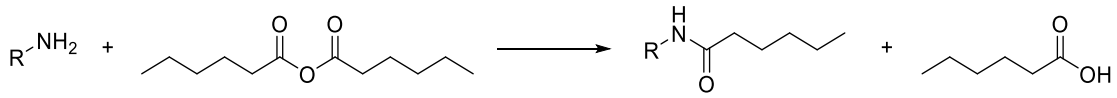


Figure 4-1. Derivatization reaction of amines using hexanoic anhydride to form amides

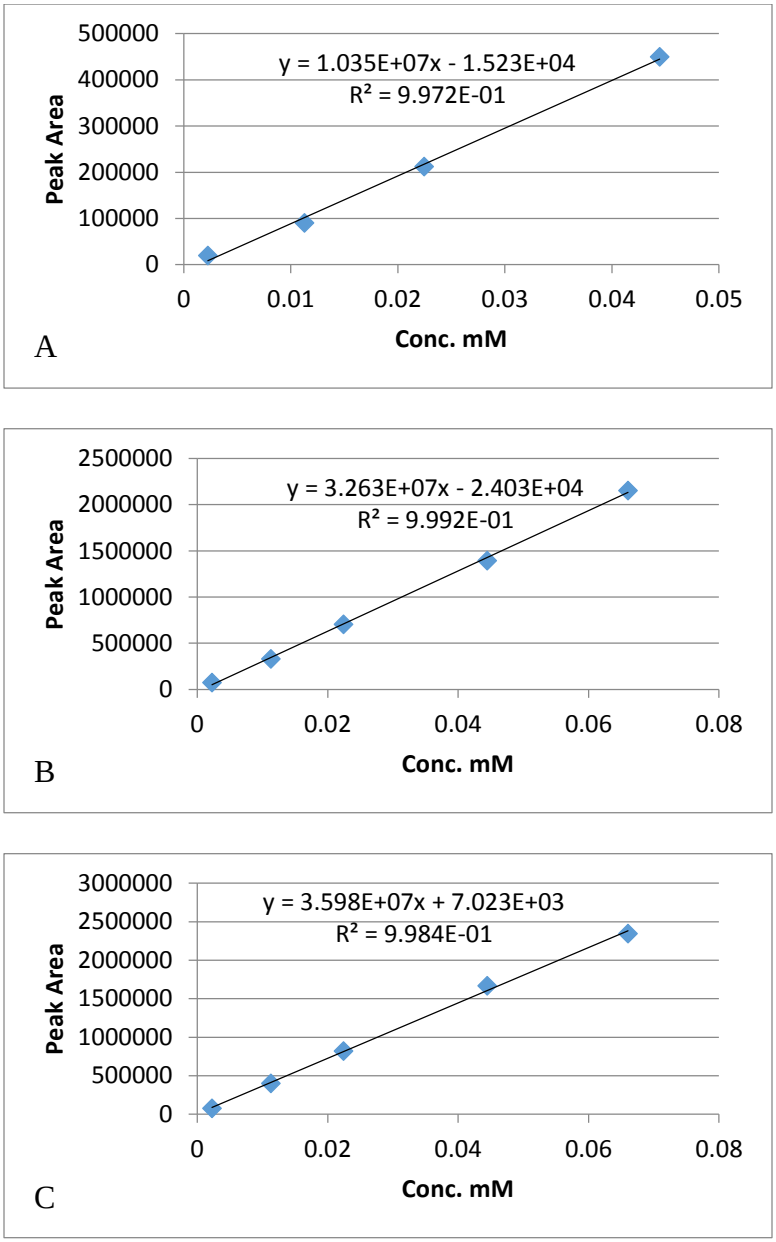


Figure 4-2. GC-MS calibration curves for (a) 4-bromoaniline, (b) 4-bromoaniline butyric chloride derivative and (c) 4-bromoaniline hexanoyl chloride derivative

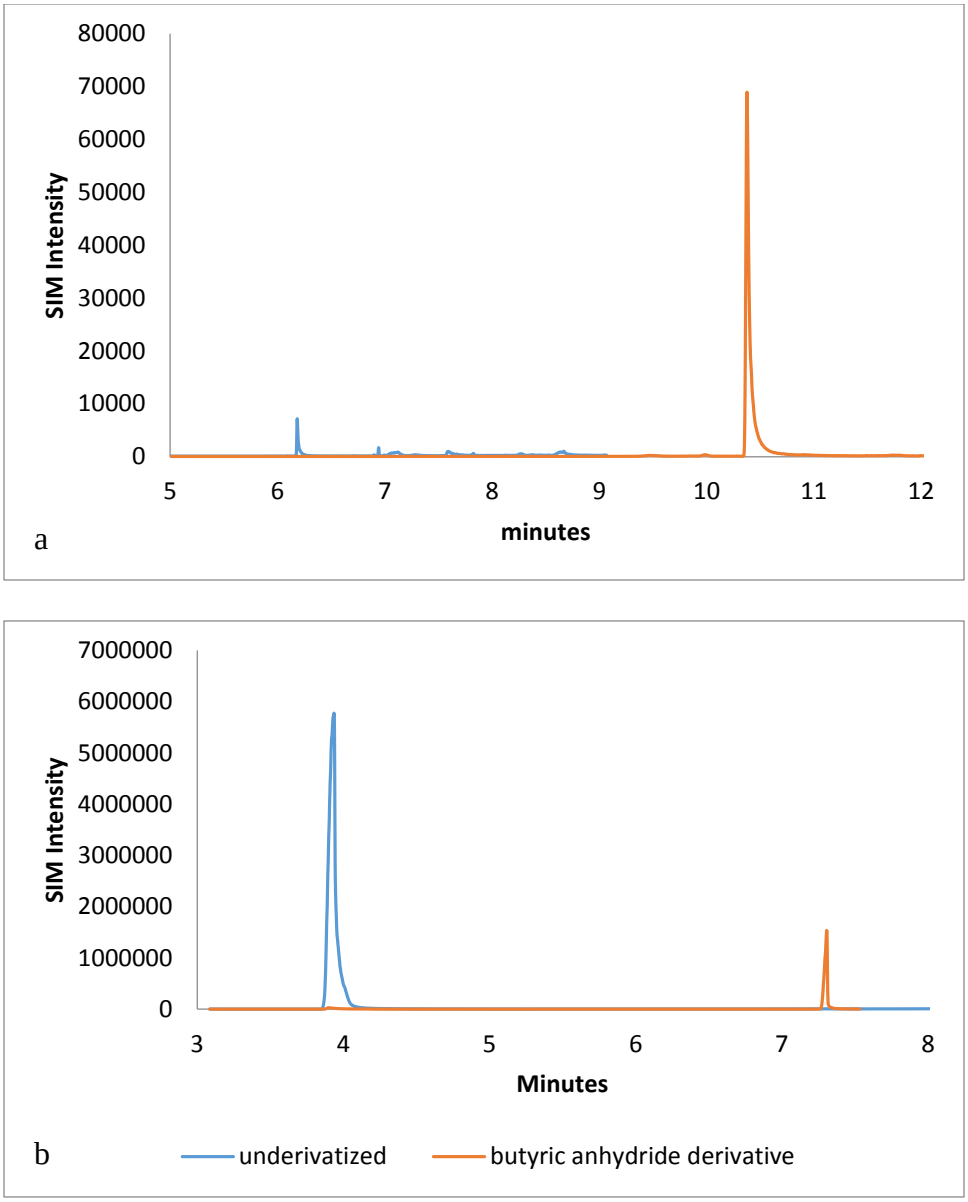


Figure 4-3. Derivatization of a) 4-bromoaniline and b) o-anisidine with butyric anhydride(orange) leading to the disappearance of the underivatized peak(blue).

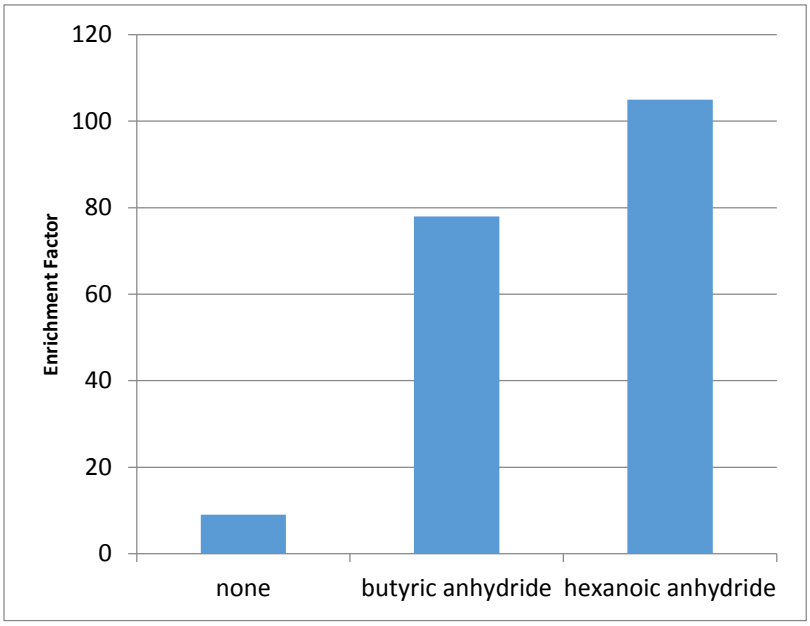


Figure 4-4. Enrichment factor of 4-bromoaniline extraction using A) no derivative B) butyric anhydride C) hexanoic anhydride using 200 µL of 1:1 HFIP:THF for extraction on a 4 mL aqueous solution

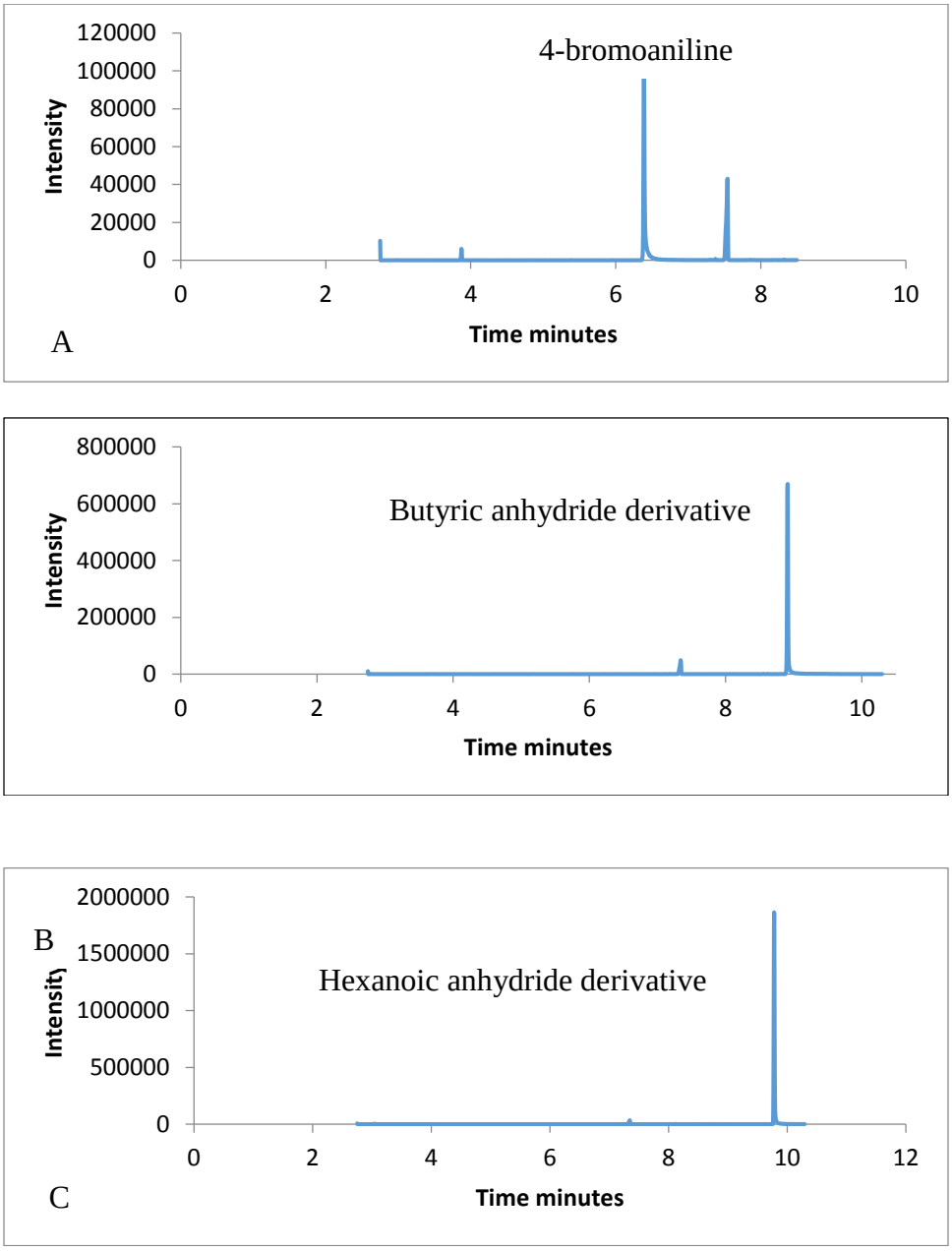


Figure 4-5. GC-MS SIM chromatogram of 22 μM 4-bromoaniline standards A) underivatized B) butyric anhydride derivative C) hexanoic anhydride derivative. Peak before 8 minutes is from stabilizer present in THF.

