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Spontaneous Amide Bond Formation of Amino Acids in Aqueous Solution

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Spontaneous Amide Bond Formation of Amino Acids in Aqueous Solution

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by

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Accepted for _____Highest Honors_____
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Abstract

Previous studies in Dr. Kranbuehl's laboratory at the College of William and Mary have shown that carboxylic acids and amines react spontaneously to form amides. These results prompted questions about whether amino acids, which possess both carboxylic acid and amine functionality, would also spontaneously form amide bonds, creating peptides. For this study, aqueous amino acid solutions were created and placed in a 120°C oven. Samples were taken over time and analyzed both quantitatively with a gas chromatograph mass spectrometer and qualitatively with an ion trap mass spectrometer. This dual analysis method allows for the measurement of amino acid concentration and the detection of small quantities of polypeptides.

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Chapter 1: Introduction

Amide bonds are crucial to every form of life. Without them, proteins would not form, and, consequently life as we know it would not exist without protein-based enzymes to catalyze life-sustaining reactions. Amino acids joined together by amide bonds, called peptide bonds in this context, are called peptides and are the basic building blocks for proteins. Amide bonds do not form only between amino acids, however, and can form between any amine and carboxylic acid functional group. The reaction involves the loss of water and the formation of an amide group.

Figure 1: Amide Bond Formation

Natalie Stinton (William and Mary, Class of 2007), a former student in Dr. Kranbuehl's Laboratory, investigated the spontaneous amide bond formation between simple amines and carboxylic acids. Jordan Walk (William and Mary, Class of 2008) joined the study in the spring of 2007, and he continued and expanded the project. For this study, aqueous solutions of an amine (such as methylamine, ethylamine, etc.) and a carboxylic acid (such as valeric acid, acetic acid, cyclohexanoic acid, etc.) were made in a 1:1 ratio and sealed in glass capillary tubes. The tubes were placed in 100°C and 120°C ovens. The capillary tubes were removed from the oven and broken to retrieve samples, and the samples were analyzed with gas chromatography to track the concentration of reactants (focus on carboxylic acid depletion) and product (amide formation).

Stinton and Walk found that the equilibrium of these reactions favors the amide product and not the amine and acid reactants. Most of the reactions reached equilibrium

within about 200 days. From the data collected, Walk calculated the equilibrium constant, Gibbs free energy, and other kinetic data for each reaction.

Upon completion of the simple amine and carboxylic acid study, Walk began investigating whether or not amino acids in aqueous solution would also spontaneously form amide bonds (also called peptide bonds for amino acids). This is a valid hypothesis because amino acids possess both amine and carboxylic acid functional groups. The study he started showed some interesting preliminary results, to include the detection of glycine dipeptide and possibly tripeptide. The project, however, also had a few problems, such as erratic quantitative data. For many reasons, further investigation was warranted. My project picks up where Jordan Walk left off. I studied peptide bond formation for single amino acids in aqueous solution, and I expanded the project to include the study of bond formation between different combinations of amino acids.

The four amino acids that Walk and I focused on are alanine, glycine, proline, and valine. These amino acids were chosen because they are easily obtainable and relatively inexpensive.

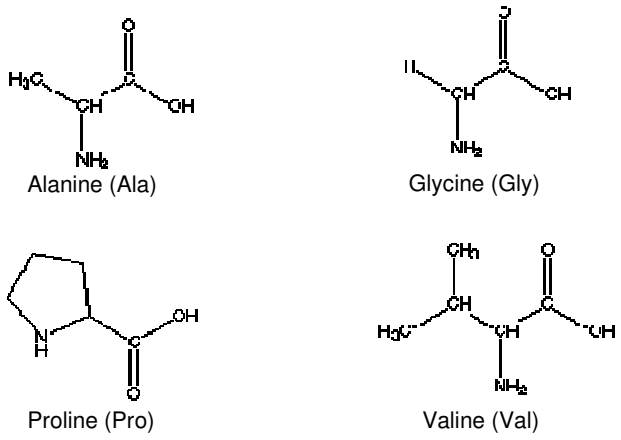

Figure 2: Amino Acid Structures

The study of spontaneous amide bond formation between amino acids has been active for many years. What follows is a brief description of how the field began and where it stands today.

In correspondence to a friend, Charles Darwin, of *On the Origin of Species* fame, suggested that some protein-like compound could have been created in the warm oceans of the early earth and then undergone further changes that may have resulted in the origin of life[1]. This established a "protein first" theory of the origin of life, which spurred a plethora of investigations and experiments. The possibility of spontaneous abiogenic amino acid formation was established by Alfonso L. Herrera[2] and Stanley L. Miller[3] in the early to mid twentieth century. To my knowledge, the subject of spontaneous amide bond formation between amino acids was first addressed in the 1950s by Sidney W. Fox.[4]

In numerous studies, Fox successfully generated short polymers of amino acids connected by peptide bonds. These abiogenically created amino acid chains are termed "proteinoids."[5] The method used by Fox entailed exposing a mixture of amino acids to dry heat until they formed a dry, white polymer.[6] As of yet, I have not found any articles that describe an investigation into whether or not amino acids spontaneously form amide bonds in aqueous solution. Fox discovered that "the lengths and the amino acid sequences of proteinoids are not random but are determined largely by the composition of the amino acid mixture and other reaction conditions."[7] He also established that large concentrations of glutamic acid and aspartic acid, the only two dicarboxylic amino acids, aided in the formation of the proteinoids.[8] Since these two amino acids are "relatively dominant throughout the proteins...of plants and animals,"[9] it is to be expected that their presence ought to be beneficial for the formation of these protein-like compounds.

In the mid twentieth century, various researchers in this field suggested that, once formed, proteinoids may act as catalysts and promote the formation of more proteinoids like themselves.[10] This effect was investigated and verified by Fox, who tied the catalytic properties to the formation of "microspheres."[11] These microspheres are formed when proteinoids are placed in an aqueous solution, and the amino acid chains form bubble-type structures consisting of a proteinoid shell surrounding an aqueous interior.[12,13] These microspheres, according to Fox, posses a variety of weak catalytic properties, one of which is catalysis of "further peptide bond synthesis."[14] Interestingly, these microspheres also share some characteristics with living cells (selective diffusion, budding, division, etc.), and have been referred to as "protocells."[15]

After Fox's discovery of proteinoid microspheres, the scientific community began investigating these structures in a variety of different contexts, to include biomedical research into their use as a means of drug delivery. Other researchers established that these protocells could be converted into more modern structures.[16] More recently, scientists have been examining meteorites and other interstellar materials for chemical combinations and compounds that may have resulted in amino acid or proteinoid formation.[17]

I have not yet found any articles that describe an investigation into whether or not amino acids spontaneously form amide bonds in aqueous solution. The work of Everett Shock, however, does theoretically address the thermodynamics of dehydration reactions (i.e., peptide bond formation) in aqueous solutions at high temperatures and pressures.[18] According to Shock's calculations, peptide bonds between amino acids can form at elevated temperatures, which gives hope for this project.[19] Shock even claims that

"condensation of complex organic molecules may be energetically favored in hydrothermal solutions."[20] If these reactions are favored, then perhaps, with time and the right reaction conditions, amino acid peptides will dominate the solutions that were initially entirely comprised of monomers.

Chapter 2: Experimental Methods

Preparation of Samples

In this project, four amino acids, alanine, glycine, proline, and valine were studied. In the single amino acid studies, each amino acid was dissolved in 10 mL of deionized water at near saturation levels. This technique provides the maximum number of amino acid molecules and increases the likelihood that two molecules will collide and react. Since each amino acid studied has a different solubility, they are all at different concentrations. Future work on this project, once equilibrium is established, could use these initial concentrations to determine and compare reaction rates.

In the combination studies, two amino acids were dissolved in the same solution at equal concentrations. Alanine and valine were paired together, and glycine and proline were put together. These combinations were chosen because the paired amino acids have peaks that do not overlap during gas chromatograph- mass spectrometry analysis.

For all studies, dry amino acid was massed out and quantitatively transferred to a 10 mL glass volumetric flask. Deionized water was added, and the solutions were then sonicated until the amino acid fully dissolved. The solutions were then diluted to full volume. Each 10 mL aqueous amino acid solution was transferred to a glass pressure tube and placed in a 120°C oven.

In previous studies, glass capillary tubes were used as reaction vessels. While capillary tubes work, they require labor-intensive set up, cumbersome equipment, and an abundance of storage space. Due to the size of the laboratory's oven, a capillary tube must be cut to short lengths, limiting the volume of sample it can hold to less than 1 mL.

In addition, each capillary tube must be broken to retrieve a sample and cannot be reused, creating waste. Therefore, one capillary tube must be filled with solution and sealed in the beginning of the study for each sample that is to be taken for the duration of the study. When all of the sample-filled capillary tubes have been broken to retrieve data, the study must either end, or new tubes must be set up. This system, while accurate, is immensely inconvenient and wasteful.

In the fall of 2007, the capillary tube system was abandoned in favor of ~12 mL glass pressure tubes made by Ace Glass Incorporated. These tubes have thick glass walls that can withstand the high pressures and forces associated with holding aqueous solutions at elevated temperatures. The pressure tubes have Teflon screw caps that enable the researcher to take a sample and then reseal the tube for continued use. This allows one solution and one tube to last for an entire study and provide far more samples with much less waste than the capillary tube system.

When this project was started in the fall of 2007, the aqueous solutions began turning from their original clear color to a pale yellow shade. This color change could be attributed to oxidation of the amino acids by dissolved oxygen in the water. To combat this undesirable result, the solutions were restarted and bubbled with argon gas to remove the dissolved oxygen. The argon bubbling was repeated every time the tubes were opened and resealed. No solutions have turned yellow since this procedural step was adopted.

Two different techniques were used to track the formation of amide bonds. Gas chromatography mass spectrometry, coupled with the use of an internal standard, provided a way to quantitatively measure the concentration of unreacted amino acid over time. Ion trap mass spectrometry was extremely useful in qualitative analysis of the

amino acid solutions. The preparation and analysis procedures for these methods are discussed next.

Gas Chromatography Mass Spectrometry Method

Derivatization Method and Explanation

Gas chromatography mass spectrometry allows the researcher to separate compounds in a sample solution by their boiling point, and then scan the solutions to determine the mass of compounds and compound fragments present. In order to do this, a gas chromatograph mass spectrometer (GCMS) must be able to attain temperatures high enough to boil off the compounds to be analyzed. Unfortunately, amino acids as well as the polypeptides formed from them have boiling points well outside the range of most GCMS instruments. In order to analyze the amino acid solutions with GCMS, they must be "transformed" or derivatized into more volatile compounds.

Many scientific articles describe derivatization methods for analysis of amino acids. However, the vast majority of these methods are incompatible with aqueous solutions, making them useless for this investigation. Other methods require harsh conditions- high pressure, extreme temperatures, dangerous chemicals, and lengthy reaction times. Attaining the equipment necessary to perform these methods was unrealistic.

In 2007, Zampolli, et al., published a method for the derivatization and analysis of amino acids via GCMS.[21] This method was initially developed for the separation and identification of extra-terrestrial amino acid samples aboard spacecraft. It uses an "alkyl chloroformate–alcohol–pyridine mixture"[22] to transform the amino acid starting material

into an ester that can be easily analyzed with the GCMS method. The conditions are "soft," meaning the reaction can be run at room temperature and atmospheric pressure, and the reagents are easily obtainable and require no special handling or precautions. The reaction is also very fast- a sample can be fully derivatized and ready for injection into the GCMS in less than 15 minutes. These qualities make the method described by Zampolli, et al., ideal for the budget, resources, and time restraints of an undergraduate study in a college research laboratory.

In order to make the GCMS analysis quantitative, an internal standard is used. By mixing each sample for analysis with the same, known amount (both volume and concentration) of a standard compound, the instrument response to the sample compounds can be compared to the response to the known standard. For GCMS, the compounds appear as response peaks, and the area of those peaks is used to measure the concentration of each compound. The internal standard used needs to be similar to, but still distinguishable from the compounds that are being studied. For this research, methyl laurate (methyl dodecanoate) was chosen as an internal standard. To make up the internal standard solution, 25 μL of methyl laurate was diluted in 50 mL of acetonitrile (it is insoluble in water). A new solution was prepared each week samples were taken to minimize potential contamination.

Since the method requires very small quantities of each reagent, the equipment used had to be suited for those requirements. Small ~1 mL plastic snap-cap eppendorf microtubes were used as reaction vessels, and a 10-100 μL adjustable volume pipette was used to measure and dispense reagents. This pipette was a valuable tool for this project

because it is accurate and, since the tips are disposable and easily replaceable, there's no need to rinse or clean the pipette between reagents, minimizing contamination.

In preparation for derivatization, 100 μL of aqueous amino acid sample solution was diluted to 10 mL with deionized water. To start the derivatization process, 25 μL each of this diluted amino acid solution and the internal standard were transferred to the eppendorf tube. Then, 60 μL of 2,2,3,3,4,4,4-heptafluoro-1-butanol (HFB), 15 μL of pyridine, and 15 μL of methyl chloroformate (MCF) were added. HFB and MCF were chosen because of their use in the Zampolli, et al., procedure and because perfluoronated products are very volatile and have short retention times on the GCMS column, making the entire procedure faster.[23] Pyridine, while commonly used as a solvent, probably acts as a base and deprotonates the amino acid in this reaction. Upon addition of MCF, the solution bubbles violently and becomes slightly warm. Flasks are capped at this point and sonicated for approximately one minute to ensure that the reaction runs to completion. Occasional venting is necessary to release the gas byproduct and relieve pressure in the tubes.

The derivatization mechanism is not totally understood. Zampolli and his colleagues, however, do present a reasonable hypothesis as to what reactions occur. Pyridine, while commonly used as a solvent, probably acts as a base and deprotonates the amino acid in this reaction. The HFB and MCF react with the amino acid to create a mixed anhydride intermediate (product A in Figure 3).[24] This intermediate apparently then loses carbon dioxide. This may explain the bubbles that are released upon addition of MCF. Along with the decarboxylation, the intermediate may also undergo a substitution reaction with the alcohol, HFB, and exchange the alkyl group from MCF for

that of HFB. This gives the "main" product (product B in Figure 3) according to Zampolli.[25] If the intermediate does not under go this exchange, byproduct C is formed (Figure 3). It is possible that these two potential products account for the splitting of the GCMS amino acid peak in glycine, alanine, and valine. However, the fact that the proline peak does not split is still mystifying. Perhaps the ring structure of the R-group for this amino acid prevents one of the two possible reactions from occurring, but this is merely a hypothesis.

