ABSTRACT

Rates and Equilibria of Vial-In-Vial Vapor Diffusion for Common Laboratory Solvents

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Vapor diffusion crystallization is the chief method of crystallization used throughout the world for preparing X-ray crystals. While the extensively studied hanging drop method remains the most popular, almost no research has been done, despite its frequent use, on the vial-in-vial method, which can be effective for both preparative and analytical amounts unlike the hanging drop method. Indeed, the literature contains no mention of either the kinetics or equilibrium for vial-in-vial vapor diffusion, and the rules for proper technique remain largely empirical.

Included herein are the results of an extensive study of the rates and equilibria governing vial-in-vial vapor diffusion. A large table is included which lists various combinations of laboratory solvents which researchers can consult when selecting solvents for their system. We attempt to characterize the underlying forces which drive vapor diffusion, and we propose two tentative methods of modeling the kinetics data to provide a quantitative measure of the rates and/or equilibria. Finally, we examine common variations in configurations which may affect the researcher's results.

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RATES AND EQUILIBRIA OF VIAL-IN-VIAL VAPOR DIFFUSION FOR COMMON LABORATORY SOLVENTS

A Thesis Submitted to the Faculty of

Baylor University

In Partial Fulfillment of the Requirements for the

Honors Program

By

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Tables and Figures

To my mentor, Dr. Charles Garner,

who could have opened a fast-food chain

with all of the nuggets (of wisdom)

he has imparted to students.

Thank you to Baylor University

for use of her facilities,

and her wonderful library program,

without which this endeavor

would not have been possible.

Finally, thank you to all those

who offered their expertise and advice

when we were lost.

CHAPTER ONE

Introduction

Crystallization is an important step for obtaining pure samples of compounds for preparative or analytical needs.[1,2,3,4] Obtaining crystals is especially valuable for X-ray crystallography. The pioneers of which, William Henry Bragg and his son Lawrence Bragg (the youngest ever winner), were awarded the Nobel Prize in Physics for their work in 1915.[5] Since then, many important discoveries have relied on crystallization to elucidate the structure of molecules. For example, it was X-ray analysis of crystals which confirmed Watson and Crick's structure of DNA in 1973.[6] The United Nations designating the year 2014 as the International Year of Crystallography to highlight the importance of crystallography gives testament to the integral role crystals and crystallography has played in modern science.[7]

1.1 *Commonly Used Terms*

In the interests of readers without a scientific background, common jargon used in this paper will be briefly introduced. 'Solubility' is a measure of the quantity of solute that will dissolve in a solvent and is a critical property pertaining to growing crystals. The general term 'solute' is used to refer to the solid being dissolved, while the term 'solvent' refers to the liquid doing the dissolving. Using table salt and water as an example, the salt is the solute and the water is the solvent. The salt and water together is termed a 'solution.' When some solute dissolves into a solvent successfully, we say the

solute is ‘soluble’ in the solvent, to varying degrees. Salt will not dissolve in vegetable oil and so is ‘insoluble’ in oil. When another solvent can mix into a solvent, we say the two are ‘miscible.’ Water and oil do not mix and so are ‘immiscible.’ ‘Concentration’ refers to the amount of salt currently dissolved in solution *relative* to the amount of solvent. So, if solvent volume decreases while the amount of solute is held constant, concentration will still have increased. When no more solute can be dissolved in solvent, the solution is then ‘saturated.’

1.1.1 *Supersaturation*

For any given solid/liquid combination, the amount of solute needed to reach saturation varies depending on many factors. One of simplest to control is temperature. The higher the temperature, the more solute can be dissolved (almost universally), and conversely, the lower the temperature, the less. When a solution is saturated, no more of that solute can dissolve, but heating up a saturated solution will make it no longer saturated. Conversely, when a solution is saturated and then cooled to below starting temperature, the solution is temporarily ‘supersaturated,’ where solute concentration exceeds its solubility, or in other words, more solute is in solution than should be. It is during this time that the dissolved solute tries to reform crystals to reach normal saturation. Under these conditions, the crystallization rate can often be irreproducible, depending on the presence of nucleation sites. To illustrate, making rock candy is a fun experiment which exhibits crystallization, where a string is submerged in a jar of hot sugar water. Over time as the solution cools, large sugar crystals coat the string which acts as a large nucleation site. This is supersaturation at work, and the process itself

mimics a version of crystallization used commonly in laboratories: hot/cold crystallization.

The same effect of supersaturation followed by crystallization can also be achieved by varying polarity. Table salt is very polar like water, while oils are nonpolar, and so table salt dissolves in water, but does not dissolve into oils. This is because the polarities of the two are too different. In common practice, polarity is a continuum. Most organic compounds have a polarity between the two extremes due to having parts which are polar and other parts which are nonpolar. The target variable for vapor diffusion in our study is to vary the polarity. By diffusing a solvent of different polarity into a solution, the overall polarity of the solution can be changed and supersaturation perhaps induced.

1.2 *Crystallization and crystallography*

Crystallography deals with determining the structure of a compound from crystals, and X-ray crystallography continues to be a particularly powerful tool to elucidate atom arrangement. For it to be useable, however, requires the researcher to obtain a pure crystal of compound, and usually creating that pure crystal is the major bottleneck of the crystallography process.[8] Obtaining a good quality crystal is often done in the laboratory via a crystallization method. The phenomenon of crystallization is a two-step process, split into nucleation and growth.[9] Beginning with nucleation, solute molecules dissolved in solution initially gather, creating a nucleation site, a fragment, where further growth can occur. An astute chef may have figured out through observation that the bubbles in boiling water form more easily on imperfections such as

scratches on cookware. Scratches are an example of a nucleation site, without which crystallization may never begin. For example, water may refuse to crystallize and form ice even at -30 °C if it lacks the presence of a nucleation site.[10] For this reason, using new vials which lack scratches for nucleation is occasionally advised against. Dust sometimes can function as a nucleation site, though "seed" crystals are more reliable and more commonly used for expediting nucleation when necessary.[11,12]

Growth occurs when solute deposits onto the surface of the eventual crystal. The solute must therefore be willing to exit solution, and this is induced by maintaining a state of slight supersaturation. All crystallizations require supersaturation, which functions as the main driving force behind nucleation and growth.[13] However, the process must be done slowly to ensure that satisfactory crystals are obtained. If crystallization occurs too quickly, then too many nuclei form during the nucleation phase, giving rise to small crystals unsuitable for crystallography.[14] Slow crystallizations hence form the largest crystals because spontaneous nucleation site formation is minimized and affords the purest crystals as the expanding crystal face has enough time to select only like molecules and exclude impurities.

1.3 *Vapor diffusion crystallization*

Vapor diffusion is a broad category of diffusion involving movement of vapors from one area to another. The hanging-drop variation is currently the most popular method, used in at least 73% of protein screening in Europe, and it has been shown to be more successful at producing crystals during the first four weeks than a competing method.[15] Vapor diffusion is a process which utilizes two solvents, one to dissolve the compound and the other to slowly supersaturate the compound, such that it begins to

crystallize. Vapor diffusion stands out due to being extremely controllable with respect to rates by adjusting the cross-sectional area or by temperature.[16] As stated earlier, a slowly changing environment is crucial to growing good crystals, and so if for instance the diffusion is noted to be proceeding too quickly, the temperature of the setup can be lowered to decrease the rate.[16] One constraint on vapor diffusion is that careful consideration must be taken towards the solvents chosen. The chosen solvent must be one that the compound of interest can dissolve in, while the second one should be both more volatile and of a different, typically lower, polarity.

1.3.1 *Hanging drop*

Of all the methods that vapor diffusion encompasses, the "hanging drop" method is the most popular for protein crystallization.[17,18] As the name suggests, the compound of interest is dissolved in a drop of solvent which is placed on a cover slip. The cover slip is then flipped upside down over a reservoir containing more solvent so that the droplet is suspended over the reservoir. The reservoir solvent, called the precipitant, is prepared such that water (usually) from the droplet will evaporate and diffuse towards the reservoir, thus increasing concentration of the protein in the drop to supersaturation levels where the compound often crystallizes. In a way, this technique is similar to a controlled evaporation.

There exists a variation to the hanging drop method called the sitting drop method. Whereas the droplet is suspended in the hanging drop method, the sitting drop method has the droplet in a less precarious position: sitting on an alcove next to the reservoir or on an island surrounded by a sea of solvent. There does not seem to be

difference between the two in efficiency, and a technique involving solidifying the reservoir solution with agarose exists to transform one variant to another.[19]

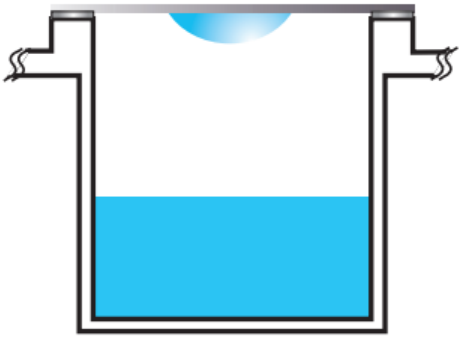

Figure 1: Illustration of the hanging drop method.[20] This method utilizes the diffusion of water from the droplet to the reservoir below to induce supersaturation.

1.3.2 *Solvent layering*

Solvent layering calls for two distinct solvents, the main solvent which has the compound of interest dissolved into it, and the antisolvent, which is to decrease the overall solubility. The compound of interest should be soluble in the solvent, but less or not soluble in the antisolvent, and the two should be miscible. There must also be a difference in polarity between the solvent and antisolvent. Solvent layering calls for the researcher to gently "layer" the antisolvent, ideally of a lower density, onto the solution. As the antisolvent diffuses into the solvent, the resulting mixture carries a lower solubility for the compound than pure solvent, thereby creating a supersaturated state which usually begins crystallizing the compound. The use of two solvents in direct contact means solvent layering is not a vapor diffusion but rather a liquid-liquid diffusion as shown in Figure 2. Solvent layering is mentioned because, in some respects, it mimics a step in the vial-in-vial method of vapor diffusion.

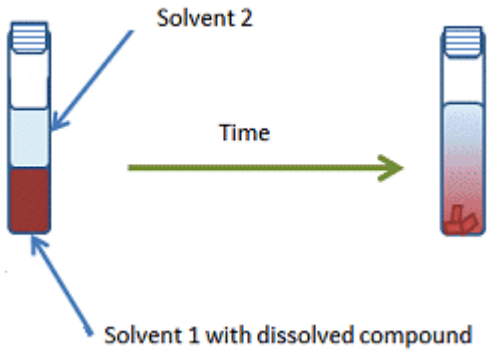

Figure 2: Illustration of the solvent layering method.[21] The gradient in color is similar to the liquid-liquid diffusion step in vial-in-vial.