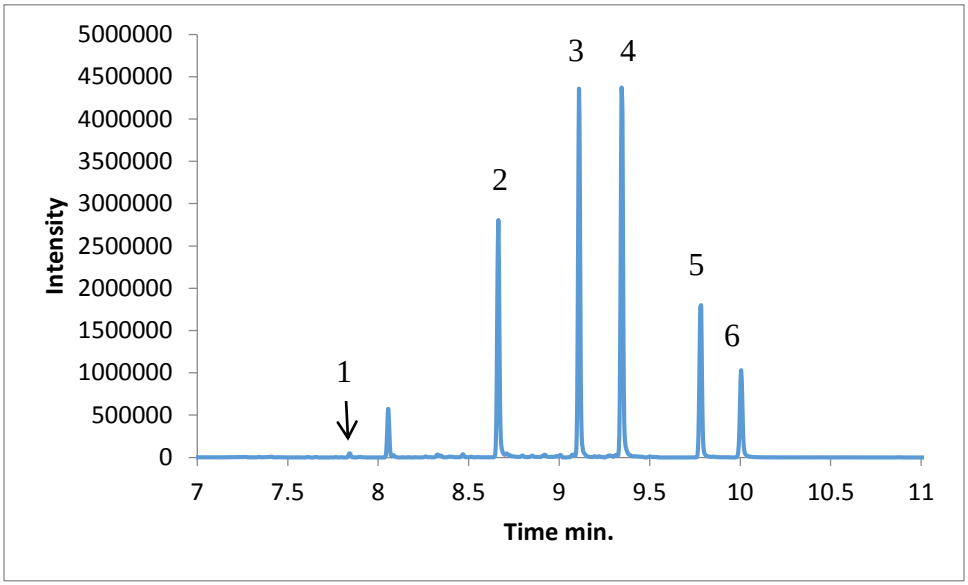


Figure 4-6. GC Chromatogram from amine derivatization extraction using 200 µL of 1:1 HFIP THF in 4mL of DI water spiked with 20 µg/L of each amines. The amide derivatives formed with hexanoic anhydride were observed for all amines; 1) hexylamine 2) o-toluidine 3) o-anisidine 4) 4-chloroaniline 5) 4-bromoaniline 6) 3,5-dichloroaniline

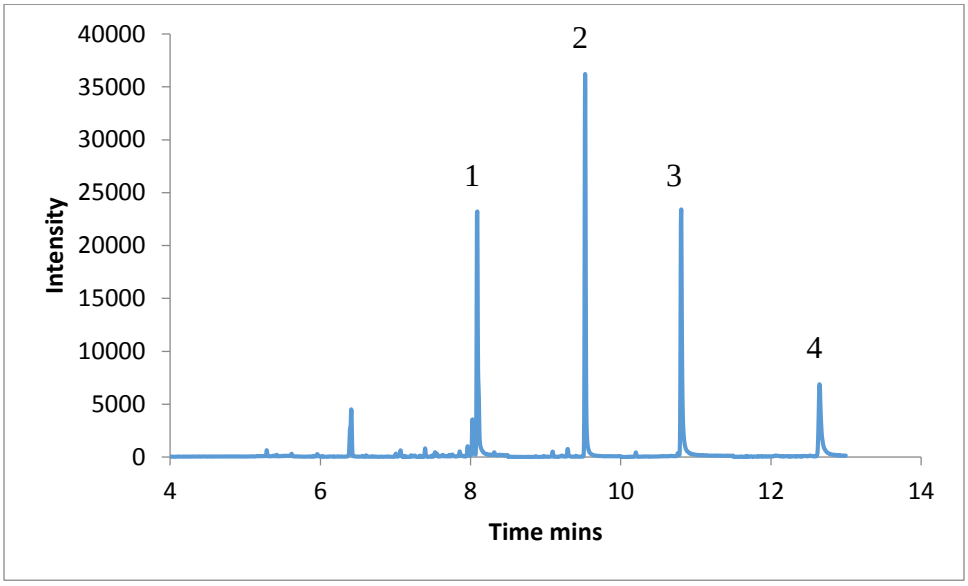


Figure 4-7. GC Chromatogram from PAH extraction using 200 µL of 1:1 HFIP:THF in a 4 mL solution. 1) Anthracene 2) Pyrene 3) Chrysene 4) Perylene

Chapter 5

Biological extractions and proteomic analysis of membrane proteins with HFIP-solvent two-phase systems

Abstract

Two-phase solutions formed by the addition of a third miscible solvent, primarily acetone and N,N-dimethylformamide(DMF), to aqueous HFIP solutions were used for proteomic analysis of integral membrane proteins (IMPs). One phase is largely aqueous and the other is composed of HFIP and the third solvent. The HFIP-solvent phase was shown to selectively extract hydrophobic protein and peptide standards while more hydrophilic proteins remained in the aqueous phase. When used to analysis mouse red blood cell membranes a higher % of proteins identified in the HFIP-solvent phase were IMPs compared to a control and the aqueous phase. The use of two-phase extraction prior to digestion alos led to an increase in the total number of proteins and IMPs identified compared to a control without initial two-phase extraction. The IMPs identified in the HFIP-solvent phase were more hydrophobic than the ones identified in the control and aqueous phase based on GRAVY values. With many of the more hydrophilic proteins remaining in the aqueous phase hydrophobic proteins that do not produce many tryptic peptides were able to be identified in the HFIP-solvent phase since these peptides are more likely to be detected without all of the peptides from more hydrophilic proteins being present. Combining high pH washing of the membranes with HFIP-solvent extraction led to a further increase in the number of IMPs identified.

Introduction

The term proteome was coined in 1994 by Marc Wilkins[1] and is defined as "the protein complement of the genome"; the study of which is termed proteomics. The goal of proteomics is to gain an understanding of the characteristics of proteins that are present in an organism such as their structure abundance, functions, etc. as well as changes in the proteins characteristics with time and physiological state. The concept of proteomics dates back to the 1970's with the "Human Protein Index" [2] which had a goal to catalogue all human proteins but was limited by the technology of the time. The ability to analyze the proteome in addition to the genome of an organism is important and poses specific challenges as the genome of an organism remains fairly constant but the nature of expressed proteins and subsequently the phenotype of the organism change with time due to chemical modification and stress. Study of the proteome makes it possible to determine how processes that affect the cells of an organism such as aging or disease are represented by changes in protein expression, thus allowing for the discovery of possible drug targets or disease biomarkers. In addition, studying the proteome allows for determination of the types of post-translational modifications of proteins which cannot be determined from the genome. This will ultimately lead to a better understanding of the genome allowing for functional annotation. The challenges of proteomic analysis originates from the large diversity of proteins in terms of abundance and physical and chemical properties that in turn leads to the needs for development novel methodologies for sample preparation, separation, structural characterization, and bioinformatics.

The field of proteomics really took off in the 1990's due to the valuable information gained from the human genome project, as well as many advances in the instrumentation

used for analysis and identification of proteins in complex samples. Early methods of protein structural analysis relied on Edman Sequencing [3] which degrades peptides at their N-terminus without disturbing the other peptide bonds allowing for determination of the amino acid sequence. The development of soft ionization techniques; matrix assisted laser desorption ionization (MALDI)[4] by Koichi Tanaka as well as electrospray ionization (ESI)[5] by John Bennett Fenn in the 1980's, allowed for ionizations of biomolecules enabling the use of mass spectrometry (MS) for protein and peptide analysis. For their ground-breaking contributions, Tanaka and Fenn shared the 2002 Nobel Prize in Chemistry. Mass spectrometry would go on to replace Edman sequencing due to its much better detection limits as well the ability to handle mixtures. The initial uses of MS for protein identification involved peptide mass fingerprinting (PMF). In this technique proteins could be identified by measuring the exact mass of the peptides produced following tryptic digestion of the protein and subsequent comparison with the theoretical masses of the peptides for proteins predicted from the genome.[6] Tryptic digestion is highly specific with trypsin only cleaving on the carboxyl side of arginine and lysine residues with few exceptions. This allows for the potential tryptic peptides of proteins to be predicted from the genome. PMF is a simple and fairly fast method requiring a database of protein sequences but suffers from shortcomings in analyzing complex mixtures of proteins and their tryptic peptides. For this reason protein mixtures from a biological system often have to be separated prior to analysis using methods such as two-dimensional gel electrophoresis (2-DE) so that spots of individual proteins can be cut from the gel and digested and analyzed individually by PMF. In order to analyze more complex mixtures of peptides and proteins; an initial separation of peptides or proteins is carried out using HPLC, which can be coupled to a mass spectrometer via an ESI source.

The most common HPLC method used is reversed phase (RP-HPLC) which separates the molecules based on their hydrophobicity. The molecules have an initial affinity for the alkyl-bonded stationary phase and are eluted as the organic component of the mobile phase is increased. When coupled to hybrid mass spectrometers, tandem MS can be used to determine peptide or protein structures. Hybrid MS instruments typically consist of two types of mass spectrometers coupled either in time or space. Tandem MS instruments are considered separated in space when the two MS components are physically separated but connected in a way that a high vacuum is maintained. Examples of tandem in space instruments would be triple quadrupole (QqQ) and quadrupole time of flight (QTOF) mass spectrometers. Tandem in time instruments incorporate ion traps which contain all of the ions in the same place. The most common type of ion trap would be quadrupole ions traps which can be either 2D or 3D. The 2D or 3D designation refers to how many dimensions the ions are oscillated while in the trap, 2D traps also known as linear traps offer larger space for trapping ions compared to 3D traps and thus have higher signal to noise and a larger dynamic range.[7] Ions within a trap can be subjected to multiple MS measurements known as MS^n and often incorporate fragmentation to gain structural information of the ions after measuring their initial m/z. An example of a tandem in time MS instrument would be the LTQ-Orbitrap where the Orbitrap is used to give high resolution measurements of initial intact peptide m/z followed by selection and fragmentation of that m/z in the linear trap and measurement of fragment ions m/z to generate MS/MS spectra. Fragmentation for MS^n analysis is often performed using low energy collisional induced/activated dissociation (CID/CAD) in which ions are accelerated into collisions with neutral gas molecules increasing their kinetic energy

which is ultimately converted to internal vibrational energy resulting in bond cleavage.[8] The mass of the resulting fragments is then measured to gain structural information.

When intact proteins are analyzed via MS it is referred to as "Top down" proteomics. In "top-down" proteomics the mass spectrometer initially determines the m/z of the intact protein which is then subjected to fragmentation the products of this fragmentation can then be subjected to further MS analysis to give structural information on the protein. Top down proteomics is good for analyzing individual intact proteins and gaining structural information from its degradation products such as any post translational modifications. Similar to PMF however top-down proteomics suffers from an inability to analyze complex mixtures of proteins for this reason a "bottom-up" approach is often used for complex protein mixtures such as cell lysate extracts. In a bottom-up approach the proteins are digested into smaller peptides prior to MS analysis typically with trypsin or other proteases. Proteins can be initially separated using 2-dimensional gel electrophoresis (2-DE) which separates proteins by their isoelectric point (pI) in one dimension and molecular weight (MW) in another. Following 2-DE the protein spots can be cut from the gel and digested prior to analysis. Another technique referred to as "shotgun proteomics" does not use 2-DE but rather the proteins are extracted and solubilized using various solubilization techniques and digested in solution.[9] The protein digest is then separated using chromatography and introduced into a tandem mass spectrometer using ESI. As peptides elute from the column and are introduced to the mass spectrometer through ESI, the mass spectrometer measures the initial peptide masses and then selects the most abundant to be fragmented and then measures the masses of the fragments generated. The MS/MS spectrum can then be searched against databases to match the spectrum to those of known sequencing and identify the peptide sequence which

can then be matched to proteins. Several different algorithms are available for searching MS/MS data against databases such as Mascot and Sequest.[10] These types of studies must be carried out on organisms that have had their genomes sequenced so that the possible peptides are known for the database search.