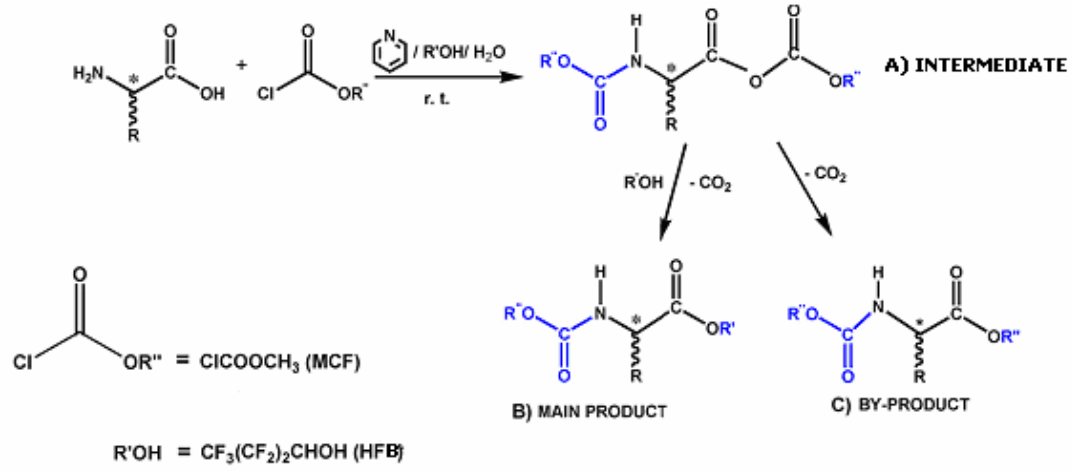

Figure 3: Derivatization mechanism[26]

The next step of the derivatization process calls for the addition of 200 µL of chloroform to extract the derivatization products into the lower organic layer. To dry the solution and pull water into the upper layer, 20 µL of saturated NaCl solution was added, and the flask was shaken to ensure thorough mixing. After waiting for the layers to separate, 1 µL of the lower layer can be injected into the GCMS for analysis.

GCMS Procedure and Method for Analysis

In order to use the GCMS instrument, there must be a flow of nitrogen gas. This N_2 tank is usually left on, so it was not a regular concern for the purposes of this project. Through the computer interface, the method named "walk amino" must first be loaded, and a sample name and directory information must be filled in. Once this is done, the "Start Run" button on the screen, along with the "Prep Run" button on the instrument itself should be pushed. As soon as the light on the GCMS becomes steady, the instrument is ready for injection and the computer interface will provide instructions on how to proceed. Approximately 1 μL of the sample solution should be injected into the front inlet (there is only one operational inlet on this particular instrument), and the "Start" button on the instrument should be pushed simultaneously to start the run at the same time the sample is injected.

Each run takes about 14 minutes to complete. The internal standard (methyl laurate) peak has a retention time of approximately 13.6 minutes, and all of the amino acids studied elute before then. All of the amino acids except proline show two GCMS peaks with similar compositions. At this point, it is still unclear as to why exactly two peaks are present, but my guess is that it is because of the side reaction in the derivatization method. Table 1 lists the elution times for all of the species studied.

Compound	**Approximate Elution Time (minutes)**	
	First Peak	**Second Peak**
Alanine	5.2	6.0
Glycine	5.0	6.2
Proline	10.5	N/A
Valine	8.2	8.4
Methyl Laurate (IS)	13.6	N/A

Table 1: GCMS Elution Times

For each GCMS run, the spectrum was integrated to determine the retention time of each peak and a printout of the spectrum and a percent report of the peaks were obtained. The percent report details the retention time, height, and area for each peak. The areas of the amino acid peaks were compared to that of the internal standard peak for each run, and the concentration of each amino acid was determined based on previously made calibration charts.

To make a calibration chart, four solutions, each at a different concentration level up to that used for the study, were made for each amino acid. For these solutions, dry, pure amino acid was massed out and dissolved in deionized water. Sonication was used to ensure complete solvation. These solutions were derivatized with the same amount of internal standard as the "real" study and run through the GCMS following the standard procedure. The ratio of amino acid peak area to internal standard peak area was graphed against the known amino acid concentration in Microsoft Excel, and a best fit line was obtained. For the solutions with split amino acid peaks, three data sets were graphed- one for each peak individually, and one for the area of the peaks combined. In general, the summed peak areas showed the most linear trend. The best fit line for these calibration charts provides an equation that defines the GCMS detector response of each amino acid in terms of the internal standard response. These charts and equations will be discussed further in the results section.

Ion Trap Mass Spectrometry Method

Since the GCMS analysis only provides quantitative data about how much unreacted amino acid is left in solution, a separate method was needed to track what kinds of products were actually being formed. A quantitative approach to measuring

polypeptides was not found, but the use of ion trap mass spectrometry (IT-MS) allowed for qualitative detection of products.

To prepare samples for analysis by IT-MS, 2.5 μL of aqueous amino acid solution was diluted to 10 mL in a glass volumetric flask with deionized water. To this, 1 μL of acetic acid was added to protonate the compounds in solution. This protonation step is crucial because the IT-MS only detects ions. Unlike with GCMS, no derivatization is necessary and this dilute protonated solution can be injected directly into the instrument.

The IT-MS used is in the laboratory of Dr. J.C. Poutsma of the Chemistry Department at the College of William and Mary. It is operated through a computer interface. The instrument scans sample solutions and provides a spectrum of all of the mass to charge ratios (m/z) present in the solution within the selected m/z range. Peaks with relatively high abundances are identified by the analysis program, and the rest are left unlabeled.

To operate the IT-MS for the purposes of this experiment, the method "kranbuehl2" must first be loaded on the computer interface. When this is complete, a yellow "pause" button should be visible on the screen. If, instead, there is a green "play" button, then the green button must be clicked. This pauses the flow of solution into the instrument and allows for the solution to be changed or refilled. At this point, approximately 500 μL of sample protonated amino acid solution can be withdrawn into a glass syringe. The syringe is then loaded into an automated syringe pump set to 1000 μL/hour. A capillary tube that feeds into the instrument can then be attached to the tip of the syringe. The tube connector should be pushed onto the tip gently, but firmly. If the connection is too tight or too loose, the flow will not be correct and the solution being

analyzed may form droplets at the connection point. This can be fixed by removing the connector from the syringe and trying again.

Once the syringe is in place and connected properly, the yellow “pause” button on the computer interface should be pushed. It will change to a green “play” button and the syringe pump will begin injecting solution into the instrument for analysis. In a matter of seconds, a mass spectrum of the sample solution will appear on the computer screen. This spectrum will fluctuate over time, but an average spectrum can be taken for analysis purposes.

The peaks of interest for this project are those m/z peaks of the protonated single amino acids, dipeptides, tri and other polypeptides, along with dimers, trimers, and perhaps other by-products. The mass of the protonated amino acids is the molecular weight of the acid plus one mass unit (the proton). The dipeptide mass is calculated by multiplying the molecular weight of the amino acid by two, subtracting 18 mass units (the mass of the water lost during the formation of the peptide bond), and adding one mass unit to account for the proton needed for ionization. A similar calculation is performed for tripeptides and larger peptides, with the molecular weight being multiplied by the number of amino acid sub units and subtracting 18 mass units for each peptide bond required to make the compound. Only one proton mass is added to each polypeptide, regardless of the size of the molecule.

Dimers form when two single amino acid molecules become joined by intermolecular forces, such as hydrogen bonds. These compounds do not lose any atoms in joining together, and they are very different from peptides. Their mass is calculated by

multiplying the molecular weight of the amino acid by two (for dimers) or three (for trimers), etc., and then adding to this one mass unit for the ionizing proton.

Table 2 displays the m/z ratios of the peaks of interest for each amino acid studied.

	m/z ratio				
Amino Acid	**parent**	**dipeptide**	**tripeptide**	**dimer**	**trimer**
Alanine	90	161	232	179	268
Glycine	76	133	189	151	226
Proline	116	213	311	231	346
Valine	118	217	316	235	352

Table 2: Ion Trap Mass Spectrometry m/z Ratios

In this study, dimers and trimers and the like have been referred to as "by-products," suggesting that they are seen as failed reactions or of little interest. This is not true. It is possible that these dimers form as precursors to peptide bond formation between amino acids. If possible in future years, the formation of dimers should be investigated and tracked along with the formation of peptides to see if there is any correlation.

Chapter 3: Results and Discussion

Gas Chromatography Mass Spectrometry Results for Amino Acid Studies

Note on Amino Acid Studies Started in Spring 2008

In the spring of 2008, Jordan Walk started aqueous alanine, glycine, proline, and valine studies. He took 3 to 4 samples for each amino acid and ran them through the GCMS. This data was graphed in Excel. He also used Excel to create a calibration chart for each amino acid. When I took over the project in the fall of 2008, I wanted to keep monitoring these original solutions to see how the reactions progressed over a long period of time. To continue the analysis, I needed these original Excel spreadsheets including data and charts. Unfortunately, over the summer of 2008, all of these Excel files were lost due a data transfer mishap from Jordan Walk's old laptop (on which all files were stored) to his new computer. None of the GCMS spectra printouts were saved, either. I have a copy of his thesis with the final calibration and sample charts in it, but the file is in pdf format and the charts cannot be easily extracted and include none of the data used to build each chart.

In the wake of this very unfortunate event, I began storing the spectra hard copies in a well-labeled binder and saving all Excel-based data analysis on both my laptop and the laboratory desktop, in addition to a USB flash drive. With the data and analysis backed up in several places, the data is protected in the event that one computer crashes or somehow loses files.

Still wanting to continue study of the spring 2008 solutions, I decided to attempt to recreate Jordan's calibration charts and data analysis based on data files saved in the

computer attached to the GCMS instrument. This proved extremely tedious because for some amino acid studies, there were fewer spectra saved than there were points in Jordan's charts. Also, for the calibration charts, many more spectra were saved for each amino acid than were used to create the calibration charts. There was also some confusion about the concentration of the calibration solutions, since this information was not listed in the file name of the spectra located. I was also unable to locate Jordan's laboratory notebook to double check the concentrations of solutions made. Determining which data sets he used and attempting to reassemble all of these charts took several months of the fall semester.

Recreated and New Calibration Charts

Due in part, perhaps, to the confusion over which points to use and the concentration of solutions, the calibration charts I was able to create from Jordan's data had slopes similar to but still different from those found in his thesis. I decided to analyze the sample data he took in the spring by comparison to my recreated calibration charts, and to analyze any data points I took beginning in the fall of 2008 by new calibration charts made by myself and Kendall Meyer.

Figures 2, 3, 4, and 5 show the recreated calibration charts used for analysis of the data points collected in the spring of 2008. For the amino acids with split GCMS peaks (alanine, glycine, and valine), the area of the first peak alone was compared to the methyl laurate (ML) internal standard peak for calibration and quantitation purposes. According to Jordan Walk, this peak ratio had the most linear trend.[27] A linear fit line is shown for

each chart. The corresponding equation is used for quantitation of amino acid in GCMS analysis of sample solutions.