1.3.3 *Vial-in-vial*

The focus of our research, the vial-in-vial vapor diffusion method is a technique where a small, uncapped vial containing solvent with dissolved solute is placed in a larger vial. The outer vial is filled with a second solvent, capped, and then left to sit. The solvent placed in the outer vial is given many names: antisolvent, precipitant, and non-solvent has also been seen.[16] We will be using the term antisolvent to differentiate from the hanging drop method, which prefers precipitant. Over time, as illustrated in Figure 3, the antisolvent vapors diffuse into the inner vial and join the inner solvent, increasing the inner vial volume and modifying the polarity of the solvent. The outer antisolvent is almost always a more volatile solvent so that the diffusion proceeds from outer to inner, and, like the solvent layering method, the two solvents should be miscible. Many more empirical rules of thumb regarding combinations and setup exist, though whether they are all true or not remains to be seen. For example, one precaution is that the walls of the

two vials should not be allowed to touch so that antisolvent cannot travel up the sides into the inner vial by capillary action.[1,22]

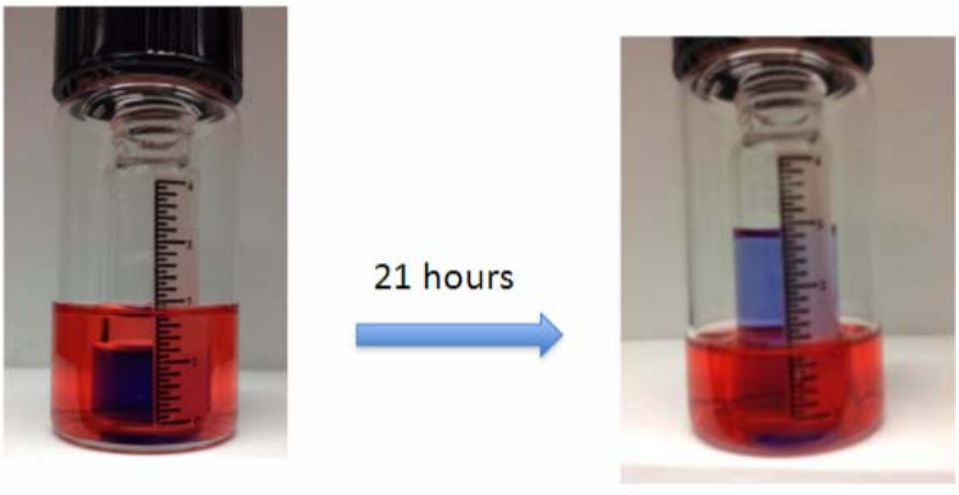

Figure 3: Illustration of the vial-in-vial method taken from our lab. Colored dye was used solely for better visualization.

1.4 *Quantitation of the vial-in-vial method*

Despite the vial-in-vial method's extensive use, little is understood about the underlying factors that govern its kinetics and equilibria. Heuristics used in laboratories remain largely empirical, though some study into effective solvent combinations have been done.[23] Nevertheless, while quantitation on kinetics of water-vapor equilibration for the hanging drop method exists,[24] no such study for vial-in-vial was found. Virtually no quantitation of rates or equilibrium in such diffusions exists in the literature, only passing mentions.[3] We here systematically studied vial-in-vial vapor diffusion to identify combinations that perform well, to determine their rates and equilibria, and to estimate their viability for growing high-quality crystals. Due to the nature of the hanging and sitting drop methods, not much compound can be crystallized, restricting them to analytical use only. Vial-in-vial (along with solvent layering) can process larger

quantities, making these methods useful for preparatory work. The main factors considered were rate of diffusion and extent of diffusion at equilibrium. Rate tells researchers how long the crystallization process is expected to take. The extent of diffusion is directly related to the change in polarity and subsequent likelihood of recrystallization because of the disparity in polarity between the two solvents. We hope our research will serve as useful reference to researchers who can save time and resources not having to test combinations and setups themselves, and we hope our research generates further interest in the field as many intriguing discoveries have been made.

CHAPTER TWO

Methods

2.1 *The two-vial system*

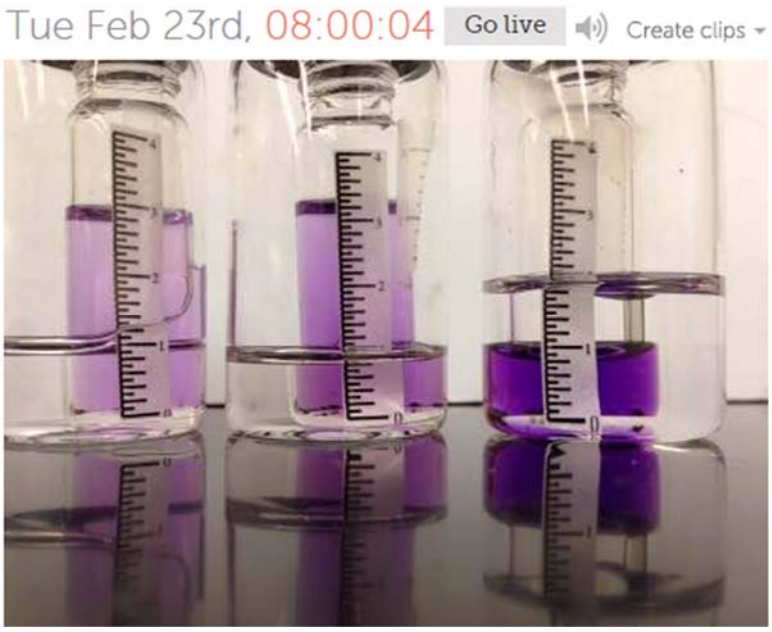

Figure 4: Example of our two-vial system. Diffusion of 5.0 mL of pentane, ether, and heptane into 1.0 mL of benzene is shown. The presence of 4,6,8-trimethylazulene facilitates scale-reading.

The two-vial system in Figure 4 was the most commonly used setup in this study. To focus on the kinetics of the two solvents only, diffusion studies were done without solute at room temperature (21 °C). The absence or presence of solute was found to not appreciably affect the kinetics (See Chapter 3.9.6). The inner vial was a 1-dram vial (inner diameter 15 mm, height 45 mm, total volume 5.0 mL) equipped with a calibrated scale with marks every 0.1 mL up to 4.0 mL, and the outer vial was a 20 mL scintillation-

type (inner diameter 28 mm, height 57 mm, total volume 24 mL). The inner vial was usually charged with 1.0 mL of a higher-boiling solvent and the outer vial was charged with 5.0 mL of a lower-boiling solvent. A tiny amount of 4,6,8-trimethylazulene (TMA), a purple organic dye, was added to the inner solvent for clarity. FD&C blue #1 food color was used if water was the solvent. In a few cases, Reichardt's dye (Betaine 30),[25] bought from Sigma Aldrich, was used in place of TMA to visualize polarity changes. The outer vial was tightly capped, and the system was sometimes weighed before and after the experiment to monitor for vapor leakages. Multiple studies were done simultaneously to maximize time efficiency.

2.2. *Graduated cylinders in TLC jars*

We encountered numerous combinations which diffused well beyond 4.0 mL, the limit of the small-scale setup. In these cases, to quantify the equilibrium or maximum diffusion of a combination, a large-scale setup utilizing 10 mL graduated cylinders (VWR 14212-512) and a thin-layer chromatography (TLC) Jar (Chemglass CG-1181-03) were employed. The 10 mL cylinders had been cut to approximately 1 cm above their highest gradation to keep uniform cross-sectional area. Inert foam or wood holders were used to support the cylinders. The height added by these holders had no effect on results per further study.

However, it was found that three graduated cylinders side-by-side as in Figure 5 had possible effects on neighboring diffusions and could skew results. It is unknown whether the solvents directly affected each other or merely competed for antisolvent vapor. For future experiments where the inner solvents were sufficiently different, one graduated cylinder per TLC jar was used. The larger setup has allowed quantification of

more extensive diffusions such as diethyl ether into dimethylformamide, and 25 mL graduated cylinders were used for a few even larger scale studies.

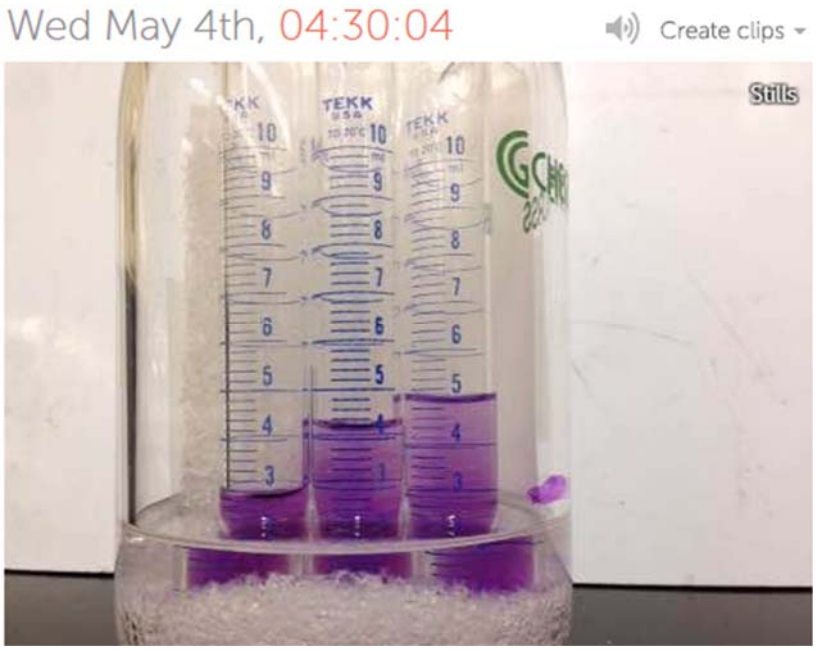

Figure 5: Example of our large-scale setup. 50 mL of diethyl ether into 1.0 mL of acetonitrile, dimethylformamide, and toluene, respectively. The graduated cylinders are kept upright with unreactive foam. One cylinder per jar was also commonly used.

2.3 *Camera*

An iPhone 5 with a time-lapse app was used as the camera. A wooden holder was made to hold the camera for stability. Focused lighting was initially provided, and its presence was found to not appreciably affect the kinetics, but it was later found that the overhead ceiling lights were sufficient and provided smoother lighting. The camera was generally set to take a photo every hour, and setups were allowed to run anywhere from 48 hours to 300 hours, depending on the rates and kinetics of the combination. Photos/videos were retained in cloud storage and can be provided upon request.

CHAPTER THREE

Results and Discussion

Antisolvent	Boiling Point (°C)	Polarity	Vapor Pressure 20°C (hPa)
acetone	56.2	0.355	240
diethyl ether	34.6	0.117	587
pentane	36.1	0.009	573
methanol	64.6	0.762	128
ethanol	78.5	0.654	59
benzene	80.1	0.111	101
acetonitrile	81.6	0.46	97
water	100	1	17.5

Solvent	Boiling Point (°C)	Polarity	Vapor Pressure 20°C (hPa)
dichloromethane	39.8	0.309	475
acetone	56.2	0.355	240
chloroform	61.7	0.259	210
methanol	64.6	0.762	128
tetrahydrofuran	66	0.207	200
ethyl acetate	77	0.228	97
ethanol	78.5	0.654	59
benzene	80.1	0.111	101
2-methyltetrahydrofuran	80.2	0.136	136
acetonitrile	81.6	0.46	97
water	100	1	17.5
toluene	110.6	0.099	29
dimethyl formamide	153	0.386	3.5
dimethyl sulfoxide	189	0.444	0.61 (25°C)

Table 1: Common laboratory solvents and their properties, sorted by boiling point. Polarity values are based on the normalized $E_T(30)$ scale.[25]

3.1 *Best solvents and antisolvents*

In general, of all the common laboratory solvents tested, diethyl ether and pentane were found empirically to be the greatest antisolvents, exhibiting the greatest extent of diffusions, in terms of rate and equilibrium. This was expected as diethyl ether and

pentane have the lowest boiling points and extremely high vapor pressures (See Table 1), traits which are often related to rates. As such, most of the combinations studied involve diethyl ether or pentane.

There was more variation for the inner solvent. Benzene and toluene, both highly nonpolar, were receptive to some antisolvents. Water, extremely polar, expressed slow with moderate equilibrium diffusions, generally. The other solvents studied with emphasis were acetone, acetonitrile, methanol, ethanol, dichloromethane, dimethylformamide, and tetrahydrofuran. These solvents on the polarity spectrum between polar and non-polar displayed different behaviors often regardless of differences in vapor pressure and boiling point. Only few trends regarding the correlation between solvent, antisolvent, and rates or equilibrium were evident from the data.