Prior to LC-MS/MS analysis, proteins must first be extracted from the medium of interest and then digested to generate peptides. The initial step in the process is to homogenize the sample of interest to disrupt the cells and allow for the proteins to be extracted. Cell disruption can be done through mechanical, physical or chemical methods.[11] Following cell disruption, the proteins must be extracted and solubilized prior to being digested for in-solution digestion or separated using 2-DE. The protein extraction and solubilization step is where the work presented here is focused. Traditional methods of protein extraction and solubilization involve the use of chaotropes or detergents. Chaotropes disrupt hydrogen bonds and hydrophilic interactions allowing proteins to unfold and expose ionizable groups to the solution. The most common chaotrope used for protein solubilization is urea which is used at a high concentration of 5-9M to disrupt protein secondary structure.[12] Other common chaotropic agents used are thiourea and guanidine hydrochloride. Because urea disrupts protein secondary structure solutions must be diluted prior to digestion with enzymes such as trypsin to around 2M or less to prevent deactivation of the enzyme. Detergents act by disrupting hydrophobic interactions which enables protein solubilization. Detergents are often classified according to the ionic character of their head group and can be anionic, cationic, neutral or zwitterionic. Ionic surfactants such as sodium dodecyl sulfate (SDS) are often used to their strong ability to lyse membranes and solubilize hydrophobic proteins. Ionic surfactants can be problematic as they can interfere with downstream parts of

the analysis such as digestion, chromatography and ionization.[13] As a result, steps are taken to remove surfactants, particularly SDS, prior to digestion and LC-MS analysis. The FASP (filter aided sample preparation) method can be used to remove surfactant prior to digestion and analysis.[14] With the FASP method, the surfactant is removed using a spin-filter that doesn't allow the proteins to pass through. The proteins are then digested on the filter after which the smaller peptides can be spun through the filter. Although the FASP method is claimed to be a "universal sample preparation technique for proteome analysis" some issues still exist with its use particularly for very hydrophobic proteins.[15] Another approach for overcoming the problems presented by surfactants is to use acid labile surfactants which solubilize proteins without inhibiting enzymes and do not interfere with LC-MS analysis due to breaking down at low pH.[16] Only a few acid labile surfactants are commercially available and they tend to be expensive so they have not been as widely applied for proteomics studies as other solubilization agents. Sodium deoxy cholate (SDC) a bile salt can also be used as a mass spec friendly surfactant and does not interfere with trypsin activity at concentrations around 1%.[13] SDC is not quite as good at solubilizing hydrophobic proteins as SDS unless it is used at a 5% concentration for solubilization which must be diluted prior to digestion with trypsin. The best choice of solubilization will be dependent on what types of proteins are being targeted and the number and type of proteins identified will vary with the use of different extraction and solubilization techniques.

Proteomic analysis of membrane proteins is important because membrane proteins play many important roles in cells such as ion transport, intercellular communication, influencing cell structure and more essential roles of the cell membrane. This is why membrane proteins make up approximately 60% of drug targets while comprising only one-third of the

proteome.[17] Membrane proteins can be defined as peripheral or integral. Peripheral membrane proteins exist on the surface of the membrane and can be bound to the membrane through interactions with other membrane proteins or though interactions with the polar head groups of the membrane. Peripheral membrane proteins also include proteins that are anchored to the membrane either by hydrocarbon or lipid anchors. Integral membrane proteins (IMP's) interact with the inside of the cell membrane. A protein that fully traverses the membrane is known as a transmembrane protein and these can either be single pass or multipass. Due to the hydrophobic nature of the membrane interior the transmembrane regions of proteins are composed of 20-25 hydrophobic amino acids that form α-helices and span the membrane.[18] Increasing the number of transmembrane regions relative to the size of the protein will increase the proteins hydrophobicity but the proteins still have regions outside of the membrane that are more water soluble making them amphipathic. Transmembrane proteins can also traverse the membrane as β barrels which are less hydrophobic than α-helices since they consist of alternating polar and nonpolar amino acids. The amphipathic character of integral membrane proteins as well as their lower abundance makes them difficult to study using traditional proteomic methods. One of the main difficulties associated with proteomics of membrane proteins is solubilization. Membrane proteins are much more difficult to extract and solubilize than cytosolic proteins due to their increased hydrophobicity and amphipathic nature especially in ways that are compatible with the subsequent digestion and analysis methods. Membrane proteins are underrepresented in 2-DE because they are not soluble in the running buffers and even when solubilized, they often precipitate at their pI. Some work has been done to improve 2-DE of membrane proteins using co-solvents such as trifluoroethanol (TFE) to help keep the proteins

soluble.[19,20] With a "shotgun proteomics" approach using in-solution digestion, solubilization of membrane proteins as well as their low abundance makes them difficult to study. Even if the membrane proteins can be solubilized the large abundance of more soluble proteins will make them harder to detect during the LC-MS analysis as many more peptides will be generated by soluble proteins that could hinder the detection of peptides from membrane proteins. The transmembrane regions of these proteins will also not be detected due to the lack of trypsin cleavage sites and their high hydrophobicity causing them to be insoluble in the LC diluent.[17] The use of organic solvents and detergents during in solution digestion has been shown to improve detection of membrane proteins. Organic solvents such as chloroform/methanol mixtures [21], TFE and methanol [22,23] have all been used for the extraction of proteins. Digestion of the extracted proteins has been carried out in solution after extraction with 50% TFE and 60% methanol. Digestion in the TFE extraction takes place after 10x dilution to allow for trypsin activity. While in 60% methanol no dilution is required and trypsin remains active. Trypsin has been shown to have activity in various high % solvent solutions that could be useful for keeping membrane proteins soluble during digestion.[24]

Although trypsin is the most common protease used for digestion other digestion approaches can be advantageous when analyzing membrane proteins. Tryptic digestion of membrane proteins can be hindered due to loss of enzyme activity in solutions that are able to solubilize the proteins as well as it being difficult for trypsin to access certain cleavage sites of membrane proteins. Transmembrane regions rarely contain lysine or arginine residues since they are more polar amino acids which can result in large tryptic peptides that are difficult to analyze under typical LC-MS/MS conditions. The use of different digestion

techniques can improve detection of integral membrane proteins. One popular approach is combining trypsin digestion with Lys-C digestion. Lys-C is an enzyme that is specific to the C-terminal of lysine residues. Unlike trypsin, Lys-C is still active in solutions of 8 M urea, which would allow predigestion of hydrophobic proteins into smaller peptides that can then be subjected to trypsin digestion leading to increased identification of IMP's.[25,26] Glu-C is another enzyme that can be incorporated in a similar manner to Lys-C providing relatively similar performance.[27] Chymotrypsin is useful for membrane proteomics because it cleaves after many residues which tend to feature prominently in transmembrane regions such as phenylalanine, tyrosine and tryptophan. Although there are not many studies using chymotrypsin its utility in membrane protein identification has been demonstrated for digestion in organic solvents [28] as well as in combination with trypsin.[29] Similar to chymotrypsin the gastric enzyme pepsin also cleaves after several of the more hydrophobic amino acids. Pepsin is active at low pH's, 2-4, and its use in combination with subsequent trypsin digestion was shown to increase sequence coverage of identified IMP's in microsomal membrane samples compared to trypsin alone.[30,31] Proteinase-K is a nonspecific enzyme that has been used for what is termed "high pH proteinase-K" (hppk) in which high pH conditions are used to remove membrane associated proteins and open the membrane vesicle allowing proteinase-K to digest or "shave" the exposed portions of the IMP's which can then be used for protein identification.[32] The use of a nonspecific enzyme such as proteinase-K can complicate protein ID's because it drastically increases the database search space and can lead to a higher rate of false positives.[33] Many other enzymes are available for protein digestion although they are not extensively studied.[34,35] Chemical cleavage using cyanogen bromide (CNBr) can also be used for protein digestion. CNBr reacts with

methionine residues in acidic solutions fragmenting the peptide. CNBr cleavage is very robust and because methionine occurs at frequent intervals in transmembrane regions [36] it can improve IMP identification especially in combination with proteases.[29,37,38]

Because of the amphipathic nature of membrane proteins it can be difficult to try and target both the hydrophilic and hydrophobic regions of the proteins with a single method. Several protocols that focus on the exposed hydrophilic regions have been developed based on labeling and subsequent affinity purification known as cell surface capture (CSC). Exposed primary amines such as those from lysine as well as sugar moieties from glycoproteins can be labeled using biotin based labels and following digestion can be isolated and analyzed by LC-MS/MS for protein identification.[39,40] CSC methods have been shown to lead to high enrichment and identification of IMP's based on their exposed regions but because it requires live cells and is pretty laborious it has not been widely adopted.[41,42] Other methods for the identification of labeled glycoproteins such as Solid Phase Extraction of formerly N-glycosylated glycoproteins (SPEG) [43,44] and Glyco-FASP[45,46] have also been developed for the enrichment of exposed portions of IMP's based labeling of sugar moieties. In SPEG the diol groups of glycoproteins are converted to aldehydes which then can react with immobilized hydrazide groups on a solid support allowing non glycosylated proteins to wash through giving enrichment of glycol-proteins.[43] Methods for targeting the hydrophobic portions of IMP's have also been developed. As previously mentioned the CNBr based hppk method can be used to initially shave the exposed regions of IMP's from the cell membrane. This leaves the integral membrane regions embedded in the membrane which can then be subjected to solubilization in concentrated formic acid and CNBr digestion to digest the integral membrane regions for LC-MS/MS analysis.[32]

The complexity of IMP's can cause difficulties in their analysis and identification. Optimization of each step of the process such as extraction and solubilization, digestion and analysis can lead to increases in the number of IMP's identified. The work presented here focuses on the extraction and solubilization step. HFIP-solvent two-phase systems were explored for extraction of membrane proteins and particularly IMP's. The H-O phase being more hydrophobic is not likely to extract more hydrophilic proteins that will remain in the aqueous phase while more hydrophobic IMP's may partition into the H-O phase. This could increase IMP identification as serving as a prefractionation of the IMP's from hydrophilic proteins enriching them and allowing them to be identified more easily during LC-MS/MS analysis as their peptides will be detected more easily without as many interfering peptides form hydrophilic proteins.

Experimental

Materials

1,1,1,3,3,3-Hexafluoroisopropanol (HFIP) was purchased form Oakwood Chemical at 99% purity. N,N-dimethylformamide and Acetone were ACS grade from Fisher scientific while THF was purchased from Sigma Aldrich at ACS grade. 2-butanone was purchased from Alfa Aesar at 99% purity. Ammonium bicarbonate (ABC) was reagent plus (≥ 99%) from Sigma Aldrich. Formic acid was purchased from Acros Organics at 98+ % purity. Tris-HCl was molecular grade from Promega. Protein concentrations were measured using the Thermo Scientific™ Pierce™ Coomassie (Bradford) Protein Assay (product # 23200). Iodoacetamide (IAA) was purchased form Sigma Aldrich at Bioultra grade, ≥99% purity.

Dithiothreitol (DTT) was ≥99% from Fisher scientific. HPLC solvents water and acetonitrile were purchased from Fisher scientific at gradient grade.

Gramicidin A from Bacillus brevis was purchased from Sigma Aldrich at 90% purity. Cytochrome C from bovine heart was from Sigma Aldrich at ≥95% purity. Bovine serum albumin (BSA) was purchased at ≥96% from Sigma Aldrich. Trypsin from Bovine pancreas used for protein partitioning was ≥90% from Sigma Aldrich. Trypsin used for protein digestion was sequencing grade modified trypsin frozen in acetate buffer from Promega. CD-1 mouse red blood cells were purchased from Innovative Research (www.innov-research.com).

Methods

Partitioning of protein and peptide standards

The distribution of the proteins and peptide were observed by analyzing both phase with HPLC after extraction. Stock solutions of the more hydrophilic proteins, cytochrome C and BSA, were prepared in deionized water while stock solutions for the hydrophobic peptide, gramicidin A and membrane protein, bacteriorhodopsin, were prepared in HFIP. Calibration plots for each peptide or protein were then determined using peak areas from HPLC analysis with UV detection. Initial experiments determined distribution of proteins standards in the HFIP-DMF two phase systems. Distribution of protein or peptide samples between water and organic phase was achieved by mixing the appropriate amounts of water, HFIP, DMF and protein or peptide stock solution resulting in the formation of a two phase solution. After mixing of all reagents, the solution was vortexed for 30 seconds and rotated for 30 minutes. Following rotation the solution was centrifuged for 2.5 minutes at 1100 rpm. After

centrifugation 1 mL of the aqueous phase was removed to be analyzed by HPLC with no dilution. Due to the small volume of the HFIP-DMF phase they were diluted with isopropyl alcohol for HPLC analysis. The specific dilutions used for each extraction are noted in the text. Enrichment factor was determined by the following equation: $EF = \frac{C_{HS}}{C_i}$, where C_{HS} is the concentration in the extraction phase and C_i is the initial concentration.