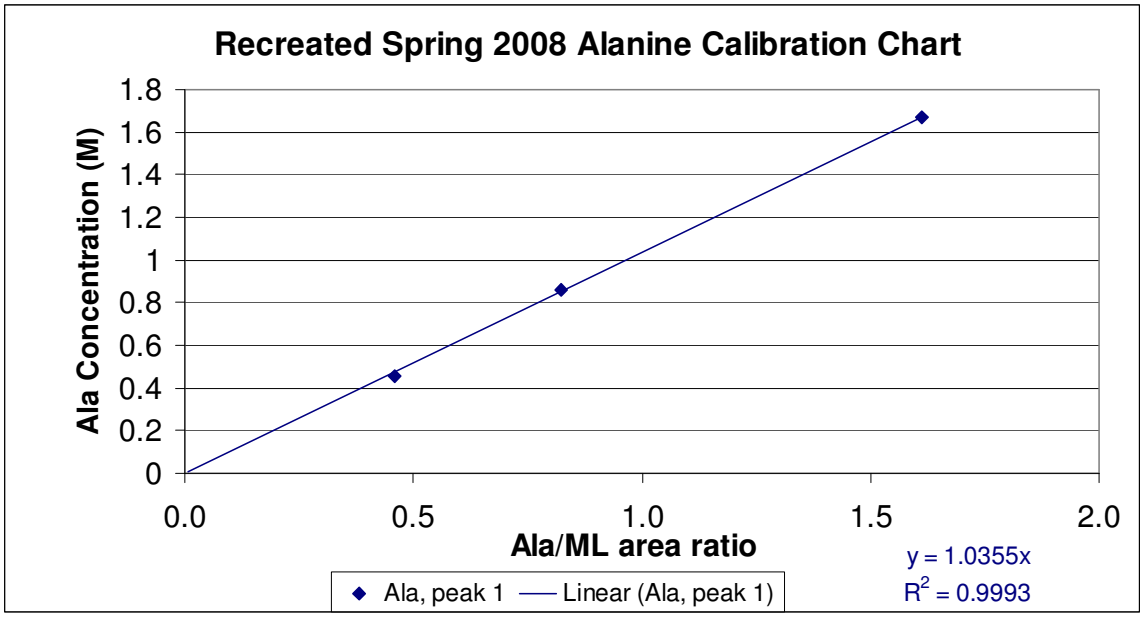

Figure 4: Recreated Spring 2008 Alanine Calibration Chart

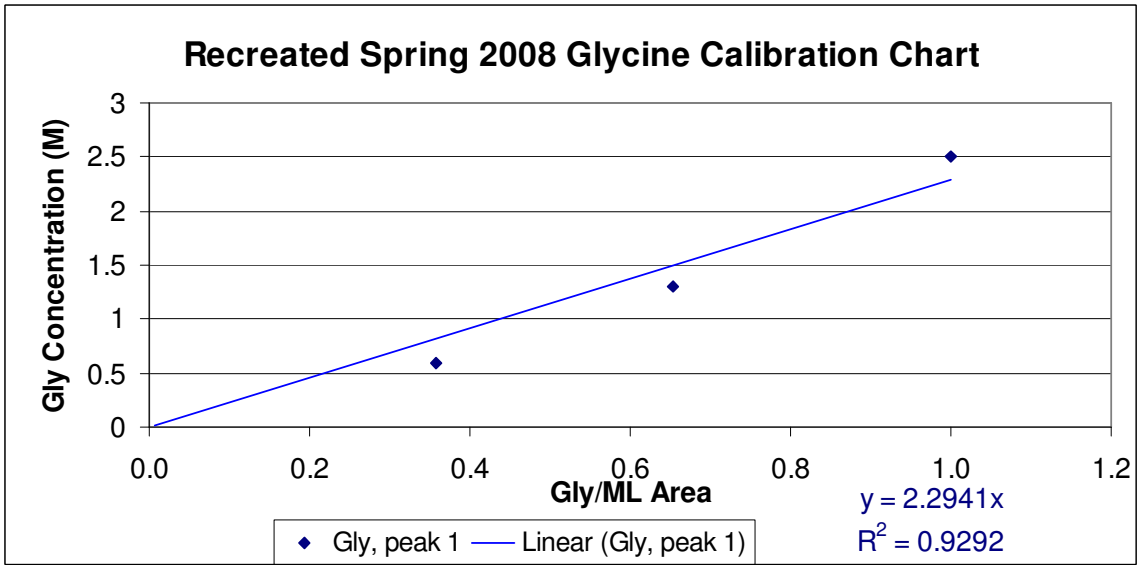

Figure 5: Recreated Spring 2008 Glycine Calibration Chart

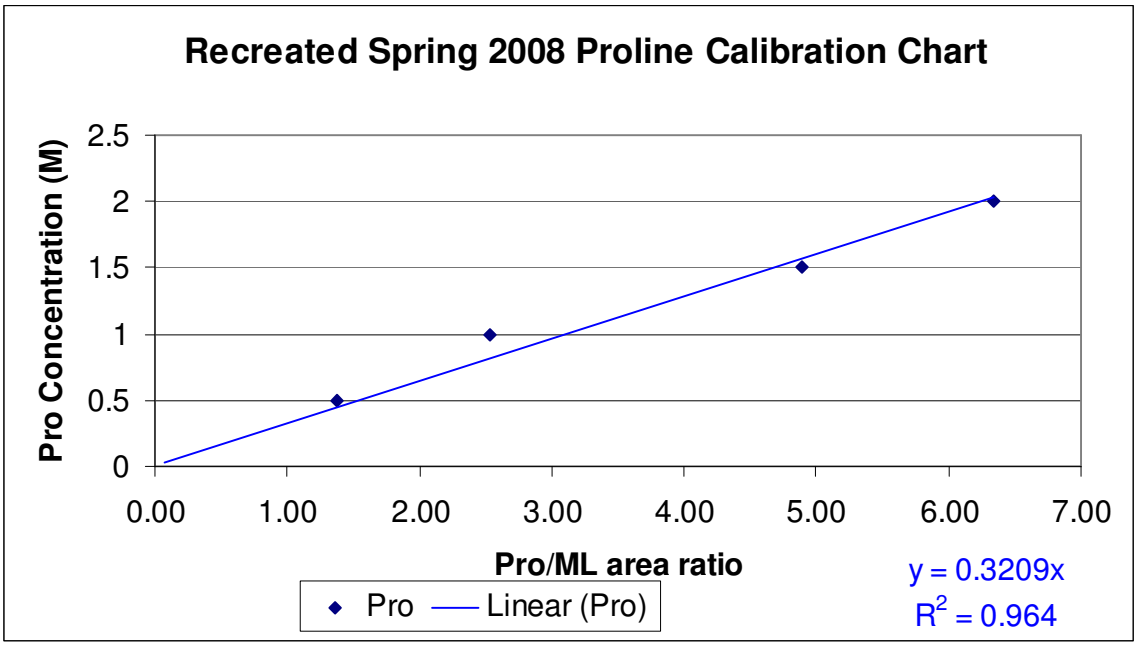

Figure 6: Recreated Spring 2008 Proline Calibration Chart

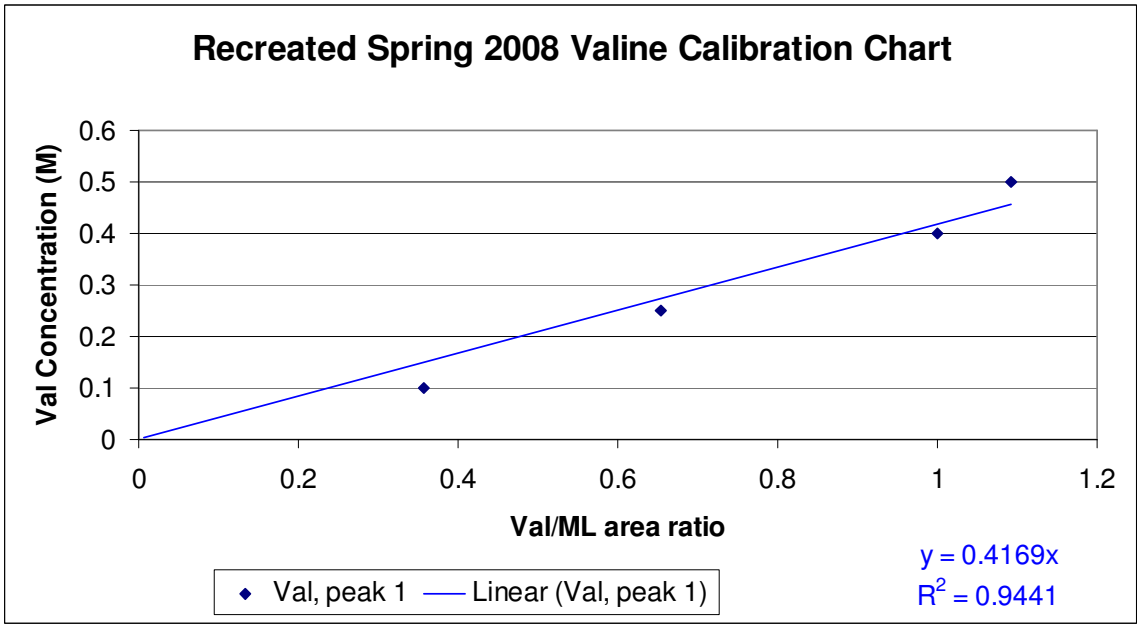

Figure 7: Recreated Spring 2008 Valine Calibration Chart

Table 3 summarizes the data from the recreated calibration charts. For each amino acid, the equation relating amino acid concentration to the ratio of amino acid peak area to methyl laurate peak area.

Amino Acid	Equation for Quantitation
Alanine	[Ala] = 1.0355 (Ala area/ML area)
Glycine	[Gly] = 2.2941 (Gly area/ML area)
Proline	[Pro] = 0.3209 (Pro area/ML area)
Valine	[Val] = 0.4169 (Val area/ML area)

Table 3: Summary of Recreated Spring 2008 Calibration Charts

Because of the confusion over the original and recreated calibration charts, I decided in the fall of 2008 to make new calibration solutions and charts. This way, I would know for sure what concentration the solutions were. I would also be able to look at the GCMS results from an unbiased perspective since I was not trying to pick data to match someone else's charts. In addition, instrument response to certain compounds can change over time as the chromatography column ages, so making a fresh calibration chart or at least double checking an old chart is a good idea every year or so.

Figures 6, 7, 8, and 9 are the calibration charts made for each amino acid in the fall of 2008. Unlike Jordan Walk's data from spring 2008, the sum of the area of the split peaks for alanine, glycine, and valine had the most linear character when compared to the area of the methyl laurate internal standard peak. Again, a linear fit line and equation are shown for each chart.

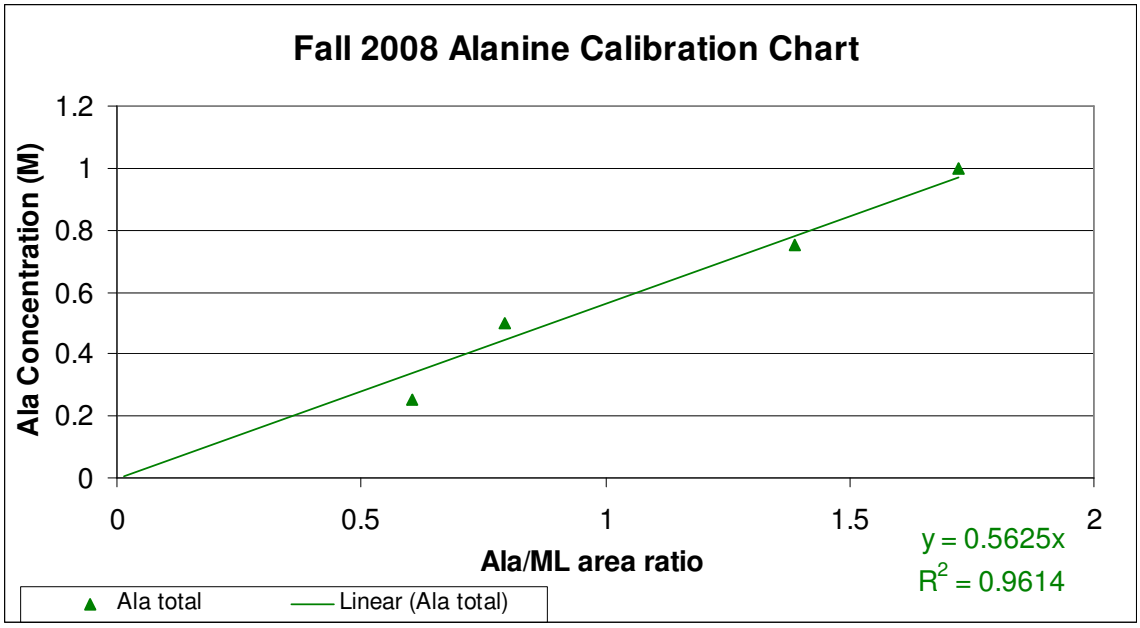

Figure 8: Fall 2008 Alanine Calibration Chart

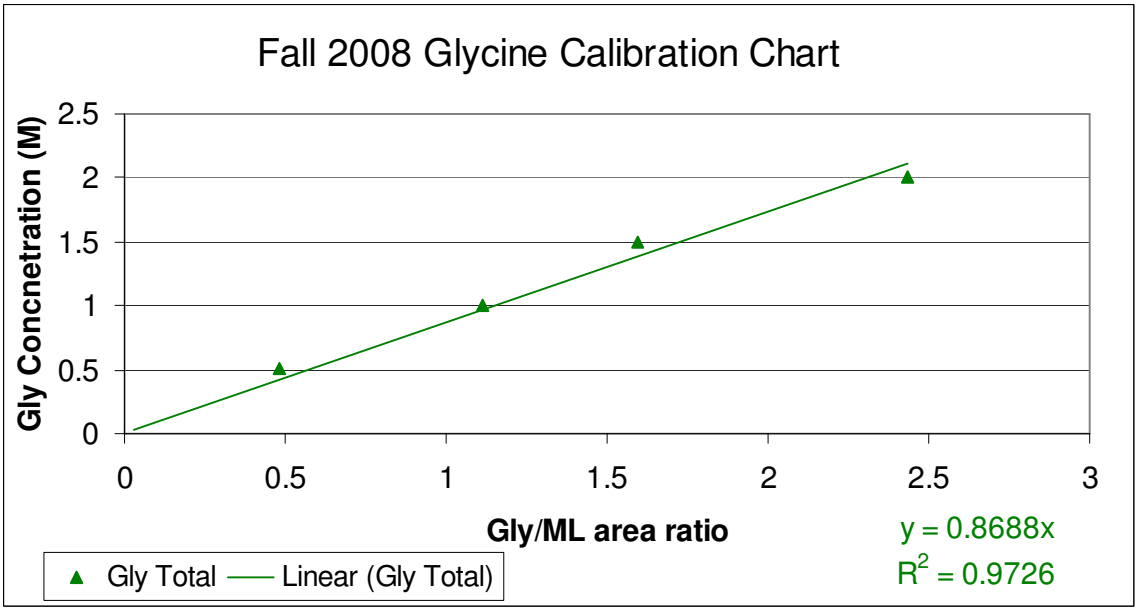

Figure 9: Fall 2008 Glycine Calibration Chart

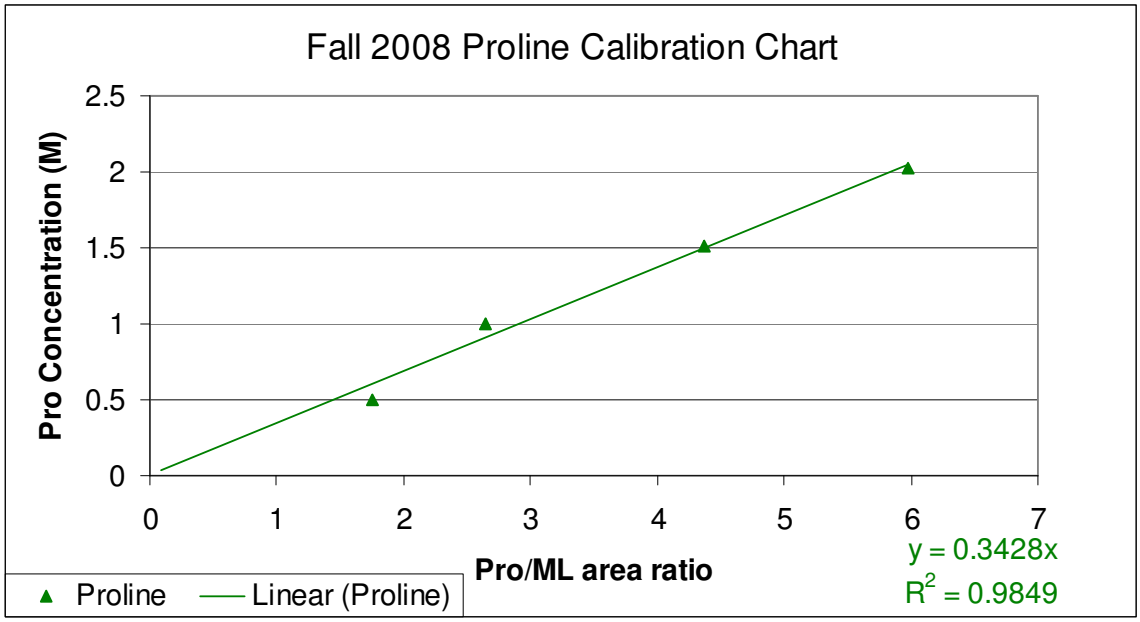

Figure 10: Fall 2008 Proline Calibration Chart

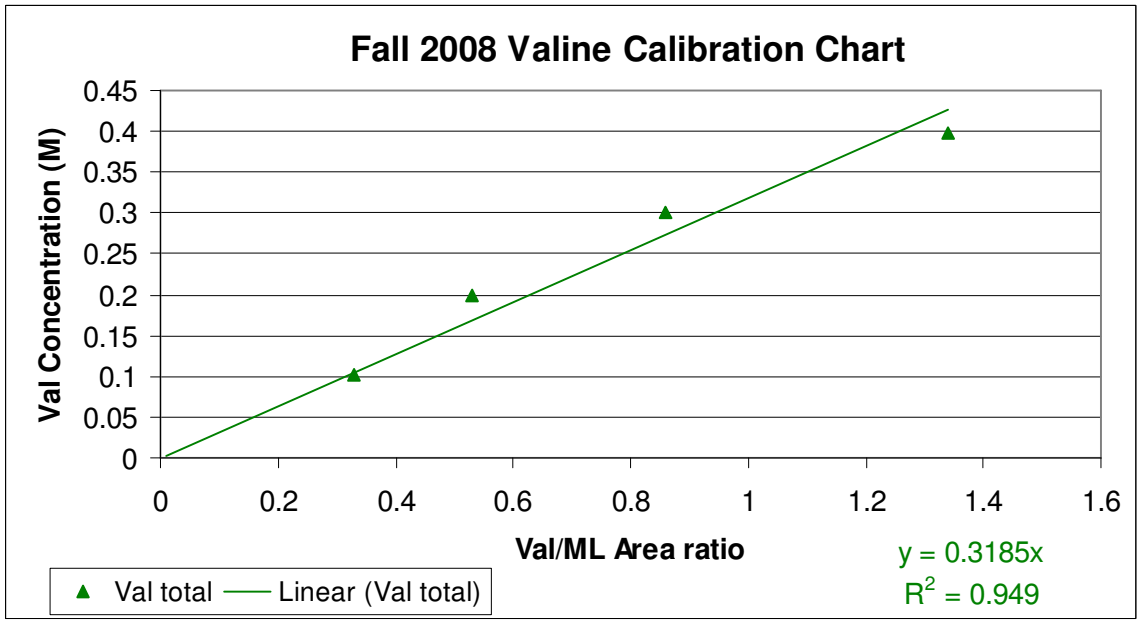

Figure 11: Fall 2008 Valine Calibration Chart

The new calibration charts created in the fall of 2008 are summarized in Table 4, following the same concepts as outlined for the recreated charts.

