SUSTAINABLE REACTION AND SEPARATION SYSTEMS

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SUSTAINABLE REACTION AND SEPARATION SYSTEMS

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For Anthony

Whose extent of chemistry knowledge consists of that titration he did once in 10^{th} grade,
but who always tried to help anyway.

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SUMMARY

Ionic liquids are being studied extensively for their replacement of organic solvents in many processes and applications. With negligible vapor pressure and no bioaccumulation, these solvents are considered environmentally friendly. However, the behavior of ionic liquids is not well understood, particularly with CO_2. Several researchers have documented depressed melting points when using ionic liquids with CO_2 and this study explores that phenomenon both quantitatively and molecularly.

With the increased prevalence of protein and enzyme chemistry, it is necessary to have separations that are as cheap and effective as non-biological separations while being benign enough that they do not denature the proteins and enzymes. This can be done using water-miscible solvents for extraction. By adding salt, two phases form that are similar enough in composition so as to be safe for the enzymes -- yet different enough that they yield better separations than those currently in use.

The last part of the research deals with converting waste into a useful product. In the case of asymmetric catalysis, the catalyst is recycled both to prevent its disposal into the environment and to save companies money. The final project produces an additional revenue stream for a brewery by using near-critical water to extract valuable chemicals from Brewers Spent Grain, a waste product of from the brewing process.

CHAPTER I

INTRODUCTION

The definition of green chemistry is widely accepted as "the design, development and implementation of chemical processes and products to reduce or eliminate substances hazardous to human health and the environment," as published by Anastas and Warner in *Green Chemistry Theory and Practice* in 1998. This definition has been expanded into the "12 Principles of Green Chemistry" and "12 Principles of Green Engineering," which set forth standards with which new products and processes should be measured.

The motivation behind this and other such programs is to alleviate the problems facing our society like pollution, toxic waste, bioaccumulation, and reliance on non-renewable resources. Recent legislation has also put pressure on the chemical industry to pursue more environmentally benign processes. It is up to scientists and engineers to develop technology in such a way that it has minimal impact on the environment, and to make it sustainable enough to not rely on limited resources. One area where our group has made such an effort is in reactions and separations.

Often the most expensive and wasteful step in a given process is the separation of the final product. This can come in the form of toxic or harmful solvents, high energy consumption, poor yields, chiral catalysts, introduction of complex chemicals, and remediation costs for disposing of those chemicals. This thesis focuses on sustainability of separations through use of novel solvents, tunable solvents, and benign extraction.

Chapter II deals with the manipulation of ionic solids and liquids with CO_2. Ionic liquids are at the forefront of green chemistry research due to their negligible volatility and lack of accumulation in groundwater, and they have been pursued as reaction media, separation media, and as designer solvents. The unique interaction of ionic liquids with CO_2 permits both the depression of the melting point of the ionic liquids as well as a high level of control over them. The chapter quantifies that depression and explores ways this can be beneficial in many areas of chemistry.

One method to perform chemistry naturally is to let biology do it for you. The recent developments in biotechnology and protein engineering have created the need for separations that are not only efficient, but that are also safe enough on biological systems. Chapter III explores a method for protein separation based on Aqueous Two Phase Extraction (APTE), in which the polymer is replaced with a water-miscible solvent. As currently used, ATPE has several drawbacks to industrial use, and this project aims to eliminate those.

The most expensive step in pharmaceutical processing is the separation of enantiomers. To combat this, expensive chiral catalysts are used, and often in a way that is inefficient. Using OATS (Organic Aqueous Tunable Solvents), a technique developed in our group, the catalyst can be run homogeneously for higher efficiency, and then easily separated with CO_2 pressure, allowing for recycle and a huge cost savings. Chapter IV explores the development of Salen, a chiral catalyst used for making the HIV protease inhibitor Crixivan, for use with this technique.

Chapters V and VI both deal with benign separations of ferulic acid from biomass. Ferulic acid is a valuable compound and has recently been touted as the most abundant,

renewable chemical feedstock. It is most often used as a precursor for microbial transformation, specifically for all-natural vanillin. It is found in the cell wall of many agricultural products, bound by ester bonds. In Chapter V, the ferulic acid is released from the grain by enzymes, and therefore a separation is designed for extracting the ferulic acid from the biomass while not damaging the enzymes. To be regarded as an all-natural product by the USDA, the vanillin must be only minimally processed, thus putting many restrictions on the extraction.

Conversely, Chapter VI utilizes near-critical water to release the ferulic acid, thus eliminating enzymes from the process. Near-critical water is a particularly advantageous "green" solvent because it combines the properties of an organic solvent while eliminating the waste of an organic solvent. Near-critical water is also used to convert ferulic acid to another valuable chemical, 4-vinylguaiacol. Because no enzymes are used in this process, the extraction has fewer limitations. Additionally, the ferulic acid source in Chapter VI is Brewers Spent Grain, a waste from the beer-making process. Thus, we are effectively taking a waste stream and converting a portion of it to valuable chemicals using minimal upfront capital and negligible operating costs.

Finally, Chapter VII explains the conclusions and ramifications of the presented work and offers recommendations for continuation of each of the projects.

CHAPTER II

MELTING POINT DEPRESSION OF IONIC "LIQUIDS" WITH CO_2

Ionic Liquids represent an exciting alternative to volatile organic solvents commonly used in processing. Their negligible vapor pressure suggests a reduction in emissions common to other solvents. Furthermore, ionic liquids are also being touted as "designer solvents", in which the cation and anion can be carefully tailored to produce a desired solvent property. However, many of the cation-anion combinations are not actually liquids at room temperature. Combining carbon dioxide with ionic solids produces a large melting point depression and therefore significantly increases the operating range of these solids.

An Ionic Liquid (IL) or Room Temperature Ionic Liquid (RTIL) is defined as an organic salt with a melting point below 100^{o}C at ambient pressure. Because of their negligible vapor pressure, ILs are considered by many to be environmentally benign as they do not contribute to air pollution. It has also been shown that ILs will not accumulate in the environment (Ropel, Belveze, et al 2005). For these reasons, along with their excellent organic solubility, these substances have been at the forefront of green chemistry research. Extensive studies show potential solvent applications including synthesis (Olivier-Bourgibou and Magna 2002; Wassercheid and Welton; Welton 1999; Gordon 2001), biocatalysis (Cull, Holbrey et al. 2001; Nara, Harjani et al. 2002), electrochemistry (Hyk and Stojek 1998), and separation technologies (Scurto, Aki

et al. 2002). In particular, there has been significant research in pairing CO_2 with ionic liquids (Scurto, Aki et al. 2002; Lu, Liotta et al. 2003; Cadena, Anthony et al. 2004; Anthony, Aki et al. 2004; Solinas, Pfaltz et al. 2004; Kazarian, Sakellarios et al. 2002; Niehaus, Phillips et al. 1985).

Carbon dioxide is frequently used in green processing due to its benign characteristics (Eckert, Knutson et al. 1996). From the work of Brennecke, it is known that CO_2 is the best extraction solvent for systems involving ionic liquids, because many organics are soluble in CO_2, while no ionic liquids are (Blanchard and Brennecke 2001; Blanchard and Hancu 1999). However, CO_2 has excellent solubility in many ILs, and much research has been done to study this behavior in imidazolium-based ionic liquids (Scurto, Aki et al. 2002; Lu, Liotta et al. 2003; Cadena, Anthony et al. 2004; Anthony, Aki et al. 2004; Solinas, Pfaltz et al. 2004; Kazarian, Sakellarios et a.l 2002). This phenomenon has been utilized in reference to an IL-organic solvent separation "switch" (Scurto, Aki et al. 2002), in separating CO_2 from other gases (Anthony, Aki et al. 2004), and in heterogeneous catalysis reaction media (Solinas, Pfaltz et al. 2004). It has also been shown that CO_2 can accelerate reactions in solventless synthesis through melting point depression of organic solids (Jessop, Wynne et al. 2000). Several researchers working with CO_2 and ILs have observed this depression as well (Kazarian, Sakellarios et al. 2002; Niehaus, Phillips et al. 1985; Scurto and Leitner 2005), so our group set out to elucidate this phenomenon. Leitner also showed ionic liquid-CO_2 biphasic systems for enzymatic reactions where melting point depression would be ideally suited (Reetz, Wiesenhoefer et al. 2003). Thus, CO_2 could be used as a solvent to lower the melting point and as the extraction solvent after the reaction is complete.

One of the most interesting aspects of ionic liquids is their use as "designer solvents". By choice of both the cation and anion, one can tailor the solvent to whatever properties are necessary for the given chemistry. Of the estimated 10^{18} possible ionic liquid cation-anion combinations, few have ever been synthesized and of those, many melt well above 100°C. To maximize the use of these designer solvents, control over the melting point is crucial. Creating a CO_2-IL mixture extends the liquid operating range significantly, and this study sets forth a method for quantifying melting point depression of several CO_2-IL mixtures. We have developed a system for readily quantifying the freezing eutectic of CO_2-organic salt mixtures and have done so for naphthalene and several ionic liquids, which are shown in Figure 2-1. The results indicate a high level of control over the melting point of CO_2-IL systems, thus enabling the use of many more ionic solids for solvent replacement.

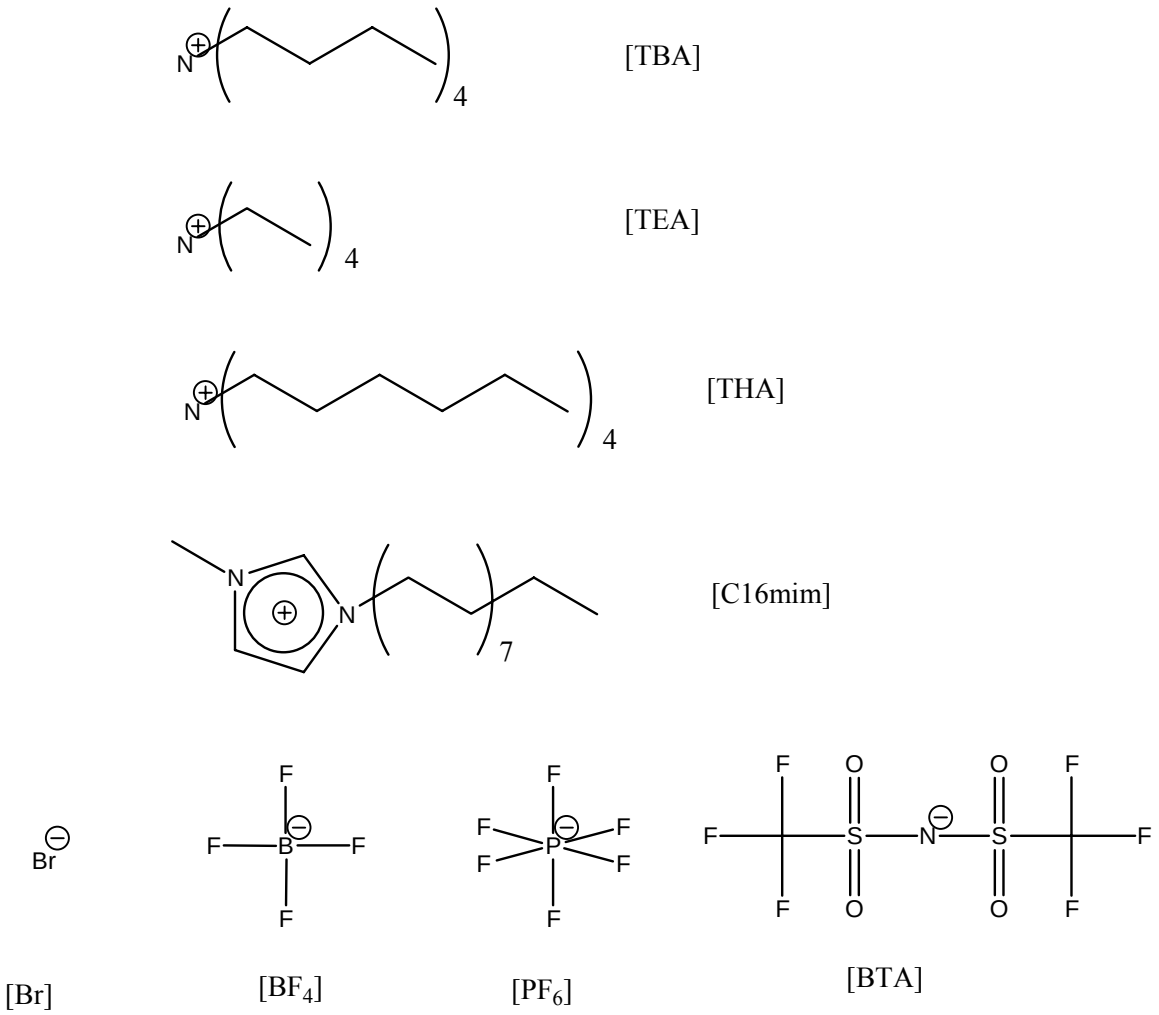

Figure 2-1: Ionic liquid cations and anions used in this work and their abbreviations.

Experimental Methods

Materials

All chemicals were purchased from Sigma-Aldrich unless otherwise noted. The naphthalene (99%), methanol (extra dry with molecular sieves, <50ppm water, Acros), and Aquastart Combititrant 1 (EMD chemicals) were used as purchased. SFE/SFC grade carbon dioxide was purchased from Airgas and filtered prior to use. All ionic liquids were further purified by drying at room temperature under vacuum of 10^{-3} torr for 48 hours and stored at room temperature under nitrogen atmosphere. The ionic liquids used were: tetrabutylammonium tetrafluoroborate [TBA][BF_4] 99+%, tetrahexylammonium bromide [THA][Br] 99%, tetrabutylammonium bromide [TBA][Br] 99%, tetraethylammonium bistrifluoromethanesulfonimidate [TEA][BTA] 99+%.

Experimental Procedure

Cheong et al measured the temperature composition of naphthalene-CO_2 using what they called the "first freezing point method" (Cheong, Zhang et al. 1996). This was done by observing the initial appearance of solid, followed by sampling from a high pressure view cell. An equation of state, paired with pressure, temperature, and volume data, provided the gas phase composition. Gravimetric measurement of the solid phase after dissolution and drying gave the other compositions. Our group developed a first freezing point method where the composition could be determined without sampling.

First, a known mass of organic solid was loaded into a variable volume high pressure view cell, and the pure solid melting point was confirmed before adding any CO_2. The cell was then loaded with a known quantity of CO_2 from a syringe pump and

allowed to reach equilibrium with temperature and pressure such that there was a single liquid phase. Next, the mixture was cooled isobarically until the first crystal formed. Generally the entire contents froze quickly thereafter. The composition of the single liquid phase is known since there are only two components and one phase. The first crystal of pure solid has a negligible impact on the overall composition and thus the composition at the freezing point is known.

After one freezing point was measured, the system was reheated back into one phase and the process was repeated. This was repeated until two consecutive data points were identical to ensure the system was at equilibrium, which can take up to 24 hours, depending on the solid.

Apparatus

All measurements were carried out in a variable-volume windowed vessel (1.59 cm i.d., 20 cm^3 maximum volume) similar to that used by McHugh (Kirby and McHugh 1999) and previously used in our group (Brown, Hallet et al. 2000). The apparatus diagram is shown in Figure 2-2. The vessel window and variable-volume piston were sealed with Buna-N O-rings. Phase boundaries were measured by visually observing the freezing point through a 2.54 cm diameter sapphire window (1.27 cm viewable area) with a CCD camera (Sony) mounted on a 0.635 cm borescope (Olympus). The borescope and video camera not only allowed for safe observation of the phase equilibria, but also provided a significant magnification of the viewable area. The binary mixtures were stirred with a Teflon-coated stir bar coupled with an external magnet. The entire cell was placed in a thermostated air bath (modified Varian 3400 gas chromatograph) with

temperature control better than ±0.5 K. Precise temperature control was not required as freezing points were induced and observed while cooling the vessel. The temperature was measured with a hand-held readout (HH-22 Omega) and thermocouple (Omega Type K) inserted into the center of the phase equilibria vessel. The thermocouple response time was on the order of seconds. The combination of thermocouple and readout was accurate to ±0.2 K and calibrated for each experiment against a platinum RTD (Omega PRP-4) with a DP251 precision RTD bench top thermometer (DP251 Omega) accurate to ±0.025 K and traceable to NIST. Back-pressure was applied to the piston with a syringe pump (ISCO 100D) operated at constant pressure. To avoid any vapor phase, the pressure was held constant at 210 bar measured with a Druck DPI 260 gauge with a PDCR 910 transducer accurate to ±0.01 bar.

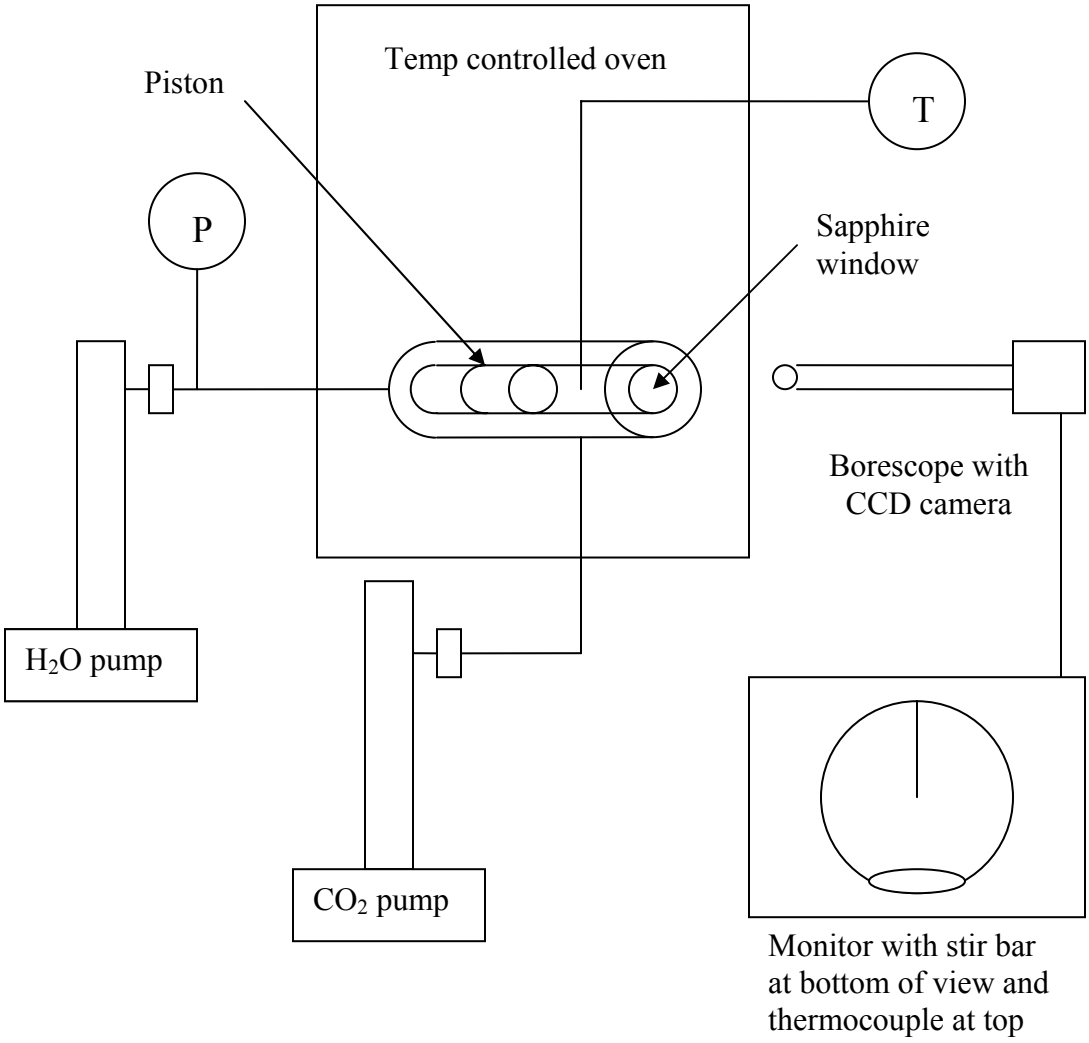

Figure 2-2: Experimental apparatus.

To ensure the validity of our new system, we conducted the new freezing point method on a naphthalene-CO_2 system. Many sources give T-P data for a naphthalene-CO_2 equilibria, but Cheong was the only to provide T-x data (Cheong, Zhang et al. 1986). As seen in Figure 2-3, our system is in excellent agreement with their data, despite the differing methods of analysis. We believe this gives credence to the new freezing point method.

After purification, the ionic solids were tested for water content. Samples were prepared by dissolving 1 gram of solid in 0.5 mL methanol. The water content of both the methanol and the dissolved solids were measured using a Karl Fisher Titrator, model DL31 from Mettler Toledo. Aquastar Combititrant 1 from EMD chemicals was used for the titrant. The results, as shown in Table 2-1, are averaged over 5 runs per solid except where noted.

Results and Discussion

Melting Point Depression

As mentioned previously, the results with naphthalene were nearly identical to those of Cheong et al (Figure 2-3). Again, Cheong used a visual freezing point combined with a sampling method to obtain composition. Our method also utilized the visual freezing point, but without the experimental complexity of sampling. Because the two data sets are nearly identical, we believe our system to be accurate. For comparison, the ideal solubility based on literature melting point and heat of fusion data (Table 2-1), and also the MOSCED solubility prediction are also included.

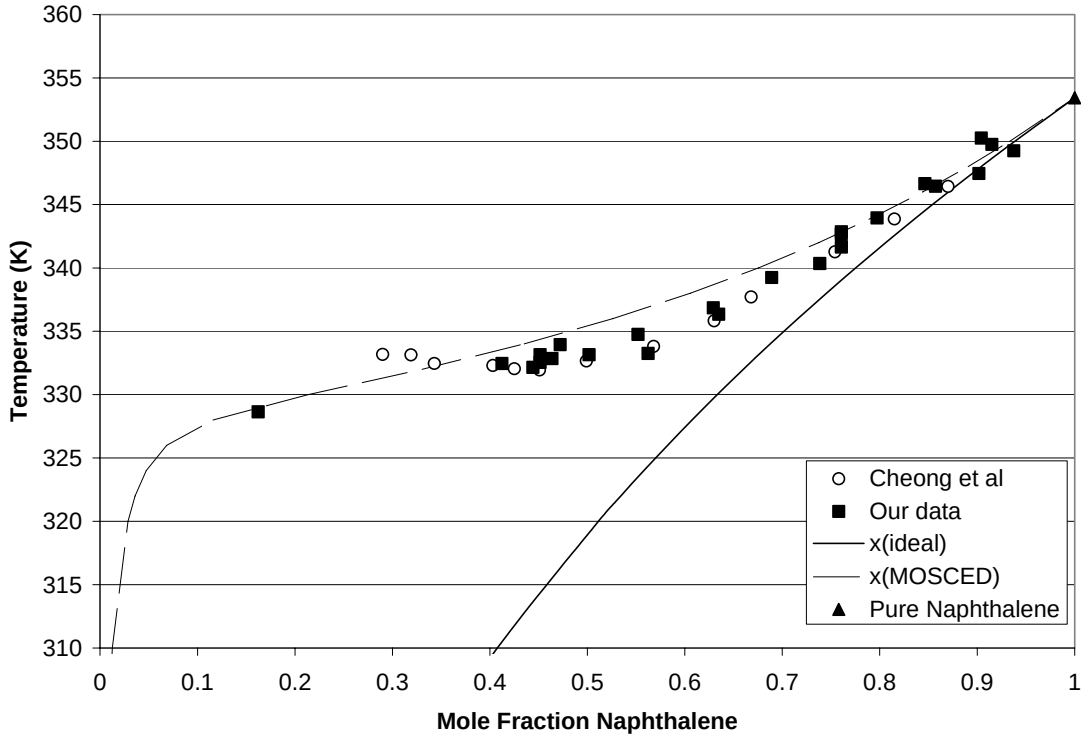

Figure 2-3: T-x diagram of Naphthalene-CO_2 with MOSCED and ideal solubility predictions

Table 2-1: Data from literature used in the ideal solubility and MOSCED predictions.

Compound	ΔH_{fus}(J/mol)	T_m (ºC)	ΔH_{trans}(J/mol)	T_{trans} (ºC)	Reference
Naphthalene	18980	80	-	-	McCullough et al
[THA][Br]	15909	100	6688/12122	32/42	Coker et al
[TBA][BF_4]	10467	160	6688	68	Coker et al
[C_{16}mim][PF_6]	500	125	37500	75	Gordon et al

The equation used for ideal solubility can be seen in Equation 2-1. MOSCED, MOdified Separation of Cohesive Energy Density, is a model developed by the Eckert group, (Thomas and Eckert 1984; Lazzaroni, Bush et al. 2005) for calculating infinite dilution activity coefficients. The model is based on five factors, dispersion (λ), induction (*q*), polarity (τ), and hydrogen-bond acidity (α) and basicity (β), which have been regressed from experimental data (Equation 2-2). The solubility line predicted in Figure 2-3 labeled "MOSCED" was calculated from the Wilson equation, with the binary interaction parameters set to the activity coefficients derived with MOSCED. Table 2-1 shows data from the literature used in these correlations.

$$x = \frac{1}{\exp\left(\frac{\Delta H_{fus}}{R}\frac{(T_m - T)}{T_m T}\right)}$$

Equation 2-1: Ideal solubility

$$\ln \gamma_2^{\infty} = \frac{v_2}{RT}\left[(\lambda_1 - \lambda_2)^2 + \frac{q_1^2 q_2^2 (\tau_1 - \tau_2)^2}{\psi_1} + \frac{(\alpha_1 - \alpha_2)(\beta_1 - \beta_2)}{\xi_1}\right] + \ln\left(\frac{v_2}{v_1}\right)^{aa} + 1 - \left(\frac{v_2}{v_1}\right)^{aa}$$

Equation 2-2: MOSCED equation for prediction of infinite dilution activity coefficients

The results for the ionic solids along with ideal solubility predictions are seen in Figures 2-4 and 2-5. In the case of ionic liquids and solids, an additional transition correction must be applied to the ideal solubility equation (Equation 2-3).

$$x = \frac{1}{\exp\left(\frac{\Delta H_{fus}}{R}\frac{(T_m - T)}{T_m T} + \frac{\Delta H_{trans}}{R}\frac{(T_{trans} - T)}{T_{trans} T}\right)}$$

Equation 2-3: Ideal solubility prediction for solid transition state.