HPLC analysis of test protein and peptide standard partitioning

HPLC analysis was done using an Agilent 1100 equipped with a UV-Vis detector. The detection of all peptide and proteins were done at 214 nm. Mobile phase A was deionized water with 0.1% (w/v) trifluoroacetic acid (TFA) and mobile phase B was acetonitrile with 0.1 % TFA (w/v) except for bacterierhopdsin where 50/50 acetonitrile and isopropyl alcohol with 0.1% TFA was used as mobile phase B. For the analysis of the three proteins extracted by the HFIP-DMF system, the column used was a Zorbax SB-C3 4.6 mm x 50 mm, and 3.5 μm particle size. For the analysis of the proteins extracted by the coacervate systems as well as gramicidin A in both systems, the column used was a Zorbax SB-C18 4.6 mm x 150 mm and 3.5 μm particle size equipped with a C18 guard column. The analysis of the BSA digest was done on the C18 column using water and acetonitrile both containing 0.1% TFA.

For BSA and cytochrome C analysis a gradient of 15-65% B over 5 minutes followed by a 3 minute equilibration at 15% B was used. Bacteriorhodopsin analysis was done with a gradient of 50 to 100% B over 10 minutes followed by a 5 minute hold at 100% and a 5 minute equilibration at 50% B. Gramicidin A analysis was done with a gradient of 75 to 100% B over 5 minutes followed by 3 minute equilibration at 75% B. All flow rates were 1.0 mL/min. For analysis of the BSA digest a gradient from 3-34 %B over 40 minutes followed by and increase

to 60% B over 10 minutes and another increase to 95 % B in two minutes with a subsequent 8 minute equilibration was used for a total analysis time of 60 minutes.

Preparation of Mouse red blood cell (RBC) membranes

RBC membranes were prepared through hypotonic lysis based on literature methods.[22,47] Approximately 3 mL of mouse RBC's was diluted 1:1 with isotonic Tris buffer (310 imOsM, pH 7.6, 4°C) to an approximate hematocrit of 50% in 50 mL centrifuge tubes. The RBC's were then diluted 7x with hypotonic Tris buffer (20 imOsM, pH 7.6, 4°C) and allowed to rest at 4°C for 5 minutes for hypotonic lysis. RBC membranes were then pelleted by centrifugation at 20,000 xg at 4°C for 40 minutes. Membranes were washed 4 times with the 20 imOsM Tris buffer with centrifugation at 20,000 xg for 40 minutes until the membranes were nearly colorless. Membranes were then suspended in 20 imOsM Tris buffer to be used for proteomics experiments or further washing using Na_2CO_3 buffer. High pH carbonate washing is an extra washing step often used on membranes to strip proteins associated with the outside of the membrane while leaving integral membrane proteins behind.[22,48] Carbonate washing was done by diluting RBC membranes 20x with 100mM Na_2CO_3 pH 11.0 4°C and allowed to rest at 4°C for 30 minutes. Membranes were then pelleted by ultracentrifugation at 100,000 xg for 45 minutes. Membranes were then washed with deionized water and pelleted again at 100,000 xg for 45 minutes. Membranes were then resuspended in deionized water and aliquoted for storage at -50°C.

Extraction of proteins from RBC's using HFIP-solvent two phase systems

For HFIP-solvent extraction of RBC membranes an amount of RBC suspension containing approximately 500 μg protein was diluted to 1 mL with 50 mM ABC buffer pH 8.5. The two phase system was then formed by adding the 150μL of HFIP and the other solvent combined at a 3:2 HFIP:solvent ratio to the suspended RBC solution. The solution was vortexed for a minute and placed in a bath sonicator for 45 minutes while being removed and briefly vortexed every 5 minutes. The solution was then rotated for 20 minutes followed by centrifugation for two minutes to separate the phases. The HFIP-Solvent (H-O) phase could then be removed from the bottom of the vial using a syringe and the aqueous phase could be collected as well. Both phases were then placed in a vacuum concentrator to remove the solvents leaving behind any proteins that were extracted. The entire proteomics workflow from extraction to database searching is depicted in Figure 5-1.

Digestion of proteins from RBC extraction

Once the protein extraction phases (H-O or aqueous) were dried by vacuum concentration the proteins were resuspended in 50 mM ABC buffer pH 8.5. 50 μL of ABC was used for H-O phases and 150 μL was used for aqueous phases because it was known that a majority of the initial protein remained in the aqueous phase. Once the proteins were resuspended, dry urea was added to a concentration of 8 M in solution. The solutions were vortexed to solubilize the urea as well as the proteins. The proteins were reduced with 10 mM DTT for 30 minutes at 37 °C and then allowed to cool at room temperature for 10 minutes. Following reduction, 20 mM IAA was used for alkylation and the solutions were placed in the dark for 30 minutes. The alkylation reaction was then quenched with 10 mM DTT for 15

minutes at room temperature. The protein mixture was diluted with the ABC buffer to get the urea concentration to 1.5 M for trypsin digestion. Sequencing grade modified trypsin was then added (8 μg for H-O phase and 10 μg for aqueous phase) and the solution was placed in a 37 °C incubator overnight. The following day 0.5 to 1.0% v/v TFA was added to stop the digestion and acidify the solution. The solution was then desalted using a Sep Pak and concentrated to near dryness in a vacuum concentrator. The peptides were resuspended in 0.2% v/v formic acid to a peptide concentration around 0.5-0.8 mg/mL determined using a nanodrop A205 method. The urea control digestion was performed in the same manner without doing the initial two-phase extraction.

LC-MS analysis of RBC digests

Peptides were separated by reversed-phase chromatography, using a nanoviper analytical C18 column (Acclaim Pep Map 100 C18 LC Columns, Thermo Scientific). Separation was performed with a binary gradient system, in which the organic and aqueous mobile phases contained 95/5 acetonitrile/water and 98/2 water/acetonitrile, respectively. The nanocolumn flow rate and injection volume was set at 300 nL/min and 5 μL (partial injection mode), respectively. For ionization, the nanoelectrospray ionization (ESI) source was utilized with a fixed spray voltage and heated capillary temperature of 2.0 V and 275 °C, respectively. Full scan spectra (AGC 3×10^4) were obtained from 350 to 2000 m/z. Data dependent MS/MS spectra (AGC 1×10^4) were collected from the five most abundant precursor ions. The dynamic exclusion time was fixed at 30 ms for separating consecutive ions. Data acquisition was set for 90 min. Xcalibur software was utilized for data processing. In CID fragmentation mode, the activation energy was set to 45%, along with an isolation width of 1.5 Da, activation Q of 0.25, and activation time of 10 ms.

Data analysis of LC-MS results and protein identification

Proteins were identified through Proteome Discoverer software (ver. 2.0, Thermo Fisher Scientific) and a mouse (Mus musculus) Swiss-Prot protein database (16831 sequences). The considerations in SEQUEST searches for normal peptides were used carbamidomethylation of cysteine as static modification and oxidation of methionine as a dynamic modification. Trypsin was indicated as the proteolytic enzyme with two missed cleavages. Precursor and fragment mass tolerance were set at ± 1.6 and 0.6 Da, and peptide charges were set excluding +1. SEQUEST results were filtered with the target PSM validator to improve the sensitivity and accuracy of the peptide identification. Using a decoy search strategy, target false discovery rates for peptide identification of all searches were <1% with at least two peptides per proteins, and the results were strictly filtered by ΔCn (<0.01), Xcorr (≥ 1.5) for peptides, and peptide spectral matches (PSMs) with high confidence with q value (<0.05).

Analysis of ID'd proteins was done primarily using UniProt Retrieve/ID mapping (http://www.uniprot.org/uploadlists/) to gain information on the number of protein integral membrane regions. The GRAVY value of the identified IMPs was calculated using the Gravy calculator from http://www.gravy-calculator.de/index.php by uploading the FASTA file of identified IMPs.

Results and discussion

Partitioning of hydrophilic protein standards

Cytochrome c and trypsin were used to investigate the partitioning of water soluble proteins in the HFIP-DMF two-phase system. The distribution of the proteins in both of the phases was determined by HPLC analysis of both phases (Figure 5-2). It was observed that nearly all of the cytochrome c remained in the aqueous phase regardless of the HFIP to DMF ratio. The measured concentration in the aqueous phase was very close to the initial concentration of 0.15 mg/mL. The peak for cytochrome c in the H-O phase was below the limit of detection for the instrument indicating very little cytochrome c is present in the H-O phase. For trypsin nearly an equal concentration (based on HPLC peak area) was observed in both phases when the dilution factor of the H-O phase (2X) was taken into account. With the volume of the H-O phase being roughly 5% of the total volume this would mean that around 95% of the trypsin remained in the aqueous phase. As expected water soluble proteins will remain largely in the aqueous phase rather than partition into the H-O phase.

Partitioning of hydrophobic peptide and protein standard

Gramicidin A and bacterierhodopsin were used as hydrophobic peptide and protein standards. Gramicidin A is hydrophobic peptide known for forming channels in phospholipid membranes and being an antibacterial agent[49]. Bacterierhodopsin is an integral membrane protein found in the plasma membranes of *Halobacterium halobium* that acts as a proton pump[23]. These two molecules were used to demonstrate the ability of the HFIP-DMF system to extract hydrophobic peptides and proteins and neither are water soluble. The partitioning experiments were done in the same way as with BSA and cytochrome c with the exception that the hydrophobic species were initially dissolved in HFIP rather than water.

When the two phases were analyzed by HPLC neither gramicidin A nor bacteriorhodopsin were detected in the aqueous phase. They were observed to be largely in the H-O phase as shown in Figure 5-3, which was expected based on their hydrophobicity. The calculated recoveries for gramicidin A using 3 different HFIP:DMF solvent ratios (2:3, 1:1 and 3:2), while maintaining the same total amount of solvent, were all around 100%. This indicates the enrichment is primarily determined by the % volume of the H-O phase and not slight differences in H-O composition at different ratios. Figure 5-4 demonstrates that the HPLC peak area of gramicidin A observed in the H-O phase is inversely proportional to the % volume of the H-O phase. It was also discovered during this research that by using higher %'s of HFIP with no other solvent that phase separation could be observed. These type of solutions were less selective than the Aq/H-O system since HFIP is able to solvate a wide range of proteins the addition of the organic solvent seemed to increase its selectivity towards more hydrophobic proteins.

HFIP-DMF extraction of a protein digests

The ability of the HFIP-DMF system to selectively extract hydrophobic peptides was tested on a Bovine Serum Albumin (BSA) tryptic digest. BSA is a 69 kDa protein that is soluble in water and contains 78 cleavage sites for trypsin so it could generate 80 tryptic peptides with complete digestion. BSA was digested using the 8M urea method and an HPLC trace of the generated peptides was obtained and shown in Fig 5-5 A. A majority of the peptides eluted within the first 30 minutes of the gradient. Since this is reversed phase HPLC an increase in retention time should correlate to increasing hydrophobicity of the peptides. A HFIP-DMF two phase solution was then formed by adding 50 uL each of HFIP and DMF to the aqueous BSA digest solution. Both phases were subsequently analyzed by

HPLC. Fig. 5-5 B and C show the peptides maps in aqueous and H-O phases from the two-phase solution. A majority of peptides remained in the aqueous phase and almost none of the earlier eluting peptides were observed in the H-O phase. The later eluting peaks after 30 minutes showed enrichment relative to either the initial digest or the aqueous phase especially with the dilution of the HFIP-DMF phase taken into account. The more pronounced partitioning of more hydrophobic peptides into the HFIP-DMF phase illustrates the selectivity of these systems in extracting hydrophobic peptides.