Amino Acid	Equation for Quantitation
Alanine	[Ala] = 0.5625 (Ala area/ML area)
Glycine	[Gly] = 0.8688 (Gly area/ML area)
Proline	[Pro] = 0.3428 (Pro area/ML area)
Valine	[Val] = 0.3185 (Val area/ML area)

Table 4: Summary of Fall 2008 Calibration Charts

GCMS Analysis of Amino Acid Studies Started Spring 2008

In the fall of 2008, I retrieved what data files I could from the amino acid studies started the previous spring. These spectra and peak areas were analyzed using the calibration charts I recreated from the recovered spring 2008 calibration data. I did not use the new fall 2008 calibration charts for this particular analysis because they reflect the GCMS response to the compounds in the fall, while the older charts more accurately reflect the response of the instrument during the time period that the data samples in question were collected. These spring 2008 data points are shown in blue diamonds in the charts below.

I took new data from these older studies this year to track the depletion of amino acid over longer periods of time. These new spectra were analyzed with the calibration charts and equations created in the fall of 2008. These newer points are shown in pink squares in the charts below.

-Alanine

The aqueous alanine solution started with a concentration of 1.48 M, which is close to the amino acid's aqueous solubility of approximately 1.9 M.[28] Only two GCMS data points from the spring could be located. Figure 10 shows the "old" and "new" data points for this study.

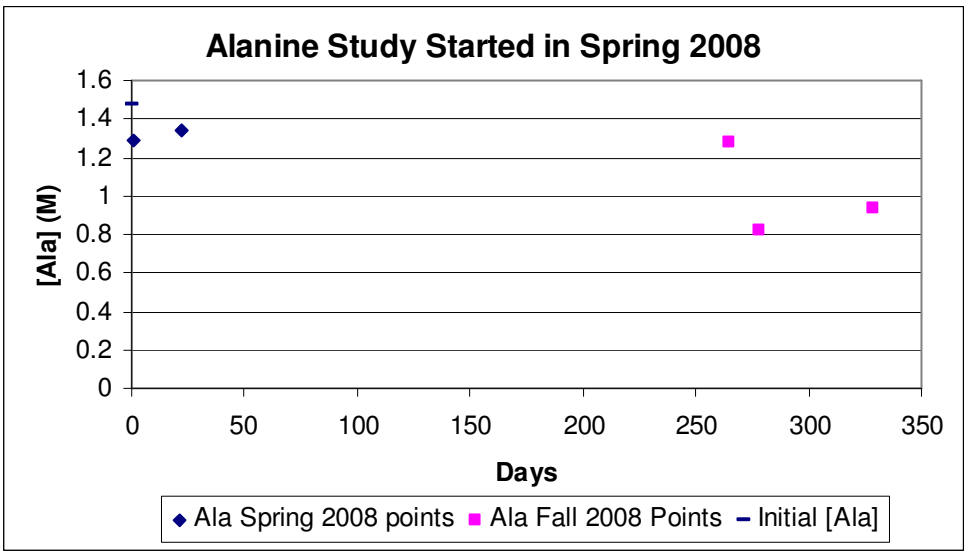

Figure 12: Alanine Study Started in Spring 2008

It is interesting to note that all of the data points collected show a concentration below the original known concentration of 1.48 M, including the day 0 reading. This could indicate an error in the initial calculation of alanine concentration, or an error in the quantitative analysis method. It could also be explained by human error. Perhaps the pressure tube was not inverted or mixed well enough to ensure an even distribution of amino acid before a sample was taken, or a bubble was in the pipette tip, resulting in less solution being derivatized and analyzed than expected.

Overall, the data suggests that the concentration of alanine is decreasing slowly over time. The points do fluctuate a great deal, however, so this conclusion may be incorrect.

-Glycine

The glycine solution started in the spring of 2008 had an initial calculated concentration of 2.59 M. Glycine's solubility in water is approximately 3.2 M.[29] At the concentration chosen, as with all of our other amino acid solutions, the amino acid is relatively concentrated by its own standards, but the concentration is still low enough that it is not difficult to get the dry amino acid to dissolve.

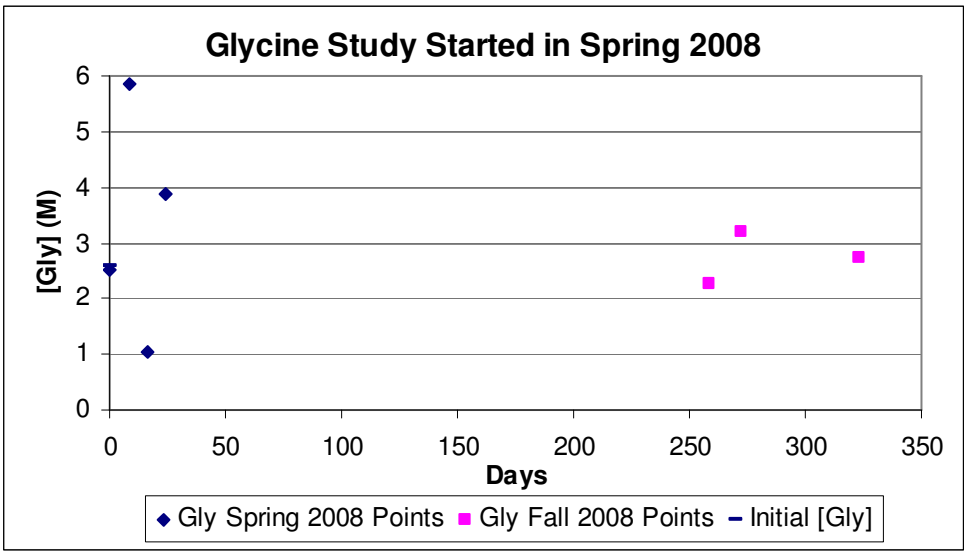

Figure 13: Glycine Study Started in Spring 2008

There is obviously something wrong with the points taken in the spring of 2008 for glycine. The day 0 point seems to be acceptable, as it is very close to the actual measured initial concentration of the solution, but after that the points become erratic. These apparent errors could be due to many of the same issues mentioned earlier for alanine.

The data points taken beginning in the fall of 2008 are much more within the acceptable range. The last two points are still above the initial measured concentration, but not nearly to the extreme of those from the spring of 2008. The glycine solution overall appears to show very little, if any, decrease in amino acid monomer concentration.

-Proline

Proline has a solubility around 14 molar in water, which is much higher than most, if not all, other amino acids.[30] We considered making a solution at a concentration near this phenomenally high solubility, but that would have required a great deal of pure proline and would be rather expensive in the long run. Instead, we selected a concentration comparable to that of the other amino acid solutions. The spring 2008 aqueous proline solution began at a concentration of approximately 2 M. If funds allow, a near saturation proline solution could be tested in future years.

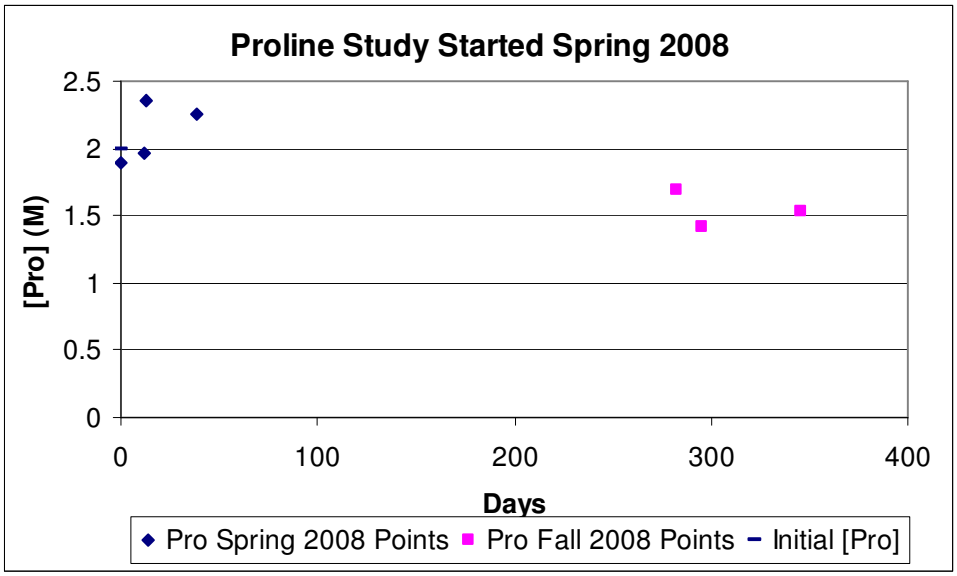

Figure 14: Proline Study Started Spring 2008

As with the other amino acid solutions, the proline study seems to be subject to some error. The early points include two above the initially calculated concentration. Little change in concentration is apparent, but there is a slight decrease in unreacted proline over time. This is similar to the alanine study.

-Valine

The valine solution created in the spring of 2008 was at an initial concentration of approximately 0.473 M. Since proline's solubility in water is around 0.75 M[31], the concentration chosen is a good compromise between maximum solubility and ease of solvation.

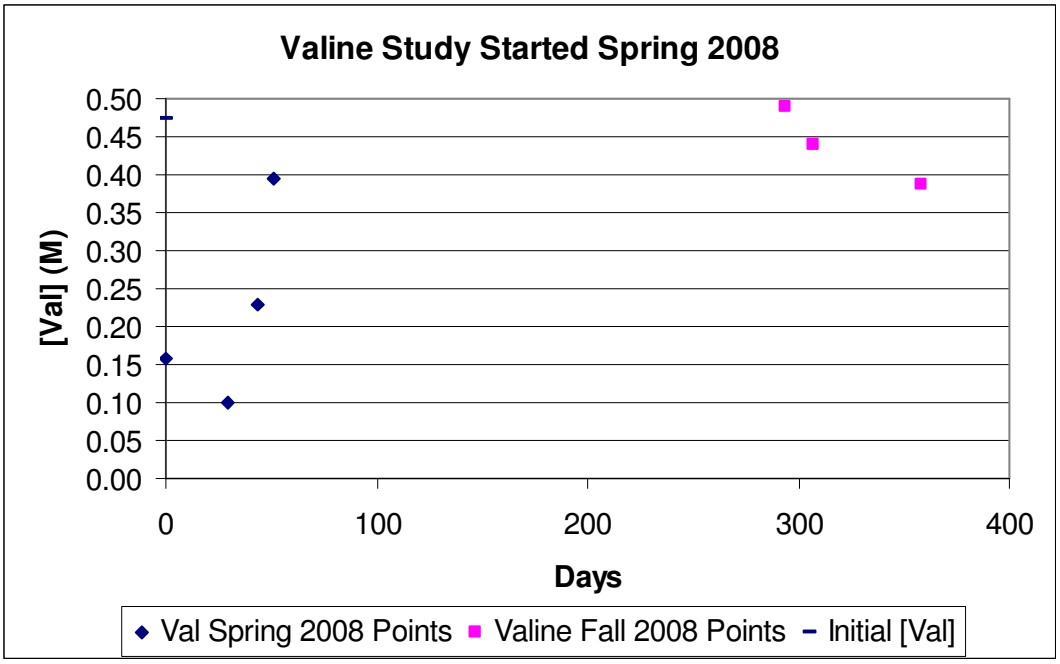

Figure 15: Valine Study Started Spring 2008

As with the early glycine study, the spring 2008 data points taken for valine are very scattered and don't seem to make any sense. The first three are well below the calculated initial concentration. These errors may be explained by poor sampling and derivatizing techniques, or could just be odd flukes in GCMS response. These errors seem to be prevalent among the data points taken in the spring of 2008.

The samples taken from the fall of 2008 onward are with in the range of the original calculated concentration. Again, these points show a relatively stable solution of

unreacted amino acid. Valine does seem to exhibit a slight decrease in concentration around day 300.

GCMS Analysis of Amino Acid Studies Started Fall 2008

After questionable and confusing results with the studies started in the spring of 2008, I decided to start fresh amino acid solutions at comparable concentrations to see if the apparent errors could be avoided or corrected with a fresh and careful start. These solutions would also provide data for the time span left blank by the study started the previous spring. This break was due to the summer vacation, during which no one was present to take samples from any amino acid solution. These new studies were all started on November 11, 2008. The same procedure for starting solutions was followed, to include massing pure dry compounds, diluting to 10 mL with deionized water, sonication, transfer to a pressure tube, bubbling with argon gas (to prevent oxidation, as previously discussed), and storage in a 120° C oven. These new solutions were analyzed using the calibration charts created in the fall of 2008.

-Alanine

The fresh alanine solution was made at a concentration of 1.50 M. Figure 14 displays the data points from GCMS analysis of this solution.

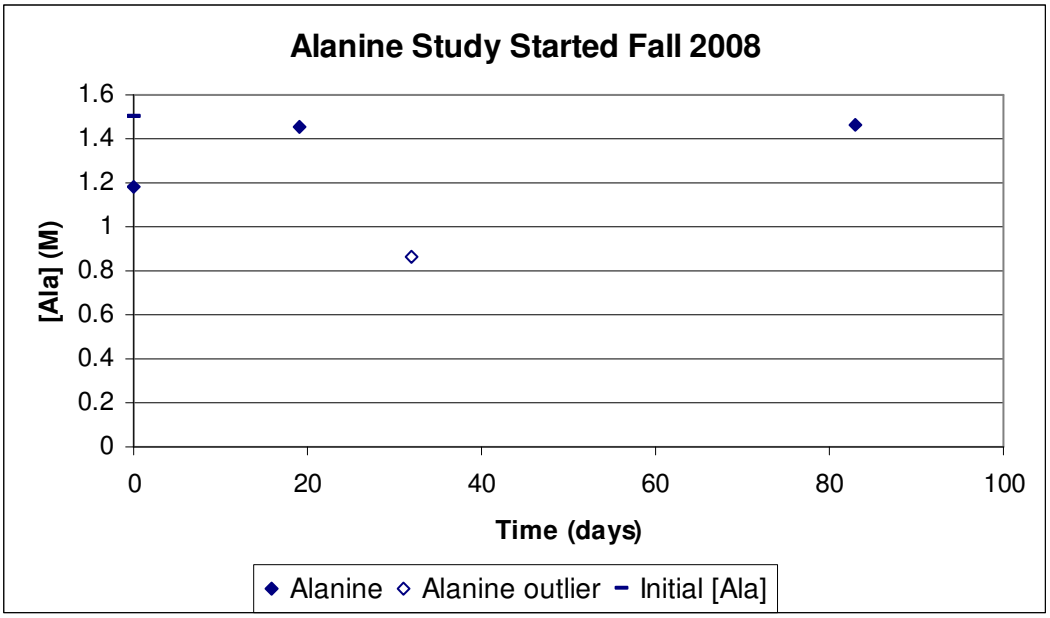

Figure 16: Alanine Study Started Fall 2008

Unfortunately, these data points are a bit scattered like in the earlier studies. However, the day 32 reading seems a bit low in several of these studies. If this one point is excluded, the solution seems steadier. The study appears to show very little loss of monomer, unreacted, alanine. This observation aligns with the previous study, but with more data points and more exact information about the initial concentration of the amino acid and the calibration solutions, the findings are better supported.

-Glycine

A 2.51 M glycine solution was made in the fall of 2008. The following chart outlines the quantitative date collected.