3.2 *Chemical affinities*

In addition to the effect of vapor pressures, there are clearly chemical affinities involved, particularly in the extent of the diffusions. For example, while pentane (B.P. = 36.1 °C) has a low boiling point and high vapor pressure, it refuses to enter dichloromethane (B.P. = 39.8 °C). Admittedly, dichloromethane itself has a remarkable vapor pressure, yet diethyl ether (B.P. = 34.6 °C) enters dichloromethane with ease.

For a given outer solvent, closely related inner solvents in terms of structure exhibit a trend where the higher boiling one accepts a greater degree of diffusion. Examples are that diethyl ether diffuses more extensively into chloroform than dichloromethane (2.94 mL vs 7.33 mL at equilibrium), into ethanol than methanol (5.18 mL vs. 3.64 mL at equilibrium), and into toluene than benzene (9.00 mL vs. 7.07 mL at equilibrium). Likewise, pentane diffuses more extensively into 2-methyl-tetrahydrofuran

than into tetrahydrofuran (4.08 mL vs. 3.34 mL at equilibrium) and into toluene than into benzene (5.11mL vs. 4.26mL at equilibrium). This pattern seems to only hold true usually with closely related solvents.

3.3 *Boiling points and vapor pressure*

One general rule that was confirmed is that the antisolvent must be of a lower boiling point than the inner solvent. While there is some leeway, such as benzene (80.1 °C) barely diffusing into ethanol (78.5 °C), no exceptional violation was found. In fact, if the lower boiling solvent is placed on the inside, such as with heptane (98 °C) into benzene, the diffusion proceeds in the expected direction, with benzene diffusing out of the inner vial into the outer vial (See Figure 3). In general, we expect lower boiling antisolvents to give the fastest diffusions. This is shown in Figure 6.

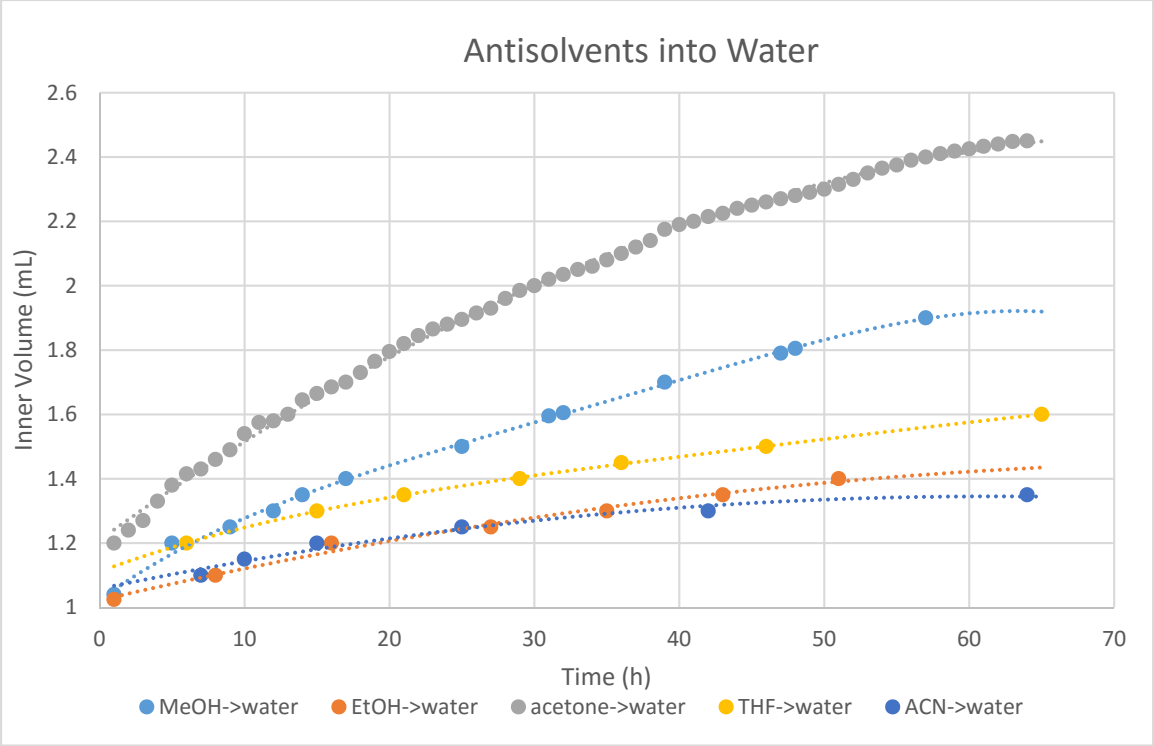

Figure 6: Boiling point shown to play a considerable role in determining equilibrium. The antisolvents in order of fastest to slowest diffusion into water: acetone (56 °C), methanol (64.6 °C), tetrahydrofuran (66 °C), ethanol (78.5 °C), acetonitrile (81.6 °C). A correlation between equilibrium and boiling point is seen.

3.4 *Polarities*

It is also important to keep track of polarity and its change because polarity change is what affects the solubility of one's desired compound. Polarity change was estimated from a weighted average of the polarities of the individual solvents. Polarity values of solvents were taken from Reichardt's paper using the normalized $E_T(30)$ scale.[25] For example, the polarity of acetone is 0.654 and the polarity of ethanol is 0.355. Assuming 0.79 mL acetone diffused into 1.0 mL of ethanol:

$$ratio_{solvent} * polarity_{solvent} + ratio_{antisolvent} * polarity_{antisolvent}$$

$$= \frac{1.0\ mL}{1.79\ mL}(0.654) + \frac{0.79\ mL}{1.79\ mL}(0.355) = 0.522$$

The percent polarity change would then be:

$$\frac{polarity_{final} - polarity_{initial}}{polarity_{initial}} 100\% = \frac{0.522 - 0.654}{0.654} 100\% = -20\%$$

A combination which affords extensive diffusion will still provide inadequate crystals if the polarity change is not significant enough. Diethyl ether into benzene is one such example, which despite having an increase factor of four only results in a polarity change of 4.0% at 24 hours. However, the weighted average method assumes no inner solvent diffuses into the outer solvent. We will see this is not quite true later and that monitoring polarity change may be more difficult than expected.

3.5 *The master diffusion table*

Our data are compiled into a master diffusion table below. Shown are the volume increase factors (VIF) every 12 hours up to 60 hours, the polarity of the mixture at 24 hours and its corresponding percentage change, and the extrapolated equilibriums from both modeling methods. The associated half-life is based on first-order treatment. The table is organized in alphabetical order, starting with the antisolvent and then the inner solvent. As a final note, controls were done, and it was found that an antisolvent would not diffuse into an empty vial or into a vial of the same solvent.

Small scale setups		Volume Increase Factor						% change	Veq	Veq	half-life
Antisolvent	Solvent	12h	24h	36h	48h	60h	pol @ 24h	@ 24h	1stOrder	reciprocal	(hours)
acetone	ethanol	1.47	1.79	1.94	1.98	2.1	0.522	-20%	2.10	2.29	7
acetone	methanol	1.51	1.63	1.71	1.77	1.8	0.605	-21%	1.80	1.94	7
acetone	water	1.48	1.74	1.97	2.14	2.29	0.726	-27%	4.21	4.86	99
acetonitrile	water	1.15	1.25	1.28	1.31	1.33	0.892	-11%	1.38	1.45	19
benzene	dimethylformamide	1.37	1.62	1.82	1.98	2.08	0.281	-27%	2.53	2.75	32
benzene	dimethylsulfoxide	1.29	1.48	1.57	1.71	1.82	0.336	-24%	2.15	2.4	34
benzene	ethanol	1.28	1.33	1.37	1.4	1.42	0.519	-21%	1.42	1.47	7
benzene	methanol	1.15	1.16	1.17	1.2	1.2	0.672	-12%	-	1.23	-
diethyl ether	acetone	1.97	2.19	2.29	2.34	2.38	0.226	-36%	2.40	2.55	11
diethyl ether	acetonitrile	2.5	3.1	3.44	3.54	3.64	0.268	-42%	3.83	4.22	13
diethyl ether	benzene	2.69	3.29	3.61	3.79	3.92	0.115	4%	3.96	4.35	14
diethyl ether	chloroform	3.01	3.7	4.03	4+	4+	0.155	-40%	4.18	4.75	10
diethyl ether	dichloromethane	1.91	2.3	2.5	2.56	2.63	0.201	-35%	2.72	2.94	12
diethyl ether	dimethylformamide	2.37	3.24	3.85	4+	4+	0.200	-48%	5.10	5.76	20
diethyl ether	dimethylsulfoxide	1.65	2.16	2.61	3	3.4	0.268	-40%	-	5.52	-
diethyl ether	ethanol	2.56	3.18	3.55	3.8	3.97	0.286	-56%	4.18	4.68	16
diethyl ether	ethyl acetate	2.59	3.28	3.65	3.87	4	0.148	-35%	4.15	4.66	13
diethyl ether	methanol	2.55	3	3.33	3.43	3.41	0.332	-56%	3.50	3.86	15
diethyl ether	toluene	3.3	4.03	4+	4+	4+	0.113	14%	4.37	4.99	8
ethanol	water	1.1	1.17	1.23	1.28	1.31	0.950	-5.0%	1.95	2.10	122
methanol	water	1.22	1.4	1.57	1.72	1.82	0.932	-6.8%	3.65	4.17	112
pentane	acetone	1.32	1.4	1.44	1.45	1.45	0.256	-28%	1.37	1.45	10
pentane	chloroform	1.78	2.32	2.67	2.9	3.06	0.117	-55%	3.50	3.95	20
pentane	benzene	2.55	3.12	3.45	3.62	3.75	0.042	-62%	3.87	4.26	14
pentane	ethyl acetate	2.01	2.4	2.64	2.79	2.9	0.100	-56%	3.06	3.54	16
pentane	ethanol	1.61	1.86	2.02	2.11	2.2	0.356	-46%	2.31	2.65	15
pentane	tetrahydrofuran	2.22	2.65	2.82	2.97	3.05	0.084	-60%	3.15	3.34	14
pentane	toluene	2.81	3.56	3.97	4+	4+	0.034	-65%	4.36	5.11	12
tetrahydrofuran	water	1.27	1.37	1.45	1.51	1.58	0.786	-21%	1.77	1.91	34
water	dimethylformamide	1.06	1.09	1.11	1.13	1.15	0.437	13%	1.52	1.61	136
water	dimethylsulfoxide	1.1	1.13	1.17	1.21	1.24	0.508	14%	2.10	1.77	210

Large scale setups		Volume Increase Factor						% change	Veq	Veq	half-life
Antisolvent	Solvent (inner)	12h	24h	36h	48h	60h	pol @ 24h	@ 24h	1stOrder	reciprocal	(hours)
diethyl ether	acetonitrile	-	2.77	2.94	3.02	3.05	0.241	-48%	3.07	3.14	8
diethyl ether	benzene	2.92	3.78	4.08	4.63	4.9	0.115	4.0%	6.03	7.07	30
diethyl ether	chloroform	2.94	3.94	4.59	5.13	5.53	-	-	6.14	7.33	22
diethyl ether	deuterated chloroform	2.91	3.9	4.49	4.95	5.39	-	-	6.03	6.27	22
diethyl ether	dimethylformamide	2.68	3.78	4.57	5.26	5.82	0.188	-51%	9.97	13.4	56
diethyl ether	dimethylsulfoxide	1.85	2.38	2.77	3	3.35	0.254	-43%	5.05	5.46	50
diethyl ether	ethanol	2.5	3.2	3.62	3.9	-	0.285	-56%	5.11	5.18	30
diethyl ether	ethyl acetate	2.68	3.39	3.8	4.12	-	0.150	-34%	5.20	5.20	30
diethyl ether	methanol	2.47	2.92	3.14	3.26	3.38	0.338	-56%	3.40	3.64	9
diethyl ether	toluene	3.31	4.45	5.28	-	-	0.113	14%	9.80	9.00	63

Table 2: The master diffusion table. This table contains a general summary of all data collected. Alterations in configuration are not included. Volume Increase Factor is defined as the ratio of inner volume at a certain time divided by initial inner volume. This term was created because of configurations that do not use 1.0 mL initially.