Mouse red blood cell proteomic analysis

Mouse red blood cells (RBC's) were used to investigate the use of Aq/H-O systems for extraction of more complex protein mixtures and subsequent proteomics analysis. RBC's are essentially "sacks of hemoglobin" where hemoglobin plays a vital role in oxygen and carbon dioxide transport for organisms.[50] Proteins within the membrane of RBC's play important roles in providing the shape and deformability of the RBC's which allows them to travel through small capillaries and deliver oxygen throughout the body. RBC transmembrane proteins also serve important roles in ion and water transportation through the cell as well as being involved in interactions with other cells through cell signaling.[51] Disorders in the RBC membrane proteins can lead to several diseases making RBC IMPs important drug targets.[52] Membrane fractions of mouse RBC's were prepared by hypotonic lysis which removes most of the hemoglobin present. These membrane fractions serve as good systems to study the usefulness of HFIP-solvent two phase systems due to their relative simplicity; having much less cytosolic proteins present that could interfere with the analysis of membrane proteins, which is the goal of the experiment. Comparisons were initially made between HFIP-solvent systems formed using different solvents to see if certain solvents

offered different selectivity or improved performance. Extractions on 1mL RBC suspensions were performed using two-phase solutions made from 150 μL of total solvent (HFIP + other solvent) at a 3:2 mole ratio of HFIP:solvent. The H-O phase extracts were separated from the aqueous phase and dried. They were then solubilized using 8 M urea and subjected to in-solution digestion with trypsin. Following digestion the peptides were analyzed by LC-MS and the results in terms of total proteins and integral membrane proteins (IMP's) identified are shown in Table 5-1. These results show that the butanone systems showed the best results in terms of total proteins identified, number of IMP's identified and the % IMP based on the total number of proteins identified in the H-O phase. When compared to the urea control all of the H-O phase extracts showed relative enrichment of IMP's as a higher % of their proteins identified were IMP's with butanone being the highest. A urea control was performed on the same RBC's by following the same urea solubilization and trypsin digestion procedure without doing the initial Aq/H-O two-phase extraction. As expected the urea control identified a larger number of total proteins due to the much larger number of more hydrophilic proteins indicating selectivity by the HFIP-solvent system. There was fairly little overlap among the IMP's identified in the H-O phases with the butanone phase having 26 unique proteins from the other 3 H-O phases indicating different selectivity with changing solvents. The total number of proteins identified in the H-O phases is much lower than urea due to a large number of proteins remaining in the aqueous phase of the two-phase solution. Digesting both phases is likely to give a more complete picture of all of the proteins present within the sample as demonstrated in the next section.

Digestion of both phases from RBC extractions in HFIP-solvent two-phase systems

In order to gain a better understanding of how proteins partition in the HFIP-solvent two-phase systems both the aqueous and H-O phases were digested. Table 5-2 shows the results of digesting both phases from 1:1 HFIP-solvent extractions with DMF and acetone in terms of total proteins and IMP's identified. The aqueous phases from both two-phase extractions contained a much larger number of total proteins compared to their corresponding solvent phases based on the number of proteins identified after digestion. More IMP's were identified in the Aqueous phases for both two-phase systems. Due to the fewer number of total proteins in the H-O phases, the % of IMP's in both H-O phases is higher than that of the control and aqueous phases showing enrichment of IMP's. Figure 5-6 A and B depicts the overlap of total proteins and IMP's between the aqueous and H-O phases from the HFIP-Acetone extraction as well as the urea control. Figure 5-6 A shows that the largest overlap between the three digest is between the aqueous phase and urea control with the H-O phase having a higher % of unique proteins. This illustrates some selectivity by the H-O phase and the benefits of using HFIP-Solvent two phase extraction as a pre-fractionation technique in order to gain increased protein identification. This same trend follows for the IMP's identified with the H-O phase digest having less overlap than the other two (Figure 5-6 B). While the urea control has a good number of unique proteins separate from both the aqueous and H-O phases, the use of the two-phase extraction leads to an overall greater number of proteins identified. For example when the two phases from the HFIP-Acetone extraction are combined 380 total proteins are identified compared to 312 for the control and 74 IMPs were identified compared to 61 for the control. The average properties of the IMP's from the urea control as well as both phases of the HFIP-solvent extractions are compared (Table 5-3).

The GRAVY scale was developed by Kyte and Doolittle in 1982 and is used to measure the hydrophobicity of polypeptides or proteins.[53] They assigned hydropathy values to each amino acid and the GRAVY score for a peptide sequence is calculated by averaging the hydropathy value for all of the amino acids in the sequence. A more positive GRAVY value represents a more hydrophobic peptide or protein while negative values are more hydrophilic. The average GRAVY scores for proteins identified in the H-O phases are much higher than those of the control and aqueous phases. This indicates that more hydrophobic proteins are enriched in the H-O phase. Because IMP's can be single or multipass and vary widely in size they can also vary widely in hydrophobicity since they have hydrophobic and hydrophilic regions. Since a typical transmembrane regions is around 21 amino acids long a single pass transmembrane protein that is 1,000 amino acids long will be much less hydrophobic than a single pass transmembrane protein that is 100 amino acids long since the hydrophobic transmembrane region makes up a much smaller percentage of the total protein. So while both the control and aqueous phases identify large numbers of IMP's the ones identified in the H-O phase are more hydrophobic on average. The IMP's identified in the H-O phase also exhibit higher pI values which likely results from some of the more hydrophobic amino acids (AA's) having higher pI values. The average length of the proteins identified in the H-O phase is shorter than those of the control and Aq phases. Thus, the H-O phases tend to extract smaller, more hydrophobic proteins although some larger proteins were only observed in the H-O phase but they typically contained several transmembrane regions indicating that hydrophobicity is the primary driving force behind partitioning in the HFIP-solvent two-phase systems. Figures 5-7 A-C depicts the distribution of IMP's identified in the control as well as the Aq and H-O phases from the HFIP-Acetone extraction

based on the GRAVY values of the individual proteins. The GRAVY values for IMP's identified in the H-O phase are shifted toward more hydrophobic values compared to the control and aqueous phase. Two IMP's with GRAVY values over 1.0 were identified in the digest from the H-O phase (accession #'s Q9CR60 and P63082). Both of these proteins are fairly small at 15.4 and 15.8 kDa containing several transmembrane regions. Many more IMP's with negative GRAVY values were identified in the control and aqueous phase digest. The digestion and LC-MS methods used for this analysis are fairly standard and not geared toward the identification of transmembrane regions. So for small proteins containing several transmembrane regions they are being identified by their non-membrane portions which are relatively small. This further supports that the HFIP-Solvent two phase system is enriching these very hydrophobic proteins excluding more hydrophilic proteins and aids in their identification. Larger more hydrophilic proteins will generate many more tryptic peptides that can be detected during the LC-MS analysis. Even if the peptides from the very hydrophobic proteins are present in the digest it is likely they will not be detected because of all of the peptides from the more hydrophilic proteins or that their signal will be too small to achieve good MS/MS data, leading to a less reliable protein identification. Table 5-4 shows some of the analytical information in terms of how many peptides and unique peptides were detected on average for the IMP's in the different digest as well as the % coverage for the proteins. These results show that on average the IMP's in the H-O phase were identified based on fewer detected peptides which would be expected based on their smaller size and more hydrophobic nature. The previously mentioned proteins, Q9CR60 and P63082, with GRAVY values over 1.0 were both identified based on a single peptide which was form their non-transmembrane regions. Figure 5-8 shows the amino acid sequences for Q9CR60 and

P63082 along with their transmembrane regions and trypsin cleavage sites with the detected peptide highlighted in yellow. The figure shows that many of the possible tryptic peptides contain portions of transmembrane regions which may hinder digestion or detection. Many of the non-transmembrane tryptic peptides are too small to give very useful information for protein identification as larger peptides such as those used for identification will give better MS/MS data and are more likely to be unique resulting in a reliable identification. So for small highly hydrophobic proteins such as these a standard RPLC-MS/MS method is reliant on being able to detect a small number of peptides with good MS/MS data in order to identify these proteins. These peptides were not detected in the urea control and aqueous phase and if they were present good enough MS/MS data to identify the protein was not obtained due to the abundance of peptides from more hydrophilic proteins. Figure 5-9 shows the LC-MS total ion chromatograms from the aqueous and H-O phase digest of the HFIP-Acetone extraction. The aqueous phase digest appears to show a more even distribution of peptides throughout the chromatogram which is expected since more proteins are identified in the aqueous phase and the larger more hydrophilic proteins should generate more peptides for detection.

HFIP-Solvent extraction combined with high pH washing

High pH washing using pH 11.0 sodium carbonate buffer is often used to remove contaminants and peripheral membrane proteins from the cell membrane surface.[54] This enriches the IMP's and leads to better identification and also opens membrane vesicles releasing compounds trapped within. The effect of high pH carbonate washing on protein identification in RBC membranes was examined using the Aq/H-O two-phase extraction

system. The results (Table 5-5) show that when high pH washing is combined with HFIP-solvent extractions an increase in the number of identified IMP's is observed in both the aqueous and H-O phases. All digests, including the urea control show increased % of total proteins identified IMP's, which is expected as the washing step strips many of the non IMP's from the membrane allowing the IMP's to be detected more easily. This is the reason that the total number of proteins ID'd in the aqueous phases decreases significantly compared to the prewashed samples. The proteins identified from the high pH washed membranes includes most of the ones identified from the Aq/H-O extractions without high pH washing as shown in figure 5-10. This indicates that selectivity remains the same but by removing more non IMP's with the carbonate washing more peptides from IMP's can be detected leading to higher identification rates. The GRAVY values of the identified IMP's show the same trend where the value is higher in the H-O phases compared to the aqueous phases and the control. Histograms of the IMP GRAVY distribution follow very closely with those from the non-washed RBC data (Fig. 5-11) Both HFIP-Solvent extraction and high pH washing lead to increased identification of IMP's and when used in combination, led to even higher identification of IMP's. By removing many of the more hydrophilic proteins, the IMP's can be identified more easily in both phases because there are fewer peptides from the hydrophilic proteins.

Conclusions

Aq/H-O two-phase solutions were used for extraction of proteins. More hydrophobic peptides and proteins were shown to preferentially partition into the H-O phase while hydrophilic proteins remained largely in the aqueous phase using protein and peptide standards. HFIP-solvent two-phase extraction leads to an increase in IMP identification from

RBC membranes. IMP's were detected in both the aqueous and H-O phases but the ones detected in the H-O phases were more hydrophobic with their transmembrane regions making up more of the total protein length on average. The detection of these hydrophobic IMP's was typically based on the detection of a small number of peptides during LC-MS analysis. So by enriching these hydrophobic IMP's relative to more hydrophilic proteins that would generate more detectable tryptic peptides, increased identification was observed. The combination of high pH washing with HFIP-solvent extraction leads to a further increase in IMP identification in both phases while maintaining the same type of selectivity. Aq/H-O two-phase extraction shows promise as a method for identifying very hydrophobic membrane proteins for proteomic purposes. The results could likely be further improved by optimizing digestion and LC-MS conditions for membrane proteins such as allowing for detection of transmembrane regions.

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Tables

Table 5-1. Comparison of proteins identified from digested RBC extracts from H-O phases formed using different solvents. Phases formed by adding 90 µL HFIP and 60 µL solvent to 1 mL RBC suspension in 50mM ammonium bicarbonate buffer, pH 8.5.