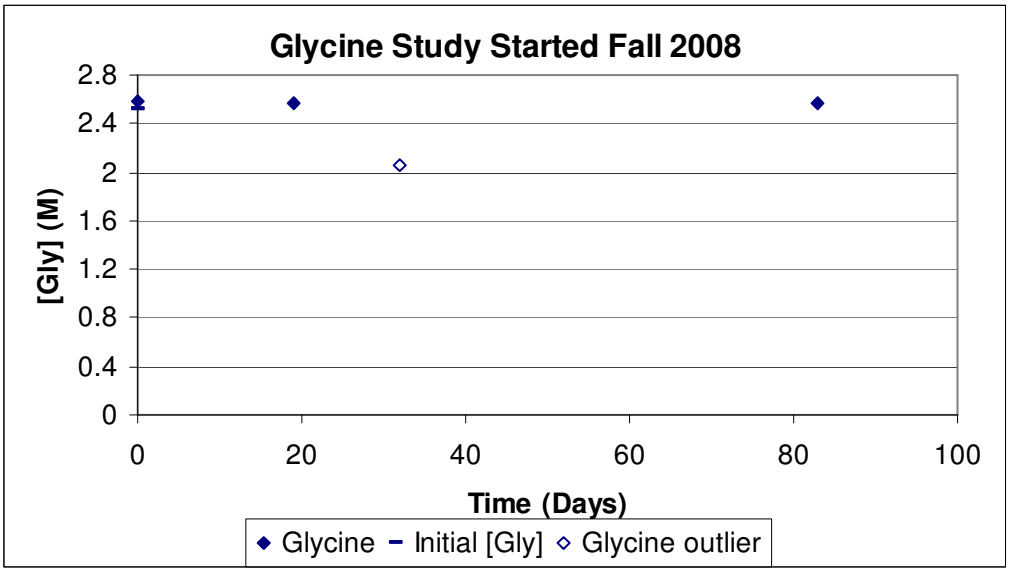

Figure 17: Glycine Study Started Fall 2008

As with the new alanine solution, the day 32 point seems a bit low. However, the other points are very linear and remain very close to the initial concentration of the solution. The data suggests that glycine monomers do not react very quickly since the solution does not show a significant decrease in glycine concentration. Such a decrease would indicate that some major reaction was taking place.

-Proline

The "new" fall 2008 proline solution was prepared at an initial concentration of 2.00 M.

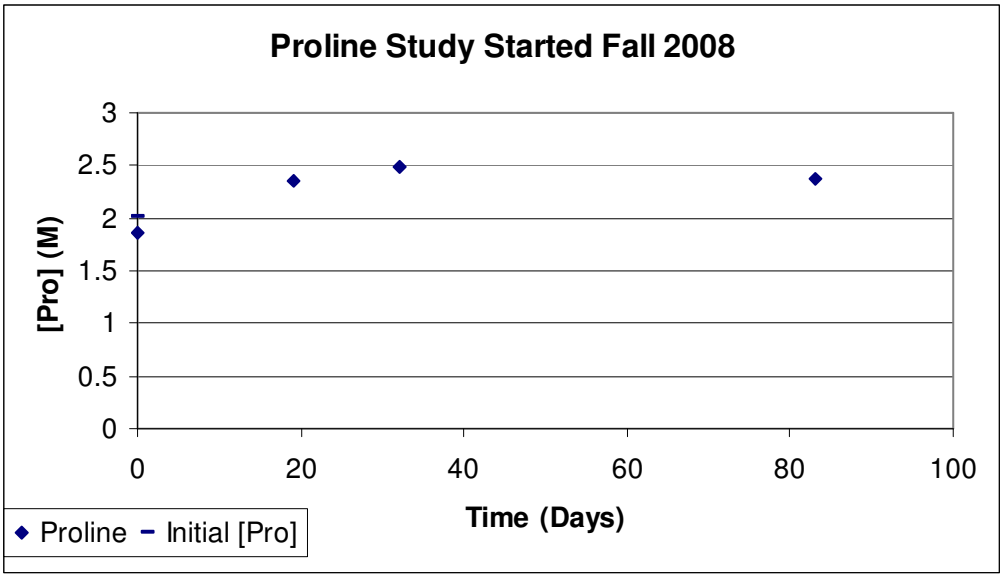

Figure 18: Proline Study Started Fall 2008

The analysis gives three points above the initial concentration and shows a similar trend as with the spring 2008 data. Overall, the data points show a relatively stable concentration of monomer proline over the time span studied. As with the other amino acid solutions, this insignificant decrease in concentration over time indicates that, either the reactions occurring are very slow, or that the monomer is highly favored at equilibrium.

-Valine

The fall 2008 aqueous solution of valine had an initial concentration of 0.502 M.

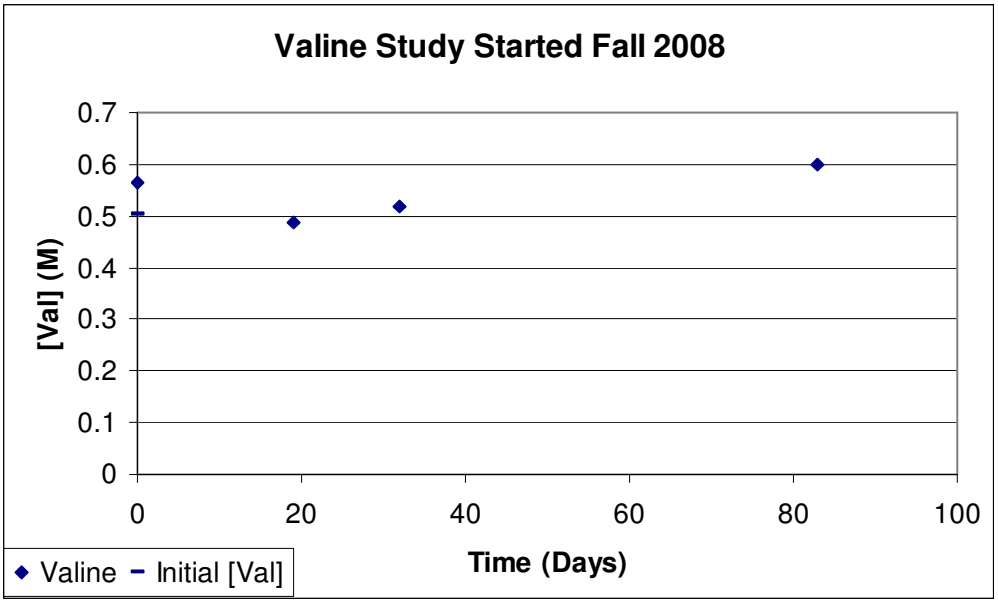

Figure 19: Valine Study Started Fall 2008

This study, so far, gives more believable data than the one started in the spring of 2008. However, as with other solutions, the data shows several points above the initial calculated concentration. The data does show an insignificant decrease in proline monomer concentration over the time studied.

-Alanine and Valine Combination Study

This solution was created in February of 2009, following the same procedure that is outlined for single amino acid solutions. The initial alanine concentration was 0.403 M and the initial valine concentration was 0.396 M. The concentrations of the amino acids were set at this level because valine's solubility is less than that of alanine, and the effect of having both amino acids in solution together on each amino acid's solubility was

unknown. So, a modest concentration was selected. It is important that each amino acid be near the same concentration in solution so that neither is favored over the other for reactions due to concentration alone.

The individual calibration data for alanine and valine were used to analyze this combination solution.

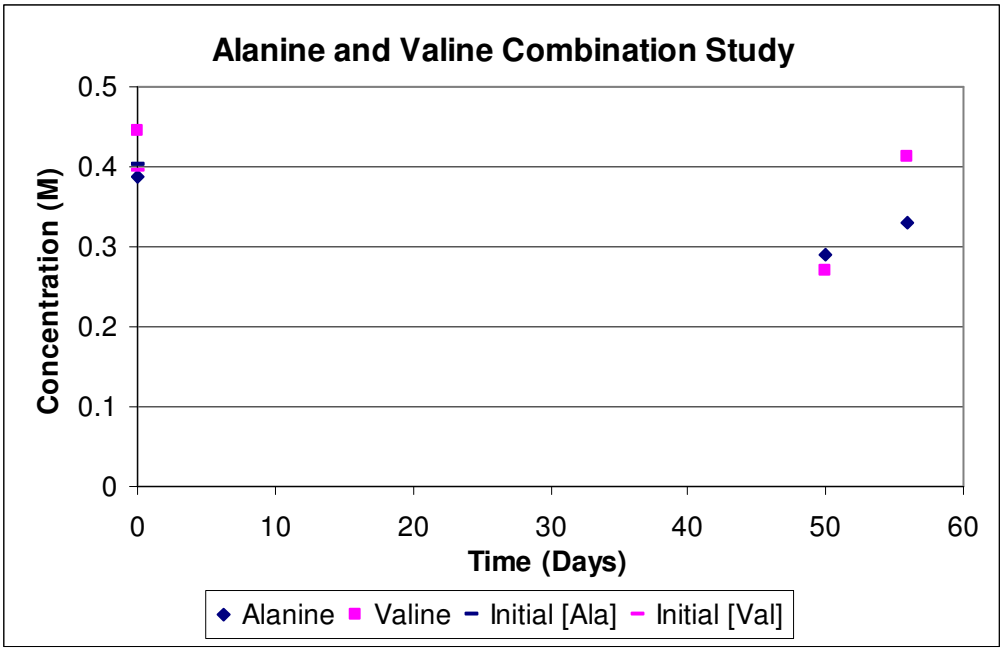

Figure 20: Alanine and Valine Combination Study

At day 0, the concentration for both amino acids reads close to the initially calculated value, although valine is a bit high. The concentrations don't seem to change much by day 56, but there is a noticeable "dip" at day 50. This anomaly could be explained by the fact that the sample pulled on day 50 did not result in a reading at all the first time it was run through the GCMS and it had to be re-done. There is a chance that there was some extra human error or some sampling "blip" associated with that particular pull. Further samples will determine if the trend follows the day 50 data or the day 56 data.

-Glycine and Proline Combination Study

This solution was also created in February 2009 following the same procedure outlined for single amino acid solutions. The initial glycine concentration was 1.01 M, and the initial proline concentration was 0.999 M. Again, these amino acids were put into solution at approximately equal concentrations to ensure that concentration itself did not favor one amino acid over the other.

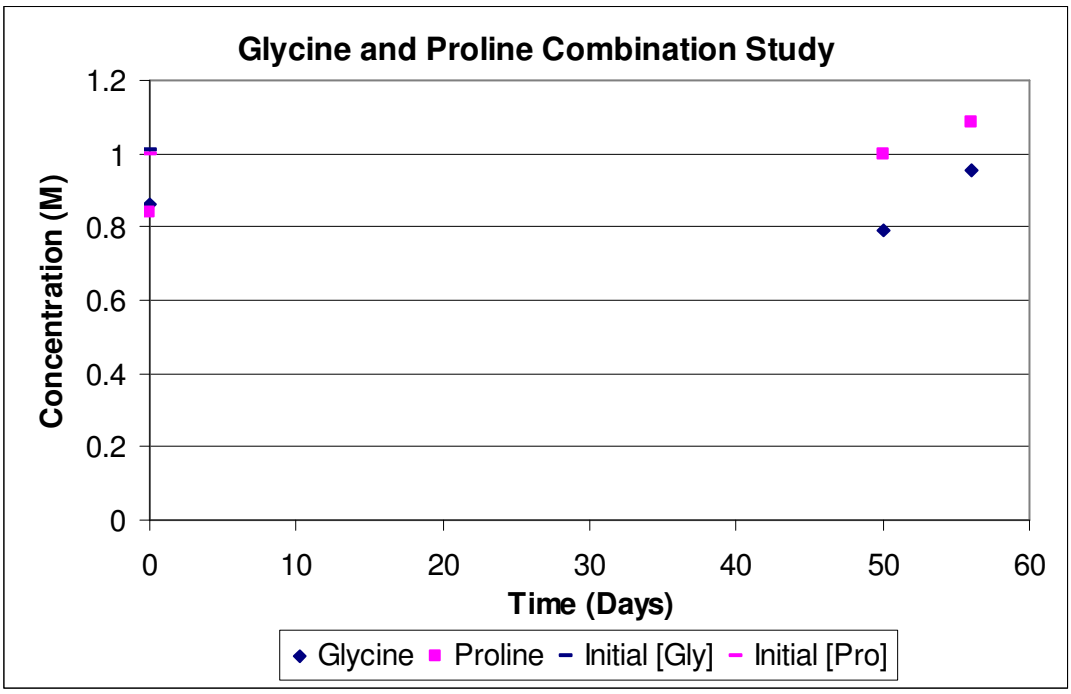

Figure 21: Glycine and Proline Combination Study

At day 0, the concentration for both amino acids reads a bit lower than the initially calculated value, but the concentration of each amino acid is close to the other. Later samples show the concentrations spreading apart, which could indicate that glycine is being incorporated into peptides and dimers and such preferentially over proline. However, this preliminary conclusion warrants further investigation with the Ion Trap Mass Spec. It is also interesting to note that the concentrations of glycine and proline seem to be increasing over time. This trend appears in several studies, however, and may

be a reflection of a sampling or measurement problem rather. Further investigation should shed light on this.

Ion Trap Mass Spectrometry Results for Amino Acid Studies

The IT-MS provides a qualitative look at the amino acid sample solutions. With this information, we can determine what products are being formed and what reactions are taking place. Eventually, a quantitative method could be found or developed for detection of products in the IT-MS, but for now, the instrument is being used for qualitative purposes only.

For the IT-MS analysis, I am looking for trends in the formation of products over time for each amino acid without distinguishing between new and old studies. Since this analysis is qualitative and not quantitative, I feel it is more acceptable to "combine" the data taken from the studies started in the spring of 2008 and those started in the fall. I do not have many spectra from the spring of 2008 (the ones I do have are pulled from Jordan Walk's thesis), and the studies started in the fall of 2008 are not very old yet, so by looking at all the spectra together, I can get a more full picture of what is being formed from day 0 nearly continually to however long the older study has been active. Of course, samples from the newer studies will need to keep being pulled to confirm that the new studies are reacting in the same way as the earlier ones.

The method for calculating the masses of species of interest for each solution was outlined in the methods chapter. A summary of these values for each amino acid is given at the beginning of the section for that amino acid below. The variety of m/z ratios that are present that do not correspond to our species of interest could represent fragments of

those compounds, impurities in the system, or residual wash fluid used to rinse out the IT-MS instrument between runs.

An example mass spectrum is shown here for each study. Additional spectra are located in the Appendix.

-Alanine

Amino Acid	m/z ratio				
	parent	**dipeptide**	**tripeptide**	**dimer**	**trimer**
Alanine	90	161	232	179	268

Table 5: Summary of Alanine Mass Spectrum Peaks of Interest

In the alanine spectra obtained from the IT-MS, I looked for the peaks listed above. Some were in great enough relative abundance to be labeled specifically on the spectrum. Other peaks were much smaller and were not specifically labeled. For these, I estimated, from the graph, which ones were there.