3.6 *The gold standard: diethyl ether into dichloromethane*

Dichloromethane is one of the most common solvents in organic chemistry, and unlike the three other most popular solvents, ethyl acetate, acetone and hexanes, dichloromethane is not nearly as flammable, though it has some toxicity. Diethyl ether

into dichloromethane was once considered by us to be the "gold standard" for vapor diffusions, being commonly used by research groups and exhibiting many traits one would expect from a diffusion. The rate of diffusion for this combination is fast, requiring only two days to reach a modest equilibrium of around 2.8 mL. We have since discovered many better diffusions, and now diethyl ether into dichloromethane is the bar for defining good solvent combinations. A table of the best combinations which have greater polarity change than the diethyl ether into dichloromethane standard is included at the end of this paper (Table 3).

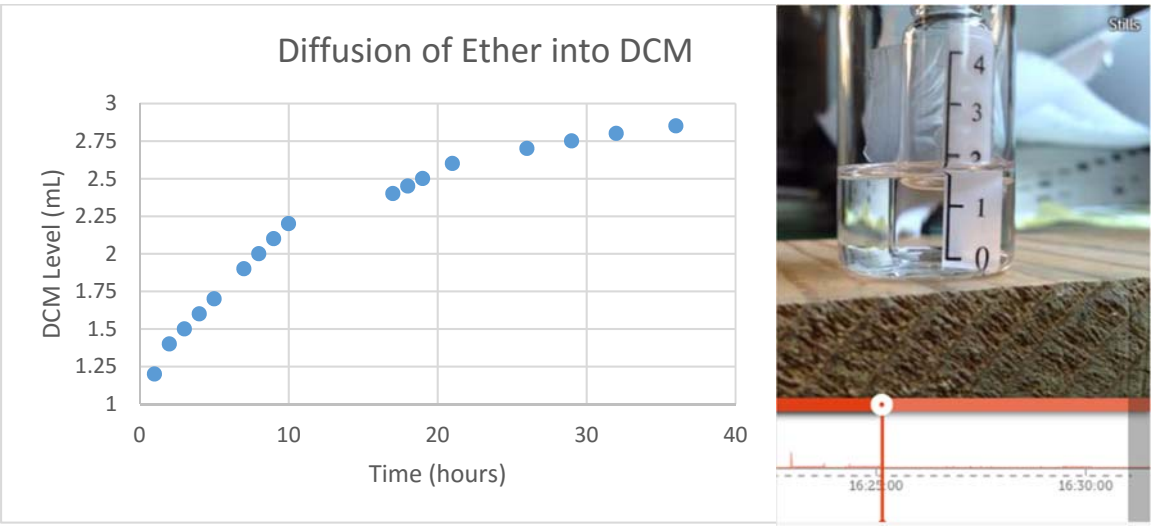

Figure 7: Our gold standard of vapor diffusion, Sept. 4, 2015. The first experiment we ever did, diethyl ether into dichloromethane was considered a good representative model for the ideal diffusion.

Though the paper scale used in Figure 7 initially had increments only every milliliter, extra markings were made afterwards for more accurate readings, reducing the number of gaps in between data points. A single, large gap of missing data points can often be seen in our data, hours 11 to 16 in Figure 7. This is due to time periods where the antisolvent's meniscus eclipses the inner solvent's meniscus, making accurate

readings impossible. We are confident that this minor impediment does not interfere with our conclusions.

The data shown in Figure 7 can be traced using either a logarithmic graph and an exponential graph with decent correlation. It was discovered early on however that not all combinations can be modeled with basic functions, and so we began searching for a better method of analyzing the data. This led to developing the first-order treatment and the double reciprocal plot, discussed later.

3.7 *Reversals*

An interesting phenomenon was observed with combinations that reached equilibrium. Rather than net diffusion approaching zero, in many cases it was noted that the inner solvent volume would begin to distinctly decrease. The reversal phenomenon was initially thought to have been caused by leaks in the vial's seal. Vials were weighed before and after diffusion, and no significant difference in weight was found, suggesting the reversal was not due to a faulty seal. It is currently unknown why this occurs. In theory, the system is equilibrating to achieve equal vapor pressures in both the inner and outer vials.[24] At short diffusion times, equilibrium is approached by the more rapid diffusion of the volatile outer antisolvent, aiding the vapor pressure of the inner vial. But at long diffusion times, the less-volatile inner solvent will have had enough time to begin to equilibrate to lower the vapor pressure of the outer vial. However, this predicts the eventual formation of identical mixtures in the inner and outer vials, which clearly did not happen with diethyl ether into dichloromethane, which was observed to reverse completely after an extended period (about a month perhaps after equilibrium was achieved) to the point where all the dichloromethane had left the inner vial. (Figure 8).

Figure 8: Complete reversal of 3.0 mL diethyl ether into 1.0 mL dichloromethane. Left alone for a month after equilibrium, the inner vial is completely empty save for dregs of 4,6,8-trimethylazulene, which cannot diffuse as it is a low-volatility solid at room temperature.

We naturally became curious about whether reversal occurred only after equilibrium has been reached or if the inner solvent was constantly diffusing into the outer. A sample of outer solvent from pentane into ethyl acetate was taken a week after equilibrium was reached and examined by proton NMR spectroscopy. It was found that a considerable quantity of ethyl acetate had diffused out into pentane: 1 molecule of ethyl acetate for every 4.3 molecules of pentane. This poses a minor problem as polarity changes recorded in the master diffusion table assume no diffusion from the inner vial. Quantifying the movement of both solvents complicates matters enormously, but is

significant only at longer times, and for now that endeavor will be set aside for another paper.

3.8 *Modeling the Data*

The advantages of developing a formula to model the kinetics of the reaction is that equilibrium could then be estimated and (for first-order processes) a half-life can be reported. Half-lives are convenient as they provide researchers with an easily used number to track their diffusion, rather having to consult the table every time, and they indicate how much time will be needed before the reaction is 50% complete. Half-lives are multiplicative, so after two half-lives the diffusion would be 75% done. For any process that has a defined half-life, five half-lives are required for that process to be considered complete (97%) by convention.[26]

3.8.1 *First order kinetics*

We chose to study whether these diffusions obeyed the simplest type of kinetics, first-order. The basic first order equation says that the speed of the reaction is determined by the concentration of only one chemical species. The equation (rate law) for a general first order reaction is:

$$rate = k * [A]_t$$

Where k represents a constant that adjusts for temperature and several other variables unique to the process in question, and $[A]_t$ denotes concentration of a compound, A, which is either a reactant or product, at a certain time t. If we integrate rate, which is the change in concentration over change in time, with respect to time, we get the integrated rate law:

$$Ln[A]_t = -kt + Ln[A]_0$$

Where $[A]_0$ represents the initial concentration of A. However, this simple first-order treatment assumes the process in question goes to completion, which in mathematical terms means that reactant A approaches zero or product A approaches unity. Equilibrium processes do not go to completion, and a different mathematical form was required for our purposes. After consulting a kineticist, the modified equilibrium first-order expression for A as a product became:

$$Ln([A]_{eq} - [A]_t) = -kt$$

Note that in this expression, the Ln term approaches zero, as it did in the original first-order expression.

3.8.2 *First order treatment*

Replacing concentration with volume to make it applicable to vapor diffusion, the equation used was:

$$Ln(V_{eq} - V_t) = -kt \qquad or \qquad Ln(V_{eq}) = -kt + Ln(V_t)$$

A graph of $Ln(V_{eq} - V_t)\ vs\ t$ should give a straight line of slope $-k$. However, the equilibrium volume, or maximum volume, is often not conveniently observed within a reasonable amount of time. Using the fact that $Ln(V_{eq} - V_t)\ vs\ t$ gives a linear line, we studied a "best-guess" approach for estimating V_{eq}, where the correct value was assumed to be that which maximizes the linearity. This approach assumes first-order behavior. Figure 9 below shows the sensitivity of the linearity (measured as r^2) versus various trial values of V_{eq}.

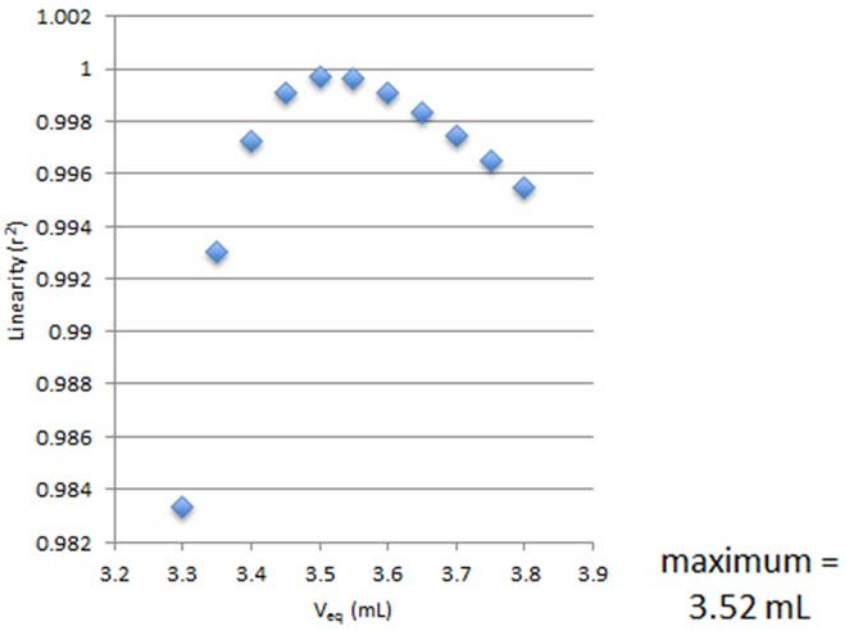

Figure 9: Linearity as a function of equilibrium for pentane into chloroform. The "guess" or trial value which maximizes linearity is the one provides the most accurate half-life. Here, 3.52 mL is the projected equilibrium for pentane into chloroform.

We applied this method to all diffusions to estimate the equilibrium positions and afterwards the half-lives. Half-lives are found by the equation:

$$t_{1/2} = \frac{Ln(2)}{k}$$

Where k is determined by a graph of $Ln(V_{eq} - V_t)\ vs\ t$, which has a slope $-k$, example shown below in Figure 10.