Sample	# proteins	# Integral membrane proteins	% Integral membrane
Urea	312	61	19.6
DMF	69	22	31.9
Acetone	98	28	28.6
THF	83	27	32.5
Butanone	120	47	39.2

Table 5-2. Total proteins and IMP's identified in both phases form HFIP-solvent two-phase extractions compared to a single phase urea control

Sample	Phase	# proteins	# Integral membrane proteins	% Integral membrane
Urea control	N/A	312	61	19.6
HFIP-DMF	Aq	305	54	17.7
HFIP-DMF	H-O	69	22	31.9
HFIP-Acetone	Aq	299	48	16.1
HFIP-Acetone	H-O	153	40	26.1

Table 5-3. Averaged properties of integral membrane proteins identified in both phases from HFIP-solvent extractions as well as a urea control

Sample	Phase	GRAVY value	pI	Length (# amino acids)
Urea control	N/A	0.01	6.72	539
HFIP-DMF	Aq	-0.03	6.64	638
HFIP-DMF	H-O	0.26	7.75	443
HFIP-Acetone	Aq	-0.04	6.45	494
HFIP-Acetone	H-O	0.29	7.75	406

Table 5-4. # of peptides detected and % coverage of IMP's identified in digest from urea control and HFIP-Acetone two-phase system

Sample	Phase	# peptides	# unique peptides	% coverage
Urea control	N/A	6.2	5.8	16
HFIP-Acetone	Aq	6.5	6.3	18.4
HFIP-Acetone	H-O	3.2	2.9	13.8

Table 5-5. Total proteins and integral membrane proteins identified in a urea control as well as digest from HFIP-Solvent two-phase extractions of high pH washed mouse red blood cells along with the average GRAVY value of ID'd integral membrane proteins

Sample	Phase	# proteins	# Integral membrane proteins	% Integral membrane	GRAVY value IMP's
Urea control	N/A	281	101	35.9	-0.01
HFIP-DMF	Aq	204	89	43.6	0
HFIP-DMF	H-O	113	47	41.6	0.19
HFIP-Acetone	Aq	194	63	32.5	0.02
HFIP-Acetone	H-O	148	62	41.9	0.26

Figures

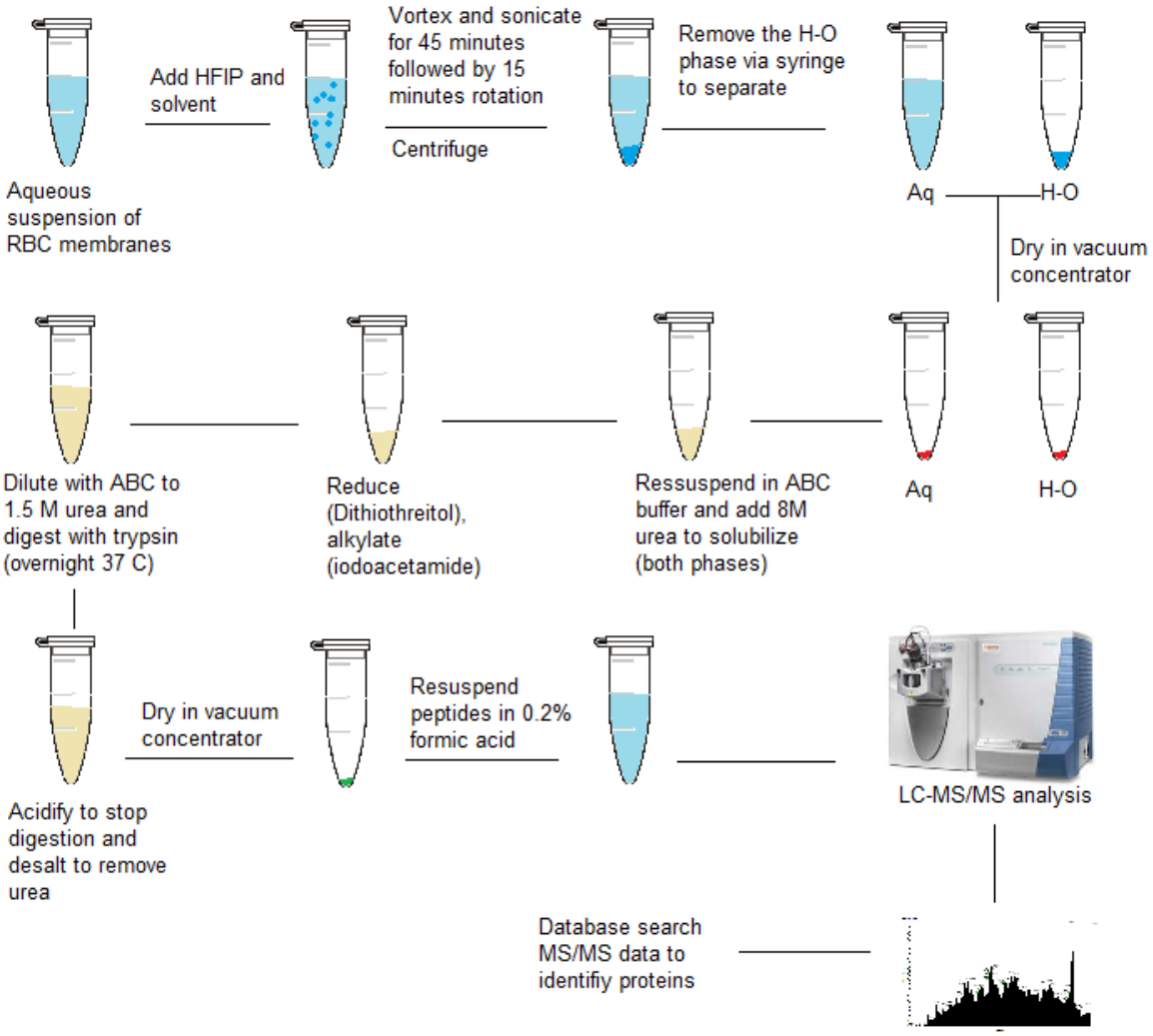


Figure 5-1. Proteomics workflow for analysis of mouse red blood cell membranes using Aq/H-O two-phase extraction

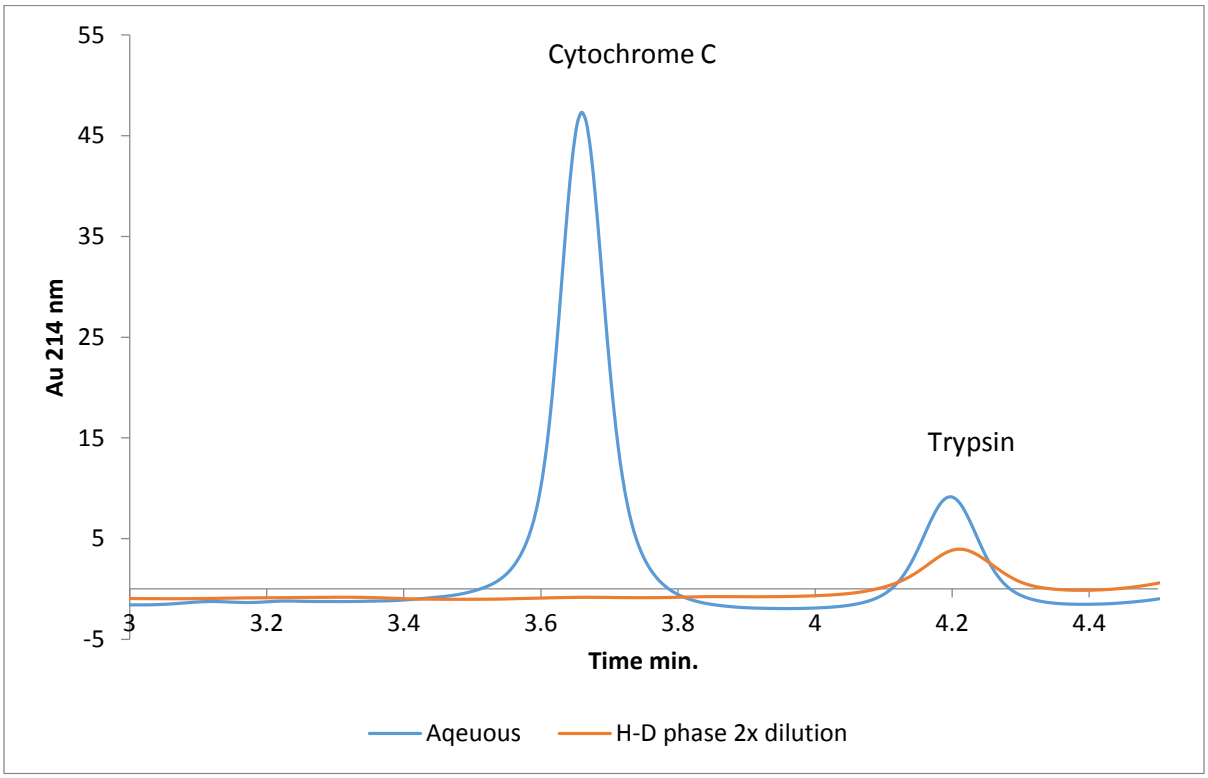


Figure 5-2. HPLC chromatogram from a 1:1 9% HFIP-DMF extraction of a 2 protein mixture containing cytochrome C and trypsin. Aqueous phase = blue, H-O phase with 2x dilution = red

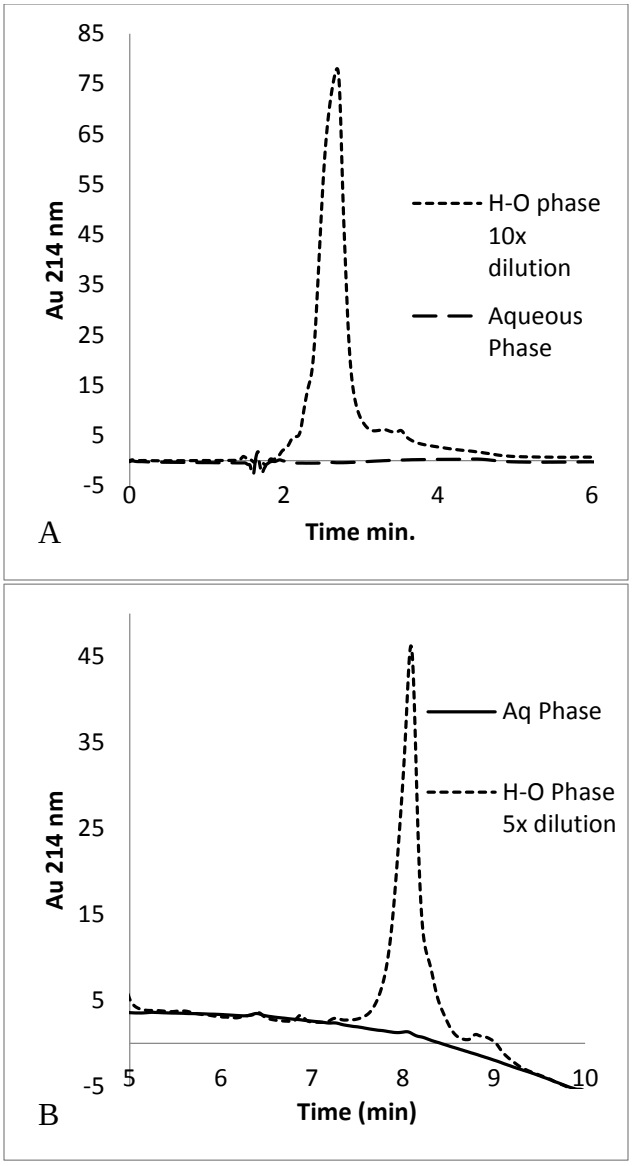


Figure 5-3. Partitioning of hydrophobic molecules A) Gramicidin A and B) bacterierhodopsin in HFIP-DMF two-phase system based on HPLC chromatograms

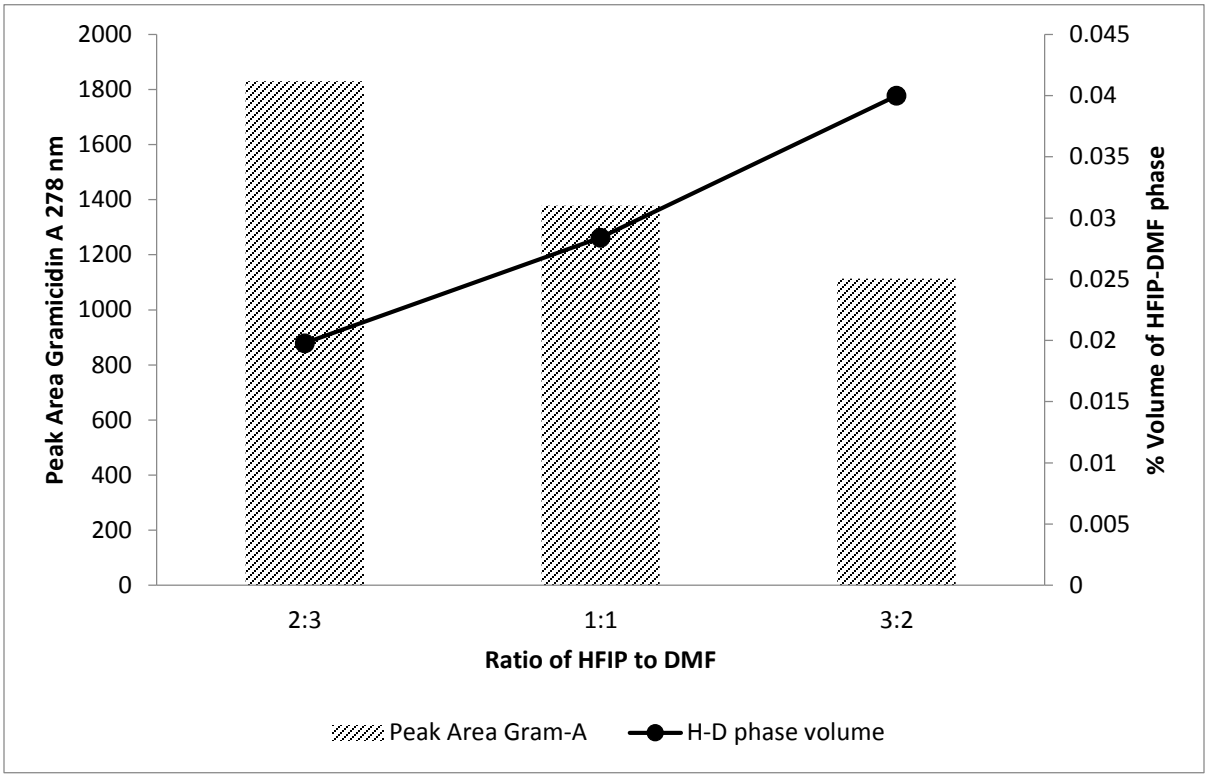


Figure 5-4. Relationship between the volume of the HFIP-DMF phase and the observed peak area of Gramicidin in the HFIP-DMF from a HFIP-DMF two-phase extraction