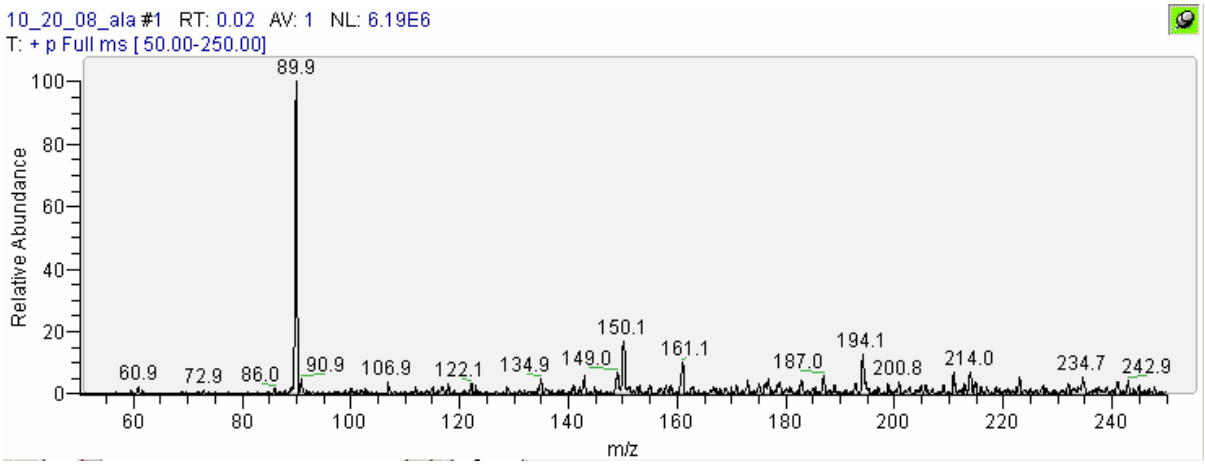

Figure 22: Alanine IT-MS Spectra, Day 222

The m/z ratios that are very close to the expected values (i.e., within 0.2 m/z) are assumed to be that expected peak. From this spectra and others, we found that there were labeled m/z peaks at approximately 90, 161, and 179, showing that both dimers and

dipeptides are being formed. Some spectra also showed small peaks around m/z 232, but since the intensity was not high enough to warrant a label, the actual m/z ratio of that peak cannot be determined yet. It is possible, however, that a small amount of tripeptide is being formed in the alanine solution.

-Glycine

Amino Acid	m/z ratio				
	parent	dipeptide	tripeptide	dimer	trimer
Glycine	76	133	189	151	226

Table 6: Summary of Glycine Mass Spectrum Peaks of Interest

The same analysis technique as described for alanine was used for glycine.

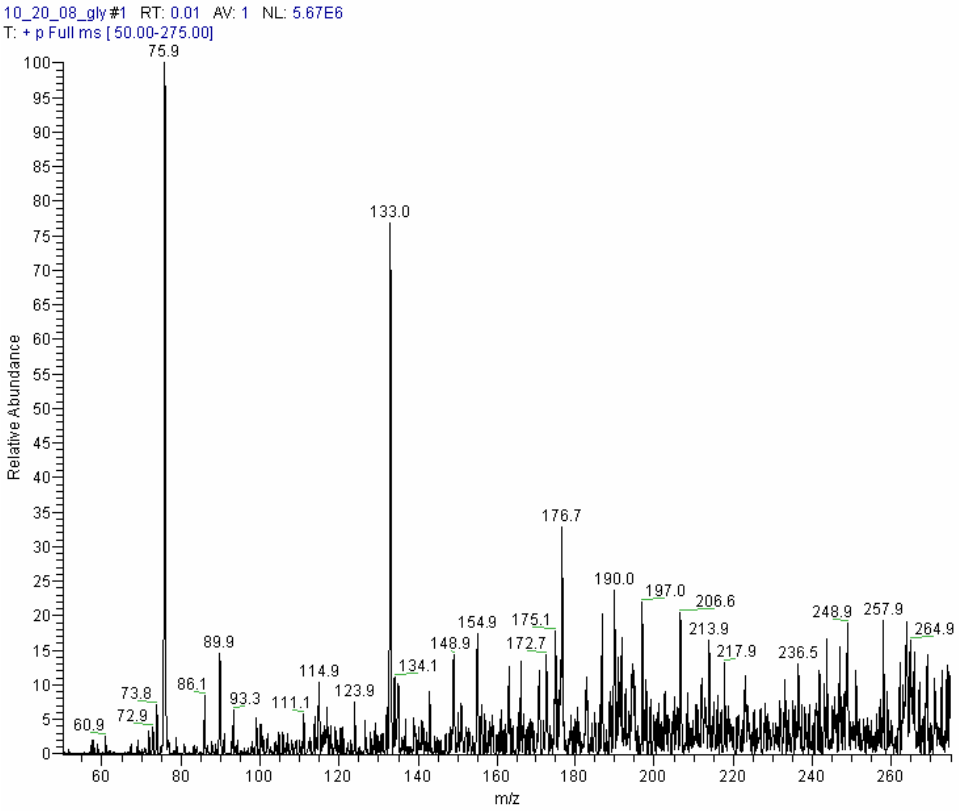

Figure 23: Glycine IT-MS Spectra, Day 216

From this spectrum and others, the glycine study has shown IT-MS peaks at approximately m/z 76, 133, and 151. These peaks correspond to glycine in monomer, dipeptide, and dimer form. Smaller unlabeled peaks have been detected around m/z 189

and 226, suggesting that both trimer and tripeptide are being formed in solution, but at very small concentrations.

-Proline

Amino Acid	m/z ratio				
	parent	**dipeptide**	**tripeptide**	**dimer**	**trimer**
Proline	116	213	311	231	346

Table 7: Summary of Proline Mass Spectrum Peaks of Interest

The same analysis technique as described for alanine was used for proline.

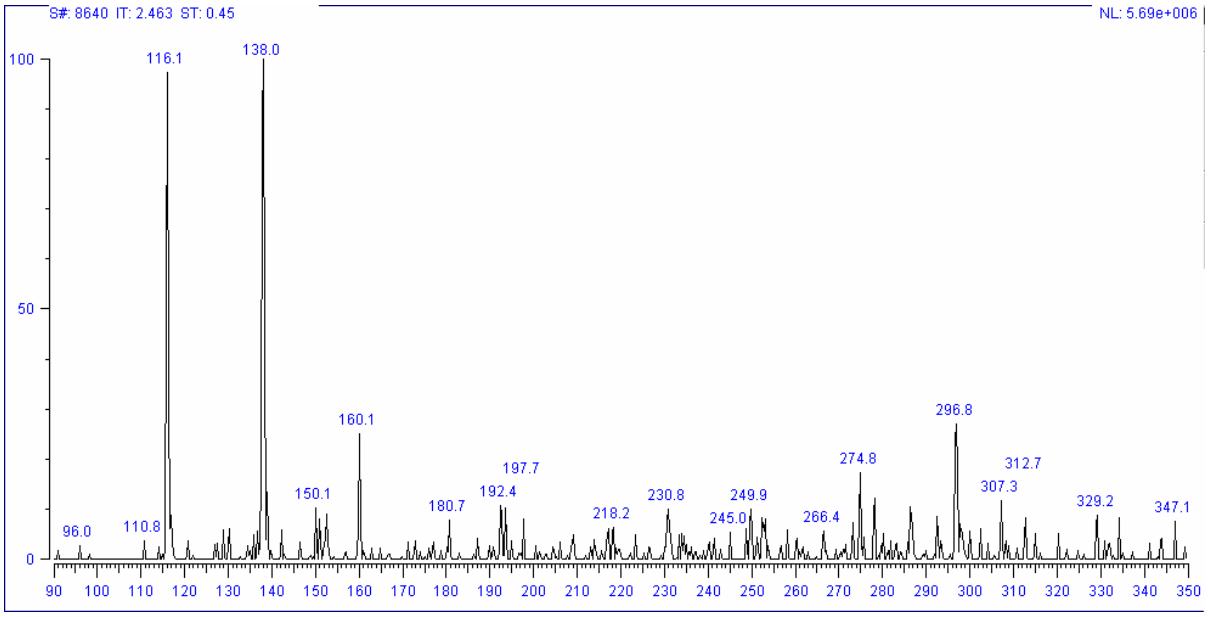

Figure 24: Proline IT-MS Spectra, Day 346

The various spectra collected for proline show labeled m/z peaks at 116, 213, 231, 311, which represent the presence of proline monomers, dipeptides, tripeptides, and dimers. A small unlabeled peak is present near m/z 346 in a couple spectra, suggesting the possible formation of minor amounts of proline trimer.

-Valine

Amino Acid	m/z ratio				
	parent	dipeptide	tripeptide	dimer	trimer
Valine	118	217	316	235	352

Table 8: Summary of Valine Mass Spectrum Peaks of Interest

The same analysis technique as described for alanine was used for valine.

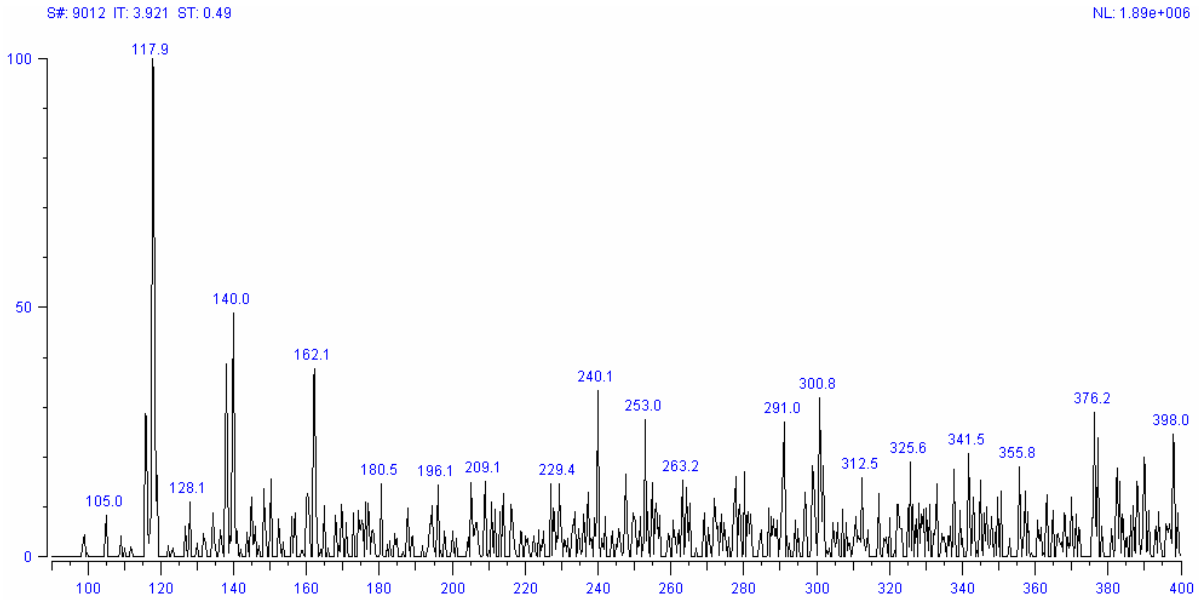

Figure 25: Valine IT-MS Spectra, Day 358

The valine spectra collected show m/z peaks at approximately 118 and 235, indicating that valine monomer and dimer are present in the sample solution. Smaller unlabeled peaks were present around m/z 217, which may represent the formation of a small amount of dipeptide in the valine solution. As with all of these solutions, samples should continue to be run through the IT-MS to monitor the presence of all of these peaks and track the formation of various products.

-Alanine and Valine Combination Study

Amino Acid	m/z ratio				
	parent	**dipeptide**	**tripeptide**	**dimer**	**trimer**
Alanine	90	161	232	179	268
Valine	118	217	316	235	352
Combinations					
A-V dipeptide	**A-V dimer**	**A-A-V tripeptide**	**A-V-V tripeptide**	**A-A-V trimer**	**A-V-V trimer**
189	207	260	288	296	324

Table 9: Summary of Alanine and Valine Combination Mass Spectrum Peaks of Interest

For the alanine and valine combination study, we looked for a wide variety of product and reactant peaks in the IT-MS spectra. We continue to look for the same peaks of interest from each individual amino acid study, but we are also looking for the peaks that represent new combination products that may form. All of these possible m/z peak ratios are listed in table 9 for the alanine and valine combination study. It should be mentioned that for the combination trimer and tripeptide m/z ratios, the three monomers could come together in any sequence order to produce the ratio given.

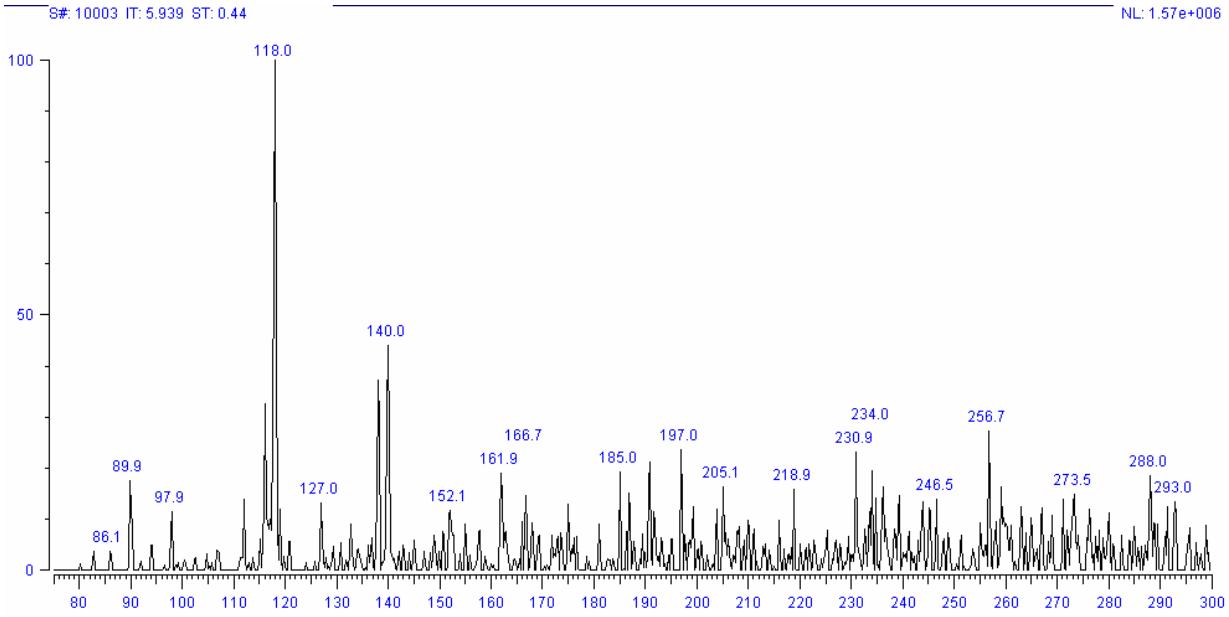

Figure 26: Alanine and Valine Combination IT-MS Spectra, Day 0

This initial spectrum shows monomer peaks for alanine and valine at approximately m/z 90 and 118, respectively. The peaks are not equal in intensity, even though the sample solution is known to have approximately equal concentrations of each of the two amino acids. This phenomena illustrates one difficulty with using IT-MS for quantitative purposes, but perhaps with further research this issue can be overcome.