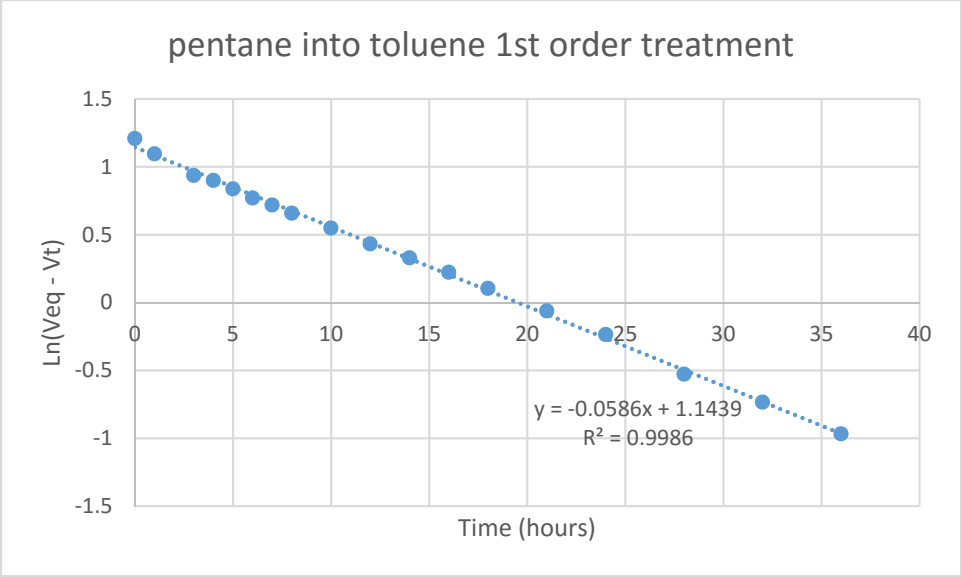

Figure 10: Finding half-life through first order treatment. k as shown is 0.0586 h^{-1}. Hence, $t_{1/2} = \frac{Ln(2)}{0.0586\ h^{-1}} = 11.8$ hours.

In all cases, r^2 values of > 0.99 were obtained. However, when compared to observed equilibrium values, it became evident that this approach generally underestimated the equilibrium value by perhaps 20% and overestimated the half-life by a similar amount.

3.8.3 *Double reciprocal plot*

Enzyme kinetics was modeled in biochemistry by researchers Leonor Michaelis and Maud Menten, whose formula related rate of reaction with concentration.[27] Further down the road, another application of Michaelis-Menten kinetics was derived, creating Lineweaver-Burk plots, which uses variables from the Michaelis-Menten model.[28] Sometimes referred to as a double reciprocal plot, a Lineweaver-Burk plot graphs $\frac{1}{rate}$ against $\frac{1}{concentration}$, which mathematically causes the x and y-intercepts to contain important information regarding the specific enzyme's kinetics. At the suggestion of

Professor Bryan Shaw, we prepared plots of $\frac{1}{volume}$ against $\frac{1}{time}$. At the y-intercept, $\frac{1}{time}$ equals zero, meaning time is theoretically infinity. Thus the y-intercept of our graph gives the reciprocal of volume after an infinite amount of time, effectively giving a prediction as to the V_{eq}. The last few points of acquired data are used to create a linear equation and estimate V_{eq}. Figure 11 below illustrates this method.

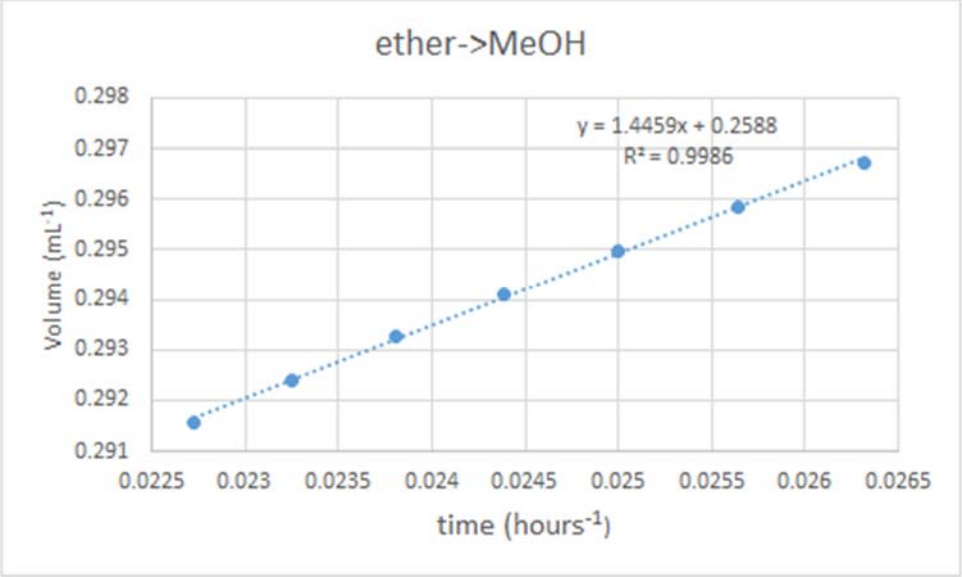

Figure 11: Double reciprocal plot of last seen data points from diethyl ether into methanol (MeOH). The y-intercept is 0.2588 mL^{-1}, so the predicted V_{eq} is $\frac{1}{0.2588 mL^{-1}}$ or 3.86 mL.

Afterwards, a half-life may be estimated by determining the time necessary for a solvent combination to reach halfway to equilibrium volume starting from the initial volume. However, the half-lives given by this approach do not appear consistent.

It is unclear which method better approximates the true equilibrium of a solvent combination. Both methods have cases where the extrapolated equilibrium better fits empirical data than its counterpart.

3.9 *Variations in configuration*

It is likely that researchers consulting our paper will often be using different setups for their purposes. Hereon we provide a preliminary look at the effect that various changes in configuration will have on the rates and equilibria stated in the master diffusion table.

3.9.1 *Effect of cross-sectional area*

Slow crystallization affords the best crystallization, but it was found often to be the case that the combinations which had the highest equilibrium values occasionally were too fast. It is for this reason that diethyl ether as an antisolvent is even sometimes advised against.[22] To slow the rate of diffusion, researchers can restrict the inner vial opening, e.g., use aluminum foil with a hole to restrict the cross-sectional area of the inner vial.[16] Naturally we were curious as to what effect this may have. Thus, caps with 2 mm, 3 mm, 4 mm, 6 mm, 8 mm, and 10 mm holes were prepared for the inner vial to simulate restricted conditions. Aside from the caps, the setup was identical to the two-vial system.

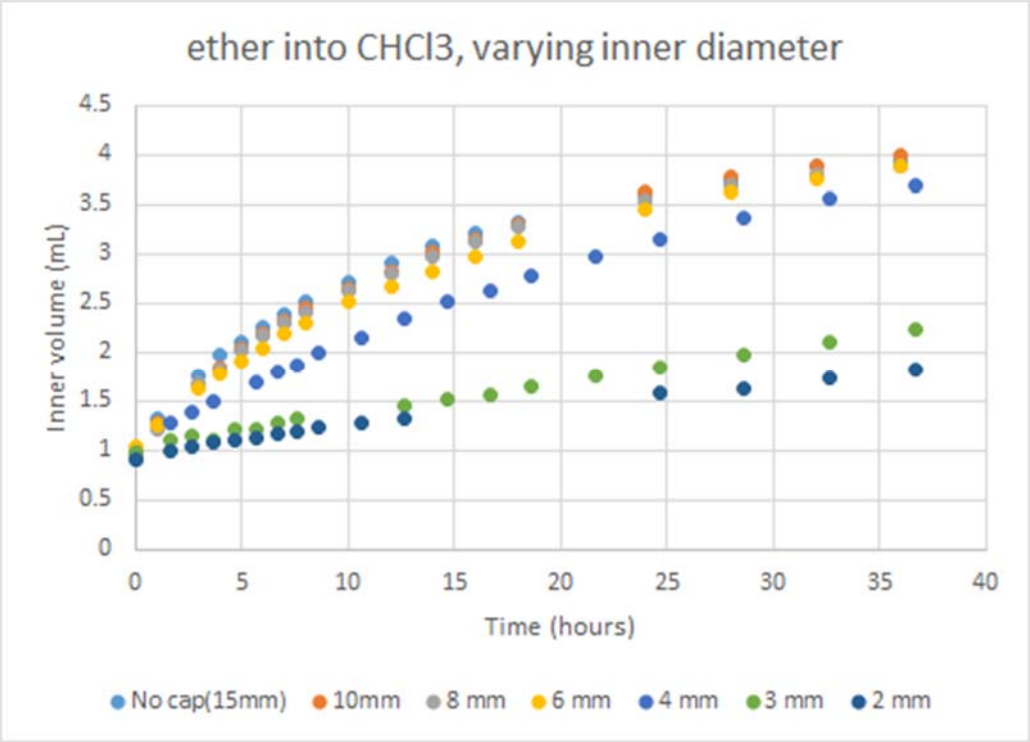

Figure 12: Diffusion of diethyl ether into chloroform ($CHCl_3$) with varying inner vial diameters. Little to no change in kinetics gives way to abrupt changes past a certain point. 15mm, 10 mm, and 8 mm show so little change that the data points overlap.

Examining Figure 12, at first, cross-sectional area of the inner vial opening has little effect on the overall rate. Hole diameters of 15 mm, 10 mm, and 8 mm display no discernable difference. There was surprisingly, however, a marked difference between 4 mm and 3 mm openings. This suggests that vapor diffusion is a two-step process, and that by changing the diameter of the opening, we have changed which step is rate-limiting. We currently propose that the outer vapor must first make its way into the vial and dissolve into the surface of the inner liquid, which is a vapor-to-liquid transition. This is followed by a liquid-liquid diffusion, identical to what occurs in a layering recrystallization (Figure 2).

The data suggest that the second, liquid-liquid diffusion step is the usual rate-limiting step. This conclusion is strongly supported by the observation that gentle stirring of the inner vial decreased the half-life by a factor of about two (Figure 13). Diethyl ether diffusing into toluene exhibited half-lives of 48 hours unstirred versus 21 hours when stirred. Likewise, diethyl ether diffusing into chloroform gave a half-life of 22 hours unstirred versus 14 hours when stirred. Note that stirring would prevent the formation of good crystals and was done here only for mechanistic reasons. This result was intriguing because the rate-limiting step in the hanging drop method was modeled to be the diffusion from solvent droplet to precipitant rather than liquid-liquid diffusion.[24]

Figure 13: Diffusion of diethyl ether into chloroform, chloroform stirred, and deuterated chloroform, respectively, in the large-scale setup. The inner solvents are sufficiently similar to permit use of a single jar. The middle cylinder contains a small magnetic bar, and the whole setup sits on a magnetic stir plate. A marked difference in rate due to gentle stirring is revealed.

3.9.2 *The effect of solvent volume*

As stated earlier, 1.0 mL of inner solvent was used in nearly all cases. Depending on quantity of product, however, the researcher seeking to use vial-in-vial vapor diffusion may find 1 mL to be impractical for their purposes. As such, studies were done where the inner volume was varied. Shown below in Figure 14 are the results of diethyl ether into dimethylformamide using different initial amounts of dimethylformamide in the inner vial and using the standard setup.