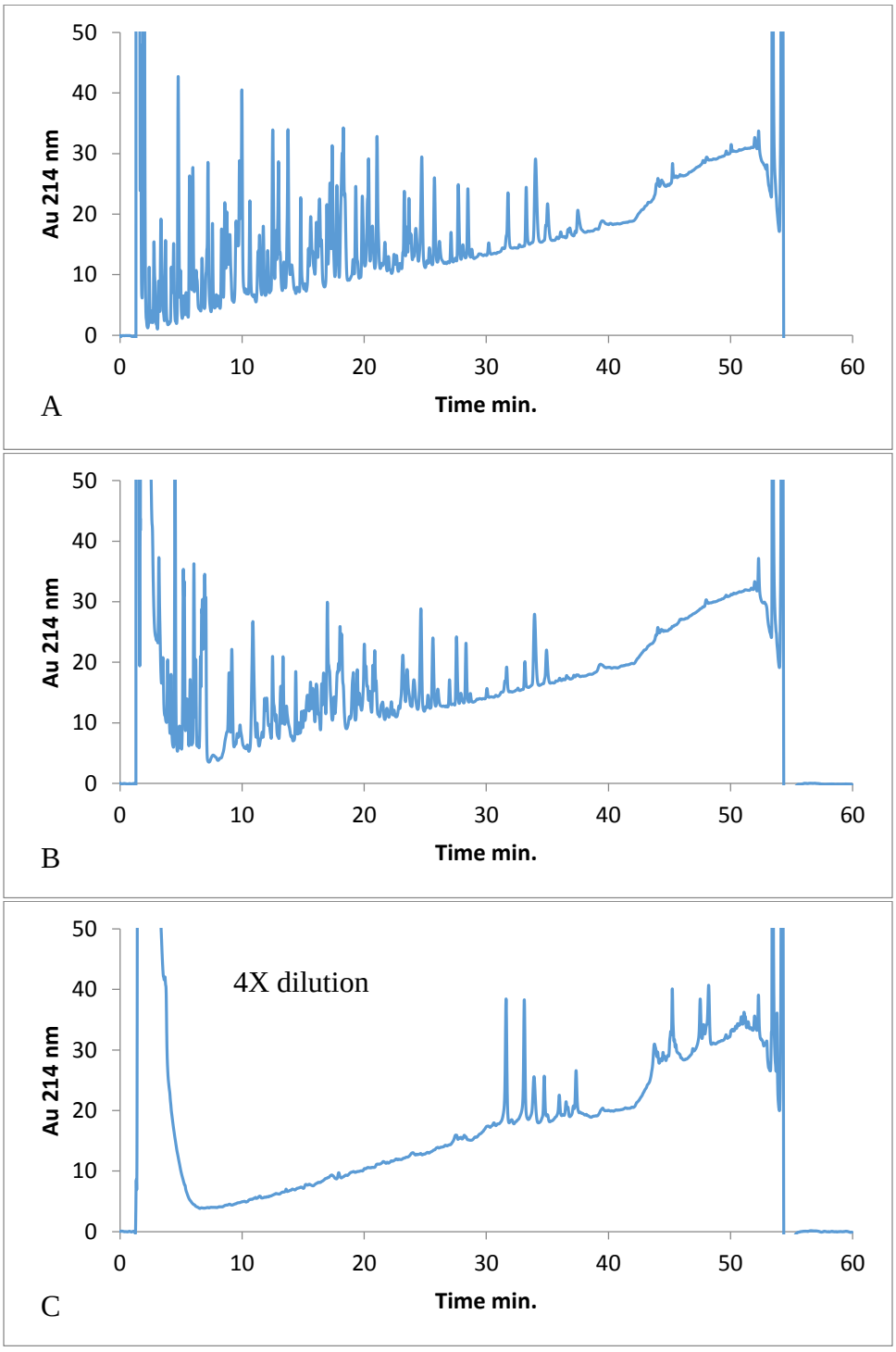
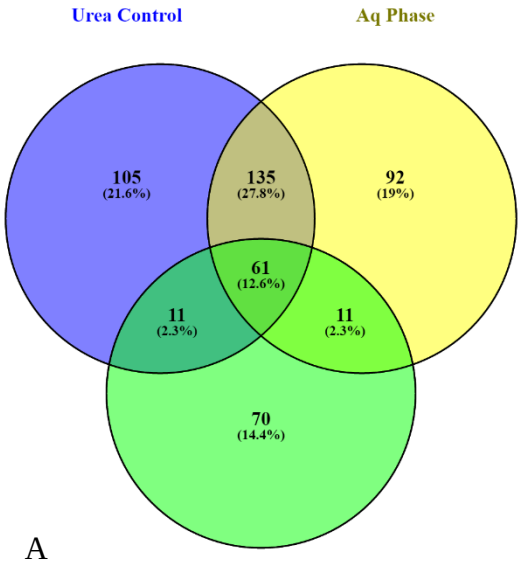
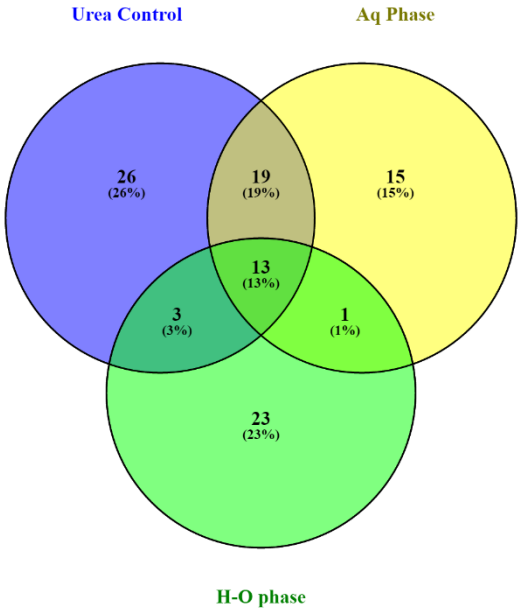


Figure 5-5. HPLC chromatogram of BSA tryptic digest A) prior to extraction and following extraction using HFIP-DMF two phase solution B) aqueous phase C) HFIP-DMF phase



A



B

Figure 5-6. Overlap of A) total proteins and B) Integral membrane proteins identified between the aqueous and solvent phases from an HFIP-Acetone extraction and a urea control digestion.

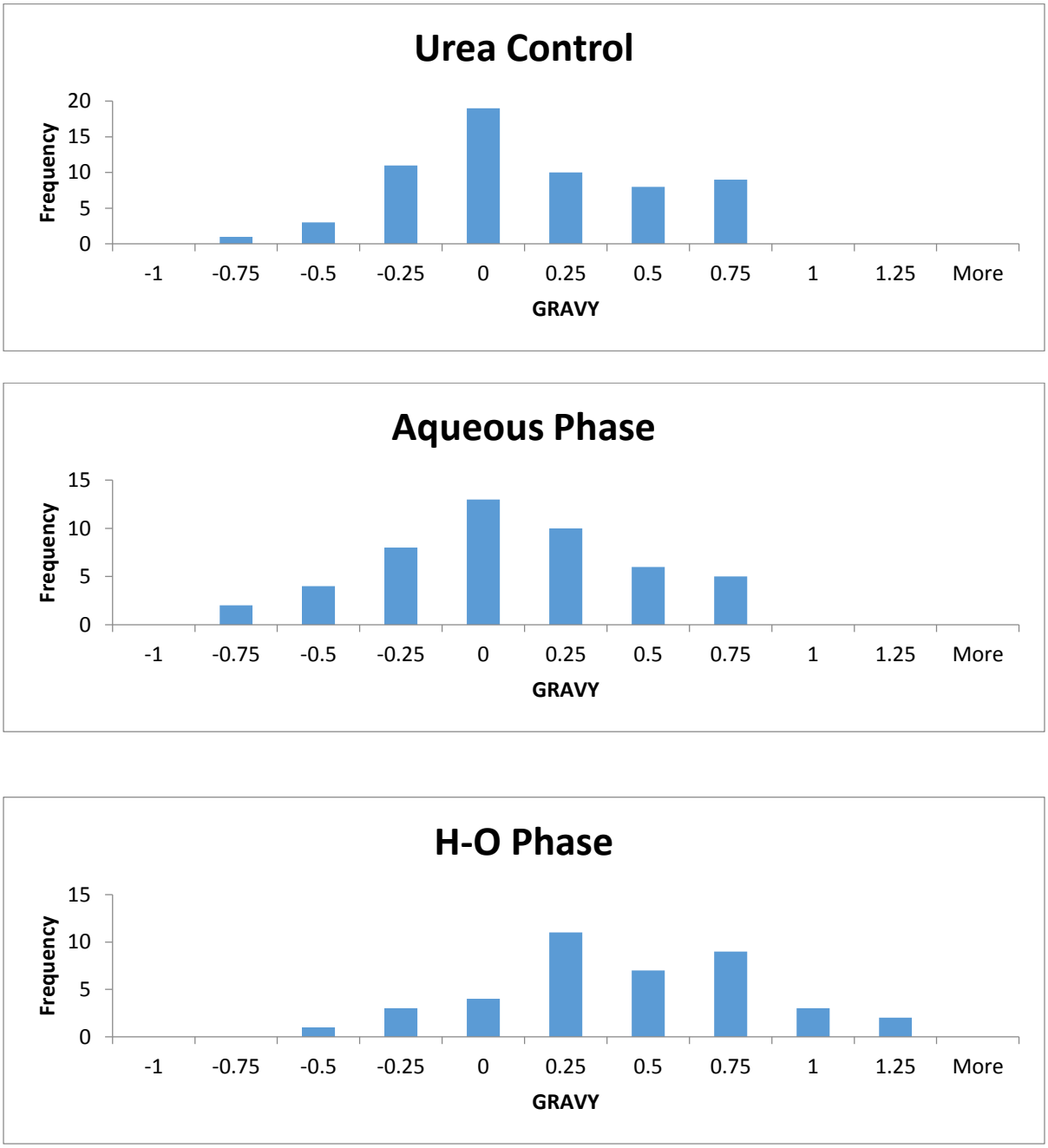


Figure 5-7. Distribution of integral membrane protein GRAVY values identified in digest from A) urea and HFIP-Acetone two-phase extraction B) aqueous phase C) H-O phase

Q9CR60:

MISLTDTQK | IGMGLTGFGVF FLFFGMILFFDK | ALLAIGNVLFVAGLAFVI

GLER | TFR | FFFQR | HK | VK | ATGFFLGGVFVVLIGWPLIGMIFE IYGFFLLFR | G

FFPVVVGFIR | R | VPVLGSLLNLPGIR | SFVDK | VGESNNMV

P63082:

MADIK | NNPEYSSFFGVMGASSAMVFSAMGAAYGTAK | SGTGIAAMSVMR | PE

LIMK | SIIPVVMAGIIAIYGLVVAVLIANSLTDGITLYR | SFLQLGAGLSVG

LSGLAAGFAI GIVGDAGVR | GTAQQPR | LFVGMILILIFAEVLGLYGLIVAL

ILSTK

Figure 5-8. Amino acid sequences for the two integral membrane proteins identified in the H-O phase from a HFIP-Acetone two-phase extraction with GRAVY values over 1.0. The transmembrane regions are highlighted green while the peptide used for identification is highlighted yellow. The sequence is broken up into potential tryptic cleavage sites.