Figure 2-4 shows that a melting point depression of 130 degrees for [TBA][BF_4] can be achieved by adding just over 1 molar equivalent of CO_2. For [THexA][Br], the melting point was reduced by 65 degrees (Figure 2-5), again with slightly more than 1 molar equivalent of CO_2. Due to equipment limitations, data at temperatures less than 25^o C were not possible. However, with an adequate cooling system, even larger melting point depressions could be expected, as the freezing eutectic could be fully quantified.

Similar curves were measured for [TBA][Br] and [TEA][BTA] but repeatability was exceedingly difficult due to long equilibration times. For instance, [TBA][Br] was allowed to equilibrate for 12 hours for a single data point, but the reading had changed after another hour. Some of this effect is due to the stability of the o-rings at high temperature and pressure in the presence of an ionic liquid. Additionally, melting point depressions of at least 42º and 80º were found for [TBA][Br] and [TEA][BTA] respectively. These data, while not repeated, are mostly in agreement with the data of Scurto and Leitner, (Scurto and Leitner 2005) who reported depressions of 23.3^o and 82^o, respectively.

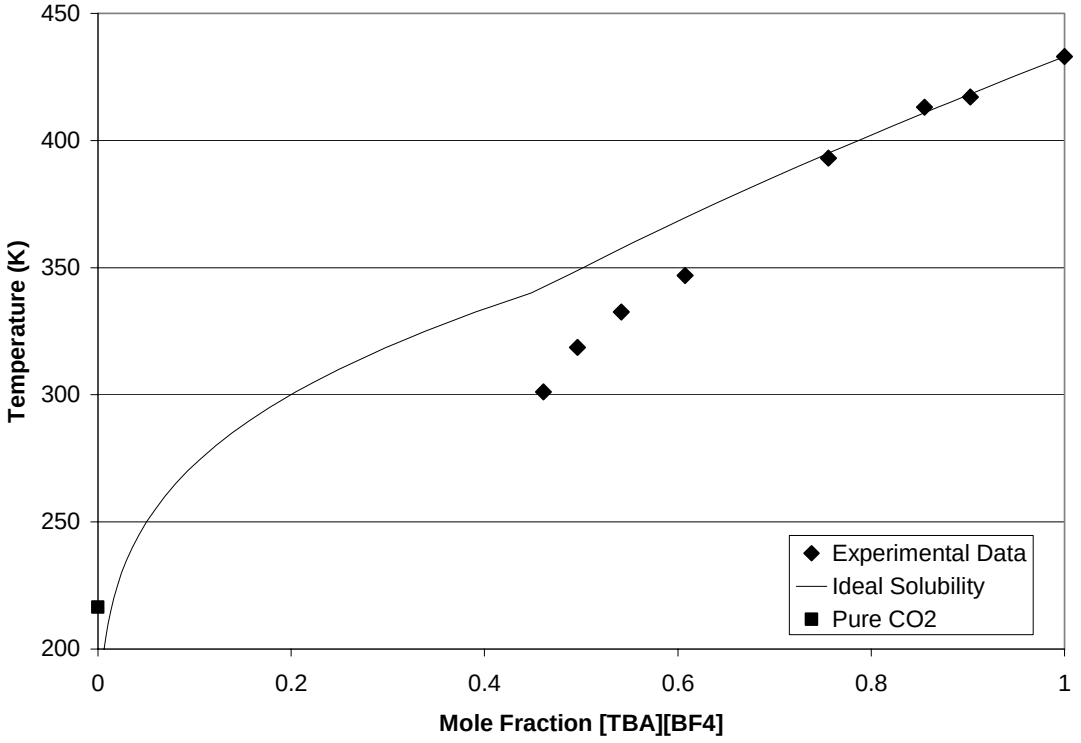

Figure 2-4: T-x diagram for tetrabutyl ammonium tetraflouroborate-CO_2 with ideal solubility prediction

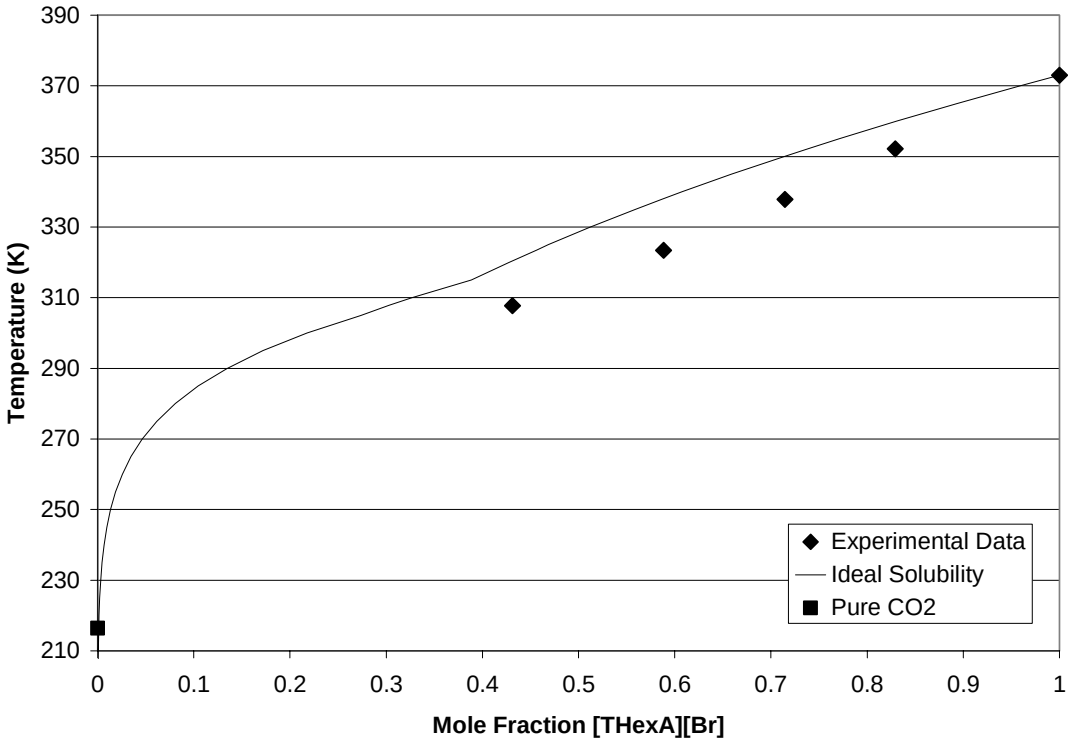

Figure 2-5: T-x diagram for tetrahexyl ammonium bromide-CO_2 with ideal solubility prediction

Negative Deviations from Raoults Law

Both of the ionic liquid systems show a negative deviation from ideality, indicating an activity coefficient less than unity. This is the opposite behavior of what was seen in the naphthalene case. In addition to [TBA][BF_4] and [THexA][Br], this trend was observed for tetraethyl ammonium bistrifluoromethanesulfonimidate [TEA][BTA] and tetrabutyl ammonium bromide [TBA][Br] also. However, data on these ionic solids are not reported here due to lack of repeatability and exceedingly long equilibration times.

In addition to the data from this study, using a single data point from Kazarian (Kazarian, Sakellarios et al. 2002) and the ideal solubility (Gordon and Holbrey 1998) for [C_{16}mim][PF_6], the same calculations were performed and also showed negative deviations from Raoults law (Figure 2-6).

These negative deviations can be attributed to the large size differences between CO_2 and the ionic liquids. This hypothesis is supported by the data of Schiller (Schiller and Gmehling 1992), and of J. E. Blanch (Blanch 1957). Schiller and Gmehling found activity coefficients for acetaldehyde and methyl formate in glycol ethers to be around 0.7. The size difference between these molecules and the glycol ethers are of similar magnitude as the ionic solids with CO_2. Blanch's thesis shows that metal acetylacetonates in benzene and toluene also have negative deviations. Again, these molecules have size differences that are similar to those in this study. In addition to the experimental data, both MOSCED and UNIFAC predict negative deviations for molecules of dissimilar size. For example, MOSCED predicts a solubility of 0.33 for eicosane in liquid ethane at 295 K, when the ideal solubility is 0.27.

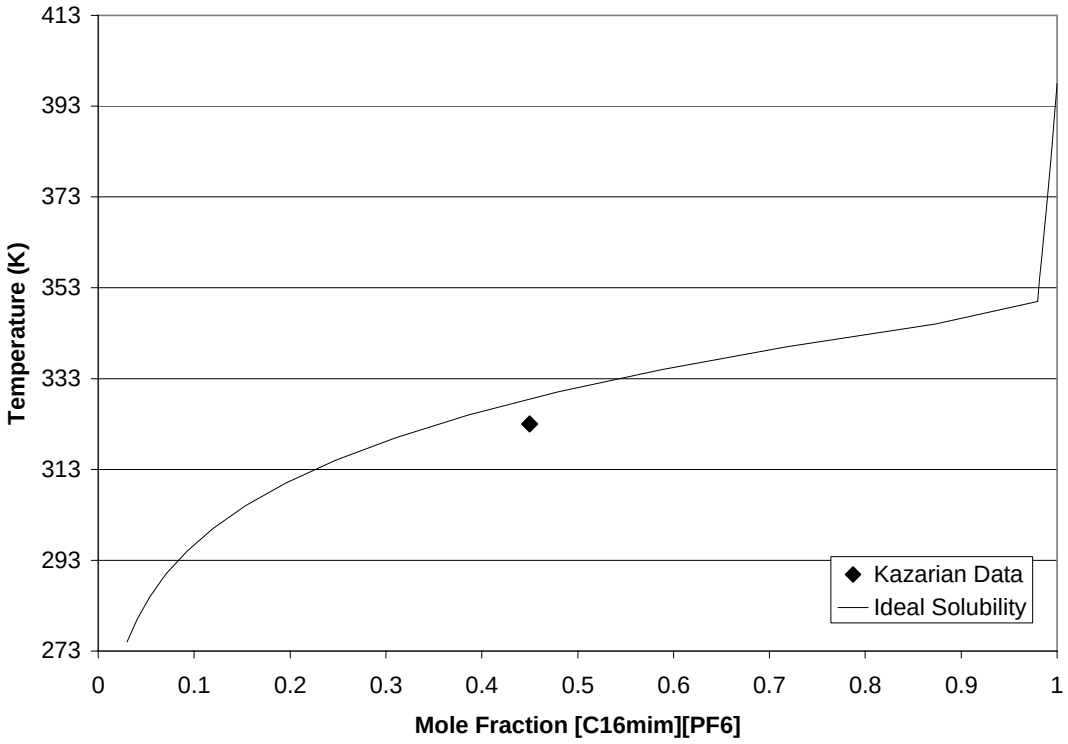

Figure 2-6: Negative deviations from ideal solubility for [C_{16}mim][PF_6]. Data point from Kazarian et al. 2002.

Water Content

It is well known that water content in ionic liquids and solids plays a large role in their behavior. Table 2-2 shows the results of the Karl Fisher analysis for all the ionic solids used here, after secondary purification. While the water content does not appear to effect the melting point depression, an apparent relationship between water content and equilibration time was seen. While [THexA][Br] and [TBA][BF_4] were relatively quick in reaching equilibrium (1-3 hours), the data for both [TBA][Br] and [TEA][BTA] was not reproducible. The long times (up to 24 hours) for equilibration for these solids led to plasticizer leaching from the o-rings, which contaminated the samples. As seen in Table 2-2, these same solids had less water than the faster stabilizing solids.

Table 2-2: Water Analysis of Ionic Solids after Purification.
[a] Only one run due to lack of material.

Ionic Solid	Average Water content (ppm)	Standard Deviation
[TBA][BF_4]	194.5	25.7
[THexA][Br]	426.5	61.2
[TBA][Br]	125.1	33.1
[TEA][BTA]	161.3[a]	-

Conclusions

Our group has developed a unique system for measuring the melting point depression of organic solids, particularly ionic solids. With known concentrations of CO_2 and ionic solid in the high pressure view cell, the contents of the cell are taken to one phase and the first crystal formation is visually observed as the temperature is reduced. With this system, no sampling is necessary, and its accuracy has been proven using naphthalene. The results for [TBA][BF_4] and [THexA][Br] of 130 and 65 degree depressions, respectively, indicate that this system can be used to significantly alter the melting point of ionic solids. This allows for optimal processing and control over system conditions. This work also indicates a negative deviation from ideality for all ionic liquids studied. This can be attributed to the large size difference between solute and solvent, and this theory is in agreement with several data sources in the literature.

Future work in this area would include measuring melting point depression for different types of ionic solids and comparison over different families of cations and anions. Also, a perturbation analysis for a given molecule, such as naphthalene, would provide insight into the molecular interactions of CO_2 induced melting point depression.

This system demonstrates the excellent accuracy and control one can achieve over the melting points of CO_2-IL systems. With this ability, it is possible to not only cater the specific cation and anions to a desired property but now also the melting point of the ionic solid. This significantly increases the possibilities for ionic liquids as solvents. The benefit of being able to accurately move the melting point can have huge advantages in separations for many reaction schemes.

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CHAPTER III

PROTEIN EXTRACTION USING WATER-MISCBILE ALCOHOLS

Aqueous Two-Phase Extraction (ATPE) has been widely studied as a benign separation scheme for proteins and other biomolecules (Walter, Brooks et al. 1985). Several interesting applications include separating human hemoglobin variants (Walter and Saskawa 1981), extracting specific proteins from plant extract (Balasubramaniam, Wilkinson et al. 2003) and delignification of hardwood (Guo, Li et al. 2002). ATPE is done by taking usually a cell broth or cell lysate and adding either, polyethylene glycol (PEG) and salt, or PEG and dextran (Walter, Brooks et al. 1985). The resulting system is biphasic with a selective but small partitioning of the desired protein into the upper PEG-rich phase. Both phases are highly water-rich and therefore create low surface tension between them, necessary for keeping proteins intact. Typically partition coefficients for ATPE range from much less than 1 to 15 (Walter, Brooks et al. 1985). Due to the low partitioning, many steps are needed to extract the protein to any reasonable yield.

There are many drawbacks to the current ATPE system. As mentioned above, the low partitioning requires multiple extraction steps, which are both costly and time-consuming. Also, because the compositions of the phases are so similar, the time for complete phase separation can be quite long (Srinivas, Barhate et al. 2000; Huenupi, Gomez et al. 1999), and the kinetics quite complex (Asenjo, Mistry, et al. 2002). Furthermore, once the protein has been extracted to the PEG phase, a new problem arises

of how to purify the protein from the PEG (Waziri, Abu-Sharkh et al. 2004). Due to these reasons, the ATPE system has yet to be used industrially. The other major barrier to commercialization is that modeling of the system is inherently complicated. Many have tried to model different systems (Jiang and Prausnitz 2000; Cabezas, Evans et al. 1989; Soares, Teixeira et al. 2003), but without a full understanding of protein folding and molecular interactions within proteins, this task is quite difficult.

One way to alleviate the problems associated with ATPE is to replace the PEG with organic solvent. In the 1950s, "counter-current distribution" was used to separate proteins with either a butanol-water or an ethanol-aqueous salt system (Morris and Morris 1976). These systems faced problems such as protein denaturation and precipitation, and with the advent of ATPE in 1958, organic solvents were nearly forgotten. Currently, organic solvents are used with a few enzymatic systems that have been thoroughly studied and known to be safe for the enzymes. However, with the introduction of protein design (Arnold 1988), fusion partners (Skosyrev, Rudenko et al. 2003), protein stabilization methods (Manning, Matsurra et al. 1995), and understanding of solvent compatibility (Toba and Merz 1997) in more recent years, the doors have been opened again for the use of organic solvents.

With a better understanding of phase behavior and protein interactions, this study was done to demonstrate the possibility of using organic solvents to extract proteins. Solvents such as tert-butanol, trifluoroethanol (TFE), and hexafluoroisopropanol (HFIP) all form two-phase systems with water upon addition of salt. The corresponding phases are of similar composition, giving the same benefits as ATPE, but without the additional downstream purification of protein from polymer. The two phases are similar enough to

be safe for the protein, yet different enough to greatly decrease the phase equilibration time. In addition, much of the phase behavior for these solvent systems has already been studied, thus making modeling easier.

Experimental Methods

Materials

With use of the Bradford Assay (Bradford 1976), any number of proteins can be studied with spectroscopy. For this study, the requirements were that the proteins be large, quite stable to organic solvents, and also be applicable to many areas of biology. Therefore, lysozyme and bovine serum albumin (BSA) were chosen to study. Lysozyme has been studied in many applications due to its inexpensiveness and ease of use, and both lysozyme and BSA have been widely used in ATPE experiments (Walter, Brooks et al. 1985). Lysozyme was purchased in lyophilized powder form at a concentration of 50,000 units per mg from Sigma. Bovine Serum Albumin was also purchased in lyophilized form at 99% purity from Sigma.

The solvents chosen for this study include tert-butanol (Sigma, 99%), 2,2,2-trifluoroethanol (TFE) (Acros Organics, 99.8%), and 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) (Aldrich, 99+%). These solvents have been used with proteins in the past and demonstrate smaller destructive effects than other organic solvents.

Experimental Procedure

All protein solutions were prepared in a 90mM HEPES buffer, and adjusted to pH 7. HEPES sodium salt, 99%, was purchased from Acros and dissolved into HPLC water

from Sigma-Aldrich. Calibrations were made for concentrations of 0, 0.087, 0.43, 0.870, and 1.30 milligrams per milliliter. The calibration standards were analyzed by the Bradford assay (Bradford 1976) with an incubation time of 12 minutes. Absorbance measurements were taken at 595 nm and corrected with a blank of 0 mg/mL protein. The calibration curves for BSA and lysozyme can be seen in Figures 3-1 and 3-2.

For the initial experiments, t-butanol was the extractant of choice for having benign characteristics and good separation. A sample of 1.5mg BSA/mL HEPES solution was made and 1mL of this solution was added to 2mL water and 3mL t-butanol. Because the initial mixture is single phase, 0.3038g sodium chloride (0.87 M NaCl) was added to induce a phase split. The samples were then allowed to equilibrate for 1 hour. A sample of 0.1mL of each phase was then taken and combined with 3mL Bradford reagent and allowed to incubate for 12 minutes. After incubation, the solutions were transferred to 3mL quartz spectrometer cells and the absorbance measured using a Hewlett-Packard Spectrophotometer model 8452A, at 595 nm. After initial testing of the phases, another set of samples were taken 45 minutes later to ensure complete phase separation.

Results and Discussion

Figures 3-1 and 3-2 are the calibration curves for BSA and lysozyme, respectively. R^2 values are 0.9992 for BSA and 0.9981 for lysozyme.

As seen in Table 3-1, the concentrations for BSA changed only negligibly between 60 and 105 minutes of equilibration time. This was therefore interpreted to be at equilibrium. The partition coefficient K was calculated using Equation 3-1.

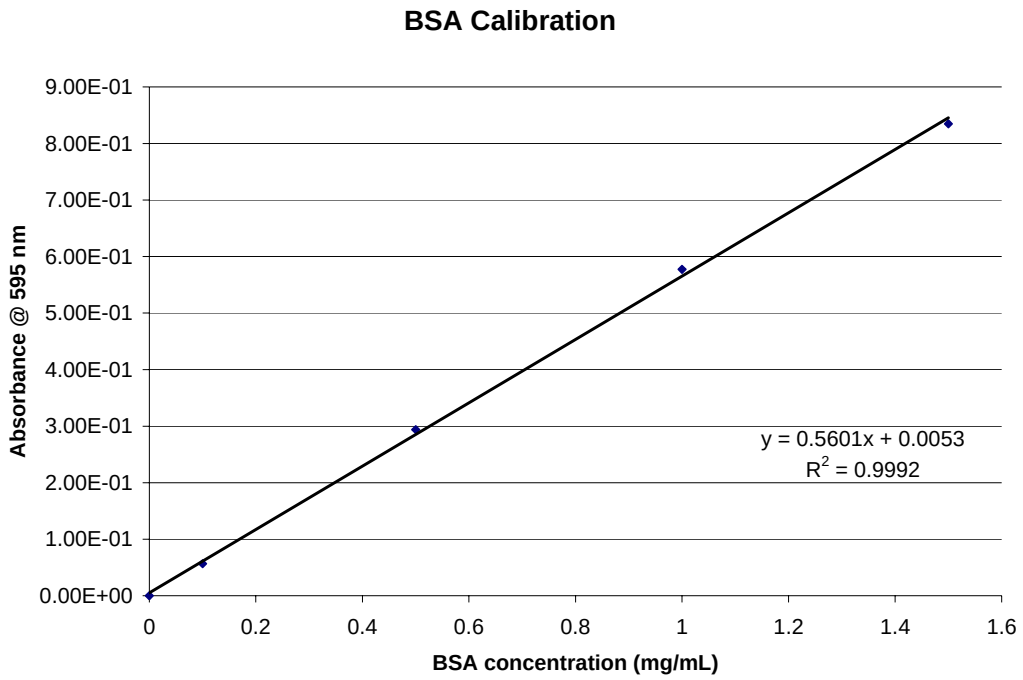

Figure 3-1: Calibration curve for Bovine Serum Albumin in HEPES buffer, pH 7.

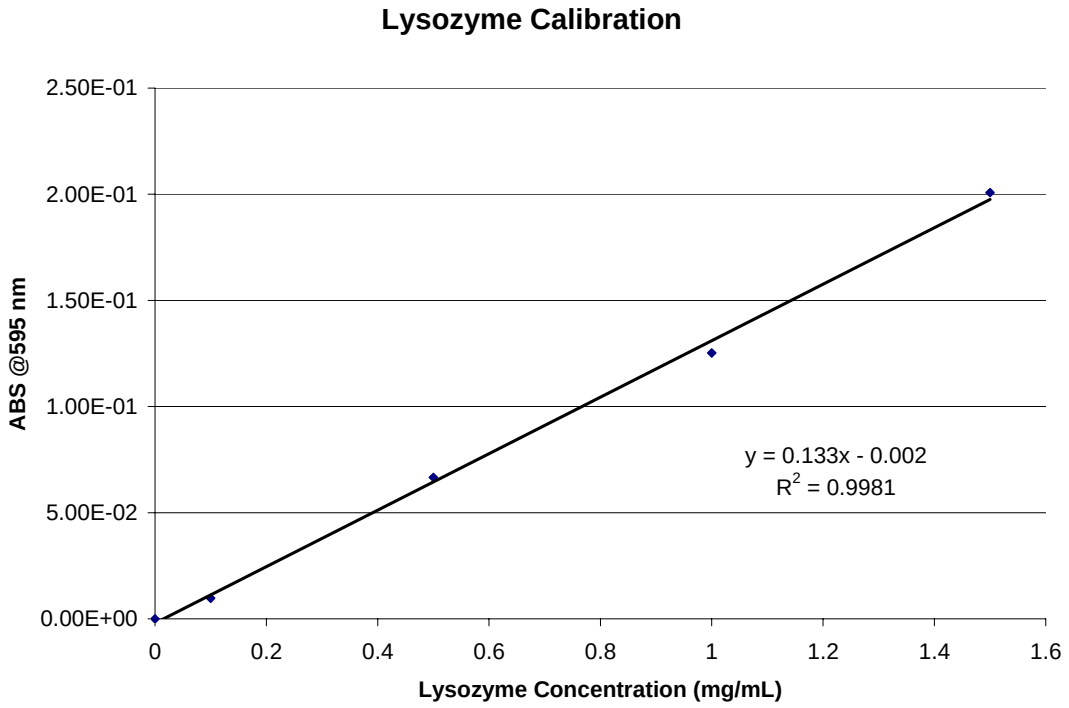

Figure 3-2: Calibration curve for Lysozyme in HEPES buffer, pH 7.

Table 3-1: Partitioning Results for BSA and Lysozyme in Tert-Butanol

Protein/phase	Equilibration Time (mins)	Absorbance (AU)	Conc. (mg/mL)	K (org/aq) experimental	K (org/aq) ATPE
BSA top	60	0.0743	0.123212	0.207805	0.0723[a]
BSA bottom	60	0.0337	0.592921		
BSA top	105	0.0761	0.126401	0.205413	0.0723[a]
BSA bottom	105	0.0350	0.615348		
Lysozyme top	60	0.0570	0.443835	0.747783	0.5[b]
Lyso. bottom	60	0.0769	0.593534		

[a] calculated from Haghtalab, Mokhtarani et al 2003, at pH 9.1 and 298K with PEG 15000 and K_2HPO_4

[b] from Haghtalab, Mokhtarani et al 2003, at pH 6.7 and 298K with PEG 4000 and 7% Na_2SO_4

$$K = \frac{protein\ conc._{organic}}{protein\ conc._{aqueous}}$$

Equation 3-1: Partition Coefficient K.