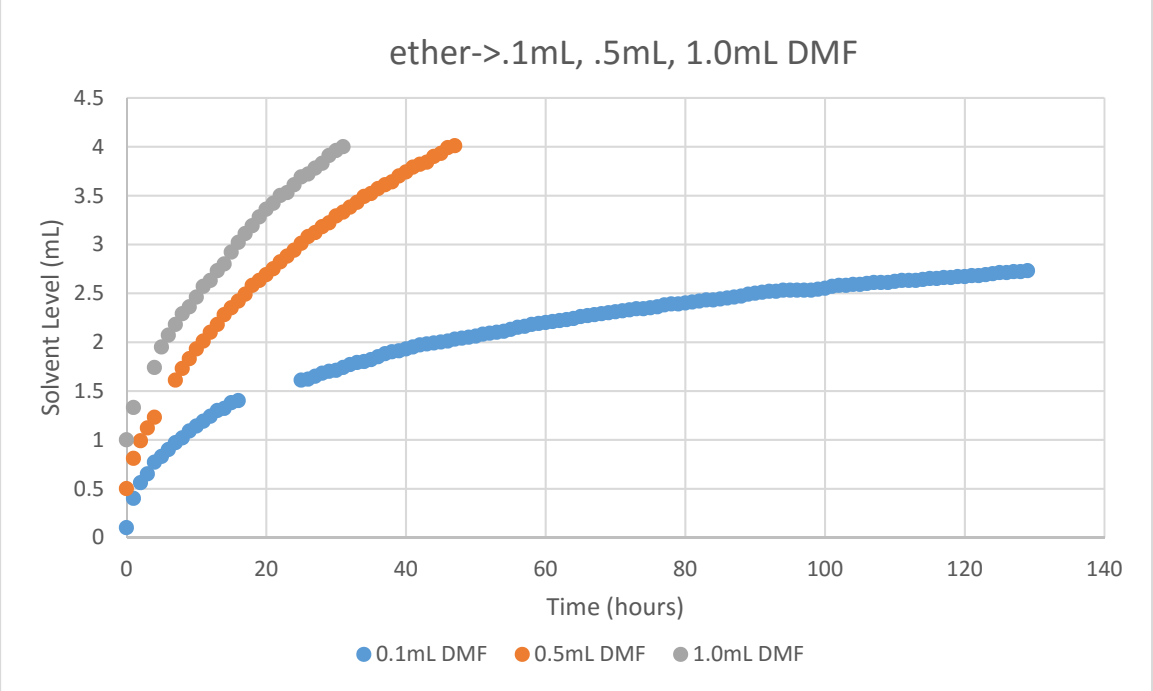

Figure 14: Diffusion of 5.0 mL diethyl ether into varying initial volumes of dimethylformamide (DMF). Data seem to suggest that the equilibrium is strongly scale-dependent, an anti-intuitive result.

The data presented in Figure 14 is astonishing in that it seems to suggest that a lower initial starting volume results in a higher increase factor. When starting with 0.1 mL, the volume of inner solvent increased by a factor of 27 with a significant polarity change of -66%. At this point, though, the inner vial is practically entirely diethyl ether.

3.9.3 *The effect of antisolvent volume*

After seeing the effect of inner solvent changes, we wondered what effect the outer solvent may have on the kinetics of diffusion. After moving to the larger setup, it became apparent that some combinations required more antisolvent than we had been initially using.

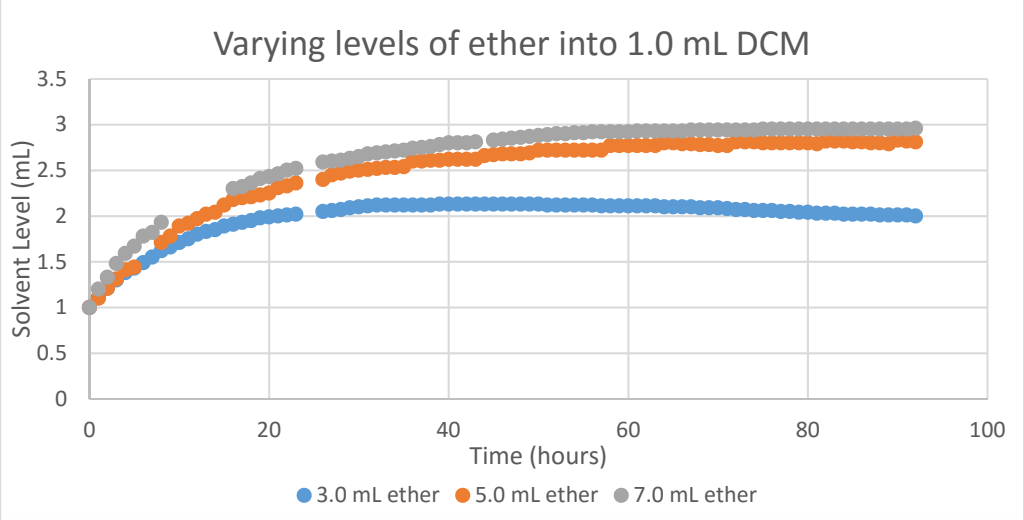

Figure 15: Diffusion of various volumes of diethyl ether into 1.0 mL of dichloromethane.

As can be seen from Figure 15, whereas excess antisolvent has little effect on equilibrium, there appears to be a point where insufficient antisolvent negatively affects the rate. For diethyl ether into dichloromethane this appears to be somewhere between 3.0-5.0 mL for 1.0 mL of dichloromethane. Further studies suggest that excess

antisolvent has no effect on the kinetics. Thus, the researcher seeking to crystallize a compound is advised to include adequate antisolvent, for extensive diffusions perhaps 5-7 times the inner solvent volume, for their crystallization. Alternatively, the volume of antisolvent could be reduced to slow rates if needed. Our findings suggest that the true maximum for a certain combination of solvents is never reached if the ratio of outer to inner is too low.

3.9.4 *The effect of temperature*

Vapor diffusions are heavily dependent on the vapor pressures of the solvents used, and vapor pressure is dependent on temperature. It comes as no surprise then that higher temperatures result in faster diffusions (Figure 16).

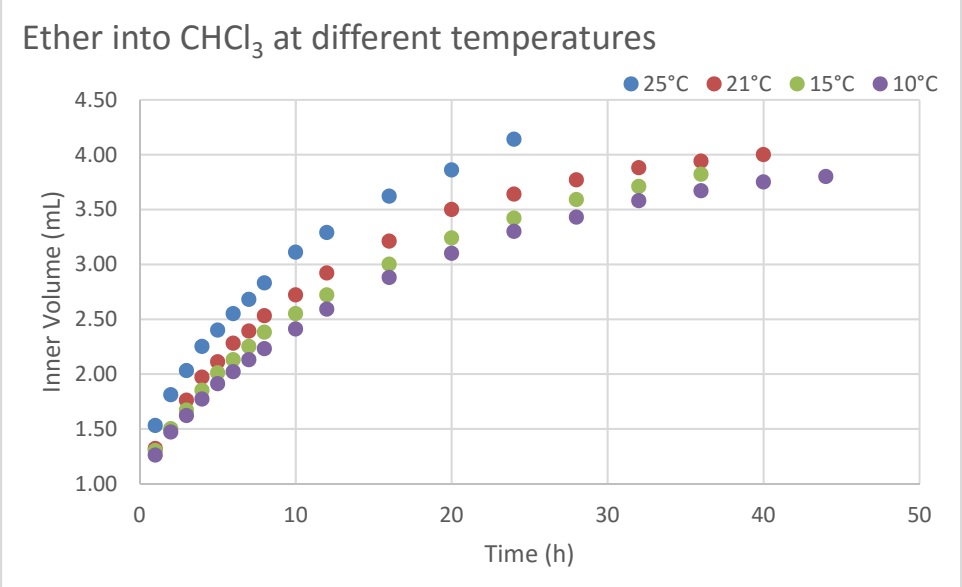

Figure 16: Diffusion of diethyl ether into chloroform at various temperatures. Room temperature in our lab was kept consistent at 21 °C. A progressive increase in rate and equilibrium values are shown, a result of increased vapor pressure from increased temperature

Temperature, however, also affects solubility. While higher temperatures may accelerate polarity change, crystallization may still not occur due to increased solubility. Furthermore, crystal morphology has been found to be temperature dependent.[16,29] It is possible that varying the temperature to alter kinetics may affect the resulting crystal.

3.9.5 *The effect of water*

Many common solvents in the laboratory are hygroscopic—they absorb water from the air. Dimethylformamide, for example, is highly hygroscopic. Hygroscopic solvents are required to be stored under an inert gas with molecular sieves to remain completely water-free. To examine the effects that water may have on diffusion, an experiment was set up involving pentane into tetrahydrofuran with 0%, 2%, and 6% water by volume. What we observe (Figure 17) is that no appreciable difference in diffusion occurs until 6% water, where only a slightly slower rate is observed. These results suggest that strictly anhydrous solvents are not required for our findings to apply.

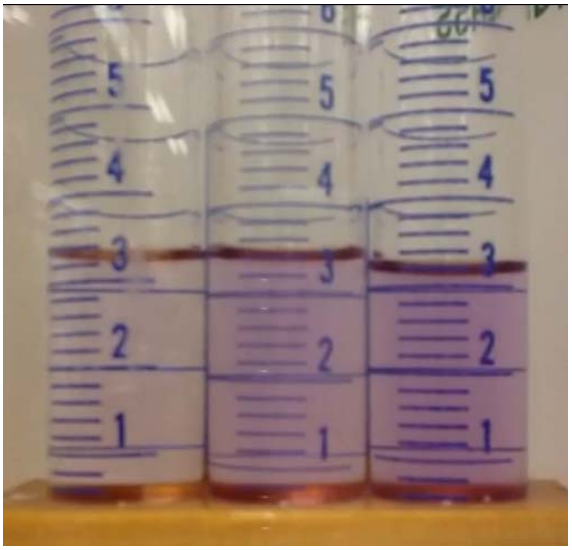

Figure 17: Diffusion of 50 mL of pentane into tetrahydrofuran with 0%, 2%, and 6% water by volume. Water is shown to have no significant effect on the kinetics

3.9.6 *The effect of solute on diffusion*

Our research was done without solute. Hence, a short study was done regarding the effects of solute on the kinetics of vapor diffusion. Several diffusions were carried out with 1 M (-)-menthol dissolved in the inner solvent. This generally had little but variable effects on the diffusion—sometimes slightly slowing it and sometimes accelerating it (Figure 18).

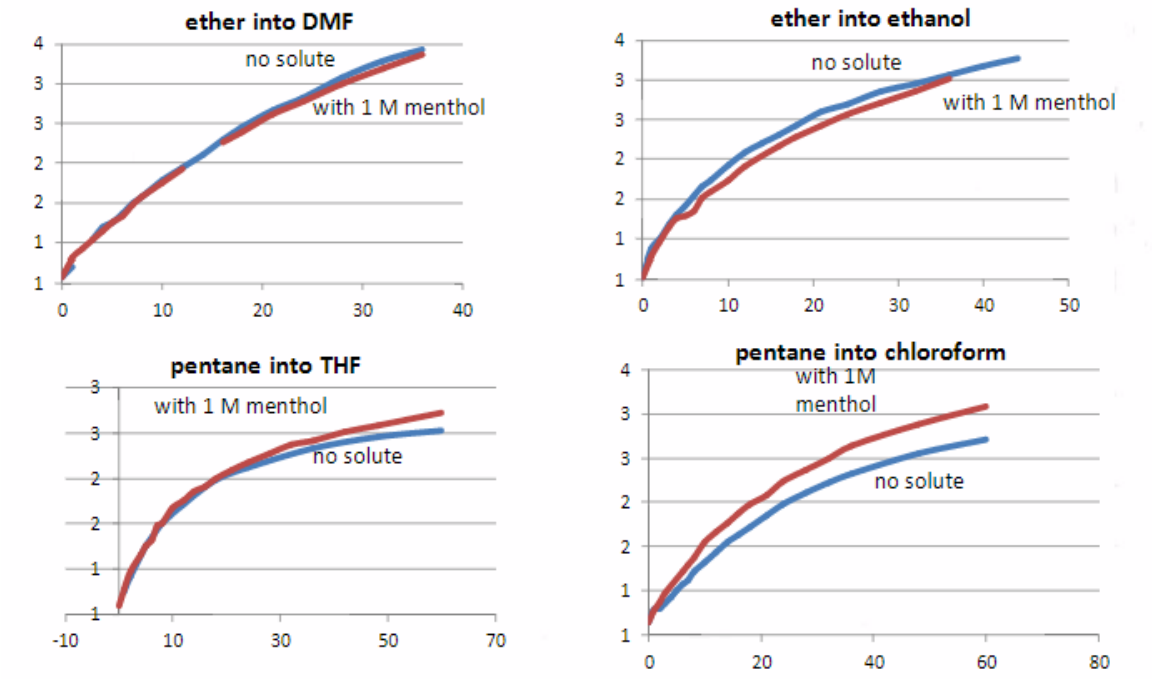

Figure 18: The various effects solute can have on kinetics. Red denotes the presence of solute, 1 M menthol. The presence of solute has only slight, variable effects on the rates.