It is also interesting to note that this spectrum shows a labeled peak at m/z 288, a ratio that corresponds to a two alanine, one valine tripeptide molecule. I find it theoretically highly unlikely that this compound could have formed so quickly upon creation of the solution, but it is possible. As with the single amino acid studies, samples from this solution will continue to be analyzed with IT-MS in order to track what peaks are present and what products are being formed.

Unfortunately, shortly after the day 0 readings were taken with the IT-MS, the instrument suffered some damage or technical difficulties and became inoperable for the purposes of this study. No further samples could be run. Once the instrument is fixed, more samples can be analyzed to continue the study.

-Glycine and Proline Combination Study

	m/z ratio				
Amino Acid	**parent**	**dipeptide**	**tripeptide**	**dimer**	**trimer**
Glycine	76	133	189	151	226
Proline	116	213	311	231	346
Combinations					
G-P dipeptide	**G-P dimer**	**G-G-P tripeptide**	**G-P-P tripeptide**	**G-G-P trimer**	**G-P-P trimer**
173	191	230	270	266	306

Table 10: Summary of Glycine and Proline Combination Mass Spectrum Peaks of Interest

The same process used to determine what peaks to look for in the alanine and valine combination study was used to identify the peaks of interest for this glycine and proline combination study. These single amino acid and combination m/z peaks are listed in Table 10 above.

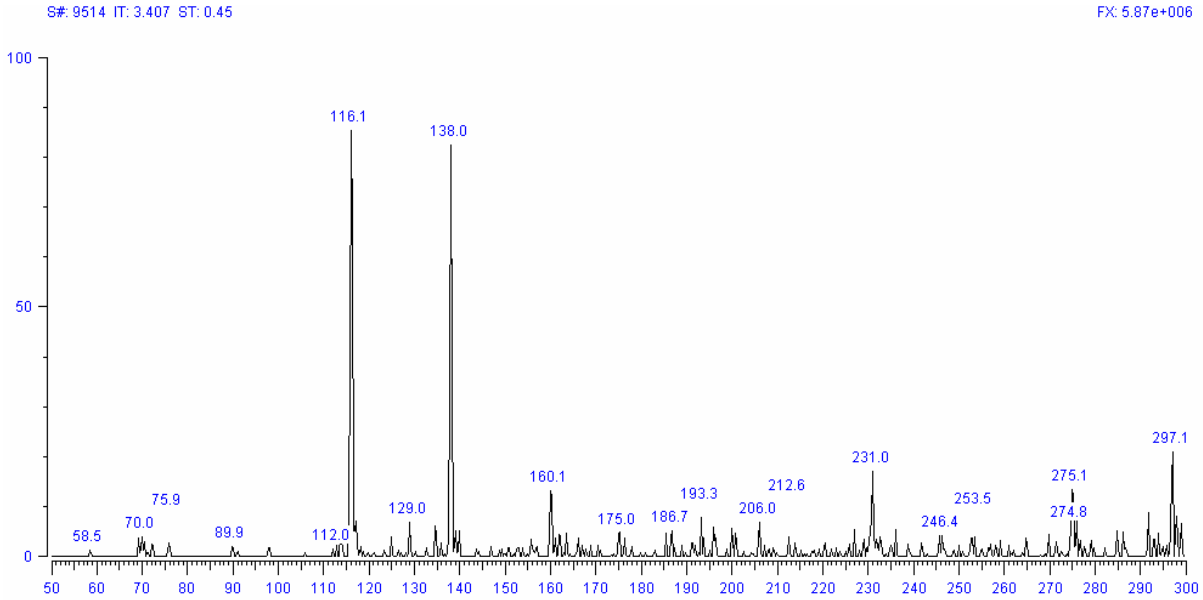

Figure 27: Glycine and Proline Combination IT-MS Spectra, Day 0

Monomer peaks for glycine and proline are present at approximate m/z ratios of 76 and 116. As with the other combination study, the peaks are not equal, even though the concentration of each amino acid in solution on day 0 is known to be approximately equal. A peak is present at m/z 231, which indicates the presence of proline dimer. There is a peak present at m/z 212.6, which may be representative of a proline dipeptide peak (m/z approximately 213), but this peak will need to be monitored over time to confirm or deny this identity. Again, due to our inability to use the IT-MS, no further samples could be analyzed this semester.

Chapter 4: Summary and Conclusions

Overall, this study proves that amino acid monomers in aqueous solution at 120°C will spontaneously form amide bonds. The rate of bond formation and the favorability of those peptides are still to be determined.

Based on quantitative analysis with the GCMS, it appears that even after several hundred days, only a very small percentage of monomer amino acid is reacting to form peptides or by-products in the single amino acid solutions. We considered the possibility that the peptide products that had formed were being derivatized along with the monomers and were eluting from the GCMS at the same time as the monomers. This would make the concentration seem constant even if products were being formed. To test this theory, we made up an aqueous solution of glycine dipeptide (Gly-Gly), derivatized it as we would any other sample, and analyzed it via GCMS. The resulting spectra showed only the methyl laurate internal standard peak at 13.6 minutes. No other peaks were present after 15 minutes, and there were certainly no peaks anywhere around where glycine is known to elute (5-6 minute range). This proves that our quantitative GCMS method is indeed measuring only the monomers in solution and not the peptide products.

Qualitative data from IT-MS analysis confirms that the monomers that have reacted are forming small amounts of a variety of dimers, trimers, dipeptides, and tripeptides. Alanine has formed dimers, dipeptides, and possibly tripeptides. Glycine spectra have indicated that dimers, dipeptides, and probably trimers and tripeptides have formed. According to IT-MS analysis of proline, monomers have formed dimers, dipeptides and tripeptides, and perhaps also trimers. Analysis of valine solutions shows formation of dimers and possibly dipeptides as well.

The fact that not much of the initial amino acid concentration is actually forming amide bonds and becoming peptides or proteinoids could be explained by several scenarios. It is possible that the rate for this type of reaction under these conditions is simply very slow. If this is the case, the concentration of monomer should slowly decrease over time. If a quantitative method for determining peptide presence is found, then the concentration of peptide in solution would rise as time goes on. Perhaps continued sampling of these established solutions over the next several years will reveal if this explanation is true. Another possibility is that the reaction equilibrium strongly favors the monomer amino acid reactants over any of the possible products. Future sampling would show little to no change in the monomer concentration and a comparable amount of peptide formation if this scenario is true. Only time and further testing will tell.

As with the single amino acid solutions, the two combination studies seem to have a fairly constant or stable concentration of monomers according to qualitative GCMS analysis. It is possible that in time, these solutions will show a greater, faster decline in monomer concentration, but further sampling must be done. Qualitative IT-MS analysis of the alanine-valine and glycine-proline solutions is still inconclusive. Only one data point could be obtained for each study before this thesis was written. However, several additional samples have been pulled and are being stored for analysis at a later date.

It should be noted that the IT-MS spectra obtained for this project are not "clean." Many peaks are present that do not correspond to the predicted m/z ratios for monomers or potential products that we looked for. It is possible that some of these peaks represent products or by-products that could be investigated, but it is far more likely that they are compounds that have lingered on the column from earlier runs. Any solution injected into

the column could leave residues behind, and, since many groups use the IT-MS for analysis of a wide variety of solutions and compounds, we may never know what some of the peaks are or which particular study that compound came from. For investigations that analyze solutions at higher concentrations that ours, the particular peaks they are looking for may "drown out" the signal from these minor impurities. With the low concentrations of our solutions, and especially our products, our spectra may never be that clear. What we can do, however, is figure out what m/z ratios we are expecting for this particular study, which we have done. Since we are using the IT-MS for qualitative analysis only, our major concern is whether or not our expected peaks are present.

In my opinion, each of the solutions from this study should be kept for future analysis, and samples should continue to be taken in order to look at these reactions over an extended period of time.

Chapter 5: Recommendations for Future Work

Other than continue sampling and monitoring the existing solutions, there are several things that can be done in the future to expand or improve this project. One major thing would be to find or develop a method for quantitatively measuring the concentration of peptide products. The method could potentially involve IT-MS, or the new HPLC instrument which should, according to some professors, be joined with a mass spectrometer sometime next year.

If the combination studies prove to be worth further analysis, it would be easy to expand them to include new amino acids and different combinations. Perhaps, if one particular amino acid is found to be particularly favored or reactive, it could be paired with a series of other acids to see if the favorability is universal, or just in comparison to the other amino acid it was paired with. It would also be interesting to put many different amino acids in the same flask to see what products are formed. Sidney Fox describes the successful creation of a liner peptide that contains 18 different amino acids in his 1960 article, "How Did Life Begin?".[32] In that particular experiment, 16 different amino acids were combined with an excess of glutamic acid and aspartic acid, the only two dicarboxylic amino acids, and exposed to dry heat until they formed a white polymer.[33] We could attempt the same type of combination, but, instead of dry heat, use our current environment of aqueous solution at 120°C.

It has been suggested by several researchers that the addition of acid may catalyze peptide formation.[34, 35] Some studies have used excesses of particularly acidic dicarboxylic acids, and others have added phosphoric acid to the reaction solutions. I have been unable to locate or access articles that specifically outline the procedure for

phosphoric acid aided peptide formation, but it seems as though a dry heat method was used. Because I have been unable to locate the original studies, I am unsure as to whether or not this method was tried for aqueous solutions, but I believe it warrants investigation. We could experiment by adding various amounts of phosphoric acid to the aqueous amino acid solutions to see if the rate of formation of peptides is increased.

Scientists have long known that clay surfaces can serve as catalysts for a "variety of reactions, including the polymerization of activated amino acids to polypeptides."[36] In 1951, J.D. Bernal postulated that the sea's low concentration of organic material may have adsorbed "on the clay mineral particles suspended in the early oceans, and that the subsequent prebiotic reactions elaborating the organic substrates were catalyzed at active sites on the clay mineral surfaces or located within the layered structures".[37] In future years, clay particles of some sort could be added to the pressure tubes to provide a potentially catalytic surface that may facilitate peptide formation.

In his book Energy and the Evolution of Life, Ronald Fox proposed that some proteinoids may catalyze the creation of more amino acid polymers.[38] According to Fox, the earliest amino acid polypeptides may have been formed through the dry heating of monomers trapped in indentations on the lava crust of the early earth.[39] When these compounds were washed back into the ocean, they may have served as catalysts for the formation of more of the same compounds. We could apply this concept to our study by adding some pre-made amino acid peptide chains to the aqueous solutions along with an excess of monomers to see if more peptides are formed faster than starting with monomers alone. These pre-made peptides could be bought from Sigma-Aldrich or some other chemical company, or, we could follow in the footsteps of earlier studies and create

our own through a dry heat process, then dissolve the resulting polymer in our sample solutions.

It should be noted that the reaction conditions we are currently using will need to be changed if pre-made proteinoids are used as catalysts. Several previous studies have shown that "catalytically active proteinoids are inactivated by heat at 100° for 20 minutes in aqueous buffer solution at pH 6.8."[40] According to this information, our method of heating the solutions at 120° C for extended periods of time would render the catalysts ineffective. To address this issue, I would suggest placing the solutions in the 70° C oven in Dr. Kranbuehl's laboratory. This lower temperature would be better suited for this potential method.

Acknowledgements and Thanks

I would like to thank my honors committee members (Dr. Christopher Abelt, Dr. Carey Bagdassarian, Dr. David Kranbuehl, and Professor Kim Whitley) for agreeing to read this paper and participate in my defense. I am sure that each of you had far more interesting things to do with your time, and I appreciate your willingness to set aside some of that valuable time for me. Thank you!

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Appendix: Additional Ion Trap Spectra