For BSA, K was found to be roughly 0.21. As seen in the chart, these values are better than those in the literature for BSA in aqueous two phase extraction. Lysozyme partitions more favorably than BSA into the organic phase, with a partition coefficient of 0.75. This value is also better than the literature value for aqueous two phase extraction under similar conditions (Haghtalab, Mokhtarani et al 2003). With optimization of this system, it should be possible to get a better separation than with ATPE, and much easier separation of protein from the organic phase.

In order to continue with this study, a test was needed for determining the state of the protein after extraction. It is well known that many organic solvents tend to denature proteins, and also that proteins partition differently upon denaturation (Jiang and Prausnitz 2000). The company Molecular Probes, sells the EnzChek Lysozyme Assay Kit (E-22013) which determines the activity of lysozyme based on a fluorescing reaction. With this assay it would be easy to determine to what degree, if any, the lysozyme has denatured. Therefore, the study was continued with lysozyme as the primary protein.

Upon further review of the literature, it was noted that the Bradford reagent was not necessary to quantify lysozyme. Lysozyme has a large number of aromatic amino acids, particularly trytophan and tyrosine (Perkins, Johnson et al. 1997), arranged such that is has a unique UV signature. Because the Bradford assay is only effective for an hour, not using the assay allowed for an overnight equilibration study to determine the exact amount of time needed for complete phase separation. This was done by taking 1.5mL TFE and 1.5mL of a 1.5mg/mL Lysozyme-HEPES solution and combining them with 0.0474 g of NaCl to induce a phase split. The mixture was shaken and poured into a 3mL quartz spectroscopy cell, where UV data was taken at 274 nm and 800 nm overnight. As seen in Figure 3-3, the system reached equilibrium in about 250 seconds. The slight jumps and oscillations in the data can be attributed to the building heating system and fluctuations in the room temperature, as the system was not temperature-controlled.

To ensure that measuring UV data for lysozyme without the Bradford assay was indeed accurate, extractions were done in the same manner as mentioned above for TFE, HFIP, and twice more with t-butanol. The experiments were run exactly as mentioned above, except without the Bradford assay. Results are shown in Figure 3-4.

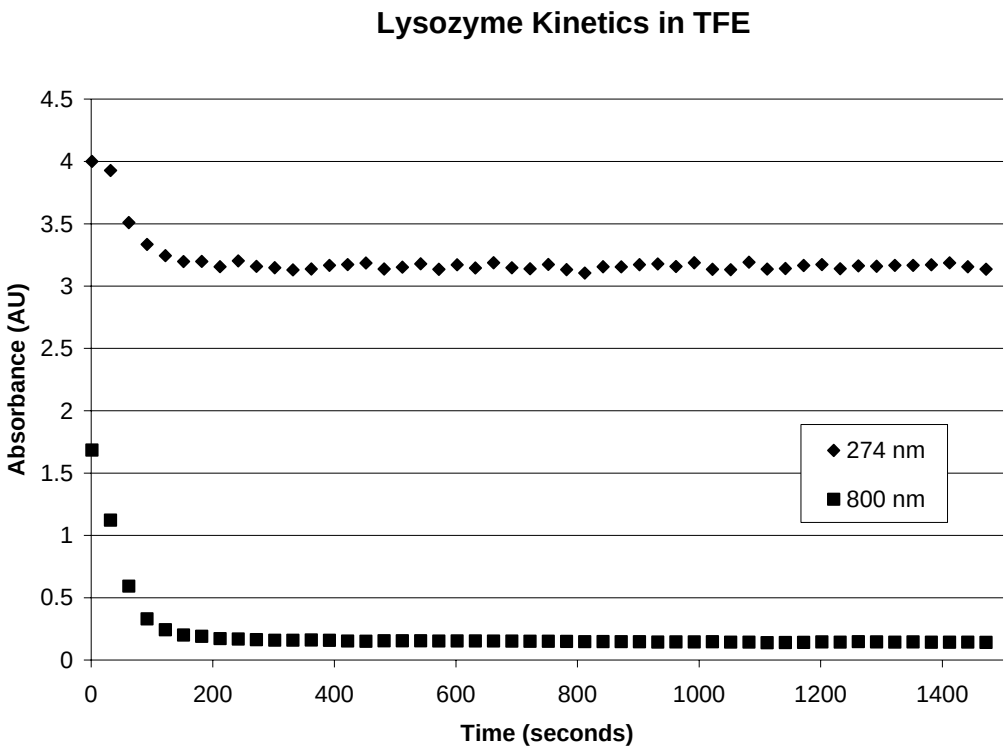

Figure 3-3: Equilibration time study for lysozyme in trifluoroethanol.

As seen in Figure 3-4, the partition coefficients without the Bradford assay are much smaller than with the Bradford assay. This seems illogical since the assay was done on samples removed from each phase after partitioning was complete. More testing is needed to determine the cause of this discrepancy.

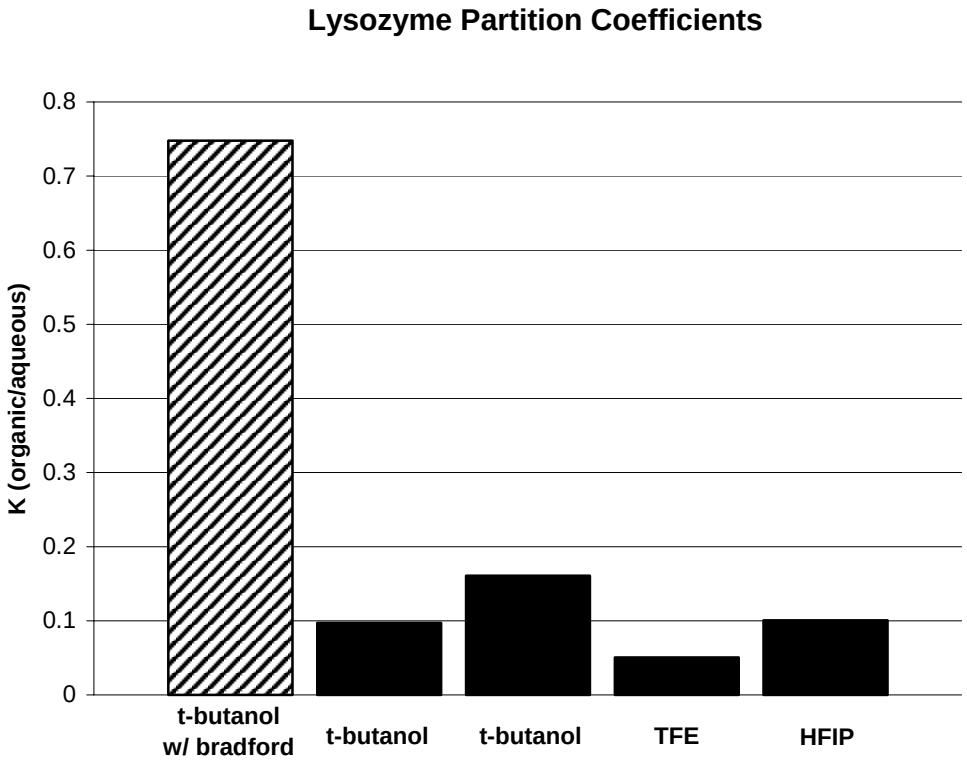

Figure 3-4: Lysozyme partition coefficients in organic solvents without the Bradford assay, as compared to the previous data with the Bradford assay.

Conclusions

The first attempts to extract both bovine serum albumin and lysozyme with tert-butanol resulted in very reasonable values. The respective partition coefficients, K, were 0.21 and 0.7, which very closely matched the literature values for ATPE. Also, due to the unique structure of lysozyme, it has a UV signature which can be quantitatively measured with spectroscopy (Perkins, Johnson et al 1997). An attempt to compare extractions of lysozyme with and without the Bradford assay shows a higher partition coefficient with the assay, K = 0.7 vs. 0.12. There appears to be no explanation for this, and further study of this phenomenon is needed to fully understand these results.

Overall, the preliminary results of the new system suggest that organic solvents could replace the ATPE system for more facile separation. Before any further testing is done though, the lysozyme activity assay is needed to determine if these solvents are denaturing the protein. If they are not denaturing, this system shows much promise and should be further developed. This particular system lends itself very well to a statistical design of experiments, with partition coefficient K and lysozyme activity being the response variables. Once optimal conditions are found, this system should be tested on cell broth and cell lysate to test the system under true conditions. Another goal would be to optimize extraction selectivity to determine which conditions would be necessary to extract a certain protein over other proteins in the cell broth.

With optimization of this system an even more effective separation is likely than what was seen in this study. If however, that is not the case and the partitioning does not improve, the system is still successful because it has eliminated the problems prohibiting ATPE from being industrialized. The water-miscible solvents provide similar enough

phase composition to be safe for the proteins, but differ enough for a quick separation time. Additionally, phase behavior of organic solvents with water is much better understood than the PEG is, so modeling of these systems would be easier. Also, in ATPE the proteins are extracted into a PEG phase which creates an even more difficult protein purification problem. In our system, the proteins are partitioned into an organic solvent which can easily be evaporated for purification, allowing for easy use on a large scale.

The success of this project means a cheaper, cleaner, and better controlled unit operation for protein purification. By eliminating all the problem factors that prohibit ATPE from being scaled up and used in industry, we are effectively taking the benefits of ATPE and combining them with the benefits of simple organic extraction. Protein purification is one of the largest expenses in pharmaceutical production and the success of this system would greatly reduce those costs, thus having a huge impact on the pharmaceutical industry.

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CHAPTER IV

PHARMACEUTICAL CATALYST PREPARATION FOR USE WITH ORGANIC-AQUEOUS TUNABLE SOLVENTS

Louis Pasteur was the first person to discover chirality when he noted that two tartaric acid molecules were identical in every way except for optical rotation (Blaser, Spindler, et al. 2001). He also recognized that many biological systems can produce enantiomerically pure compounds, but to produce them artificially a chiral agent must be introduced. Often, two enantiomers can behave in completely different ways and the differences can be critical. Enantiomeric purity in drug development became a large issue with the Thalidomide tragedy in which one enantiomer of the drug was therapeutic, while the other was toxic (Franklin 1962). Currently, modern pharmaceutical manufacturers can achieve high enantiomeric purity with the use of chiral catalysts. However, these catalysts are typically a huge expense in drug production and are often used inefficiently. The most efficient catalysis comes from running the reaction homogeneously; however this creates problems during separation. Heterogeneous catalysis, on the other hand, allows for facile separation but lower efficiency. To combat these problems, our group has developed a system called OATS: Organic Aqueous Tunable Solvents (Lu, Lazzaroni, et al. 2004).

This system was designed for combining homogeneous reaction with heterogeneous separation, thus allowing optimal reaction rates, coupled with easy recycle

and reuse of these expensive catalysts. In this technique, an organic cosolvent is added to the catalyst and reactants until they become single-phase, so the reaction can be run homogenously. When pressurized CO_2 is added the solvent and water will separate into two phases; the water phase containing the catalyst and the organic phase containing the products. The catalyst can then be recycled and its performance quantified with each recycle (Figure 4-1).

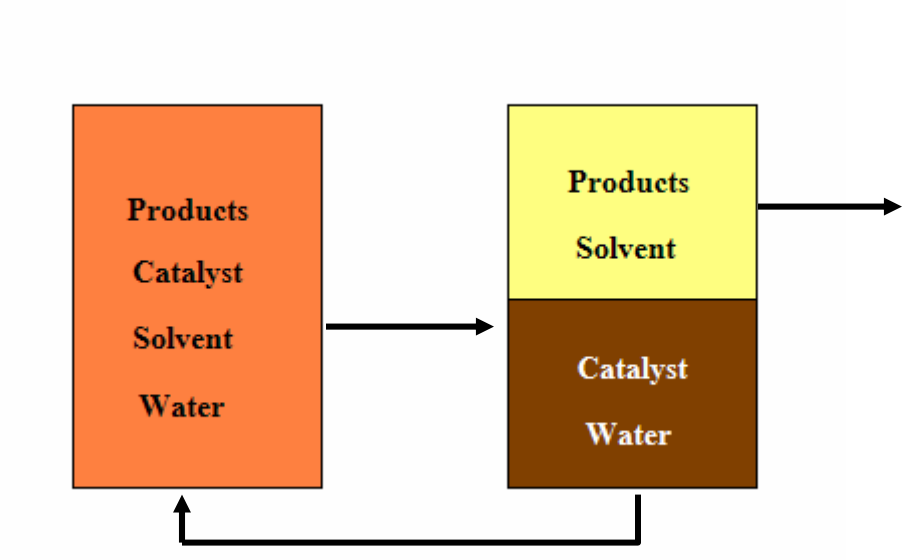

Figure 4-1: OATS system for catalyst recycle.

The specific objectives of this research included: 1) synthesis of a new water-soluble catalyst and 2) comparing yields, enantiomeric excesses, and turnover numbers for the new catalyst versus the current catalyst. When the new catalyst is determined to be as efficient as the current catalyst, the project will focus on development and optimization of CO_2-based recycling of the catalyst.

The catalyst chosen for this project is optically pure Mn-Salen, or Jacobsen's catalyst (Jacobsen, Zhang, et al. 1991), and is pictured in Figure 4-2. For our purposes, the Mn-Salen was made water-soluble via modification of two t-butyl groups (Figure 4-3). Several others have also made water-soluble versions of Mn-Salen, including Haikarainen (Haikarainen, Sipila, et al. 2001a; Haikarainen, Sipila, et al. 2001b), and Kureshy (Kureshy, Khan, et al. 2002). Mn-Salen is a highly enantioselective catalyst and has been used to produce the C-13 side chain of Taxol, an anti-leukemia drug (Deng and Jacobsen 1992), and indene oxide, a precursor to Indinavir (Figure 4-4), an HIV protease inhibitor (Senanayake, Smith, et al. 1996).

Mn-Salen has been thoroughly studied as an epoxidation catalyst. Tsutomu Katsuki wrote an excellent review of asymmetric oxidations using Mn(III) Salen complexes (Katsuki 1995). In it, he reviewed salen design, reactions, mechanisms, and other factors in the oxidation reactions. The tuning of enantioselectivity of Salen through electronic effects of the ligands was reported by Jacobsen (Jacobsen, Zhang, et al. 1991a) and more thoroughly by Palucki (Palucki, Finney, et al. 1998). Many modifications have been made to the Mn-Salen catalyst, mainly to increase steric hindrance, as demonstrated by Ahn (Ahn, Park, et al. 2001) and Sasaki (Sasaki, Irie, et al. 1994). Ana Silva performed a systematic analysis of oxygen source on various Mn-Salen complexes through the epoxidation of styrene (Silva, Freire, et al. 2004). With the many different versions of Mn-Salen and many alkenes studied for Salen epoxidation, it was difficult to directly compare our specific system to the others. Many studies used the same catalyst, but a different substrate or reaction conditions (Jacobsen, Zhang, et al. 1991b; Pietikainen 1994; Pietikainen 2001). Others used the same conditions, but a slightly different version

of Salen (Larrow and Jacobsen 1994; Pietikainen 1998; Ahn, Park, et al. 2001). The latter study by Ahn and Park matched our conditions most closely and was therefore chosen for comparison.

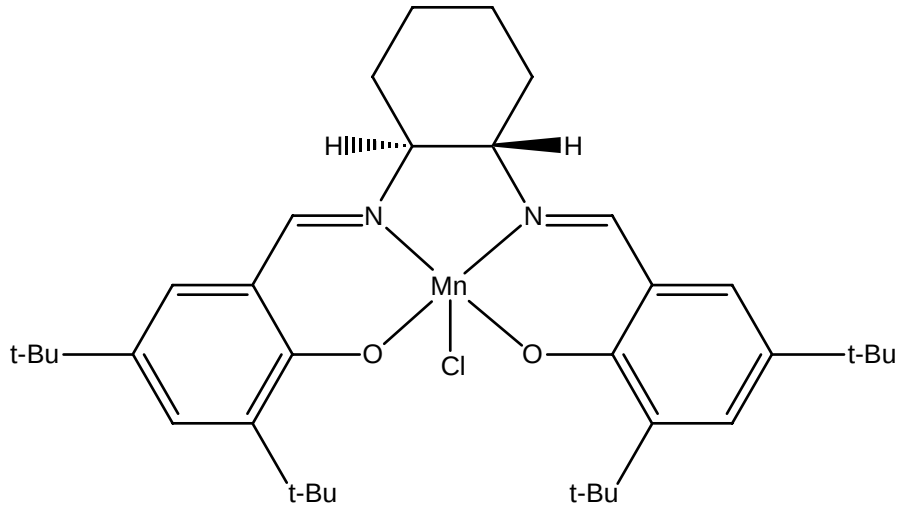

Figure 4-2: (S,S)-Mn-Salen

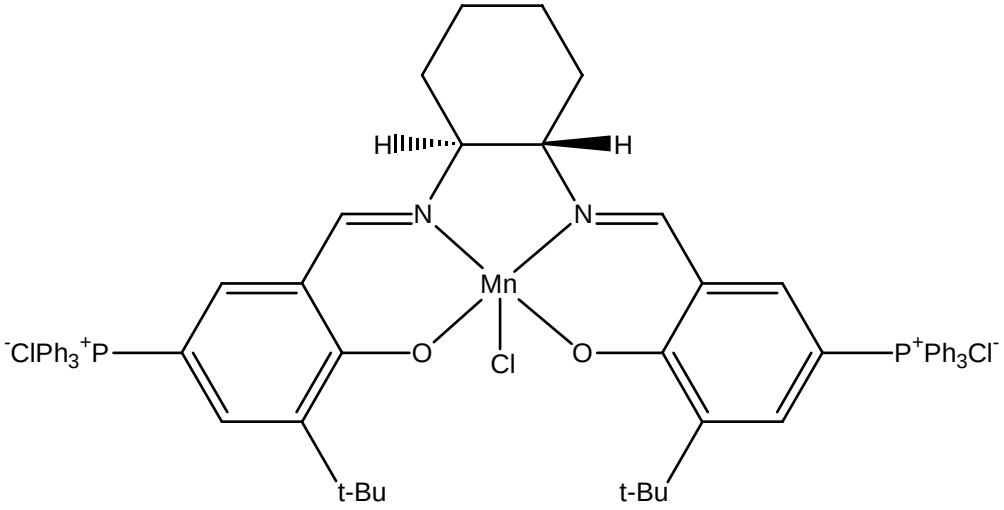

Figure 4-3: Water Soluble (S,S)-Mn-Salen

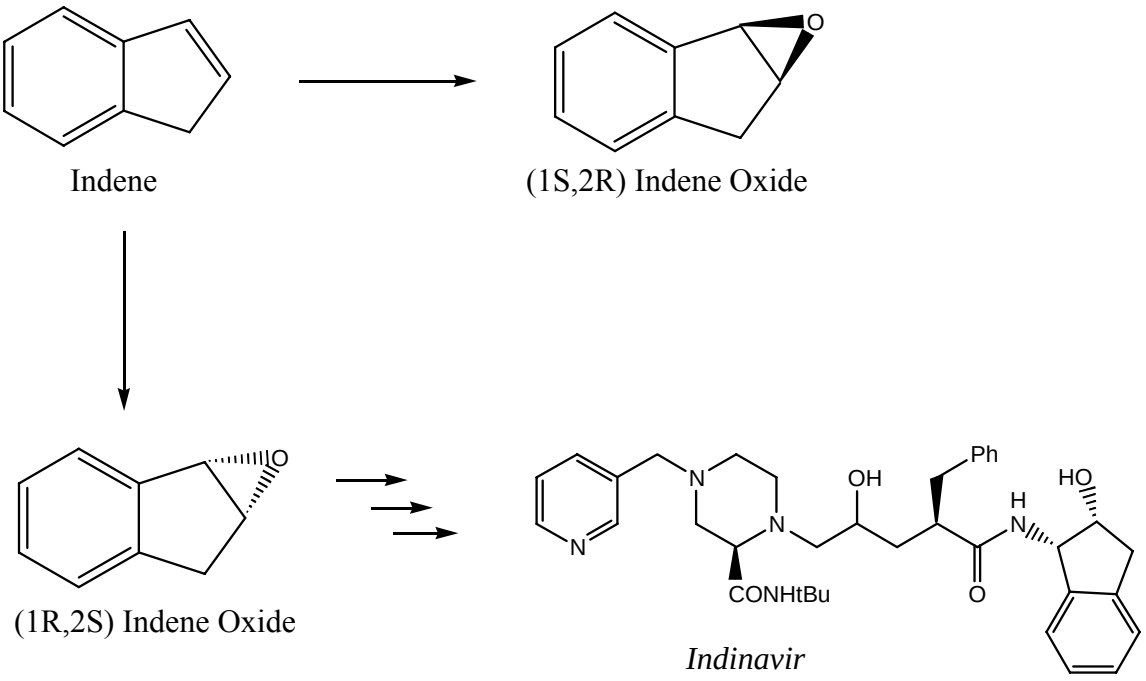

Figure 4-4: Asymmetric epoxidation of indene as a precursor to HIV protease inhibitor, *Indinavir* (Senanayake, Smith, et al. 1996).

Experimental Methods

Materials

(S,S)-(+)-N,N'-Bis(3,5-di-tert-butyl salicylidene)-1,2-cyclohexanediaminomanganese (III) chloride (Salen), also known as Jacobsen's catalyst, was purchased from Aldrich with a purity of 95%. Indene was purchased from Sigma at a purity of 98% and stored at 4°C. Sodium Hypochlorite (NaOCl) was obtained from Clorox in a solution with 6% available chlorine, or 0.8054 M NaOCl. Sodium chloride (NaCl) and sodium sulfate (Na_2SO_4) both came from Fisher with purities of >99%. The axial ligand 4-(3-phenylpropyl)pyridine N-oxide (P_3NO) was purchased from Aldrich with a purity of 96%.

Synthesis of Water-Soluble Salen

Synthesis of the water-soluble version of Mn-Salen (ws salen) was done according to the method of Haikarainen et al. (Haikarainen, Sipila, et al. 2001a). The ligand, N,N'-bis{3-tert-butyl-5-[{triphenylphosphonium)-methyl]salicylidene}-1,2-cyclohexanediamine chloride, was prepared by first reacting 2-tert-butylphenol with $(CH_2O)_n$ in the presence of concentrated hydrochloric acid. The intermediate was then reacted with PPh_3 to yield the corresponding salt. This aldehyde was then condensed with 1 equivalent of enantiomerically pure (R,R)-1,2-diaminocylcohexane. The ligand was finally complexed with Mn by refluxing with an excess of $[Mn(OAc)_2]$ in ethanol for 2-3 hours, and then treated with LiCl. The final product was characterized by ^1H and ^{13}C NMR and was in agreement with the reported literature values.

Experimental Procedure

The procedure for the indene epoxidation was taken from Jacobsen (Jacobsen, Zhang, et al. 1991b). 25mL of the Clorox bleach solution was diluted to 0.55M NaOCl with 0.05M Na_2HPO_4. The pH was then adjusted to 11.3 with 1M NaOH. Next, 26mL of the diluted NaOCl solution were added to a 250mL round bottom flask and chilled in an ice bath. Once chilled, 3g indene and 0.02 equivalents of salen (0.3281g) were added to the flask, along with 26mL methylene chloride. A condenser was used to eliminate evaporation and the two-phase reaction mixture was mixed using a stir bar. The reaction was run for 6 hours and ice was replaced as needed. Initially, the progress of the reaction was checked by sampling the brown organic layer every 2 hours which was then analyzed using thin layer chromatography (TLC).

Once the reaction was complete, another 26mL of methylene chloride were added to quench the reaction. The phases were allowed to separate and the clear aqueous NaOCl phase was discarded. The remaining brown organic phase was next washed 3 times with 50mL HPLC water, and twice more with 50mL saturated NaCl solution, each time discarding the aqueous phase. The crude product was analyzed once more by TLC and then dried overnight with Na_2SO_4. After the product was dried, it was then filtered and the methylene chloride evaporated, leaving a viscous yellow-orange liquid.

Analysis

Thin layer chromatography (TLC) was used throughout the reaction to qualitatively monitor the disappearance of indene and the appearance of indene epoxide. After the product was purified and the solvent evaporated, the sample was tested via ^1H

NMR for yield. Indene, ^{1}H, δ (d-$CdCl_3$): (m, 7.01-7.18, 4H, Ar.), (s, 3.43, 2H, CH_2), (d, 6.5, 1H, CH), (d, 6.9, 1H, CH). Indene Oxide, ^{1}H, δ (d-$CdCl_3$): (m, 7.2-7.7, 4H, Ar.), (q, 3.1, 2H, CH_2), (s, 4.2, 1H, CH), (s, 4.3, 1H, CH). Next, to determine the enantiomeric yield of the indene epoxide, the samples were run on a chiral HPLC column. The column is a Chiralcel OD-RH from Daicel Chemical Industries, Ltd. The method used a flowrate of 0.3 mL/min for 30 minutes at 30^{o}C. The mobile phase was acetonitrile/water with a gradient of 95% acetonitrile/5% water to 5% acetonitrile/95% water. UV data was taken at 220 and 255 nm.