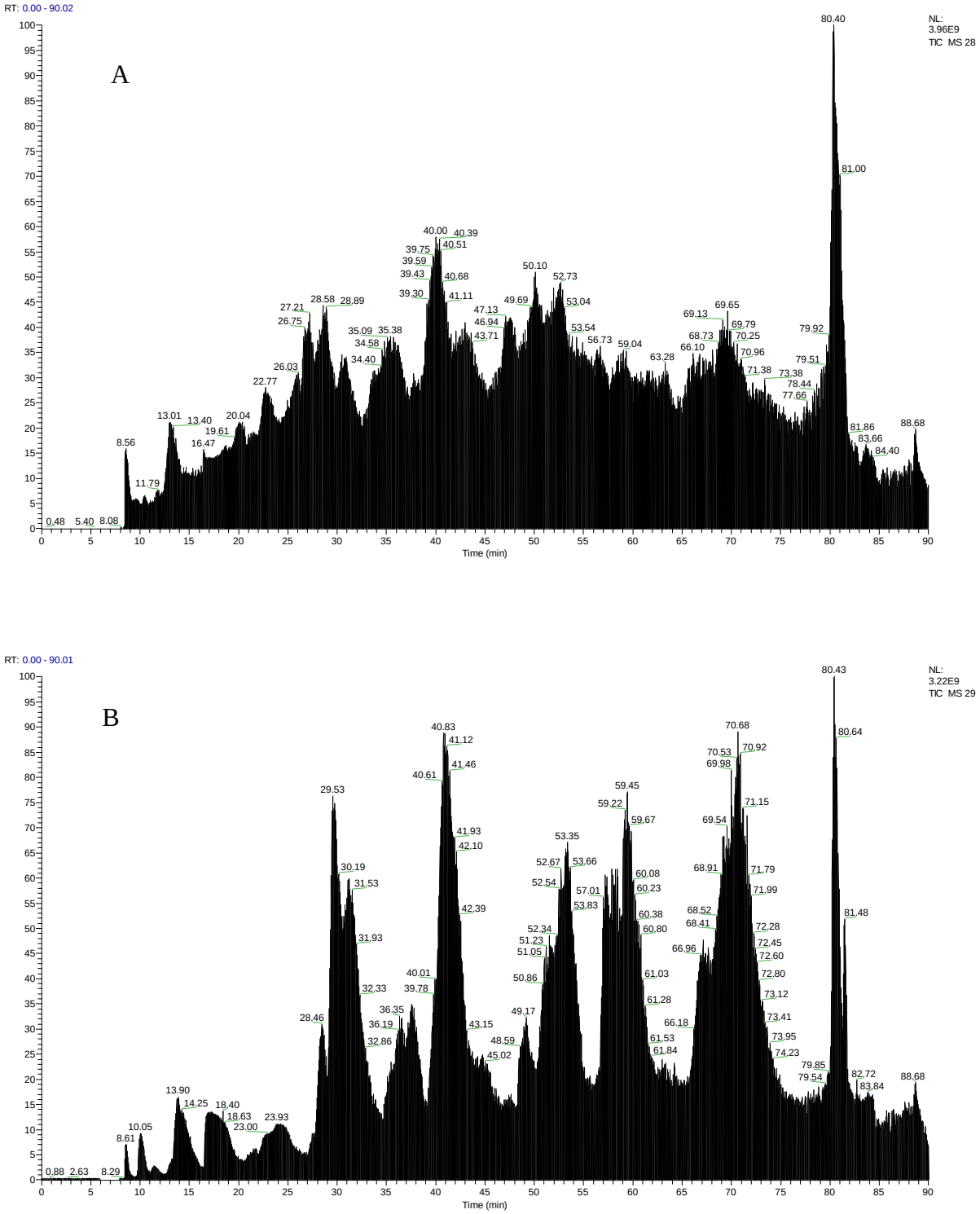


Figure 5-9. Total ion chromatograms form LC-MS analysis of A) aqueous and B) H-O phase digest of HFIP-Acetone two-phase extractions of mouse red blood cell membranes

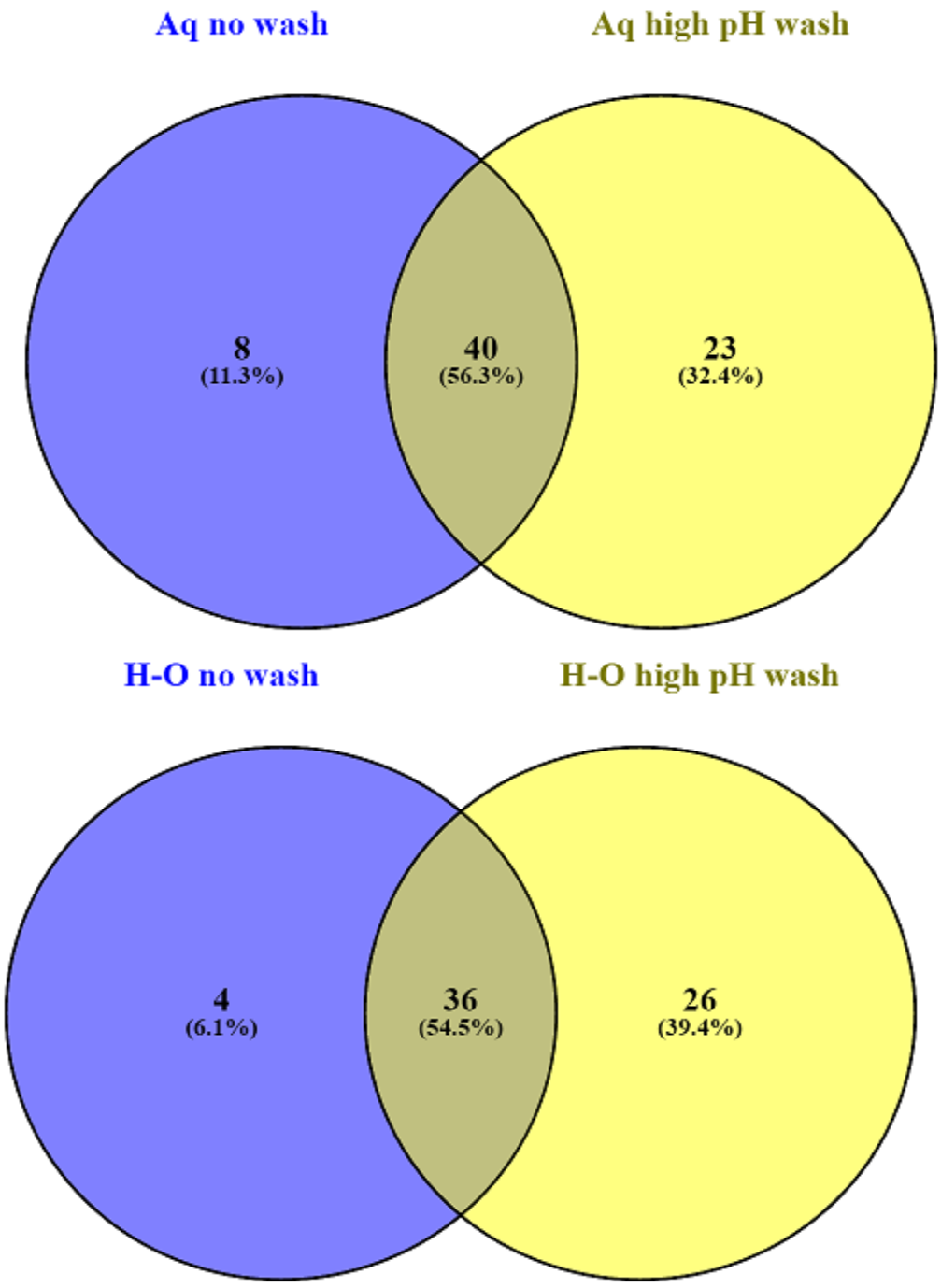
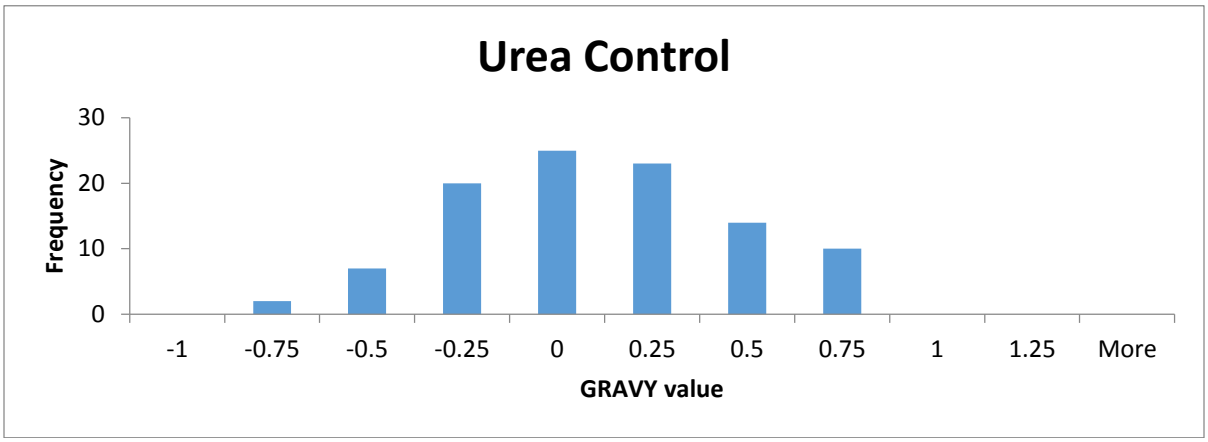
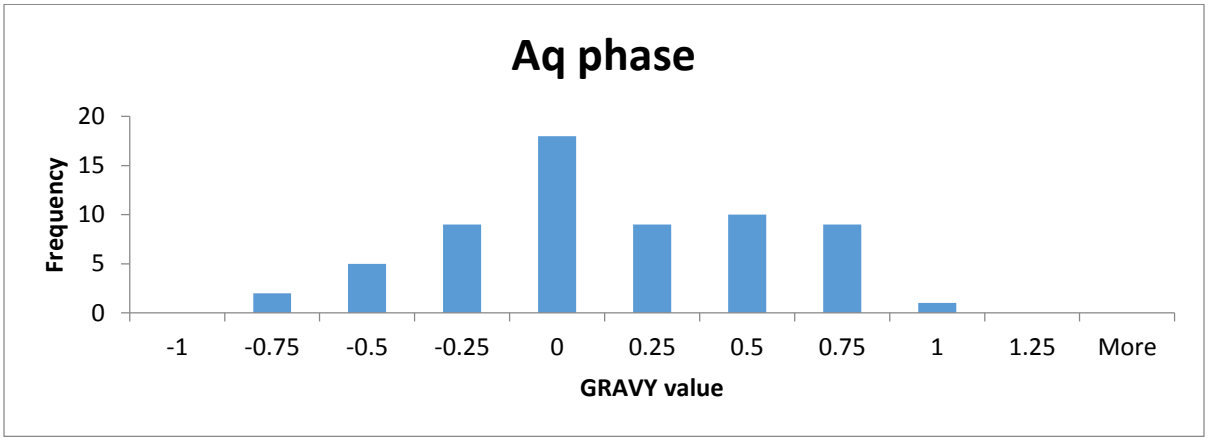


Figure 5-10. Similarity between IMP's identified in both phases from HFIP-Acetone extractions of mouse RBC membranes with and without high pH carbonate washing.





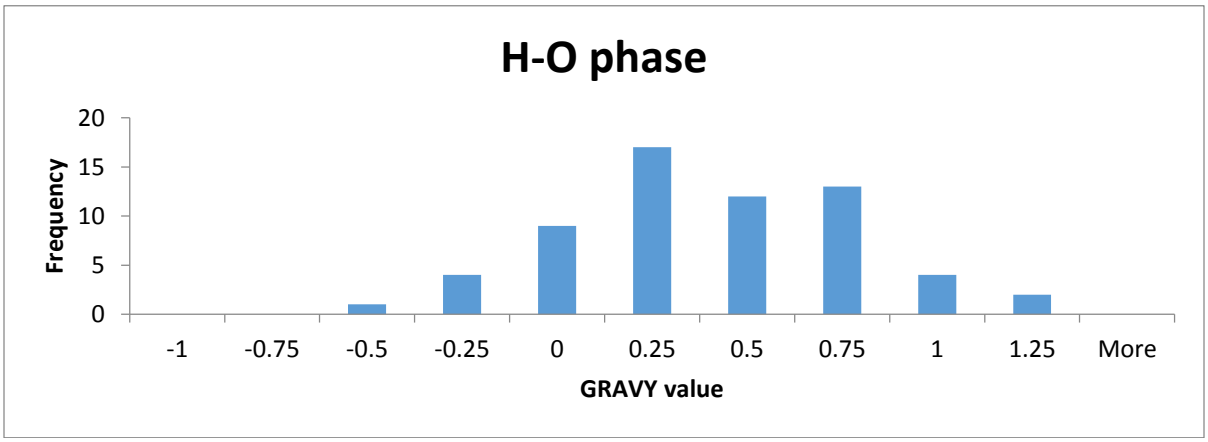


Figure 5-11. Distribution of integral membrane protein GRAVY values identified in digest of high pH washed RBC membranes from A) urea and HFIP-Acetone two-phase extraction B) aqueous phase C) H-O phase