Alanine

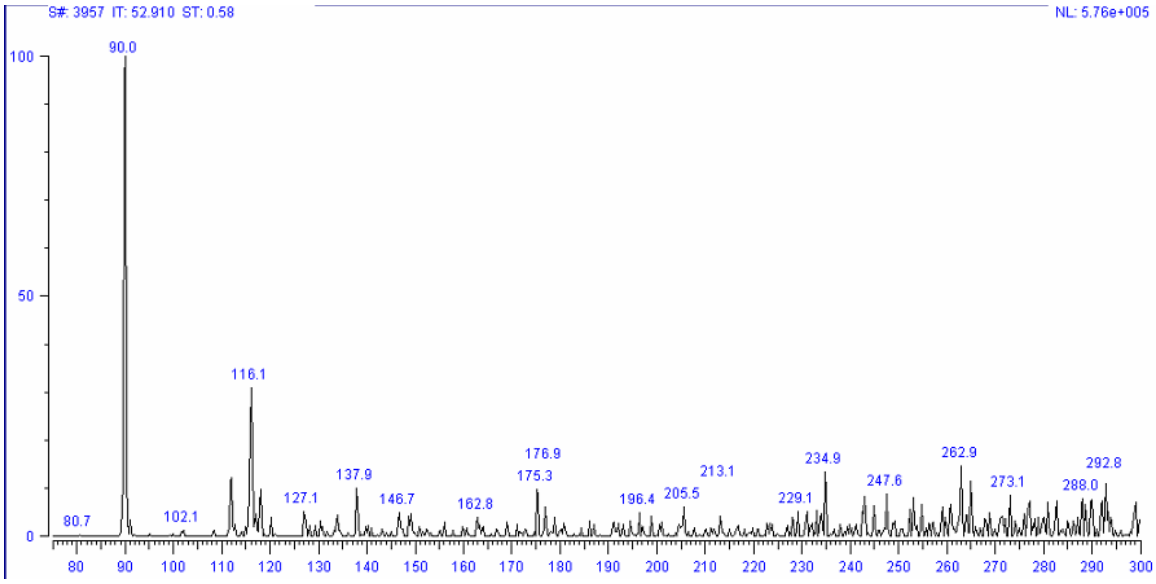

Figure 28: Alanine IT-MS Spectra, Day 0

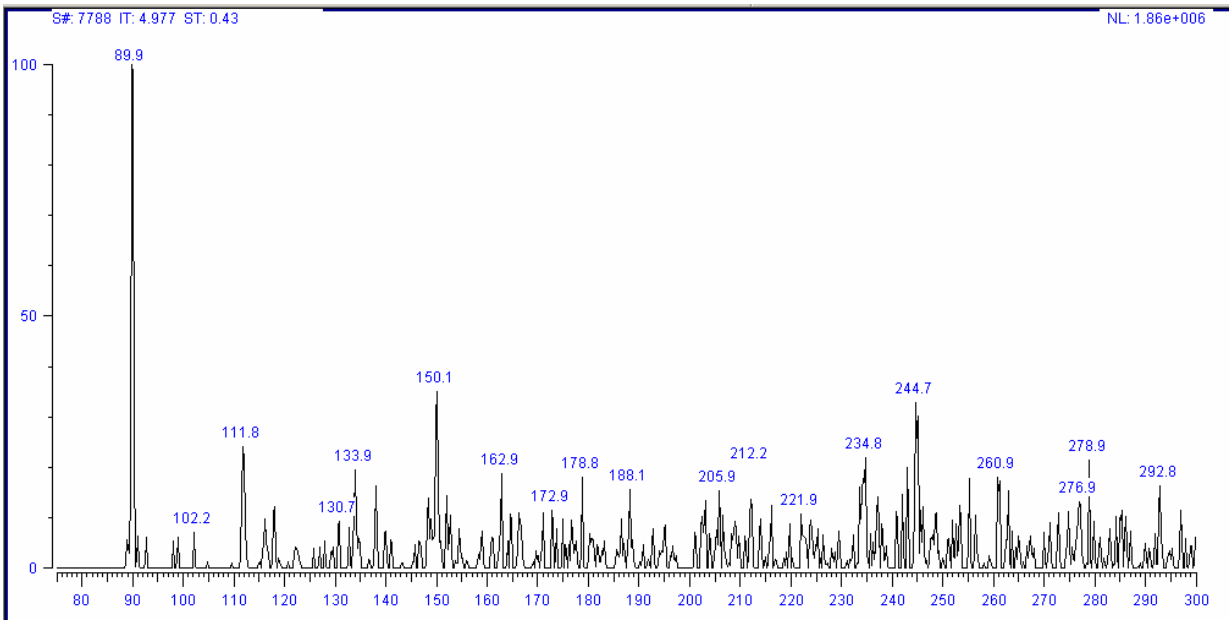

Figure 29: Alanine IT-MS Spectra, Day 329

Glycine

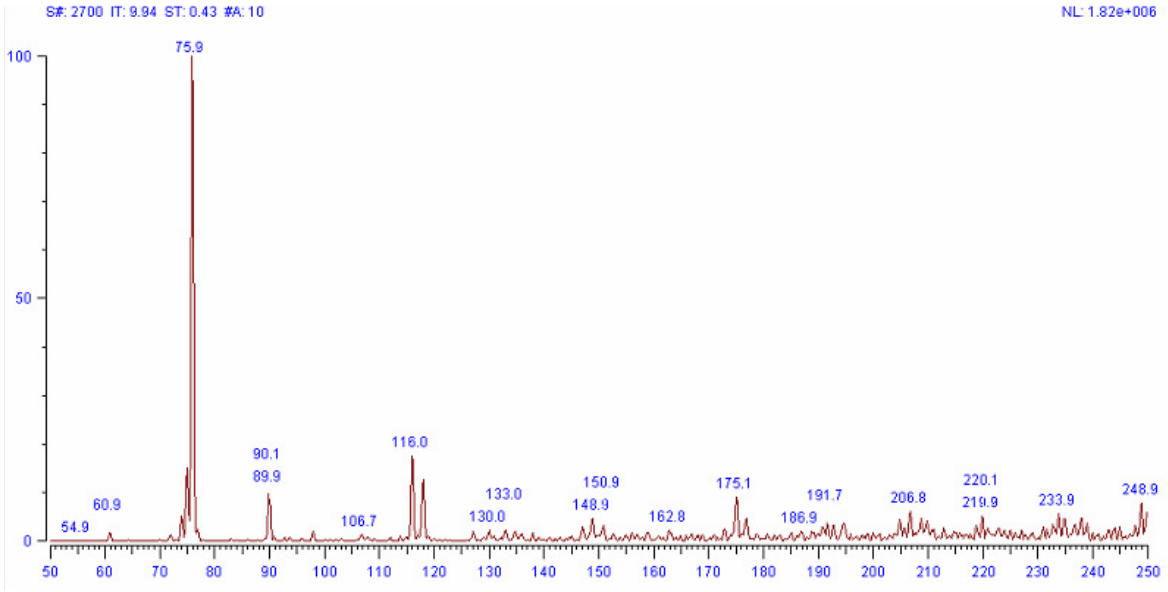

Figure 30: Glycine IT-MS Spectra, Day 0

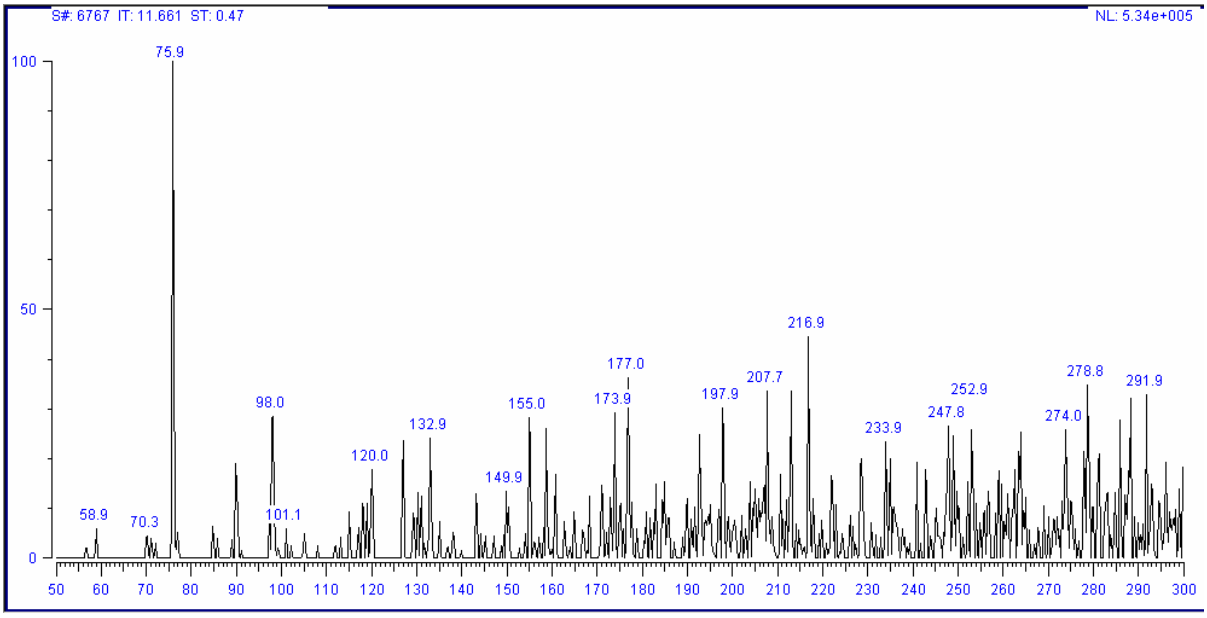

Figure 31: Glycine IT-MS Spectra, Day 83

Proline

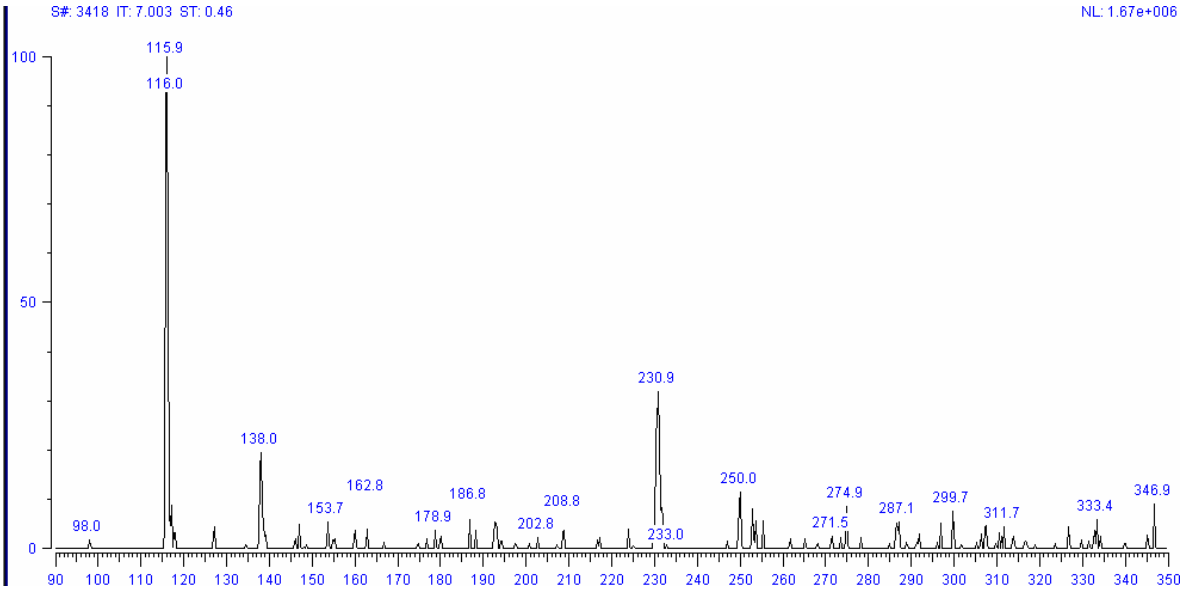

Figure 32: Proline IT-MS Spectra, Day 0

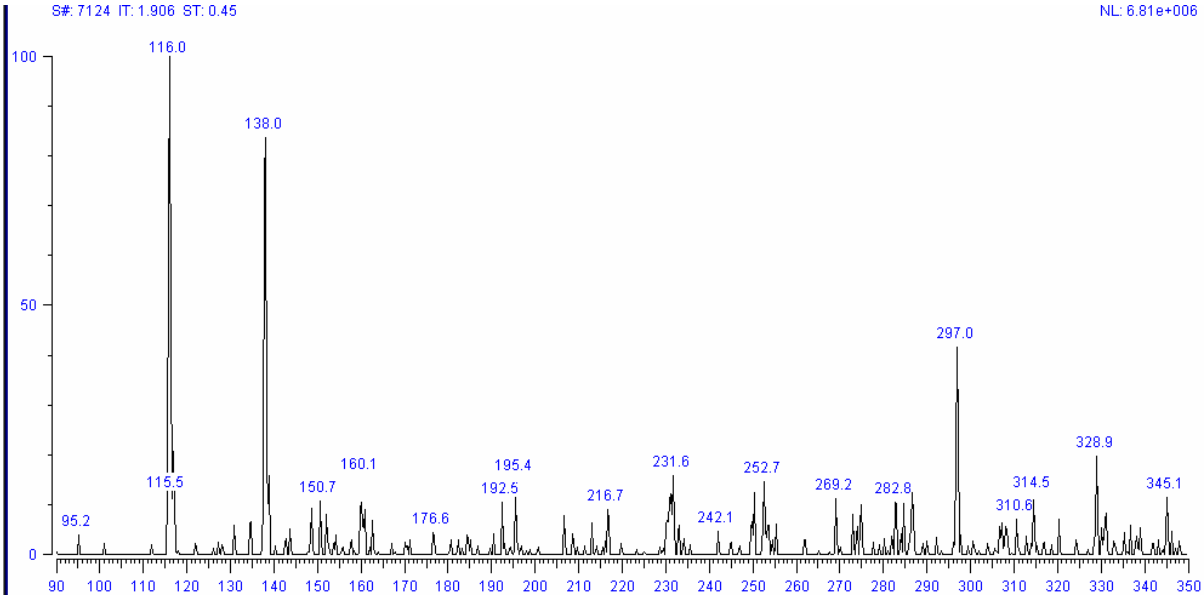

Figure 33: Proline IT-MS Spectra, Day 83

Valine

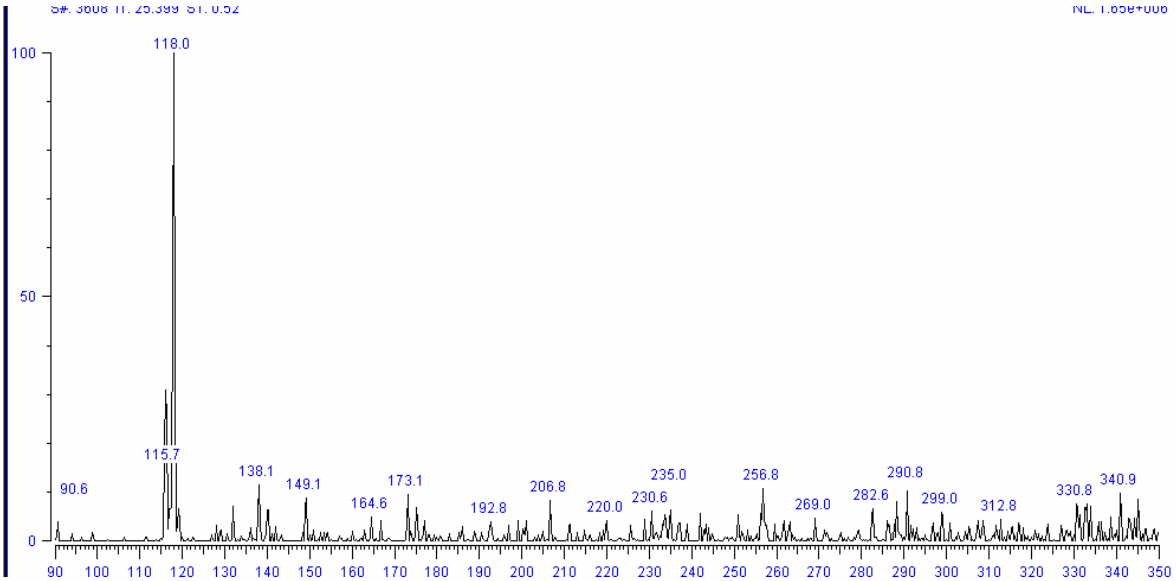

Figure 34: Valine IT-MS Spectra, Day 0

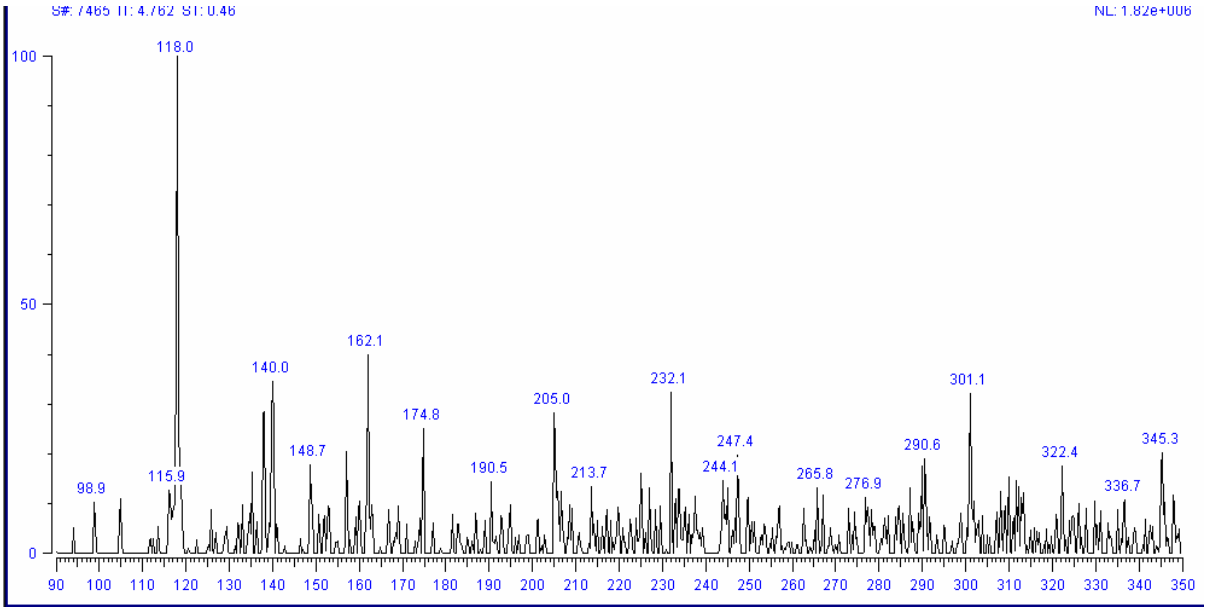

Figure 35: Valine IT-MS Spectra, Day 83

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