To obtain an isolated yield, the sample was run on a silica chromatography column. The column was prepared with hexane, and the mobile phase was composed of 95% hexane, 5% ethyl acetate, and a few drops of triethylamine to prohibit opening of the epoxide. TLC was done on all samples collected to determine the fractions containing indene epoxide. The final fractions were then dried and NMR taken to confirm the product.

Results and Discussion

After doing 5 experiments with Jacobsen's procedure (Jacobsen, Zhang, et al. 1991b)), the average yield of indene oxide was 69.0% with a standard deviation of 6.2%. The enantiomeric excess (ee), was measured for 3 of these experiments, and was an average of 38.9% and a standard deviation of 8.5%. The equation for ee is shown below.

$$ee = \frac{(A-B)}{(A+B)} \times 100, \text{ where } A \text{ and } B \text{ are enantiomers}$$

Equation 4-1: Enantiomeric Excess (ee)

The yields here were only slightly less than in the literature (~10% less), however, the ee values were significantly lower (~50%). Figures 4-5 and 4-6 demonstrate a comparison of experimental values with literature vales for the indene epoxide yield and ee, respectively.

Improving Yield and Enantiomeric Excess

One method for increasing yield in a biphasic reaction is to use a phase transfer catalyst (PTC). For the indene epoxidation reaction, using additional co-catalysts with Mn-Salen has shown to increase PTC properties (Palucki, McCormick, et al. 1995; Senanayake, Smith, et al. 1996; Pietikainen 1998). Co-catalysts such as N-methylmorpholine-N-oxide (NMO), 4-phenylpyridine-N-oxide (4-PPNO), and 4-(3-phenylpropyl)pyridine-N-oxide (P_3NO), act as axial ligands, held perpendicular to the Salen molecular plane. The proposed mechanism for this interaction suggests that the ligand helps to transfer the oxygenated Salen across the phase boundary, thus facilitating the epoxidation of the indene in the organic phase, and also stabilizing the Salen molecule.

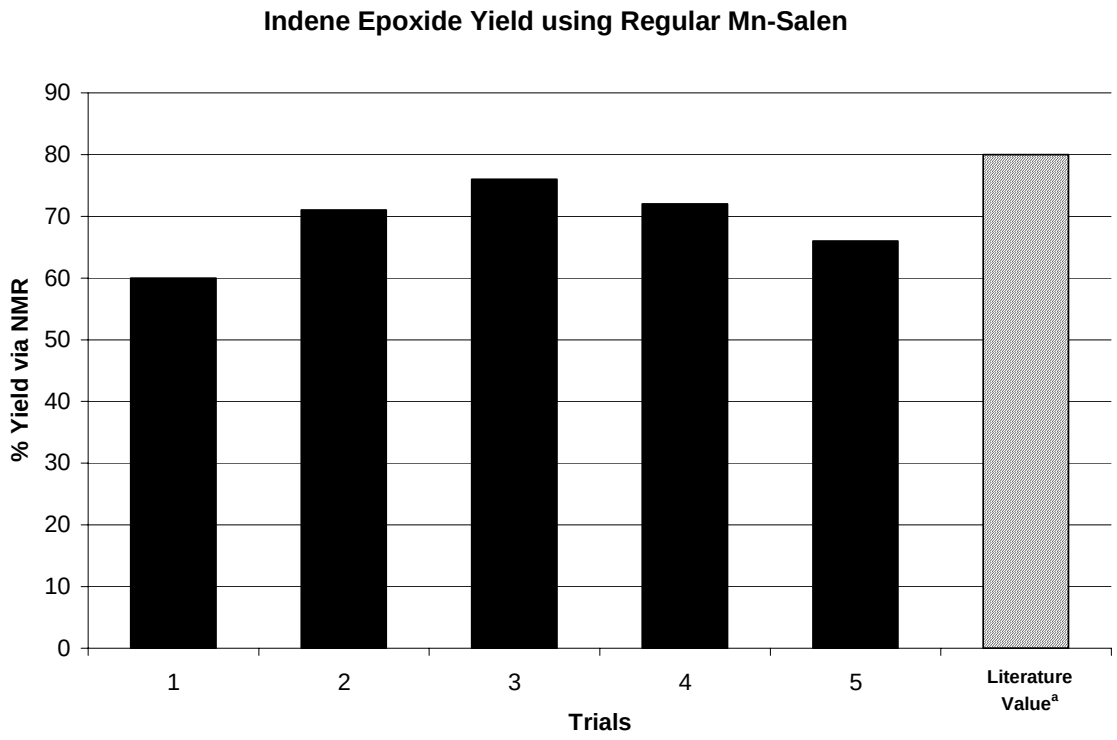

Figure 4-5: Comparison of indene epoxide yield between this study and literature values. [a]Data from Ahn, Park, et al. 2001.

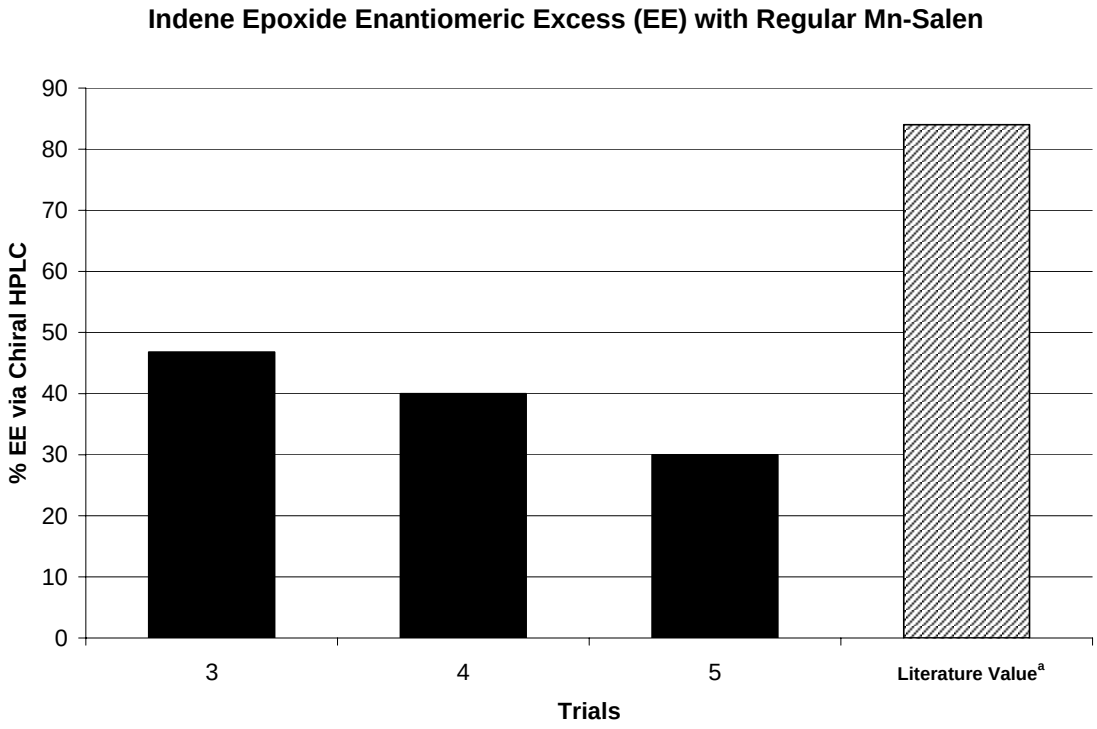

Figure 4-6: Comparison of indene epoxide enantiomeric excess between this study and the literature values.
[a]Data from Ahn, Park, et al. 2001.

In addition to the axial ligand, three other improvements were made to this reaction. It is known that ee is often increased with a decrease in temperature. Therefore, a salt-ice bath was used instead of the ice bath. Without the NaCl, the ice bath varied between 0 and 5^{o}C. With the salt-ice bath, the temperature varied between -4 and 0^{o}C, providing a lower average temperature. It is also well known that mass transfer is the limiting factor in most biphasic reactions. To increase the mass transfer, the round bottom flask was replaced with a Morton flask, and the stir bar was replaced with an

overhead mechanical stirrer. Finally, a nitrogen atmosphere was used instead of air to eliminate excess water and possible side reactions caused by it.

The next reaction was run with all of the above adjustments. P_3NO was added to the reaction mixture at 0.03 equivalents to indene. A NaCl-ice bath was used, as well as the new Morton flask, and mechanical stirring at 600 rpm. The reaction was also run under nitrogen atmosphere, and the method was adjusted slightly to that of Hughes (Hughes, Smith, et al. 1997).

The new adjustments gave an incredible increase in both yield and ee. The yield for the P_3NO reaction was 100%, as indene epoxide was the only thing detected via NMR. The ee rose to 88%. Both of these values were higher than as reported in the literature (Senanayake, Smith, et al. 1996). Despite the excellent results, the axial ligand created an emulsion which was near impossible to separate. Also, because the overall scheme of this project was to run this reaction homogeneously anyway, the use of P_3NO was not continued.

In order to decouple the P_3NO effects from the lower temperature and increased stirring effects, another reaction was run with Jacobsen's method (Jacobsen, Zhang, et al. 1991b), but with the salt-ice bath, mechanical stirring, and a nitrogen atmosphere. The results are seen in Figure 4-7. The yield does not appear to change at all in this case (less than 1%). Most noticeable, however, is the increase in ee from 39% to 85%, with only roughly a 7^oC difference in temperature. This suggests that temperature is a huge factor in this asymmetric reaction.

The same reactions were also run for water-soluble Salen (WS Salen). Only one run was done for the 3^oC and stir bar method, and results can be seen in Figure 4-8. For

WS Salen, the ee jumps from 47% to 86%, similar to that of regular Salen. However, the yield decreases from 89% to 73%. It is difficult to suggest why this might have happened, but one possible cause could be that the mechanical stirring somehow destroyed the WS Salen, seeing that it is such a large and sterically strained molecule (Figure 4-3).

Figure 4-9 shows a comparison for regular Salen and WS Salen (both at the lower temperature and greater mixing) and data from the literature for yield and ee of indene epoxide. In general, the yield is slightly lower than what is reported in the literature, but still within reason, and the ee is the same as is reported, if not better. With this proven, it would be appropriate to continue to the next step of running the reaction with WS Salen under homogeneous conditions.

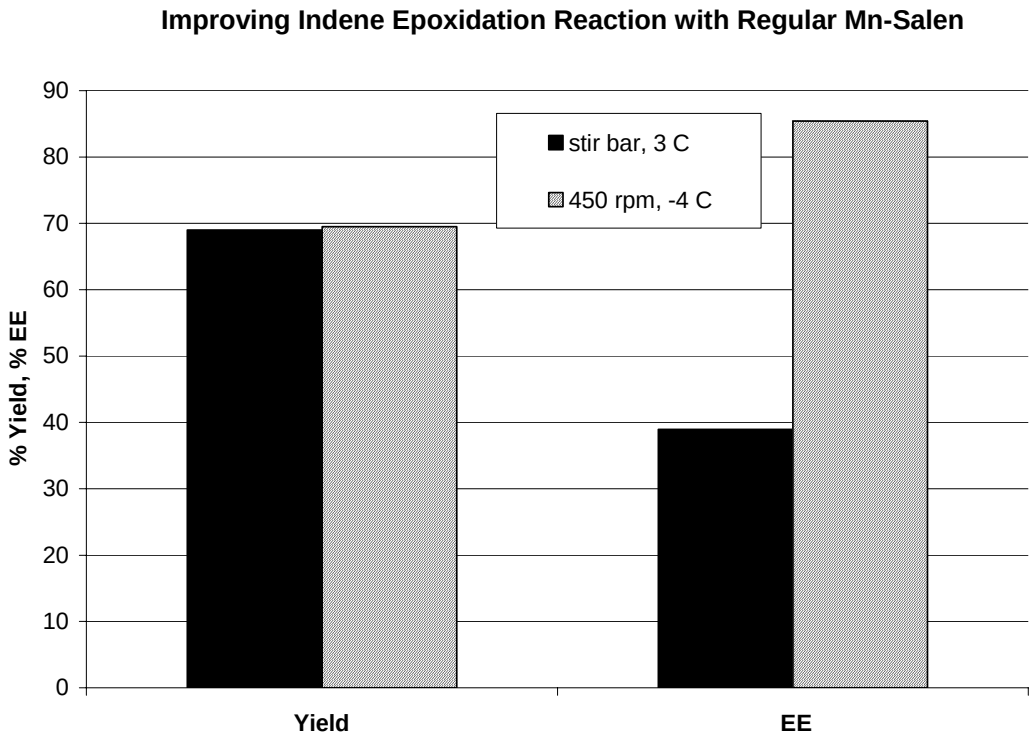

Figure 4-7: Comparison of Temperature and Mass Transfer Effects on Indene Epoxide Yield and Enantiomeric Excess.

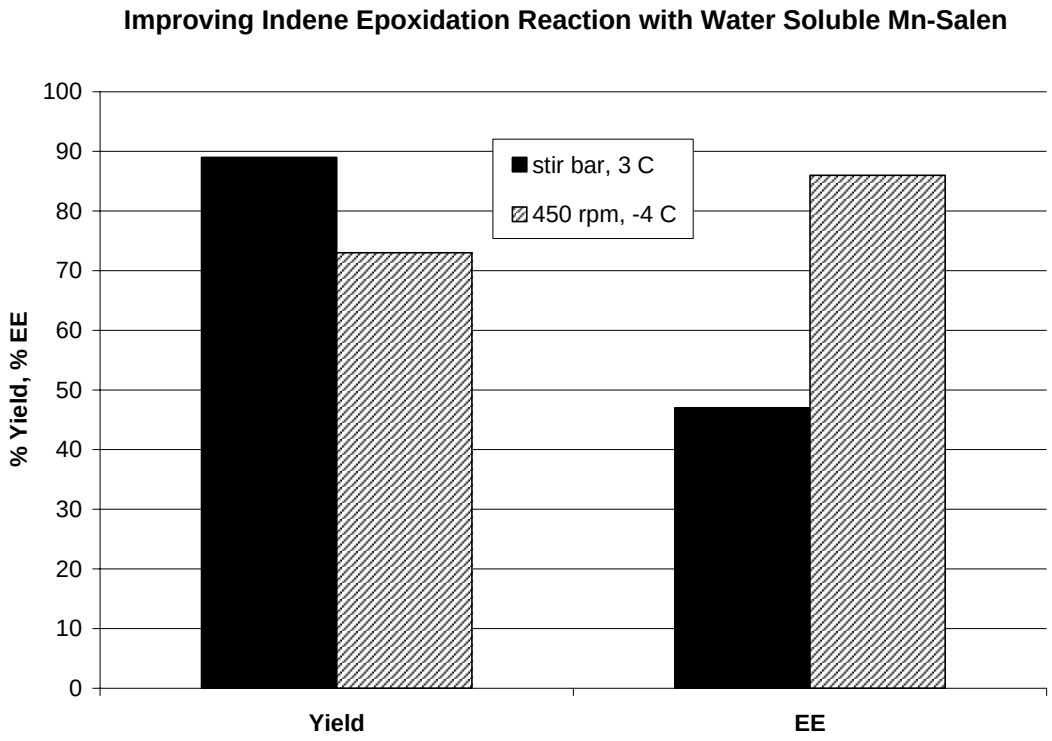

Figure 4-8: Comparison of Temperature and Mass Transfer Effects on Indene Epoxide Yield and Enantiomeric Excess with Water-soluble Salen.

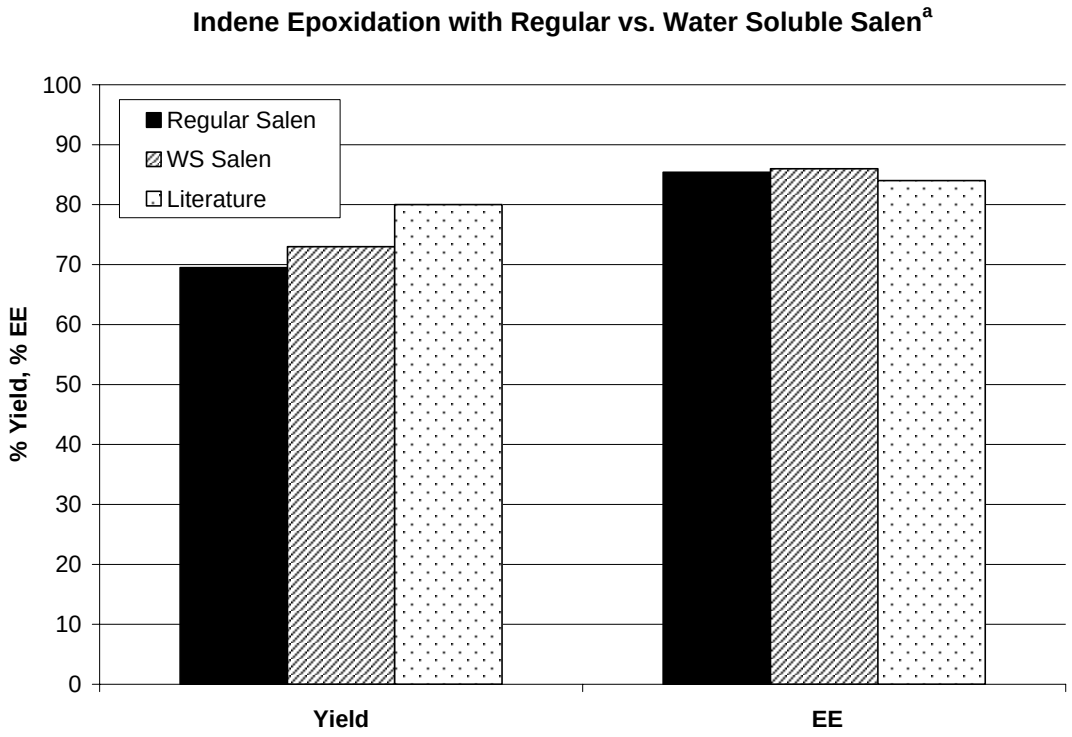

Figure 4-9: Comparison of Yield and EE for Salen and WS Salen to the literature.
[a] Literature data taken from Ahn, Park, et al. 2001, with slightly altered Mn-Salen.

Indene Oxide Stability

Due to the length of time for reaction, work-up, purification, and analysis, it was necessary to test the stability of the product over time. Epoxides are especially susceptible to breaking open and forming diols. In order to test for stability, the final product was tested nearly every day for over a week. The sample was rerun through the chiral column to determine, 1) if the epoxide was opening, and 2) if the ee was changing at all. This was calculated by monitoring the disappearance of product (indene oxide), and any change in peak ratio of the enantiomers. Results are shown in Figure 4-10. Over the course of 9 days, it appears that indene oxide is quite stable to both ring-opening and isomerization.

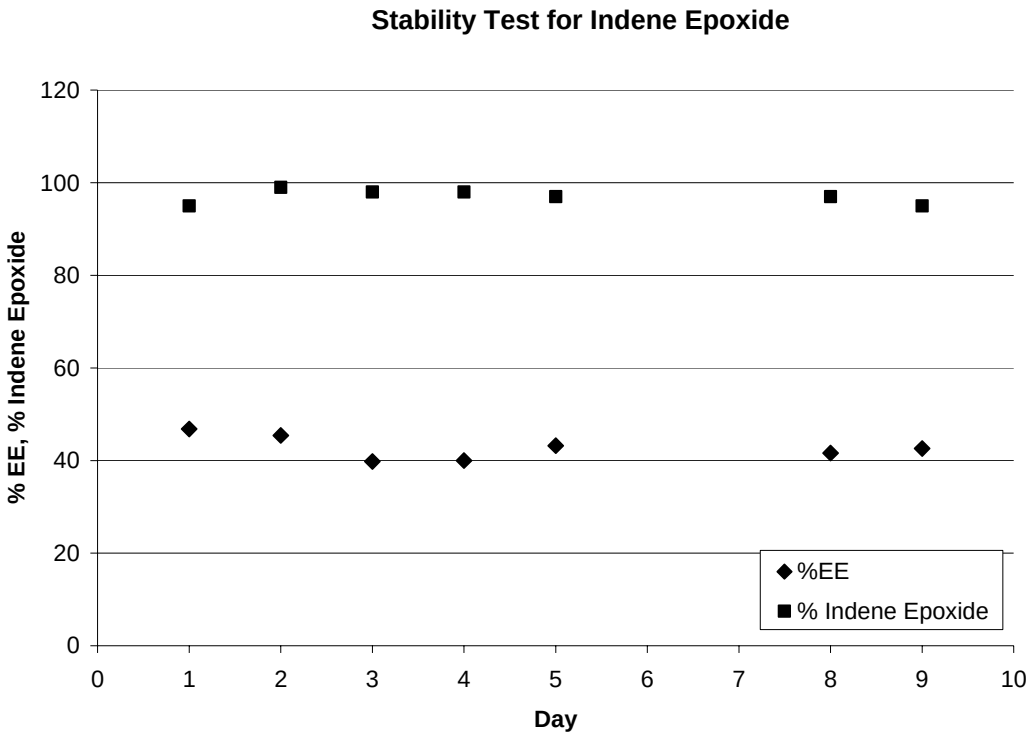

Figure 4-10: Stability of indene epoxide and ee over several days.

Catalyst Degradation

In a report by Hughes et al. (Hughes, Smith, et al. 1997), they measured the degradation of Salen as a function of mechanical stirring. Within 4 hours of stirring at 2180 rpm, the catalyst had completely degraded. For 420 rpm, similar to our experiments, the Salen was greater than 90% degraded within 13 hours. In the scope of our experiments, it took at least 6 hours to reach yields of roughly 80%. Following these parameters, the Salen would be almost completely degraded within 2 cycles. This degradation severely limits the catalyst activity and turnover numbers we would be able to get for the indene epoxidation with Salen. The cost of further developing the technology with this catalyst would outweigh the benefits reaped from only a single round of recycling. With this in mind, Salen was discontinued as the catalyst of choice, due to the apparent degradation.

In 2001, Campbell et al. (Campbell, Ashley, et al. 2001), published a dual-mode EPR study for Mn-Salen-catalyzed epoxidation of cis-β-methylstyrene, in which they explained the causes of degradation for Mn-Salen. They report that without the use of a stabilizing axial ligand, i.e. P_3NO, two or more Mn(III) centers become coupled, thus inactivating the catalyst. Since our particular reaction/separation scheme would be greatly complicated by the use of an axial ligand, the choice of a new catalyst and/or reaction is necessary.

Conclusions

Due to the reported degradation of Mn-Salen (Hughes, Smith et al. 1997), this catalyst was not the optimal choice for the organic-aqueous tunable solvents (OATS)

project. However, the synthesis of a water-soluble version of Mn-Salen was successful in maintaining similar or better yields and enantiomeric excesses as the regular Salen. It was also noted that colder temperatures (<0^{o}C) allow for much higher enantiomeric excess. In addition, for the WS Salen case, mechanical stirring appears to have produced lower yields of indene oxide. The cause of this is unknown, but catalyst degradation may be a factor.

To continue this work with OATS, a new catalyst and/or reaction scheme needs to be selected. The epoxidation reaction was chosen for its applicability to the pharmaceutical industry, and therefore should ideally remain the same. Several catalysts represent good alternatives to Salen including the Sharpless reagent, methyltrioxorhenium (MTO), and $MoO_2(acac)_2$. A more detailed discussion of these alternatives is presented in Chapter VII.

Overall, the OATS technology will be of great economic and environmental benefit to drug processing and development. Two major costs of pharmaceutical production come from catalyst losses and difficult downstream processing, which OATS will greatly reduce. The use of CO_2 also adds an attractive "green" component to the research by minimizing excess catalyst waste and potential harm to the environment, while also reducing the cost associated with disposing of waste. Because so many asymmetric catalysts are used in pharmaceutical production, the industrial candidate pool for this technique is virtually limitless.

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CHAPTER V

BENIGN EXTRACTION OF FERULIC ACID FROM ENZYME-LADEN BIOMASS

Ferulic acid (4-hydroxy-3-methoxycinnamic acid) is the major low molecular weight phenolic compound found in various cereal grains, such as wheat, rye, maize, (Sun, Sun et al. 2002), barley (Zupfer, Churchill et al. 1998), and rice (Koseki, Ito, et al. 1996). It is highly concentrated in the outer shells and cell walls of the plant, where it is bound to the arabinose backbone of the arabinoxylans through ester linkages (Faush, Kundig et al. 1963; Sun, Sun et al. 2002). Often the ferulic acid is cross-linked within the cell walls to provide added stability to the plant (Bunzel, Marita et al. 2001). Ferulic acid has a number of potential applications including antioxidant, food preservative, anti inflammatory agent, and as a food precursor (Graf 1992). It has even been studied as a potential hunger reducer and growth-depressing agent for humans and animals (Jung and Fahey 1983).