3.10 *Attempting to visualize polarity change with a solvatochromatic dye*

Reichardt's paper, from which our polarity values were obtained, was largely based on a special dye which exhibited different colors depending on the polarity of the solvent (Figure 19). Reichardt's dye absorbs in the visible spectrum, and is the basis for the normalized $E_T(30)$ polarity score.[25]

MeOH, EtOH, *i*-PrOH, MeCN, *t*-BuOH, Me_2CO, $CHCl_3$, C_6H_6

Figure 19: Reichardt's dye in various solvents.[30]

We sought to use Reichardt's dye to better illustrate the polarity changes associated with diffusion combinations, but we encountered difficulties keeping Reichardt's dye dissolved. What was expected to be seen was the color of the inner vial changing over time, from red to purple or vice versa for example. What most often happened was that the change in polarity caused Reichardt's dye to precipitate out of solution.

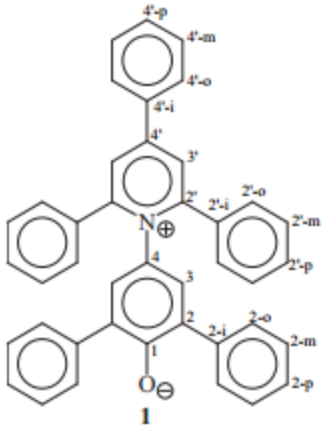

Figure 20: Structure of Reichardt's dye[30]

A further complication was that Reichardt's dye discolors in acid.[25] The deterioration of solvatochromatic effects from protonation was observed in our trial using pentane diffusing into ethyl acetate. Ethyl acetate can undergo hydrolysis by traces of water, creating acetic acid and ethanol. The proton from acetic acid can then protonate the phenoxide anion present in Reichardt's dye (Figure 20), destroying its solvatochromatic effects.

We did meet with some success, however. Diffusion of diethyl ether into methanol exhibited a dramatic red to blue transition, and diethyl ether into dimethylformamide shifted from blue to green (Figure 21).

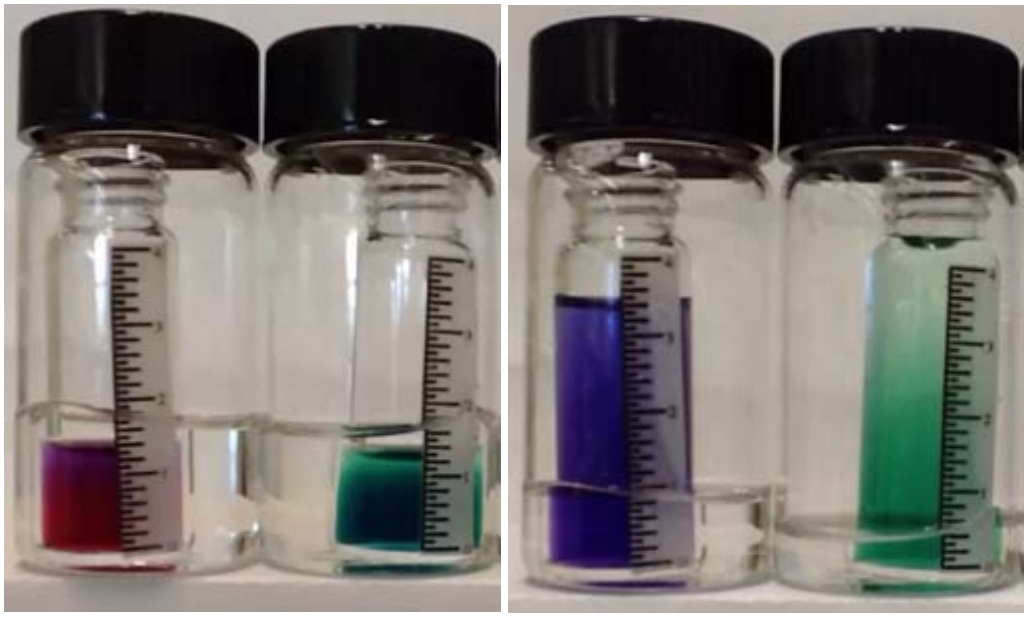

Figure 21. Reichardt's dye experiments showing polarity change. Left: Diffusion of diethyl ether into methanol (left vial) and into dimethylformamide (right vial) one hour after starting time. Hints of color change are beginning to show, and the color gradient is similar to that of solvent layering (Figure 2). Right: 44 hours after starting time, the new color is now more uniform. Right vial is beginning to discolor.

3.11 *Final remarks*

In this study, the kinetics of various combinations of solvents for vial-in-vial vapor diffusion have been laid out. Various factors controlling diffusion were explained, and a few intriguing phenomena discovered. Two possible methods of modeling acquired data were proposed, and then the effects of various alterations to configuration were examined.

There remains a large quantity of unknowns available for further study. It is still unclear why the reversal phenomenon occurs. Furthermore, the realization that there is movement of the inner solvent complicates polarity calculations at longer times. We also believe that our two models could be further refined. Finally, as solvents play a role in

crystal viability, experiments could be carried out to see which combinations prove effective at crystallizing certain compounds.

Our results were summarized and compiled into a master diffusion table containing all data deemed pertinent. We end with combinations which surpassed our gold standard, diethyl ether into dichloromethane, that we recommend for use (Table 3). Graphical representations of the master diffusion table are included afterwards. Chemical abbreviations, avoided until now, were used to conserve space.

Small scale setups		*Volume Increase Factor*									
Antisolvent	Solvent (inner)	12h	24h	36h	48h	60h	pol @ 24h	% change @ 24h	Veq (1stOrder)	Veq (reciprocal)	half-life (h)
pentane	toluene	2.81	3.56	3.97	4+	4+	0.034	-65.0%	4.36	5.11	11.8
pentane	benzene	2.55	3.12	3.45	3.62	3.75	0.042	-62.4%	3.87	4.26	14
pentane	tetrahydrofuran	2.22	2.65	2.82	2.97	3.05	0.084	-59.6%	3.15	3.34	14
diethyl ether	methanol	2.55	3	3.33	3.43	3.41	0.332	-56.4%	3.5	3.86	15
diethyl ether	ethanol	2.56	3.18	3.55	3.8	3.97	0.286	-56.0%	4.18	4.68	15.9
pentane	ethyl acetate	2.01	2.4	2.64	2.79	2.9	0.100	-56.0%	3.06	3.54	16.2
pentane	chloroform	1.78	2.32	2.67	2.9	3.06	0.117	-55.0%	3.5	3.95	20
diethyl ether	dimethylformamide	2.37	3.24	3.85	4+	4+	0.200	-48.0%	5.1	5.76	20.3
pentane	ethanol	1.61	1.86	2.02	2.11	2.2	0.356	-45.6%	2.31	2.65	15
diethyl ether	acetonitrile	2.5	3.1	3.44	3.54	3.64	0.268	-42.0%	3.83	4.22	13.2
diethyl ether	chloroform	3.01	3.7	4.03	4+	4+	0.155	-40.0%	4.18	4.75	9.9
diethyl ether	dimethylsulfoxide	1.65	2.16	2.61	3	3.4	0.268	-40.0%	-	5.52	-
diethyl ether	acetone	1.97	2.19	2.29	2.34	2.38	0.226	-36.0%	2.4	2.55	10.7
diethyl ether	dichloromethane	1.91	2.3	2.5	2.56	2.63	0.201	-35.0%	2.72	2.94	11.8

Table 3: Combinations which surpass the gold standard. The best combinations were selected based on having a greater change in polarity than diethyl ether into dichloromethane, the gold standard (>35%).

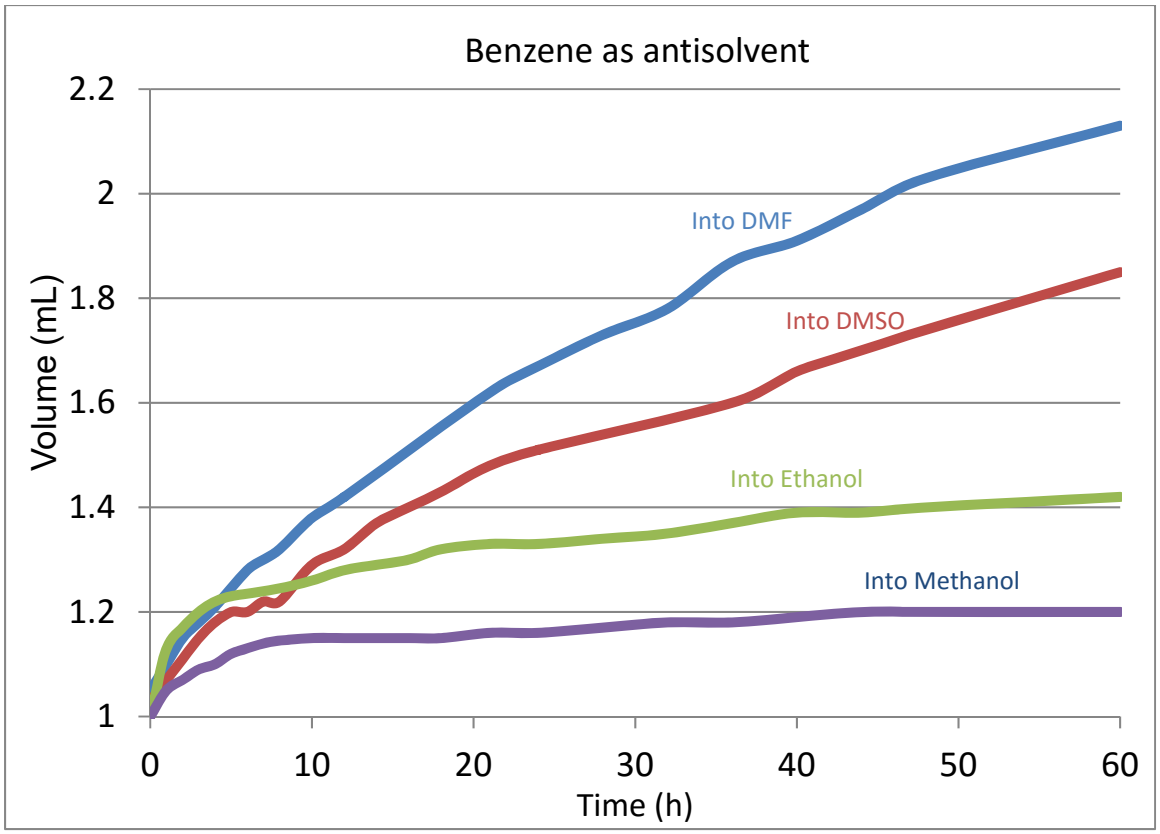

Figure 22: Comparison of all combinations with benzene as antisolvent.