One particular application of ferulic acid that is currently undergoing intense investigation is that of a precursor to vanillin. Lately, there has been an increasing demand for "all natural" products in the global market (Kavitha, Shyamala, et al. 2005), with vanillin being one of them. This is evidenced by the price of natural vanillin at $30/kg versus $13-20/kg for synthetic vanillin (Chemical Marketing Reporter 2003). The USDA loosely defines the term "natural" as "products that contain no artificial

ingredients, coloring ingredients, or chemical preservatives; and the product and its ingredients are not more than minimally processed" (Post 1999). In the recent advances of biotechnology, several microbial transformations have been studied to allow vanillin manufacture within these guidelines, and several reviews on this technology have been published (Walton, Narbad, et al. 2000; Karmakar, Sharma, et al. 2001; Walton, Mayar, et al. 2003).

The general method for making natural vanillin through microbial transformation is to use an esterase (ester-breaking enzyme) to remove ferulic acid from the plant cell walls, and then another enzyme to convert the ferulic acid to vanillin. Several fungal organisms have demonstrated high esterase activity against ferulic acid, including Aspergillus niger CFR 1005, A. oryzae CFR 232, and Rhizopus arrhizus NCIM 997 (Kavitha, Shyamala, et al. 2005), with Aspergillus niger being the most commonly used. Two frequently used enzymes for transforming ferulic acid to vanillin are Pycnporus cinnabarnums SW-0,203 (Lesage-Meessen, Lomascolo, et al. 2002) and recombinant E. coli (Torre, de Faveri, et al. 2004).

One major drawback to scale-up and commercialization of this process is the separation of a) ferulic acid from biomass, and b) vanillin from cell broth. In both cases enzymes are involved, so the separation must be safe enough to not denature the enzymes, and cost effective and simple enough for industrial use. This study focuses mainly on designing a continuous extraction of ferulic acid from biomass, but the resulting system could be adapted to the extraction of vanillin as well.

Extracting carboxylic acids is not an unknown science (Kertes and King 1986; Yang, White et al. 1991), but specifically extracting ferulic acid from biomass is. When

using an enzyme, both temperature and pH must be strictly maintained, thus reducing the power of the extraction. For instance, ferulic acid has pKa values of 4.66 and 9.09 in a 10% MeCN-water solution (Beltran, Sanli, et al. 2003). Thus, since our system is operating at pH 7, most of the acid is dissociated (Figure 5-1, form 2).

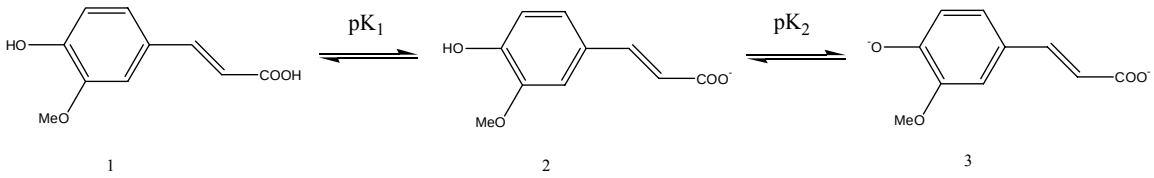


Figure 5-1: Dissociation equilibria for ferulic acid.

This is unfortunate because most organic solvents extract only one specific form of the acid. Bases will extract mostly form 1, and acids will extract mostly form 3 and some of form 2. Thus, we expect acidic solvents to work best for this study, but the challenge is to find a solvent that prefers form 2. However, when developing a continuous extraction, the partition coefficient is not of highest concern because the concentration can be enriched using a staged or multi-step operation. One such continuous extraction has been developed for wheat gliadins with a total of 56 steps (Truust and Johansson 1998).

Experimental Methods

Materials

4-hydroxy-3-methoxycinnamic acid (ferulic acid) was used as purchased from Acros Organics at a purity of 99%. All samples were buffered with a phosphate buffered saline (PBS), consisting of 150mM Na_2HPO_4 and 150mM NaCl and adjusted to pH 7

with 2.0M HCl. Both Na_2HPO_4 and NaCl were purchased from Fisher at purities of 99+%. A comprehensive list of all solvents used and manufacturer information can be found in Appendix A.

Analysis

Ferulic Acid is not stable to gas chromatography, so analysis was done via UV spectroscopy. All samples were analyzed by a Hewlett-Packard Ultra Violet Spectrophotometer model 8452A, using 3mL quartz spectrometer cells with a 1cm path length. Ferulic Acid has an extinction coefficient of 12376 L/mol-cm at a wavelength of 236 nm and a coefficient of 14452 L/mol-cm at 322 nm in water at pH 6.2 (Friedman and Jurgens 2000), so a concentration range was developed using the Beer-Lambert Law (Equation 5-1) based on those values.

$$A = c \cdot \varepsilon \cdot \ell$$

Equation 5-1: Beer-Lambert Law. A is absorbance, c is concentration, ε is the extinction coefficient, and *l* is path length.

Measurements were taken at 310 nm for ferulic acid and 500 nm for a baseline reading. The baseline was then subtracted from the ferulic acid reading, and the partition coefficient K was calculated using Equation 5-2.

$$K = \frac{A_{organic}}{A_{aqueous}}, \ A \text{ is the protein absorbance}$$

Equation 5-2: Partition coefficient K.

Experimental Procedure

A stock solution of 0.00015 M ferulic acid in PBS (150mM Na_2HPO_4, 150mM NaCl) was adjusted to 7.5 using 2.0M HCl. 6mL of the stock solution and 6mL of each solvent were combined in a glass vial and shaken until well mixed. For the water miscible solvents, sodium chloride (NaCl) was added to induce a phase split, typically between 0.1 and 1g. The samples were then shaken once more and allowed to equilibrate for 30 minutes. After equilibrating, 3mL were drawn from each phase and deposited into a 3mL quartz UV cell for analysis. If samples became contaminated with the other phase while being drawn out, a "smoking" effect occurred in the UV cell making measurements impossible. This phenomenon happened most when using the amines.

Results and Discussion

Non-polar Solvents

The first solvents tested were mostly nonpolar and aprotic. They were grouped together because they did not require any salt to be made biphasic; they are almost completely immiscible with water. These extractions were also run without the addition of PBS buffer. As seen in Figure 5-2, hexane and methylene chloride had the least partitioning, with K values of 0.004 and 0.009. Decane and chloroform were slightly higher with values of 0.015 and 0.014. Ethyl acetate had the highest partition coefficient of this group, with K = 0.22, followed by diethyl ether at K = 0.037. The basicity of the ethyl acetate is likely the reason for higher partitioning. Most of the acid is in the dissociated or salt form at pH 7 so therefore it is less likely to be soluble in the hydrocarbon solvents. Overall, the extracting power of this group of solvents is not great,

but they do have the added benefit that no salt is needed to perform the extraction, thus greatly simplifying the phase behavior.

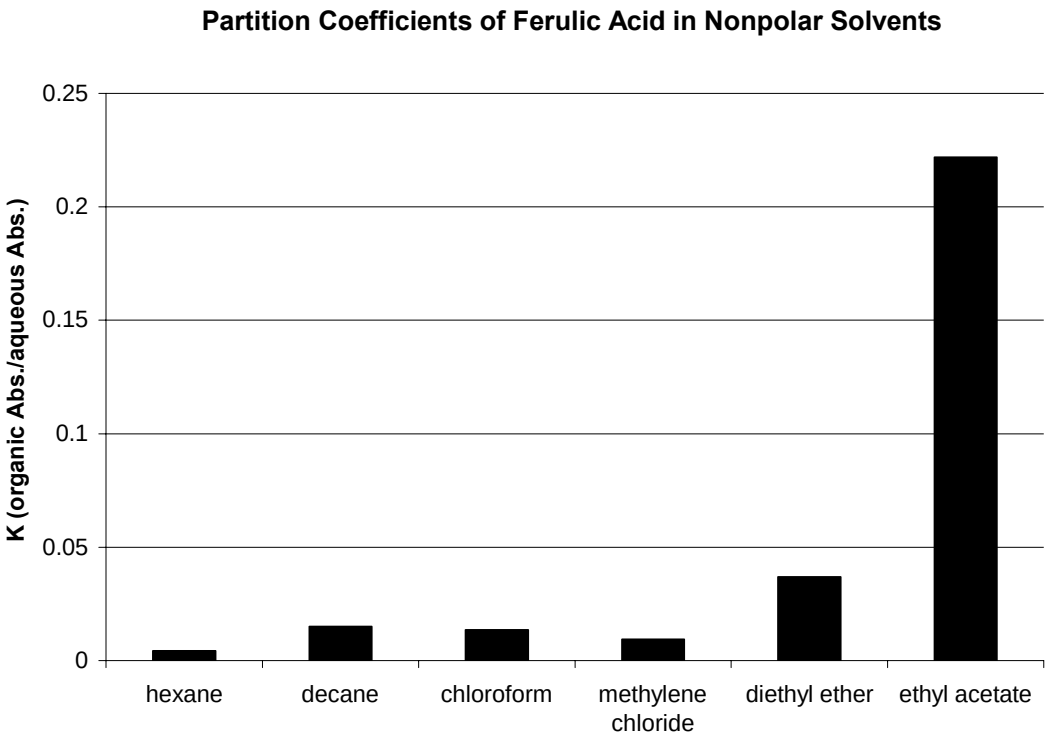


Figure 5-2: Partition coefficients (K) for ferulic acid in various nonpolar solvents, without the addition of salt or PBS.

Polar Solvents

The next solvents tested were alcohols. Each of these required the addition of NaCl to induce a phase split, and each of these solvents, except for octanol, gave higher partitioning than did the non-polar solvents. The amount of salt added to each sample along with a list of partition coefficients can be found in Appendix A. T-butanol gave the highest partitioning of this group with K = 1.30. 1-propanol and isopropanol gave the

next highest partition coefficients with roughly K= 1.16 for both. 1-Butanol, pentanol, and octanol had respective K values of 0.64, 0.39, and 0.14. Except for t-butanol, there appears to be a trend of higher partitioning with smaller molecular weight, as shown in Figure 5-3. This is in agreement with a study by Jung (Jung, Schierbaum, et al. 2000).

In addition to the alcohols listed, ethanol, dimethyl formamide (DMF), dimethyl sulfoxide (DMSO), and acetone were also tested, but addition of salt caused the ferulic acid to precipitate out of solution. Therefore, the biphasic extraction was not possible with these polar solvents. Tetrahydrofuran and acetonitrile were also attempted and were able to produce biphasic extraction. However, the stabilizers used in these solvents were UV active, thus prohibiting analysis via UV spectroscopy.

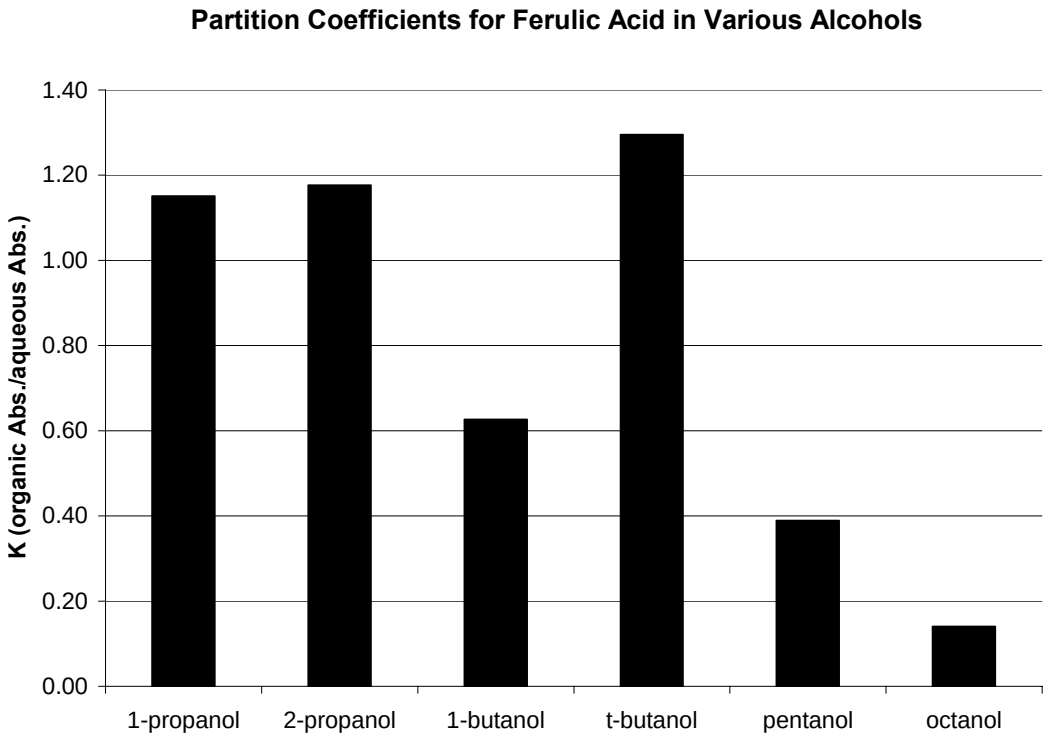


Figure 5-3: Partition coefficients (K) for ferulic acid in various alcohols with PBS buffer.

Tertiary Amines and Reactive Extraction

Partitioning of ferulic acid was next tested on the tertiary amines, trioctylamine (TOA) and triethylamine (TEA). Initially, these extractions were run with old TOA and TEA. The UV peaks suggested that the amines were somewhat dirty, so new solvents were purchased. However, rerunning the extractions with new solvents gave much lower partitioning than did the old solvents (Figure 5-4). The old TEA and TOA had K values of 1.30 and 2.02 respectively. The fresh solvents reduced the partitioning of ferulic acid by 80% for TEA and 90% for TOA. Although it was not done in this study, it would be worthwhile to determine what had contaminated the dirty amines because it was evidently beneficial to the partitioning.

In 2004, Kailas Wasewar published a review article on reactive extraction of lactic acid (Wasewar, Yawalker et al. 2004). Safely recovering lactic acid from fermented biomass is similar to the premise of this study, recovering ferulic acid from biomass. Reactive extraction is done by creating an amine-carboxylic acid complex, which then has a higher affinity for a given organic diluent. Once extracted, the amine-acid complex could then be easily broken apart and the amine could be regenerated and recycled. In the review, it was found that tertiary amines, specifically Alamine 336, were the best extractants. Longer chain alcohols were then found to be the best diluents.

Using this knowledge, reactive extraction was performed on ferulic acid with TOA as the amine, and with octanol as the diluent. As shown in Figure 5-4, the reactive extraction improved the partitioning over that with TOA alone by about 100%. Still, compared to the results for t-butanol and propanol, these partition coefficients are not very high. In another paper, Wasewar reported a distribution coefficient for lactic acid

with Alamine 336 and octanol to be 25 (Wasewar, Heesink et al. 2003), which is much higher than was found in this study. Nevertheless, the difference in partition coefficients can easily be attributed to the difference in structure of these acids (Figure 5-5). The increase in the extraction of the amine when combined with the diluent can be explained by a study by Yang et al (Yang, White, et al. 1991). Yang found that tertiary amines, as opposed to quaternary amines, can only extract undissociated acids. Because the pKa of ferulic acid is around 4.5, the extractions in this study were taking place with most of the acid dissociated. The octanol works with the tertiary amine to increase the solvation of the amine to help extract the dissociated acid, thus increasing the partition coefficient.

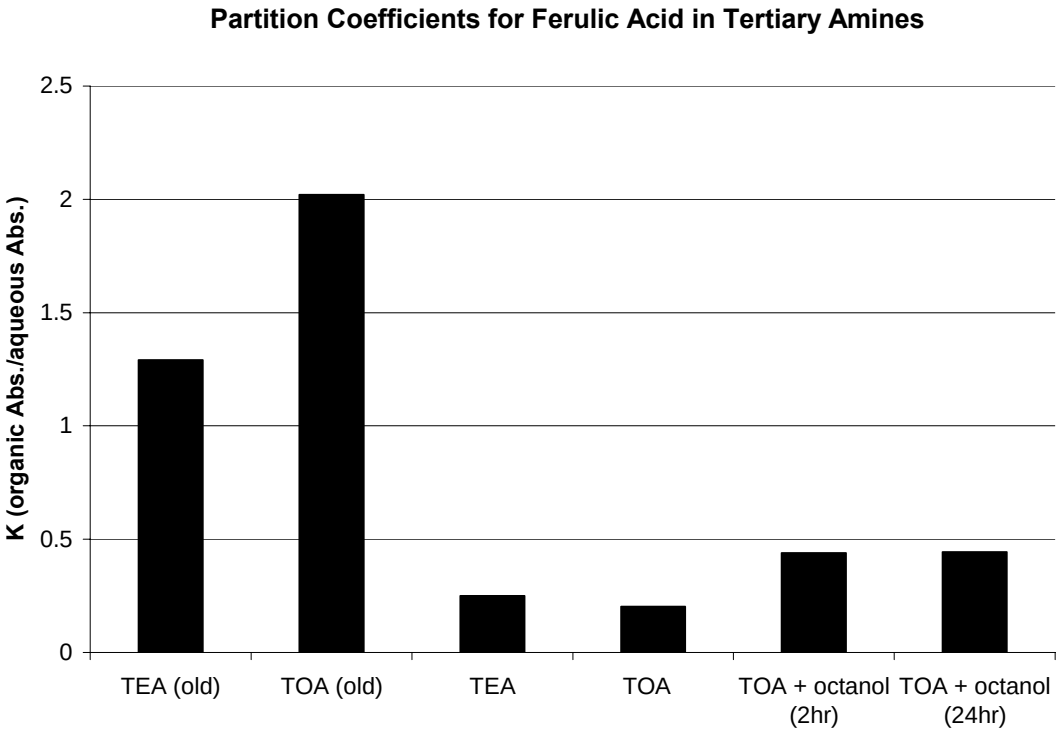
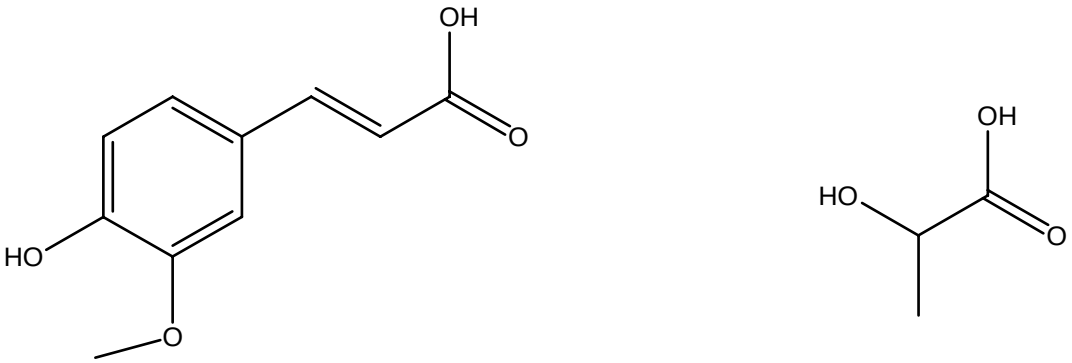


Figure 5-4: Partitioning of ferulic acid in tertiary amines, both old and new, and with octanol for reactive extraction.



ferulic acid lactic acid

Figure 5-5: Structure comparison of ferulic acid and lactic acid.

Fluorinated Solvents

Two other solvents were tested for extraction, trifluoroethanol (TFE) and hexafluoroisopropanol (HFIP). Both of these required salt to form two phases (Appendix A). Both of these solvents also gave very high partition coefficients, the highest of all solvents tested. For TFE, K= 1.59, and for HFIP, K= 2.28. These are compared to the next highest extracting solvent, t-butanol, in Figure 5-6. One possible reason for the high partitioning with these fluorinated alcohols is their hydrogen-bonding ability. Both have Kamlet-Taft acidity (α) pararmeters greater than 1, as opposed to all other solvents used in this study. An LSER regression was applied to the data, and the only correlating Kamlet-Taft parameter was acidity; however, the correlation was weak, with a R^2 value of only 0.75 (Figure 5-7).

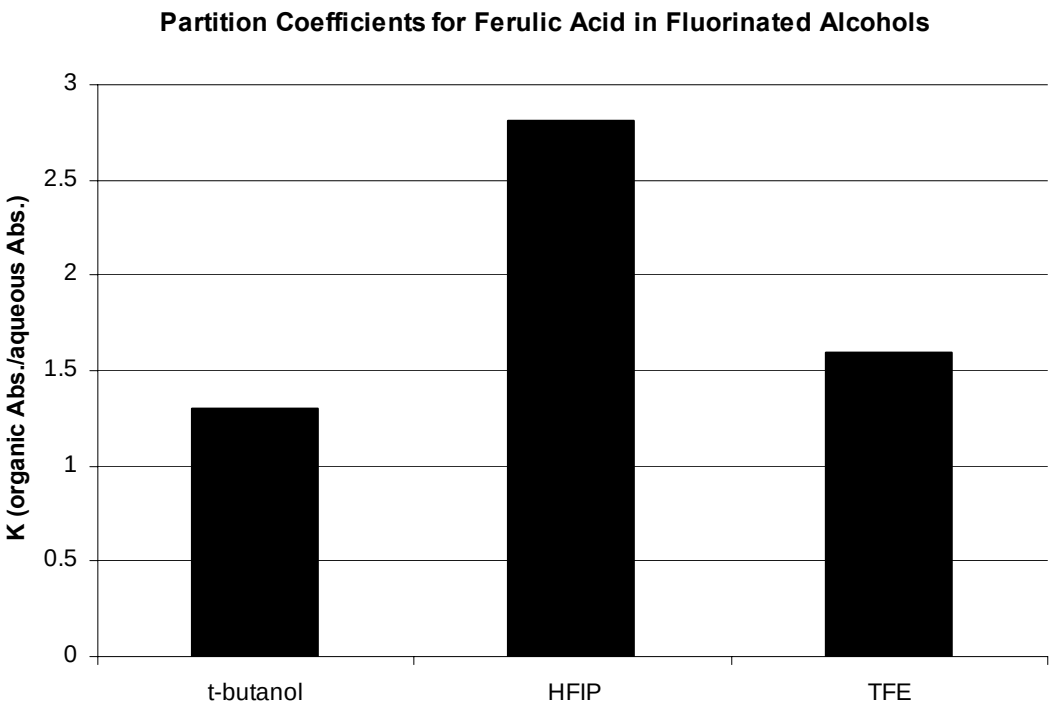


Figure 5-6: Partitioning of ferulic acid in fluorinated alcohols as compared to t-butanol.

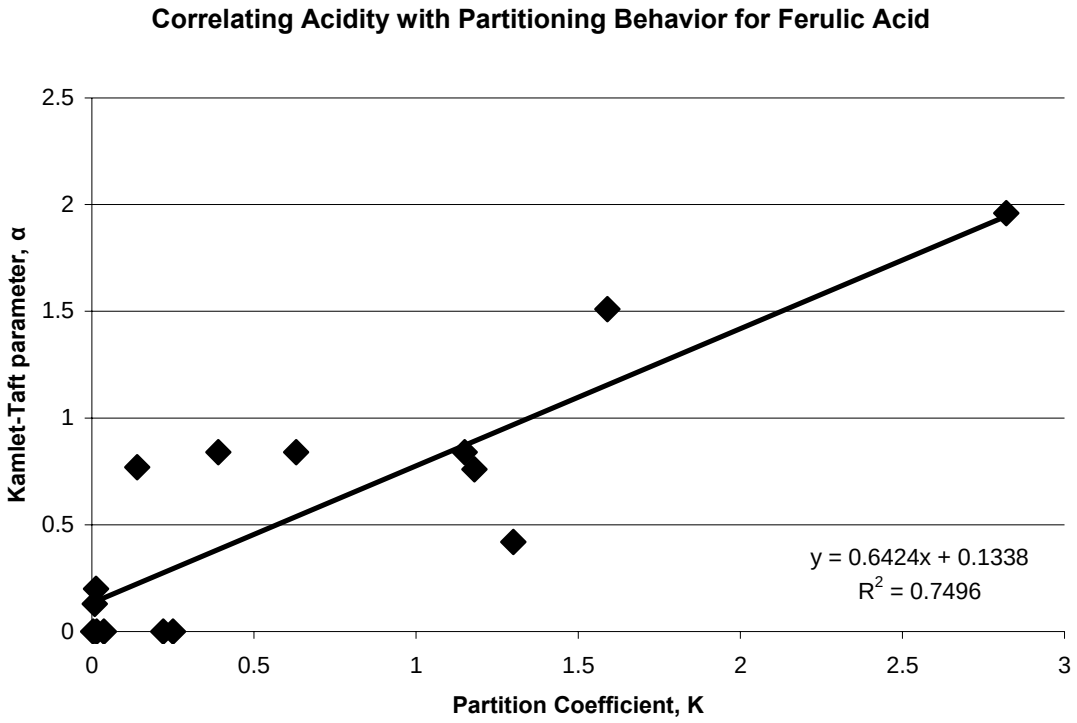


Figure 5-7: Relating Kamlet-Taft Acidity parameter (α) to partitioning behavior found in this study. Alpha values taken from Dippr database (DIPPR 801 2005).

It is important to keep in mind that there are many adjustable parameters in this system. Not only does the type of solvent effect the extraction, but temperature, pH, solvent-water volume ratio, and in many cases, the type and amount of salt used to cause a phase split are also critical. In this system everything was held constant except the solvent type, and for each case, only enough salt was added to initiate the phase split. To better optimize a system like this, a factorial design of experiments would be ideal.

Ethyl Acetate

Because this system was developed for use on a commercial scale, it was determined that ethyl acetate would be the best extractant. While ethyl acetate has one of the lower partition coefficients by far, it has several benefits that make it more useful than solvents with higher extracting power. First of all, it requires no salt to form a phase split. That means more predictable phase behavior, and elimination of the need to constantly monitor and control the salt concentration throughout the process. Secondly, there is no reaction taking place as there is in reactive extraction. This also simplifies the process by reducing the number of steps (back-extraction and regeneration of the amine), and makes the extraction kinetics easier to study and model. Also, when operated continuously, the smaller partition coefficient for ethyl acetate will not be an issue because the concentration of ferulic acid can easily be enriched with the use of stages.

To further develop ethyl acetate as the solvent of choice, a repeatability study and mass balance were done for the extraction. The same procedure was followed as described above, except that now 5mL were used instead of 6mL for each EA and FA-PBS. Results can be seen in Table 5-1, and a detailed spreadsheet is displayed in Appendix B.

Table 5-1: Results for Extraction of Ferulic Acid in Ethyl Acetate

Run #	Volume per phase (mL)	K (A_{org}/A_{aq})	Mass Balance (% FA of total in system)
1 (shown above)	6	0.222	104.9
2	5	0.175	97.6
3	5	0.175	96.9
4	5	0.173	97.1
5	5	0.188	99.7

Including the first run with ethyl acetate (also shown in Figure 5-2), the average partition coefficient is 0.187, with a standard deviation of 0.021. Excluding the first point, the average K is 0.178 with a standard deviation of 0.007. It appears that using a larger volume may increase the partitioning, but further investigation would be necessary to validate that claim. If this is indeed the case, it suggests that scale-up of this system may actually be beneficial to the partitioning behavior. However, the error in the mass balance on the first point also suggests that the absorbance may be inflated, perhaps due to overlap from another peak. The remaining mass balance values for runs 2-5 are very reasonable for this type of system, as a small amount of ferulic acid may be coagulating at the phase interface.

Ferulic Acid Degradation

One other thing to note in this study is the degradation of ferulic acid. Ferulic acid – PBS solutions are stored in a clear glass flask, sealed with a rubber septum on the bench top, at room temperature. Over the course of a few weeks, the UV signature of ferulic acid completely disappears, with no apparent precipitation observed. Consequently, the solutions had to be remade every few days. It is important to note that an identical isosbestic point to that observed by Friedman and Jurgens (Friedman and Jurgens 2000) was found (Figure 5-8), and it suggests that two forms of the acid are in solution, which is in agreement with the dissociation equilibria (Figure 5-1). In attempt to understand the disappearance of ferulic acid, NMR spectra were taken for fresh ferulic acid in d-$CdCl_3$, and again after 2 weeks. It appears that roughly 10% had degraded in this time, much less than was seen in the UV. This suggests that the water and PBS may

help to degrade the ferulic acid. It was also noticed that over time, solutions of ferulic acid in water turned yellow, while ferulic acid in methanol did not. This further indicates that water is somehow related to the degradation or alteration of ferulic acid. Several researchers have reported the photoisomerization of ferulic acid when exposed to UV light (Fenton, Mueller et al. 1978; Dimberg, Sunnerheim et al. 2001) and even when it is still attached to barley cell walls (Yamamoto, Towers, et al. 1985). Fenton et al. also reported a decrease in isomerization when storing ferulic acid in methanol, thus supporting the data in this study, and suggesting isomerization as the reason for the color change.

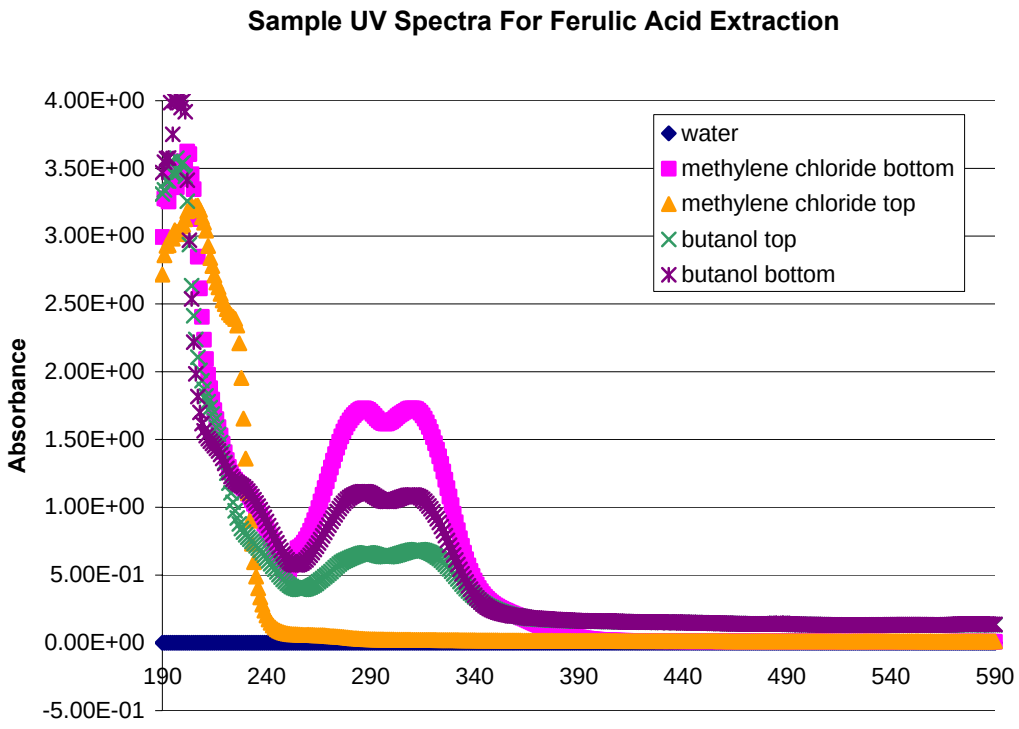


Figure 5-8: Sample UV spectra taken from this study.

Conclusions

Overall, ferulic acid does not partition very favorably into organics. Of all solvents studied, hexfluoroisopropanol (HFIP) has the highest partition coefficient at 2.28. The next highest were trifluoroethanol (TFE) with K = 1.59, and t-butanol with K = 1.30. Each of these solvents requires salt to induce a phase split though, thus complicating the system. Tertiary amines, when used with octanol for reactive extraction, also gave decent partitioning (K = 0.44), without the requirement of salt. However, the reaction kinetics, back-extraction, and regeneration of the amine also greatly complicate the system. Therefore, ethyl acetate was chosen as the best extractant for scale-up.

Repeatability and mass balance analysis for ethyl acetate gave excellent results. With a total of 5 runs, only one run appeared to be in error, as denoted by an apparent "gain" in mass, as shown by the mass balance (Table 5-1). The average partition coefficient of the other 4 runs was 0.178, with a standard deviation of 0.007, suggesting a high level of repeatability. Also for the last 4 runs, the mass balance is quite accurate, with the biggest error being 3.1%.

Along with finding the most favorable solvent for scale-up, this study found several interesting trends. First, with the exception of t-butanol, ferulic acid partitions into alcohols at a rate inversely proportional to the molecular weight of the alcohol. This has also been noted by Jung et al. in their study with alcohols as diluents in reactive extraction of carboxylic acids (Jung, Schierbaum, et al. 2000). Secondly, "dirty" tertiary amines extract ferulic acid much more efficiently than do fresh tertiary amines. The contaminants in "dirty" amines were never examined; however the difference is quite large (Figure 5-4).

Future work on this project would include a study on the enzyme that will be extracting the ferulic acid from the maize. It must be stable to ethyl acetate, and an understanding of its stability versus pH would be very useful in optimizing the extraction. All tests here were done at pH 7; however, a lower or higher pH would permit higher partitioning, should the enzyme be stable to it. It would also be useful to know the enzyme stability under different temperatures, as that would give more room for optimizing the extraction.

The success of this project will help to create a more profitable process for making "all-natural" vanillin. Developing a continuous separation unit allows for easier modeling and control of the system so that the microbial vanillin process can be scaled up and used commercially. This specific study was designed for extracting ferulic acid from biomass while not damaging the enzymes. However, as microbial processes are becoming more prevalent, benign extractions will become more indispensable. This study sets forth a framework for developing such extractions, and can be applied to many biological systems.

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CHAPTER VI

NEAR-CRITICAL WATER EXTRACTION OF VALUABLE CHEMICALS FROM BREWERS SPENT GRAIN

A recent study reports that 400 billion pounds of agricultural products are harvested every year in the United States (Food and Kindred Products 2005). Less than 50% of those harvested crops end up being used as a final product, meaning that more than 50% of that is wasted. With the investment that is put into these crops through cultivation, pesticides, and harvesting, it is poor economics to throw half of it away. Agricultural products are seen as economic renewable resources with many different uses, such as energy production, nutraceuticals, materials, and numerous other possibilities. As many of the worlds limited resources are gradually being depleted, more and more attention will be turned to agricultural products as sustainable resources.

One such sector that contributes to the large amount of agricultural waste every year is the alcoholic beverage industry. The brewing industry, combined with the wineries and distilleries, constitutes a $137 billion/year sector (Yahoo 2005). This industry supports the fermentation of wheat, barley and hops for beer, the fermenting of grapes for wine, and various other grains for alcohol. The brewing process removes a small portion of the plant and leaves behind large amounts of biomass. For the beer industry specifically, Brewers Spent Grain (BSG), consisting of fermented wheat, barley,

and hops, totals roughly 57 million pounds per year for a typical large brewery (Food and Kindred Products 2005).

One chemical with many applications and found abundantly in nature is ferulic acid (4-hydroxy-3-methoxycinnamic acid) (Sindhu and Emilia 2004; Rosazza, Huang et al. 1995). Ferulic acid is bound to the arabinoxylan by ester bonds in the cell walls of most agricultural species, and therefore also in BSG (Figure 6-1). The current price for ferulic acid is $682/kg (Sigma-Aldrich 2005). The review by Rosazza et al. advertises ferulic acid as a commodity scale, renewable chemical feedstock, and reviews the many products than can be made from it through microbial and enzymatic transformation. Most commonly used is the transformation to vanillin, as patented by Kraft General Foods (Labuda, Goers, et al. 1994), and as reviewed in Chapter V of this thesis. Ferulic acid also has multiple applications as is. It is an antioxidant (Lin, Navaratnam, et al. 1998), preventor of aroma degradation in food (Inaba, Ozaki, et al. 2003), and a potential hunger reducer (Jung and Fahey 1983).

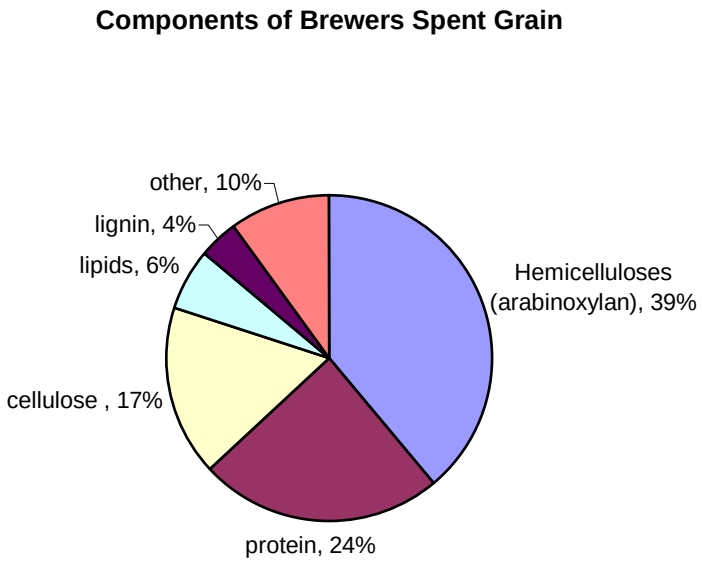

Figure 6-1: Composition of Brewers Spent Grain. Ferulic acid is bound to arabinoxylan.

The decarboxylation of ferulic acid leads to another valuable chemical, 4-vinylguaiacol (4VG). This transformation can occur through thermal degradation of ferulic acid (Fiddler, Parker et al. 1967), by acid catalysis (Johnson and Heinz 1949), or through bioconversion (Karmakar, Vohra, et al. 2000). Several mechanisms have been proposed for this transformation, yet none have been entirely conclusive (Fiddler, Parker et al. 1967; Peleg, Naim, et al. 1992; Johnson and Heinz 1949). 4VG is a flavanoid with a smoky, clove-like aroma, selling for $3,500/kg and with a US demand of 10kg/year (Sigma-Aldrich 2005). Applications of this molecule, other than natural flavoring in beer and wine, include fragrances (Higuchi, Yoda, et al. 2003), and biodegradable polymers (Sovish 1989; Hatakeyama, Hayashi et al. 1977; Iwabuchi, Nakahira, et al. 1983). The latter is an interesting prospect, considering the ever-growing use of polymers, from construction materials to pharmaceutical delivery materials.

The most common method for extracting ferulic acid from biomass is with microbes or fungal esterases (See Chapter V for full review). While it is considered a "green" and natural process, there are several drawbacks. The first is that isolating and purifying the enzymes can be a tedious and/or expensive process. Also, because most biological entities are active only at moderate pH and temperature, constraints are placed on the robustness of the process, and the system cannot be easily optimized. Secondly, the issue of product inhibition plagues many enzymatic reactions, requiring the products to be constantly removed. However, this removal process must also be benign enough to not damage the enzyme activity. One way around these issues, yet to maintain natural processing, is to use near-critical water (NCW) extraction.

Water, of course, is a completely benign processing fluid. By increasing the temperature of water to within the range of its critical point ($375^{o}C$) the properties of water change to that of a polar organic solvent (Figure 6-2). Pressure is also needed when using water above its boiling point to avoid vaporization.

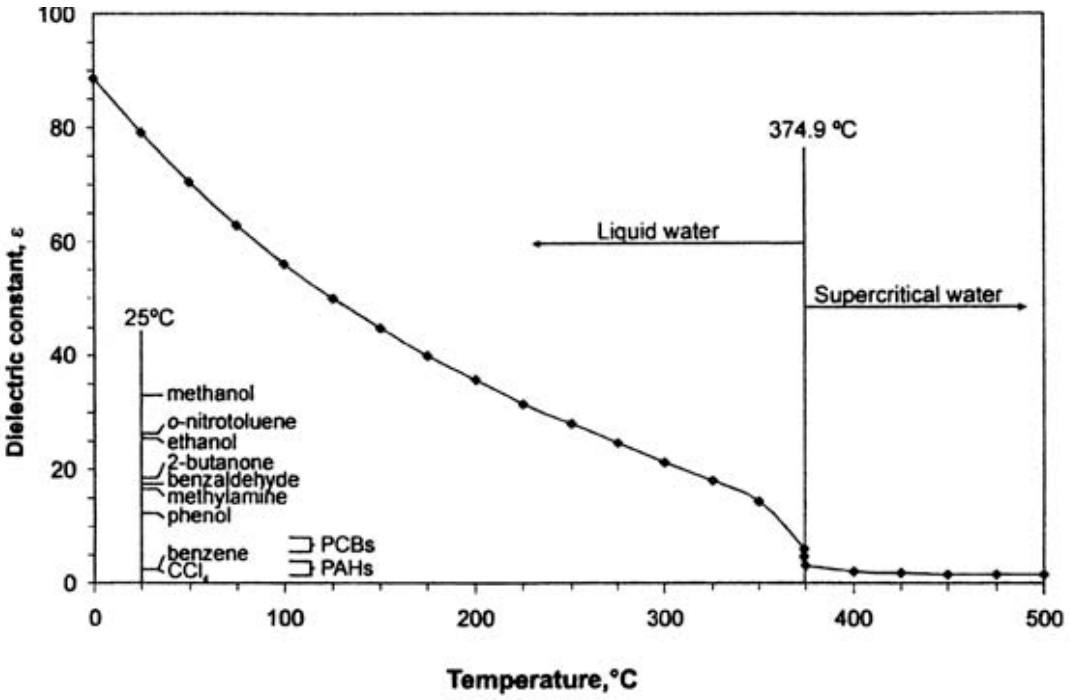

Figure 6-2: Dielectric constant of water versus temperature. (Figure from King 2004).

The method of using near-critical or sub-critical water has been used for extracting natural products (Clifford 2002; Politi, Chavez, et al. 2005), and many acid- and base-catalyzed reactions (Chandler, Deng, et al. 1997; Nolan, Liotta et al. 2003), including ester hydrolysis (Lesutis, Glaser et al. 1999). Ferulic acid is bound to plant cell walls through ester bonds (Sun, Sun, et al. 2002), and therefore creates a perfect opportunity to use NCW for its release without the use of enzymes. Furthermore, the decarboxylation of ferulic acid to form 4VG is also promoted by NCW through acid catalysis, thus making it a single step process. Finally, based on the chemical properties of both ferulic acid and 4VG, a simple liquid-liquid extraction may be used to facilitate separation (Lee, Volm et al. 1998).

This study was designed to prove the ability of NCW extraction to obtain both ferulic acid and 4VG from BSG. A statistical design of experiments was employed in an

attempt to determine the optimal extraction conditions for the recovery of both ferulic acid and 4VG.

Experimental Methods

Materials

The Brewers Spent Grain (BSG) was graciously donated by Five Seasons Brewing Company in Sandy Springs, GA. The water content of the BSG was 77% as received, and was not further treated. The water content of the BSG was included in the volume ratio calculations. Pearled barley was obtained from Kroger Foods and further ground to a fine powder with mortar and pestle. Water and Methanol, both of HPLC Chromasolv purity were purchased from Sigma-Aldrich. Acetonitrile was also obtained from Sigma-Aldrich at an HPLC gradient grade of >99.9% purity. HPLC standards were made of 4-hydroxy-3-methoxycinnamic acid (ferulic acid) (Acros Organics, 99%), 4-vinylguaiacol (Aldrich, 98%), vanillin (Aldrich, 99%), and 4-hydroxy-3-methoxybenxoic acid (vanillic acid) (Aldrich, 97%), all used as received. Resulting BSG extractants were filtered through both Whatman qualitative filters (4.25 cm) and 0.45 μm nonsterile hydrophobic fluorophore Millex-FH filters from Millipore.

Procedure

The samples of barley and BSG were weighed and deposited into 3 mL titanium reactors as pictured in Figure 6-3, and as used previously by our group (Nolen, Liotta et al. 2003; Patrick, Griffith, et al. 2001). One milliliter of HPLC water was added to each reactor and sealed with an NPT plug. The volume of these reactors should never be filled

more than 2/3 full, due to the expansion of water with increase in temperature. The reactors are then placed in the aluminum heating block for the course of the reaction. Temperature is controlled with 4 cartridge heaters (Omega Technologies Co.) and a Model CN9000A temperature controller (Omega Technologies Co.). After the extraction, reactors are removed from the heating block and placed in a room temperature water bath to quench the reaction.

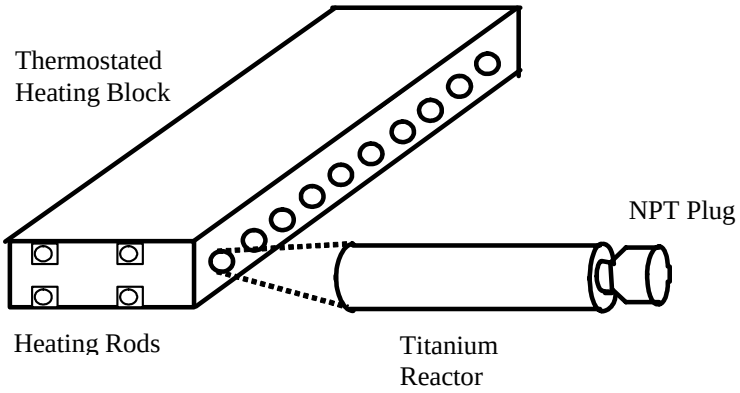

Figure 6-3: Near-critical water reactor and heating block apparatus (Nolan, Griffith et al. 2003).

Calibrations were made for the thermocouples within the heating block, and for temperature differences within the reactors at various positions along the length of the block. In these experiments, only the 6 most centered holes were used of the 10 possible holes (Figure 6-3). Previously, the time for reactor contents to heat up and time for quenching were also calculated (Lesitus 2000). Data has been reproduced in Figures 6-4.

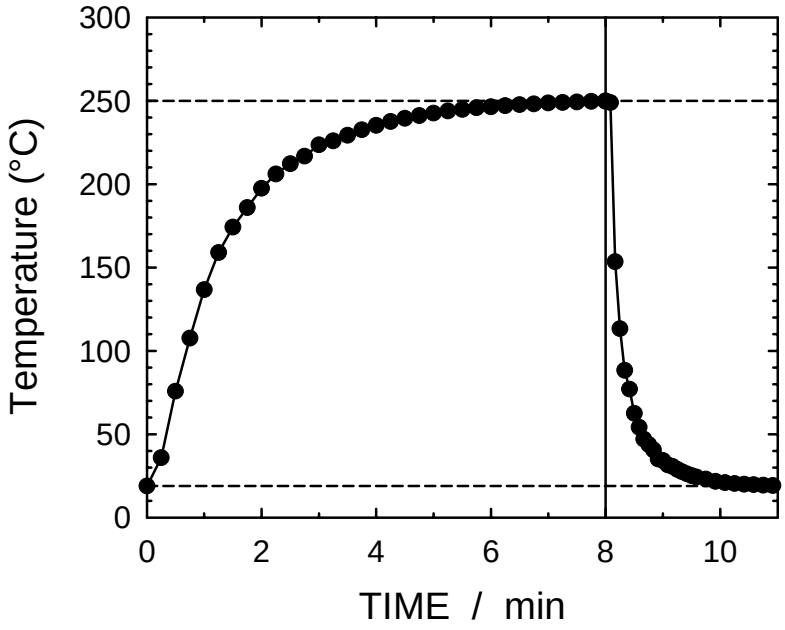

Figure 6-4: Reactor heating and cooling rates for the NCW experimental investigations (Lesutis 2000).

Once the extractions were complete and the reactors cooled, the contents were removed and diluted 3 times with HPLC methanol. Methanol was used for analysis specifically for its solubility of both ferulic acid and 4VG. The samples were then filtered with Whatman qualitative filters to remove the large amounts of biomass, and the remaining liquid was filtered once more with 0.45 µm filters for clarification. These final samples were then analyzed by LC-MS.

Analysis

Samples were analyzed qualitatively by GC-MS and quantitatively by LC-MS with UV detection. The LC column used was a Zorbax Waters Symmetry C_{18} 5um, 4.6x 150 mm column. The flow was set to 0.7 mL/min with a water/acetonitrile gradient of

100% water to 55% water in 5 minutes, 55%-50% water over 10 minutes, and 50%-0% water over a final 15 minutes. The column was set to 30^{o}C and the UV detected at 290 nm.

Standards for the LC-MS were made for ferulic acid, 4VG, and vanillic acid at concentrations of 0.01, 0.001, 0.0001, and 0.00001 mg/mL methanol. These provided calibration curves from which the concentration of the chemicals in BSG extract can be determined. An important note is that the calibration curves for LC-MS are non-linear, rendering no clear calibration equation. Therefore, some error is inherent in reading values from the curves.

Experimental Design

A statistical design of experiments was used to analyze and optimize both extraction of ferulic acid and production of 4VG. The experimental conditions can be found in Table 6-1. Time, temperature, and ratio of BSG to water were the variables tested. The response variables were yields of ferulic acid, 4VG, and vanillic acid, as determined by LC-MS.

Table 6-1: Experimental Conditions for Near Critical Water Extraction of BSG

Run	Time (min)	Temp (^oC)	Ratio (BSG:H_2O)
Blank	70	25	0.1
1	70	275	0.5
2	70	275	0.1
3	70	175	0.5
4	70	175	0.1
5	10	275	0.5
6	10	275	0.1
7	10	175	0.5
8	10	175	0.1
9	40	225	0.25
10	40	225	0.25
11	40	225	0.25
12	40	225	0.25

In addition to the statistical design, preliminary tests were run on samples of pearled barley, and again later for comparison to the BSG. A control experiment was also run on pure ferulic acid in near critical water to verify the conversion to 4VG.

Results and Discussion

Calibrations

The calibration curves for ferulic acid, 4VG, and vanillic acid were repeated 3 times each. The results are below in Figures 6-5, 6-6, and 6-7. The data points represent the average of 3 runs, and the error bars are +/- 1 standard deviation. All three curves resemble the same shape, yet a large error occurred on the 0.01 mg/mL point for ferulic acid. This point was repeated 5 times with considerable inconsistency (>22%). The 4VG calibration was the most consistent, with error not higher than 4%, and the vanillic acid nearly as consistent with the largest error at 6.4%.