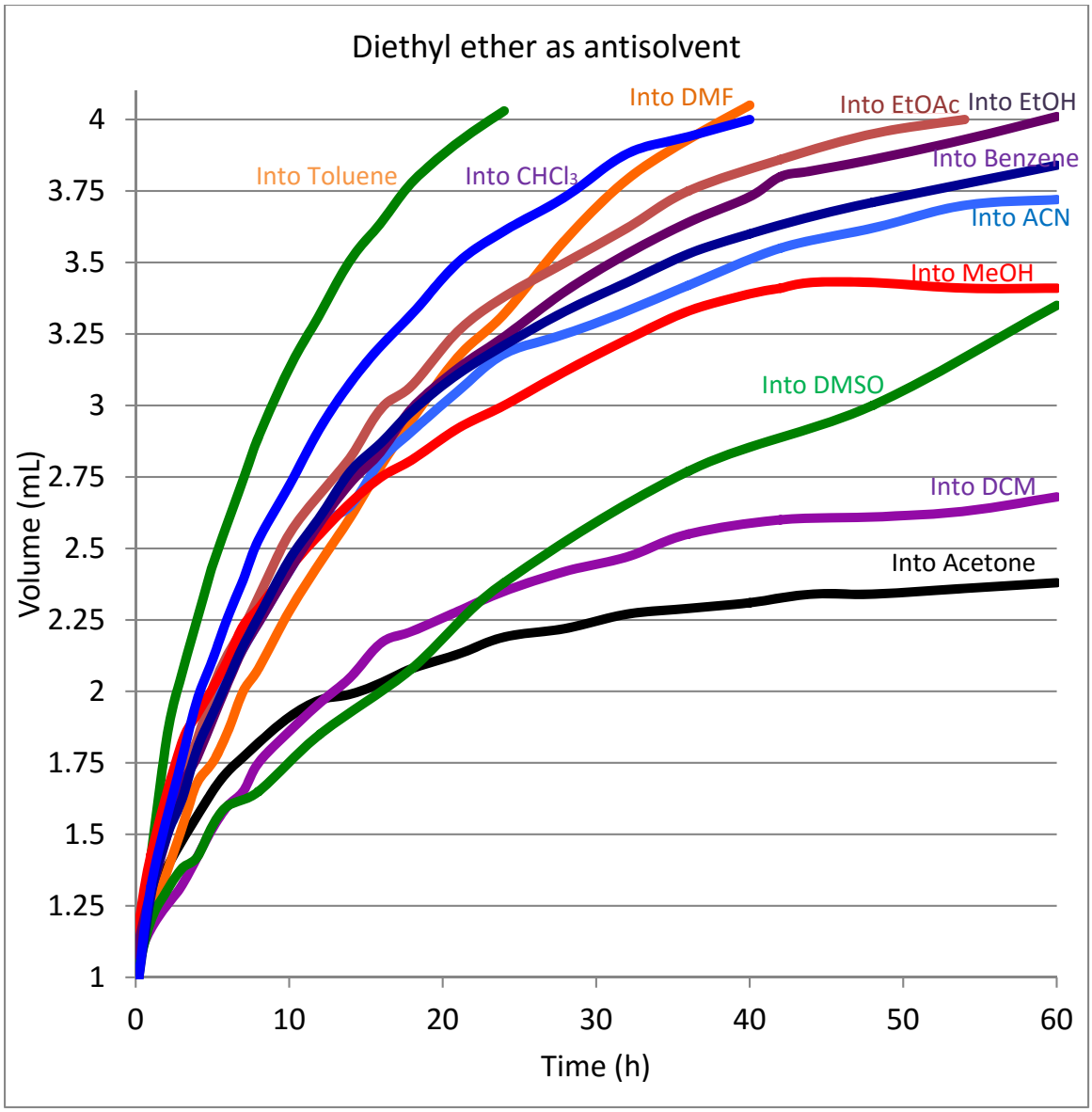

Figure 23: Comparison of all combinations with diethyl ether as antisolvent.

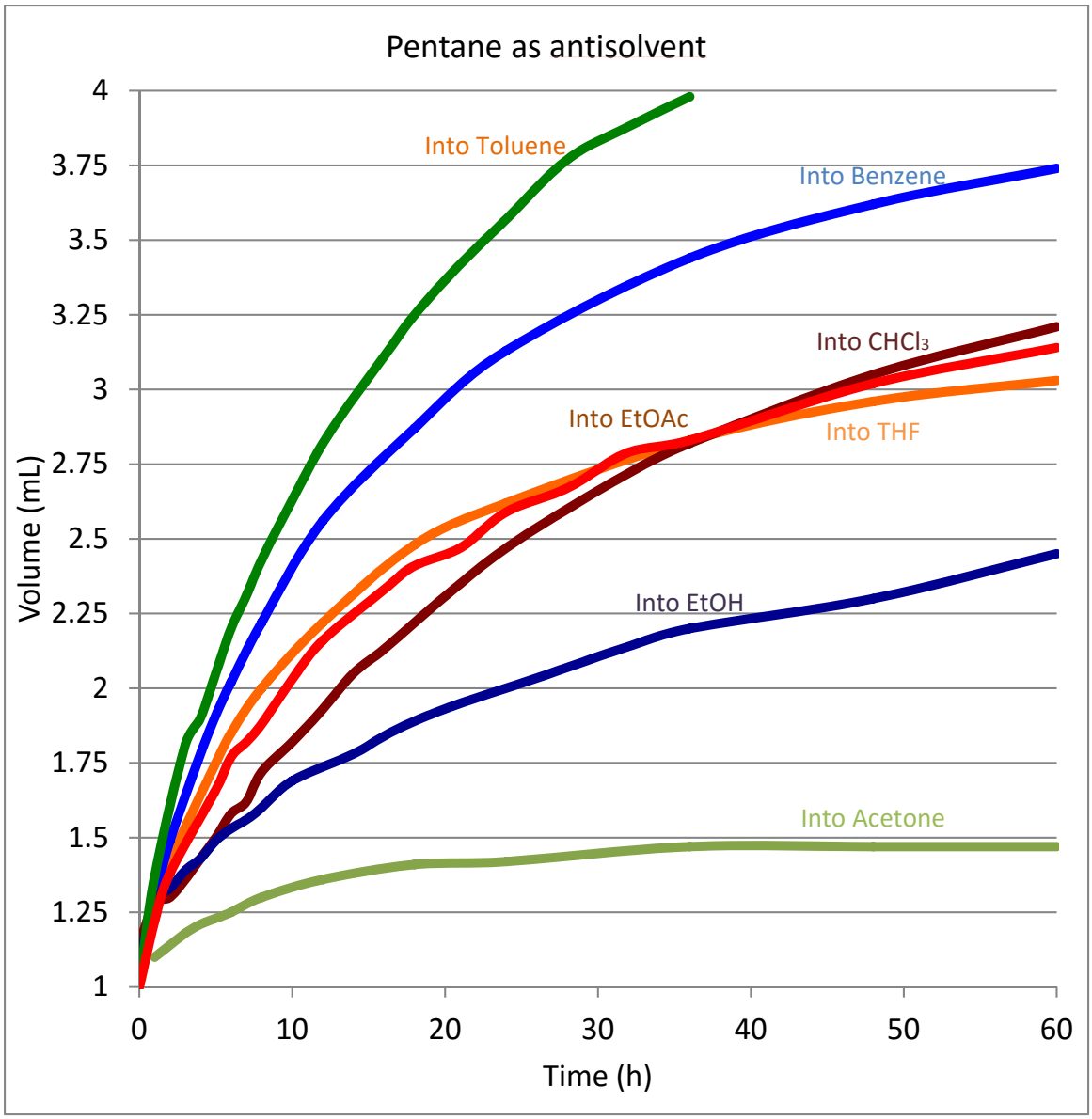

Figure 24: Comparison of all combinations with pentane as antisolvent.

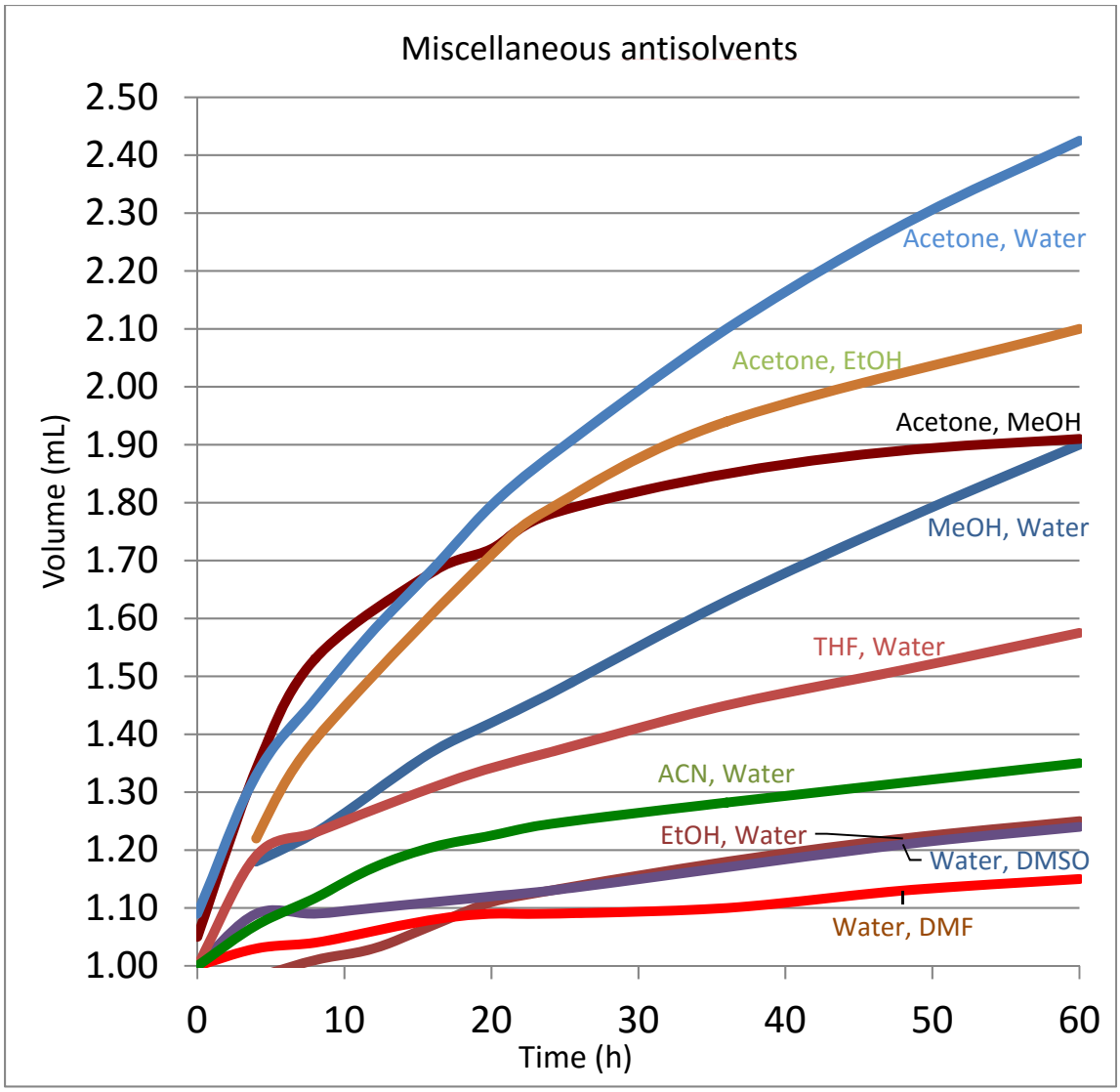

Figure 25: Comparison of all remaining combinations.

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