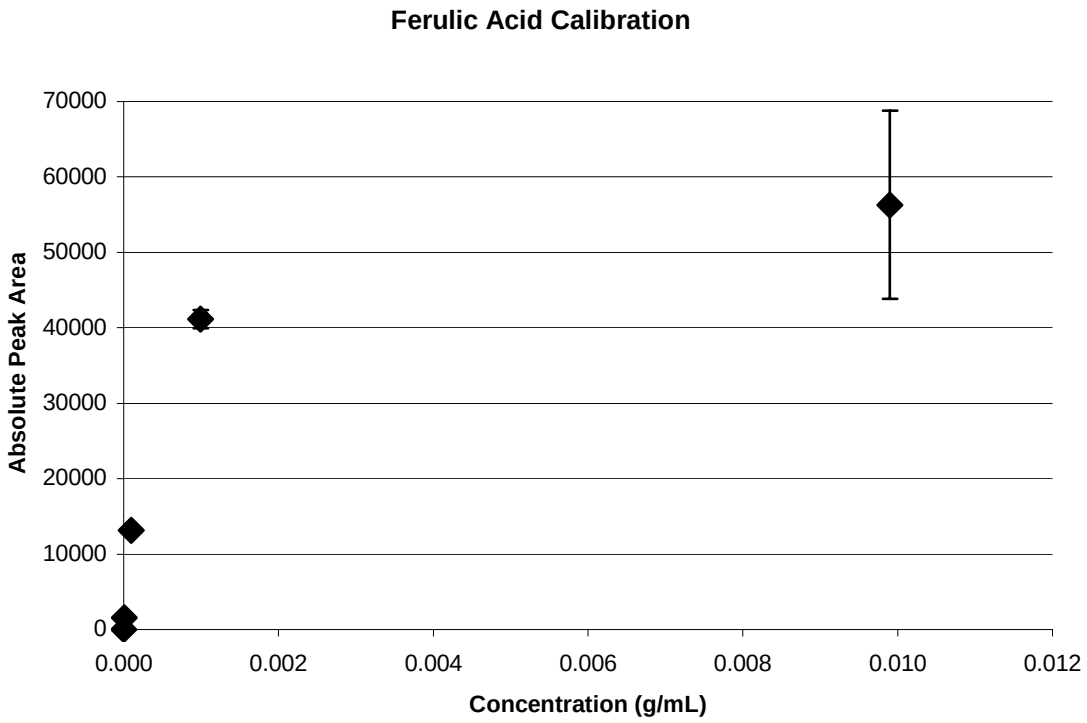

Figure 6-5: LC-MS calibration curve for ferulic acid in methanol.

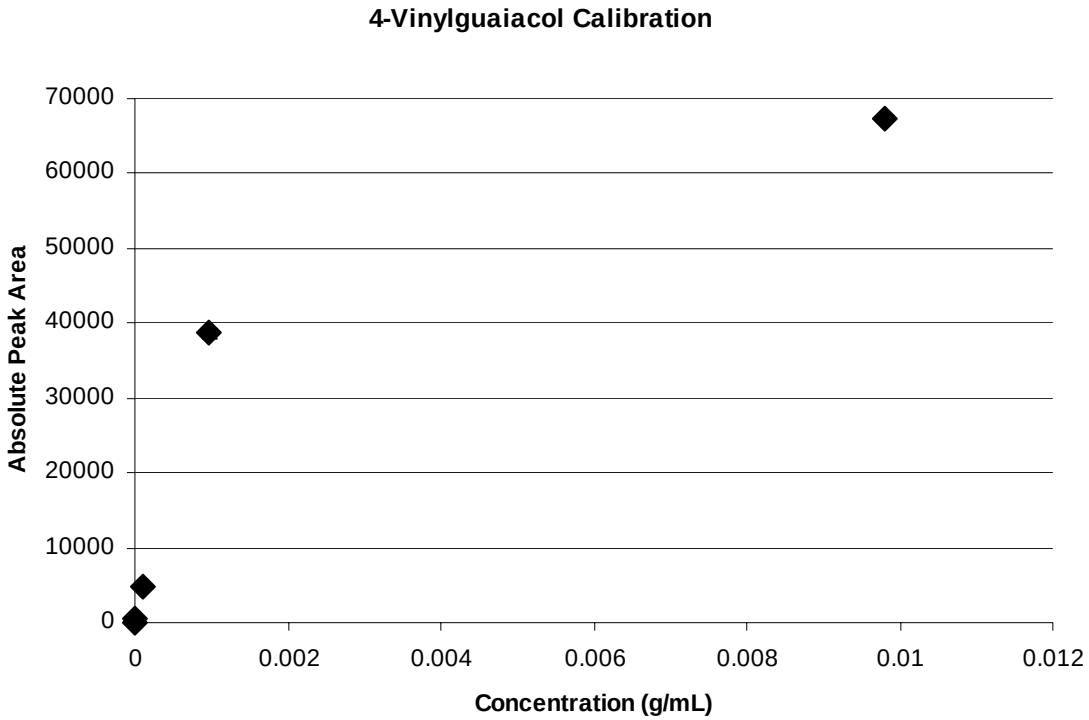

Figure 6-6: LC-MS calibration curve for 4-vinylguaiacol in methanol.

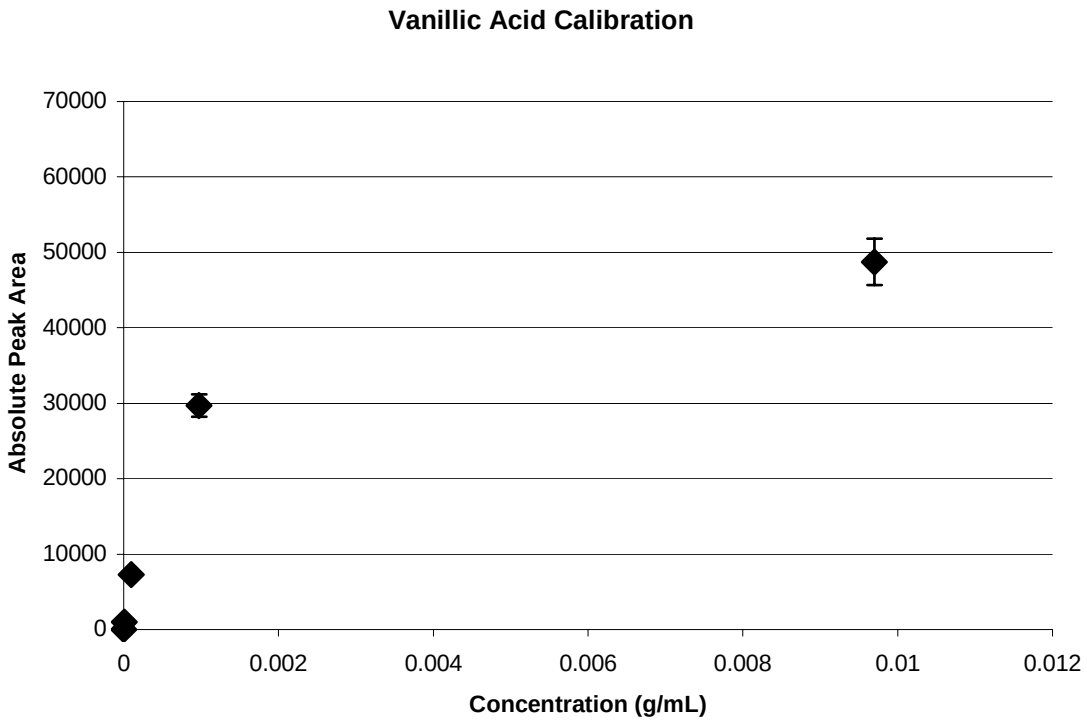

Figure 6-7: LC-MS calibration curve for vanillic acid in methanol.

The aluminum heatblock was calibrated by sealing a thermocouple inside a reactor, and comparing the reading inside the reactor to that of the heatblock. Readings were taken over a hundred degree temperature range and were taken for each of the 6 holes used for these experiments. The differences in readings between the holes differed by less than 3 degrees, and the temperature reading from the heatblock was lower than the actual temperature by ~1.4^{o}C.

Control experiment

In order to confirm the ability of near critical water to catalyze the conversion of ferulic acid to 4-vinylguaiacol, and control experiment was run. 0.1 g of ferulic acid were placed in the reactors with 1mL of HPLC water, and the reactions were run as mentioned above, for 15, 30, and 60 minutes at 200^{o}C. The reactions were run in duplicate and analyzed via LC-MS with UV detection. The results are shown in Figure 6-8.

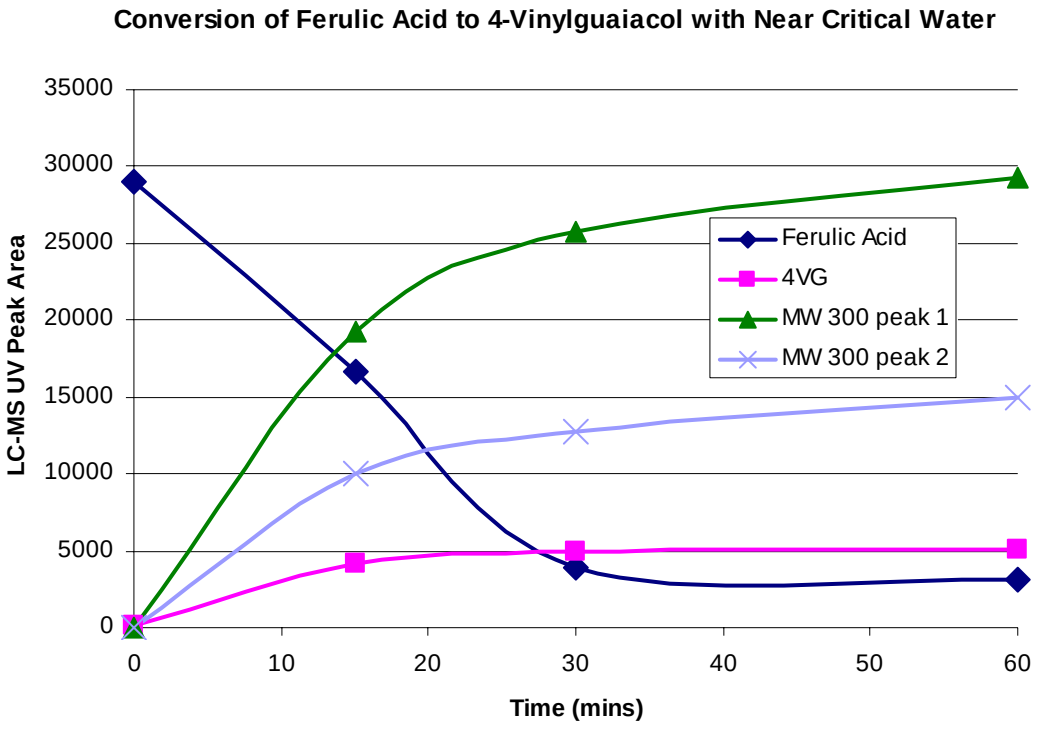

Figure 6-8: Conversion of ferulic acid to 4-vinylguaiacol in near critical water. Two unknown peaks with a molecular weight of 300 were included in the results.

As expected, the concentration of ferulic acid decreased as the concentration of 4VG increased. However, 2 other unexpected peaks were formed with a molecular weight of 300. These samples were further run through GC-MS analysis to confirm molecular weight. Though no structure was suggested, these peaks are believed to be dimers of 4VG, as the molecular weight of the peaks is exactly twice that of 4VG. The GC-MS analysis also revealed a peak with molecular weight of 450, suggesting formation of a trimer as well. An article by Rizzi and Boekley (Rizzi and Boekley 1992) shows the dimerization of 4VG as well, thus giving credence to our hypothesis. However, no evidence of these peaks was seen in the BSG extractions.

Statistical Design Results

The statistical design has been set up and analyzed according to Box and Hunter (Box, Hunter et al. 1978). The resulting yields for ferulic acid, 4VG, and vanillic acid can be seen in Table 6-2, and a full set of regression statistics can be found in Appendix C.

Table 6-2: Yield results for near-critical water extraction of BSG. (Conditions of each run can be found in Table 6-1).

Run	FA yield	4VG yield	VA yield
blank	0.00%	0.00%	-0.03%
1	2.20%	0.22%	0.70%
2	1.99%	0.44%	2.08%
3	0.39%	0.08%	0.59%
4	0.42%	0.03%	4.72%
5	1.39%	0.30%	57.71%
6	1.10%	0.38%	9.48%
7	0.16%	0.01%	0.20%
8	0.18%	0.00%	0.55%
9	1.05%	0.27%	16.88%
10	0.98%	0.26%	78.59%
11	0.97%	0.25%	71.74%
12	0.75%	0.18%	8.82%
13	0.79%	0.21%	17.76%

As seen in Table 6-2, the results for vanillic acid (VA) seem to be in error. Clearly, yields of up to 78% vanillic acid, meaning that at least 78% of BSG is vanillic acid or its precursor, seems highly inaccurate. The most obvious explanation is that the peak for vanillic acid is impure and contaminated by other BSG compounds that were not accounted for. Further evidence of this inaccuracy is conveyed through the p-value in the

analysis. Neither correlations with time, temperature, ratio, nor any combination thereof lead to a p-value less than 0.26, which suggests a poor correlation.

Ferulic acid yield was found to correlate strongly with both time and temperature. A contour plot is shown in Figure 6-9. The amount of ferulic acid extracted increases with both time and temperature, and the contour plot suggests a maximum yield of ferulic acid has not been found. The p-values for time, temperature, and the interaction of time and temperature are 0.0000695, 0.000000013, and 0.00380, respectively. P-value is an indictor of error, and a good correlation is denoted by a p-value of less than 0.01. Thus, temperature is very highly correlated, followed by length of reaction.

Temperature and BSG to water ratio are both correlated to the production of 4VG. A contour plot of these relationships is shown in Figure 6-10, which also suggests that a maximum has not been found. The p-values for temperature, ratio, and the interaction of the two are 0.000000314, 0.043, and 0.0070, respectively. Thus there is a strong correlation of 4VG with temperature, and only a weak correlation with ratio. It is odd that there appears to be no connection with time, considering that the 4VG would be expected to form only after the ferulic acid is released. This implies that the concentration of 4VG is in equilibrium, and the rate of formation of it is mostly equal to the conversion of it to other products. This is reasonable, as 4VG is believed to be the intermediate species in the conversion of ferulic acid to vanillin.

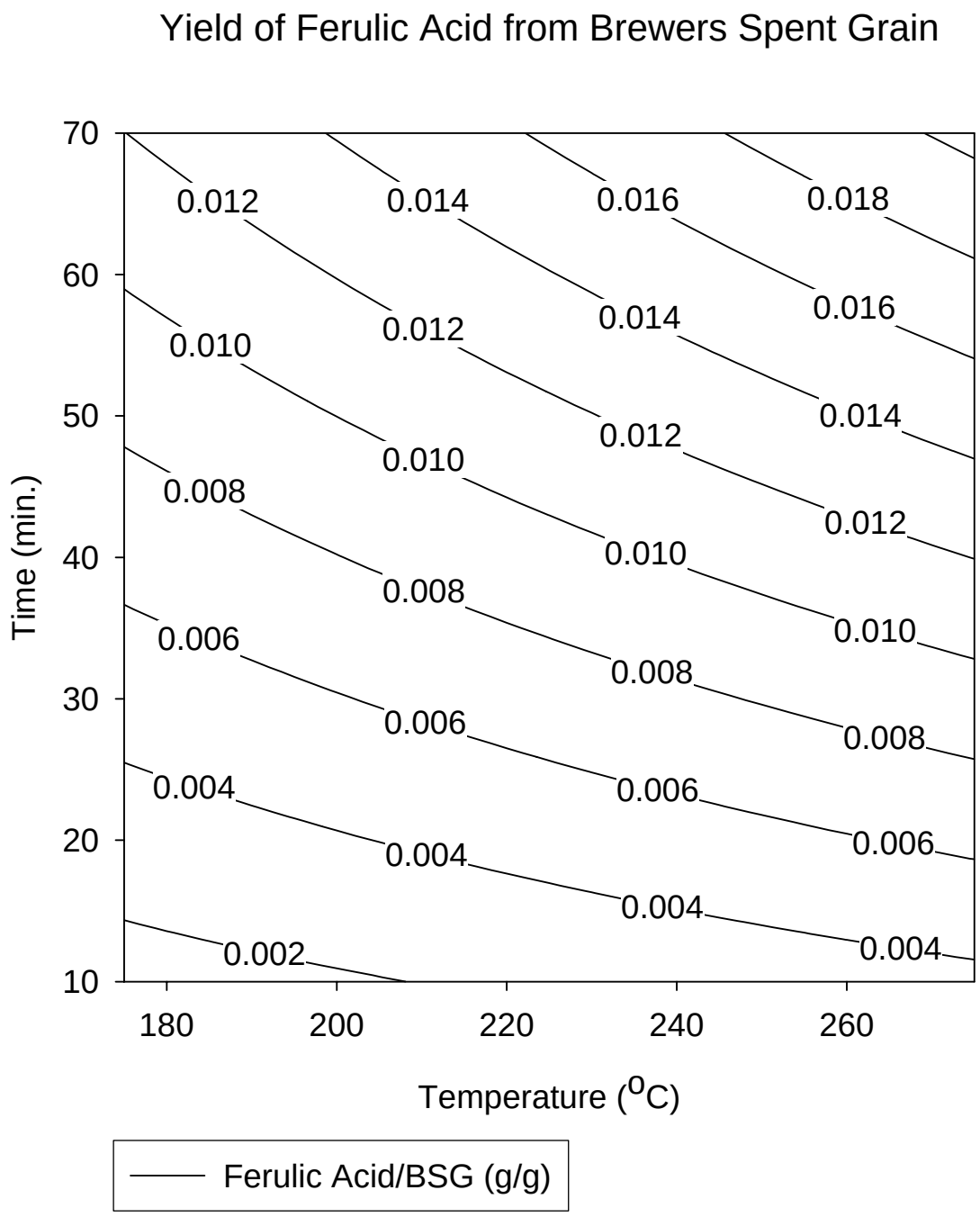

Figure 6-9: Yield of ferulic acid from BSG, correlated with time and temperature of reaction.

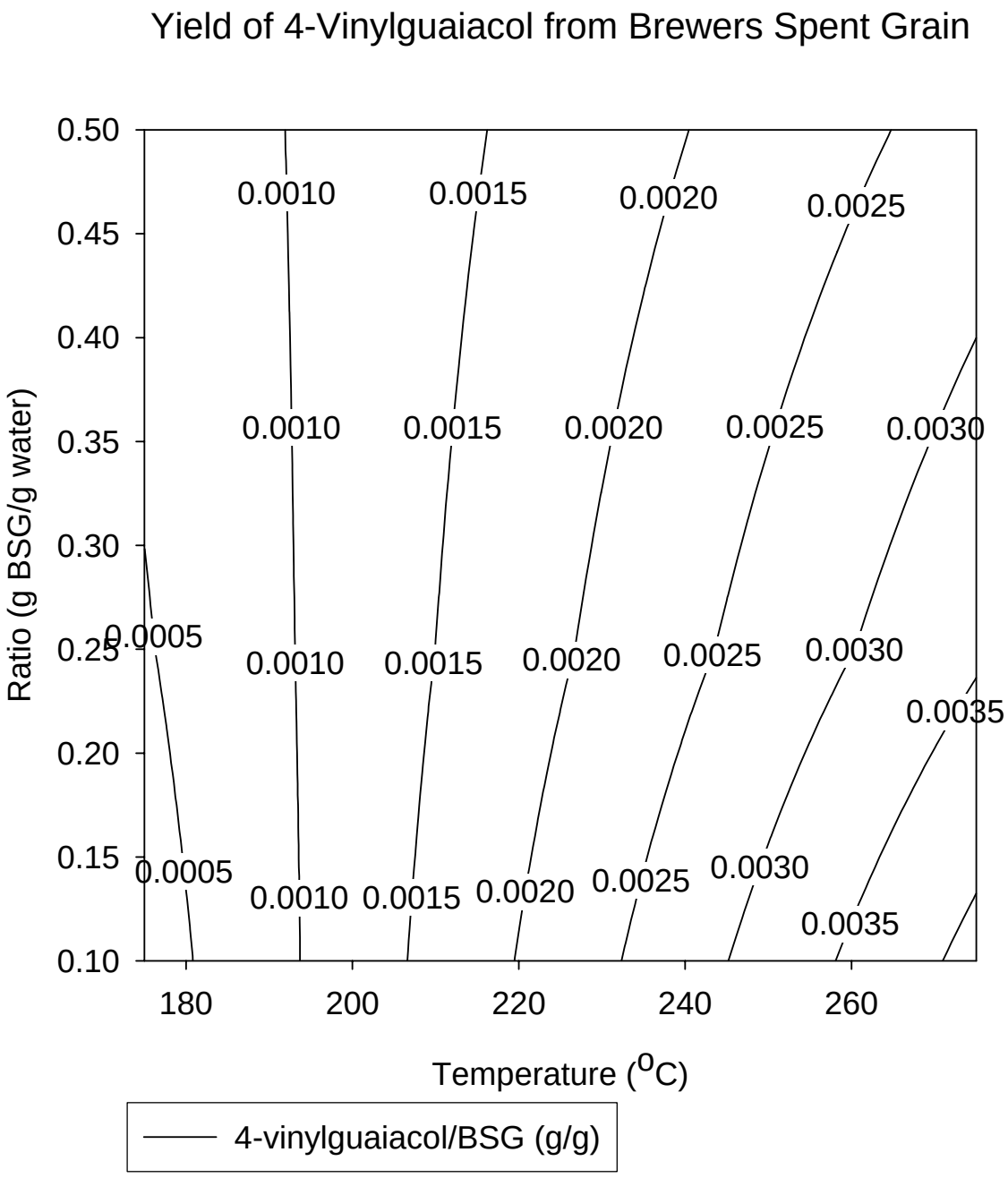

Figure 6-10: Yield of 4-vinylguaiacol from BSG, as correlated with ratio and temperature of reaction.

Table 6-3, shown below, compares the available literature data for ferulic acid content in various cereal grains. In most cases, the total FA content is determined by either alkali extraction or by microbial release. It is important to note that our values appear in some cases to be slightly higher than those in the literature. However, the components of BSG, as far as different types and ratios of grains, vary greatly depending on the beer type, and depending on the brewery. It is possible that our specific BSG had higher amounts of maize, wheat, or another grain with high amounts of ferulic acid. It is also possible, while not likely, that near-critical water performs better as an extractant than do the alkaline or microbial processes.

Table 6-3: Literature values for the ferulic acid concentrations in various grains which are used in brewing, and in BSG.

Grain	FA (ug/g)	Reference
Maize	25000	Coghe, Benoot, et al. 2004
Maize	17200	Grabber, Hatfield et al. 1998
Barley	365-605	Zupfer, Churchill et al. 1998
Barley	359-624	Hernanz, Nunez, et al. 2001
Wheat	784-1980	Lempereur, Rouau, et al. 1997
Rye	895-1174	Andreasen, Christensen et al. 2000
BSG	1860-1948	Hernanz, Nunez, et al. 2001
BSG	1800	Bartolome and Gomez-Cordoves 1999

Additional Results

To ensure the requirement of the near-critical water for the reactions, a blank was run in the same manner as the actual samples, with an extraction time of 70 minutes at room temperature, and with a ratio of 0.1g BSG/g water. The extract from the BSG

revealed no ferulic acid or 4VG, thus proving the success of the NCW extraction and the negligible effects of using methanol in the analysis.

Another variable tested was the amount of headspace, and therefore air, present during the reactions. Two other samples, #s 13 and 14, were run identically to that of experiments 9-12 of the statistical design, except that the reactors were filled almost twice as full as in the other runs, thus decreasing the amount of air in the reactors. This change did not have any effect on the ferulic acid or 4VG levels in the extractant, and this conclusion is both supported and refuted by the literature. An article by Walter Fiddler (Fiddler, Parker et al. 1967) describes the conversion to 4VG being a radical mechanism, and therefore catalyzed by air, whereas a study by Hanna Peleg (Peleg, Naim et al. 1992), found that the amount of air did not affect the amount of 4VG present. What both groups did notice however was that vanillin and vanillic acid levels were improved with air, as opposed to nitrogen. This is understandable considering the pathway of 4VG to vanillin is via oxidation. This study was unable to identify clear vanillin or vanillic acid peaks, and therefore these compounds were not analyzed for at this time.

To compare the results from BSG, 3 samples were run on pearled barley, and 2 more run with BSG under the same conditions. In two of the barley samples, the pearled barley was ground to a powder with mortar and pestle, and in the third sample, the pearled barley was used as received. Due to the fact that some ferulic acid and 4VG end up in the beer and wine products, one would expect to extract less of these products from the BSG than from the unprocessed barley. However, the results are quite the opposite (Figure 6-11).

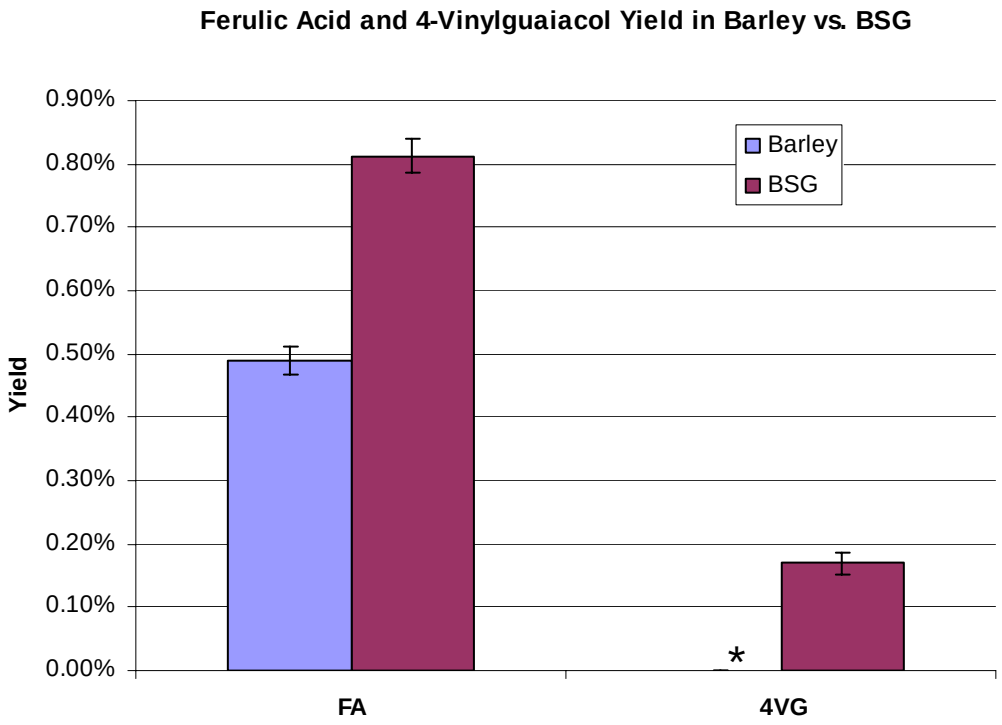

Figure 6-11: Yield comparison of ferulic acid and 4-vinylguaiacol from pearled barley and BSG. * implies no detectable product.

The yields for both ferulic acid and 4VG are significantly higher from BSG than from the pearled barley. A paper by Hernanz et al. (Hernanz, Nunez, et al. 2000) showed this same result, with BSG providing 5x the yield of ferulic acid than did the unprocessed barley. This is likely due to the composition of BSG being a mix of various types of grains, many having higher levels of ferulic acid than barley (Table 6-3). The other explanation is that the brewing process has better prepared the ferulic acid for being extracted. The comparison between ground pearled barley and the whole barley kernels was inconclusive, as the yields appeared to be the same. While these results are promising, further testing is needed to confirm these conclusions.

Conclusions

The near-critical water extraction of ferulic acid and 4-vinylguaiacol from Brewers Spent Grain was successful. However, the statistical design of experiments showed no maximum yield of either component within the scope of the parameters tested. Thus further testing is needed to optimize these yields. Furthermore, an attempt to quantify vanillic acid extraction was unsuccessful, due assumingly to an incomplete separation of peaks on the LC-MS. Control experiments verified the ability of near-critical water to catalyze the conversion of ferulic acid to 4-VG, but also led to apparent polymerization of 4VG. Lastly, a comparison of extraction between barley and BSG showed higher extraction ability for BSG. This result is corroborated by a study by Hernanz et al. (Hernanz, Nunez et al. 2000).

The next step for this project is to perform extractions at conditions that follow a given line in each of the contour plots (Figures 6-9 and 6-10) until a maximum is realized. Then, another statistical design should be created around that maximum to fully characterize the system. Once an optimal extraction is designed, the system can be scaled up to accommodate a large amount of BSG. A detailed discussion of this can be found in Chapter VII. To make this process more cost effective, the analysis method should be further developed to incorporate other high value compounds such as vanillic acid, vanillin, and syringol. Then a separation scheme, possibly fractionation, would need to be developed to isolate each chemical. For ferulic acid and 4VG, a simple organic-aqueous extraction would work, but for additional chemicals, a more complex system would be needed. Also, because beer is made in a batch process, a continuous system

would not be beneficial here; the extraction and separation would need to be run as a batch process also.

With the 57 million pounds of BSG produced in the US every year, there are plenty of resources for this process. Currently, BSG is given as feed for livestock or sometimes composted and used as fertilizer, as a brewery in South Africa does. Namibian Breweries Ltd. exists as a "fully integrated biosystem" with over 40 biochemical processes that reuse everything from heat, water, wastes, and even CO_2. The company produces 7 times more food, fuel, and fertilizer than a traditional brewery (Zero Waste 2005). While this sort of proficiency is somewhat far off, a simple, environmentally friendly extraction like NCW might not be. It could either add an additional revenue stream for a brewery, or a business could be started that buys the BSG cheaply from the breweries and then converts it to valuable chemicals with low upfront and operational costs. Either way, this process would help to eliminate some of the wasted agricultural products and create a sustainable source of valuable chemicals.

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CHAPTER VII

CONCLUSIONS & RECOMMENDATIONS

General Conclusions

The diversity of these projects has allowed this thesis to shed light on several different topics in green engineering. The study of green solvents has had a huge thrust in recent times, specifically that of ionic liquids. They have been used in countless reactions and for many different applications, and while the excitement for them is exceptional, a complete understanding of them is not. The work in this thesis has been fundamental in understanding how ionic liquids react with CO_2, and more importantly, how to manipulate them in CO_2. While this may not be as exciting a developing a new application, this discovery can be used to improve upon many already existing ionic liquid applications.

Also, with the explosion of protein and enzyme chemistry in biotechnology, typical separations can no longer be used, due to the denaturing effect many harsh solvents have on biological materials. The challenge is to design as equally effective a separation as previously used, yet have it be gentle enough to maintain biological activity. While the two studies dealing with benign extractions here are certainly not a panacea, they do have benefits over currently used biological separation techniques, in the form of reduced waste, simpler systems, and more efficient extractions. Further development of

protein-solvent interactions would provide much insight into the possibilities for separation, and should be evaluated.

One of the major drawbacks in implementation of green engineering practices is the lack of motivation of companies in industry. Many current green processes are not adopted by companies due to the high expense and inconvenience to the company. The projects involving catalyst recycle and chemical extraction from BSG demonstrate cost effectiveness in green engineering by saving the company money as well as eliminating waste streams to the environment. These types of processes are going to be the most successfully implemented, due solely to this benefit, and should serve as an example when designing green technology.

Melting Point Depression of Ionic Liquids

In this project, a method was developed for controlling the melting point of ionic solid and liquid species with CO_2 pressure. Freezing point eutectic curves were calculated for two ionic solids resulting in melting point depressions of up to 120^{o}C. Depressions of this magnitude and precise manipulation of these depressions enable better control over the many applications that are currently being developed for ionic liquids, including synthesis and separations.

To further develop this technology, more insight is needed into the molecular interactions of the ionic species with the CO_2. In this study, negative deviations from ideal solubility were found for ionic liquids in CO_2. This is reasonable considering the size difference between the cations and the CO_2. To get a better understanding of this interaction, ionic liquids of differing cation and anion sizes should be tested in the same

manner as was done here. Also, different familes of cation (imidazolium, phosphate, etc) and different types of anion (Cl, PF_6, NO_3, etc) should be tested as a comparison to the alkylammonium ionic liquids used in this study.

Another method for studying the molecular interactions of the ionic liquids with CO_2 is to do a perturbation analysis. This could be done on a given cation or even on naphthalene, the model organic compound used in this study. Slight modifications in chemical structure, as related to the change in melting point, allow for better understanding of melting point depression, and hence better design of ionic liquids and solids.

Protein Extractions using Modified Aqueous Two Phase Extraction

This study was aimed as eliminating the drawbacks to Aqueous Two Phase Extraction (ATPE) industrialization through modification of the polymer phase. By replacing the polyethylene glycol (PEG) with a water-miscible organic solvent, the phases remain similar enough to protect the proteins, yet different enough to afford a cleaner separation and improved downstream processing.

Several problems were encountered however, that call for further investigation. The first is the decrease in partitioning when lysozyme is analyzed via UV without the Bradford assay. This should not be a factor considering the assay is performed on samples removed after partitioning is complete. However, there is a large difference and accuracy of the measurements should be studied by comparison to other means of analysis, such as LC-MS. If measuring lysozyme concentration by UV alone is not accurate, then the Bradford assay will be needed for all experiments.

The next issue is the stability of lysozyme in the chosen solvents. A kit from Molecular Probes was purchased to study this effect with fluorescence; however, due to time and equipment limitations, the analysis was not completed. It is known that many organic solvents denature lysozyme, but with water-miscible solvents, the phases are of mixed composition, thus permitting a more agreeable environment for the proteins.

In order to design better extraction systems for proteins, molecular analysis of the proteins in different solvents is needed. Due to the highly complex nature of proteins, this is indeed a daunting task. However, doing a systematic test of polypeptide partitioning in various solvents would provide a basis for this understanding. Coupling these results with protein coding and protein folding models could make possible the prediction of protein behavior in different extraction media.

In either of the cases mentioned above, a statistical design of experiments lends itself to the system. Extraction variables to test include temperature, time, pH, solvent/aqueous ratio, salt type, and salt amount. The response variables would be lysozyme activity and partition coefficient, K. Given all the parameters involved with this system, a statistical design would greatly reduce the complexity and amount of time needed to optimize the lysozyme extraction.

OATS Project and Salen Replacement

Even though the asymmetric Salen catalyst was shown to be not worth recycling (Hughes, Smith et al. 1997), this project was successful in creating a water-soluble analog that performed as well as the original. The project also provided more knowledge and experience in asymmetric catalysis and improving yield and ee through temperature and

mass transfer improvements. The lessons learned can easily be applied to another catalyst or reaction system to provide a better prototype reaction scheme to be enhanced with organic aqueous tunable solvents (OATS) (Lu, Lazzaroni, et al. 2004).

The alkene epoxidation reaction was chosen for study for its relevance in pharmaceutical development. Staying with epoxidations, several other catalysts may be good alternatives to Salen, including the Sharpless reagent, $MoO_2(acac)_2$, and methyltrioxorhenium(MTO)/pyridine. The Sharpless reagent, as developed by Barry Sharpless (Katsuki and Sharpless 1980), uses (+) or (-)-diethyl tartrate, titanium tetraisopropoxide, and tert-butyl hydroperoxide in a non polar solvent to epoxidize allylic alcohols. The stereochemistry of the epoxidation is determined by the type of diethyl tartrate used, and usually has yield of 70-90% and >90% enantiomeric excess (Katsuki and Sharpless 1980). The downside to the Sharpless reagent is that the Ti/tartrate complex cannot be stored, and also the catalyst must appropriately "aged" (Besse and Veschambre 1994). Lastly, and perhaps most importantly, all the reagents involved are available commercially at low to moderate cost, thus minimizing the need for recycle.

Some of the most versatile catalysts for epoxidation of alkenes are Mo(IV) catalysts (Parshall and Ittel 1992). Great strides have already been made to design a method for recycling these catalysts, particularly $MoO_2(acac)_2$, and most research has focused on using organic polymers as supports (Dallman, Buffon, et al. 2002). A 20% loss of catalyst was found for this type of recycling, so this provides a great opportunity for OATS as an alternative to these polymers supports.

The third option is the methyltrioxorhenium (MTO)/pyridine catalyst system. In this reaction, the pyridine ligand operates as a reaction accelerant and stabilizer to both

the catalyst and the epoxide (Rudolph, Reddy et al. 1997), similar to that of P_3NO in Salen (Senanyake, Smith et al. 1996). This catalyst system has even been used with the epoxidation of indene, resulting in a 92% yield and >98% ee (Rudolph, Reddy et al. 1997). A complex system like this would need to be studied in OATS to determine if it is possible to recycle the catalyst/pyridine complex, or to recycle each separately in a two-step process. If so, this provides an excellent example of the versatility of the OATS system.

If willing to use a reaction other than epoxidation, the opportunites are limitless. Other reactions appropriate to pharmaceutical use include hydrogenations, oxidations, and aminations. One specific hydrogenation reaction is that of 2-(6'-methoxy-2'-naphthyl)acryilic acid to naproxen using an Ru-BINAP catalyst, resulting in a 92% yield and 97% ee (Wan and Davis 1993). However, the naphthyl acrylic acid may be too expensive to warrant development of this reaction (Harrington and Lodewijk 1997).

Overall the most promising alternative to Salen is the $MoO_2(acac)_2$ catalyst. It is a well-studied system and easy to work with. Further development of OATS to recycle both the MTO and pyridine ligands would also be beneficial, but would require a large investment in further developmental work. Both of these systems could provide a good model reaction and separation for the OATS technology.

Continuous Extraction of Ferulic Acid from Biomass

The goal of this project was to design a continuous extraction unit for removing ferulic acid from biomass, while not damaging the esterase enzymes. Ethyl acetate was chosen as the extraction solvent despite its low partitioning for several reasons. First, it

naturally forms two phases with water without the addition of salt, thus greatly simplifying the system. Secondly, there is no reaction as there is with tertiary amines. Lastly, the low partitioning is not an issue when designing a continuous process because the product can be enriched by increasing the number of stages. Excellent repeatability and mass balance calculations suggest this is a reproducible process for scale-up.

The largest concern facing this system currently is the stability of the enzyme. Furulyl esterases are typically used for breaking the ester bond that holds ferulic acid to the cell wall. Purifying and isolating these enzymes is not an easy task, and therefore all caution should be used to maintain their activity. This places severe limitations on the extraction process, specifically on temperature, pH, and stability towards the solvent. Once these limits have been determined, it will be possible to better optimize this system.

During the course of research, several interesting results and ideas came up. The first is that "dirty" amines, or amines that have been on the shelf for some time, are actually several hundred percent better at extracting ferulic acid than are their "clean", or recently opened counterparts. The obvious hypothesis is that the dirty amines are contaminated by superior extractant, however this was never examined. It has been noted (Yang, White et al. 1991) that tertiary amines extract only the undissociated form of carboxylic acids. In our case, most of the acid was dissociated, thus explaining the low partitioning for clean tertiary amines. One way to increase the partitioning in tertiary amines is to use an alcohol to help extract more of the acid, known as reactive extraction. Using this technique, the partition coefficients doubled from that of clean tertiary amines, but were still much lower than for the dirty amines. This suggests that whatever the contamination in the dirty amines, it be worthwhile to identify them for improvement of

extraction. This finding also implies that a combination of solvents, each targeted at different forms of the acid, may greatly improve partitioning.

Once full characterization of the method is complete, the final step would be to model the system in ASPEN or HYSYS and to develop a continuous, staged unit operation.

High Value Chemicals from Brewers Spent Grain

In this project, phenolic compounds were extracted from Brewers Spent Grain (BSG) using near-critical water. This benign extraction proved efficient at removing both ferulic acid, and its decarboxylation product, 4-vinylguaiacol (4VG) from the BSG. A statistical design of experiments showed good correlation of ferulic acid yield with time and temperature of reaction, and good correlation of 4VG with temperature and ratio of BSG to water. However, a maximum yield was not found for either of these chemicals with the conditions studied here, thus requiring more investigation.

The method for finding a maximum with statistical design is as follows. First, a line is chosen on the contour plot (Figure 6-9 or 6-10), and experiments are done in a manner following that line until the maximum is reached. Then another statistical design is done, centered on the maximum point. The resulting analysis will then provide full characterization of the system in and around the maximum. With that information, the process can be further developed to either maximize one specific chemical, or to maximize a set of chemicals. A schematic of such a system is pictured in Figure 7-1.

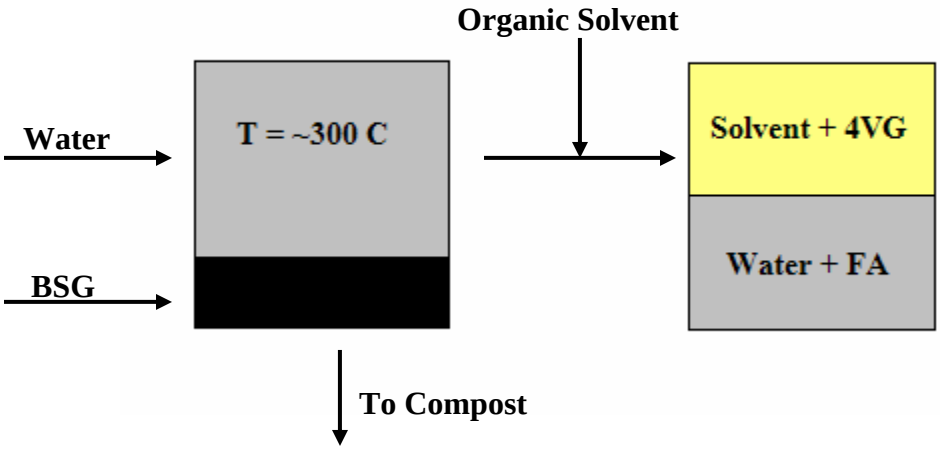

Figure 7-1: Process schematic for production of ferulic acid and 4VG from BSG.

In this study, measurements were taken for the yields of ferulic acid, 4VG and vanillic acid. However, the analysis for the vanillic acid was inaccurate, as evidenced by the resulting yields (Table 6-2), and the regression analysis (Appendix C). A more accurate LC method would allow analysis of not only ferulic acid and 4VG, but vanillic acid, vanillin, syringol, and perhaps other chemicals of high value. Also, as many chiral molecules are found in nature, the pharmaceutical world has turned its attention to the environment for insight and sources of drugs (Cragg, Newman et al. 2005). With the amount of chiral compounds produced in nature, BSG may very well be a source for the next miracle drug.

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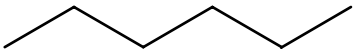
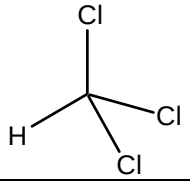
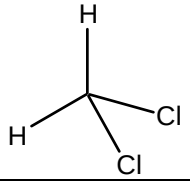
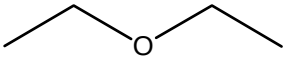
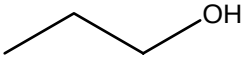
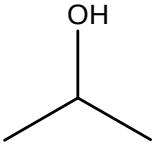
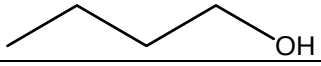
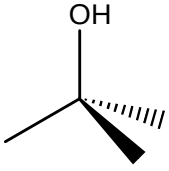
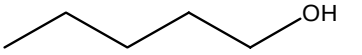
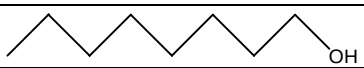
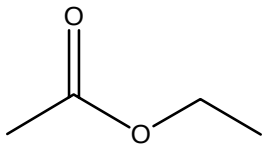
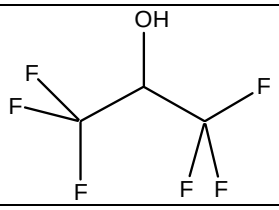
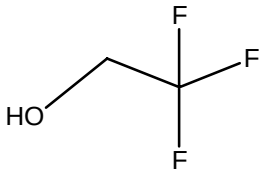
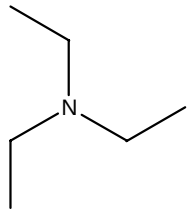
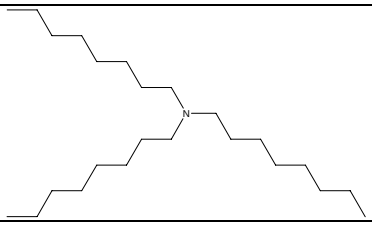
APPENDIX A

FERULIC ACID EXTRACTION SOLVENT INFORMATION

Name	Structure	MW (g/mol)	Purity (%)	Supplier	Salt Added (g)[a]	K (org/ aq)
hexane		86.2	99.7	Fischer	0	0.004
decane		142.3	99+	Sigma	0	0.015
chloroform		119.4	99.8	Acros Organics	0	0.013
methylene chloride		84.9	99.9	Fischer	0	0.009
diethyl ether		74.1	ACS	Acros	0	0.037
1-propanol		60.1	99.8	Fluka	0.387	1.15
isopropanol		60.1	99.5	Aldrich	0.8233	1.18
1-butanol		74.1	99.8	Aldrich	0	0.63
t-butanol		74.1	99	Sigma	0.2128	1.30
pentanol		88.2	99+	Aldrich	0	0.39
octanol		130.2	99+	Aldrich	0	0.14

ethyl acetate		88.1	99.9	Fischer	0	0.22
HFIP		168	99+	Aldrich	0.1375	2.82
TFE		100	99.8	Acros Organics	0.3022	1.59
TEA		101.2	99	Acros Organics	0	0.25
TOA		353.7	98	Acros Organics	0	0.20

[a] Salt required to induce a phase split between a 50/50 water/solvent mixture at 25°C

APPENDIX B

ETHYL ACETATE REPEATABILITY AND MASS BALANCE

Run	Phase	A @ 300nm	K (A_{org}/A_{aq})	Conc. (g/mL)	Phase Vol. (mL)	FA (g)	Total Mass (g)	Percent Error[a]
FA-PBS	Stock Sol.	1.9332		2.933E-05	6	1.760E-04	1.760E-04	
EA #1	top	0.3680	0.222	5.584E-06	6	3.351E-05	1.846E-04	+4.9%
	bottom	1.6591		2.517E-05	6	1.510E-04		
FA-PBS	Stock Sol.	2.1027		2.933E-05	5	1.467E-04	1.467E-04	
EA #2	top	0.3052	0.175	4.257E-06	5	2.129E-05	1.432E-04	-2.4%
	bottom	1.7480		2.438E-05	5	1.219E-04		
EA #3	top	0.3034	0.175	4.233E-06	5	2.116E-05	1.422E-04	-3.1%
	bottom	1.7350		2.420E-05	5	1.210E-04		
EA #4	top	0.3007	0.173	4.195E-06	5	2.098E-05	1.423E-04	-2.9%
	bottom	1.7401		2.427E-05	5	1.214E-04		
EA #5	top	0.3325	0.188	4.638E-06	5	2.319E-05	1.463E-04	-0.3%
	bottom	1.7648		2.462E-05	5	1.231E-04		

[a] (-) is indicative of mass lost and (+) is mass gained

	Average K	Standard Deviation of K
Runs 1-5	0.1865	0.02071386
Runs 2-5	0.1777	0.00721758

APPENDIX C

REGRESSION STATISTICS FOR VANILLIC ACID, FERULIC ACID, AND 4-VINYLGUAIACOL

SUMMARY OUTPUT FOR VANILLIC ACID

Regression Statistics	
Multiple R	0.756101
R Square	0.571688
Adjusted R Square	0.27187
Standard Error	0.23214
Observations	18

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	7	0.719281	0.102754	1.906784	0.171049
Residual	10	0.538889	0.053889		
Total	17	1.25817			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95%*	*Upper 95%*
Intercept	0.418251	0.094771	4.413293	0.001308	0.207089	0.629413	0.207089	0.629413
A	-0.08435	0.070084	-1.20356	0.256475	-0.24051	0.071806	-0.24051	0.071806
B	0.070346	0.070084	1.003734	0.339176	-0.08581	0.226502	-0.08581	0.226502
C	0.052963	0.071078	0.745146	0.473333	-0.10541	0.211335	-0.10541	0.211335
AB	-0.07667	0.070084	-1.09403	0.299593	-0.23283	0.079483	-0.23283	0.079483
AC	-0.06674	0.071078	-0.93898	0.369865	-0.22511	0.091631	-0.22511	0.091631
BC	0.064151	0.071078	0.902545	0.38799	-0.09422	0.222523	-0.09422	0.222523
Curve	-0.33276	0.11787	-2.82314	0.018063	-0.59539	-0.07013	-0.59539	-0.07013

SUMMARY OUTPUT FOR
FERULIC ACID

Regression Statistics	
Multiple R	0.987093
R Square	0.974353
Adjusted R Square	0.9564
Standard Error	0.001378
Observations	18

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	7	0.000721	0.000103	54.27236	3.7E-07
Residual	10	1.9E-05	1.9E-06		
Total	17	0.00074			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	0.008501	0.000562	15.11462	3.25E-08	0.007248	0.009754	0.007248	0.009754
A	0.002701	0.000416	6.494156	6.95E-05	0.001774	0.003628	0.001774	0.003628
B	0.00691	0.000416	16.61392	1.3E-08	0.005983	0.007837	0.005983	0.007837
C	0.000564	0.000422	1.337624	0.210644	-0.00038	0.001504	-0.00038	0.001504
AB	0.001559	0.000416	3.748168	0.003795	0.000632	0.002486	0.000632	0.002486
AC	-0.00011	0.000422	-0.25742	0.802073	-0.00105	0.000831	-0.00105	0.000831
BC	0.000677	0.000422	1.603888	0.13982	-0.00026	0.001616	-0.00026	0.001616
Curve	0.00126	0.0007	1.800829	0.101914	-0.0003	0.002818	-0.0003	0.002818

SUMMARY OUTPUT FOR
V-VINYLGUAIACOL

Regression Statistics	
Multiple R	0.973845
R Square	0.948373
Adjusted R Square	0.912234
Standard Error	0.000414
Observations	18

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	7	3.15E-05	4.51E-06	26.24254	1.16E-05
Residual	10	1.72E-06	1.72E-07		
Total	17	3.33E-05			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	0.002234	0.000169	13.20622	1.18E-07	0.001857	0.002611	0.001857	0.002611
A	7.5E-05	0.000125	0.599804	0.561981	-0.0002	0.000354	-0.0002	0.000354
B	0.00149	0.000125	11.91025	3.14E-07	0.001211	0.001769	0.001211	0.001769
C	-0.00029	0.000127	-2.31387	0.043221	-0.00058	-1.1E-05	-0.00058	-1.1E-05
AB	-0.00012	0.000125	-0.97066	0.35461	-0.0004	0.000157	-0.0004	0.000157
AC	-0.00012	0.000127	-0.96072	0.359348	-0.0004	0.000161	-0.0004	0.000161
BC	-0.00043	0.000127	-3.38408	0.006955	-0.00071	-0.00015	-0.00071	-0.00015
Curve	-0.00044	0.00021	-2.10179	0.061891	-0.00091	2.66E-05	-0.00091	2.66E-05