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RELATIVE REACTIVITY OF SIX AMINO ACIDS WITH SODIUM HYPOCHLORITE MEASURED BY GAS CHROMATOGRAPHY/ MASS SPECTROMETRY AND METHYL CHLOROFORMATE DERIVATIZATION.

Josephine Wangui Gitau

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Thesis

Presented to the

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California State University,

San Bernardino

In Partial Fulfillment

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in

Environmental Sciences

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Josephine W Gitau

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Approved by:

Brett Stanley, Committee Chair, Chemistry

Andreas Beyersdorf, Committee Member

James Noblet, Committee Member

ABSTRACT

There are 20 common amino acids that act as building blocks for proteins and each one of them differ from each other due to their chemical structure of the R group. During the process of chlorination of water and wastewaters, amino acids present have been implicated as precursors of disinfection by-products. For the analysis of amino acids Gas Chromatography/Mass Spectrometry (GC/MS) is widely used. In this research six amino acids (glycine, valine, leucine, isoleucine, methionine, and phenylalanine) were derivatized with Methyl Chloroformate and analyzed using Gas Chromatography/Mass Spectrometry without heating. The main aim of this study was to observe the reaction of the six combined amino acids when sodium hypochlorite is added at different molar ratios of 0.5, 1.0, 2.0 and 3.0. Using Gas Chromatography/Mass Spectrometry analysis peak retention times and area was observed on the GC/MS instrument. While identifying their retention time we were able to observe whether their peak areas increase or decrease for each amino acid as compared to the previous research done. From the resulting peaks, we were able to determine the decrease of amino acids concentration when reacting with different amount of sodium hypochlorite, a common disinfectant reagent. There was no observation made of chloroamino acids because they do not undergo analogous derivatization with methyl chloroformate or are thermally decomposed in the Gas Chromatography. There comparison was made on chlorinated reaction time

set by stopping the reaction with dechlorinated agent sodium bisulfite ($NaHSO_3$) and the reaction was allowed to proceed without quenching with sodium bisulfite. The derivatization method used in this study was considered to not be the general method of measuring amino acids and disinfection by-products (DBPs) since only one disinfection by-product was observed.

ACKNOWLEDGEMENTS

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CHAPTER ONE

INTRODUCTION

Background

Water treatment is used to improve water quality for the end user. This process involves 5 steps of water treatment namely coagulation, flocculation, sedimentation, filtration, and disinfection. In water treatment the water comes from waterbodies such as rivers, lakes, or groundwater.

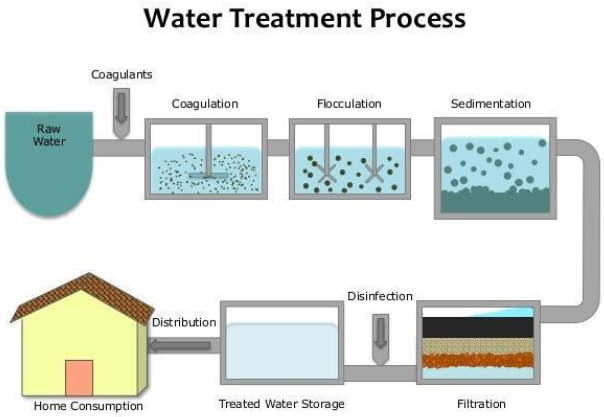


Figure 1. Water Treatment Process. (Dream civil, 2020)

Wastewater treatment on the other hand is defined as a process of improving the quality of wastewater, through removal of impurities before the water is discharged back into the environment. This wastewater comes from used water that goes through sewage system. The three steps that are involved in wastewater treatment include: primary treatment, secondary treatment, and tertiary treatment.

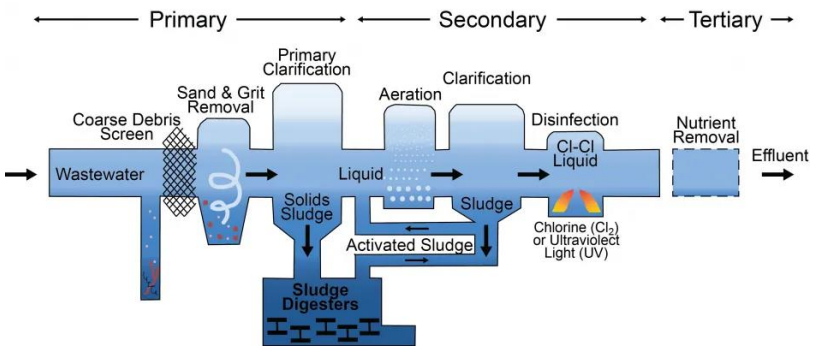


Figure 2. Wastewater treatment process. (CSS, 2021)

In natural water supplies such as groundwater and surface water, low levels of amino acids can be found influencing the wastewater treatment plants. When chlorination is done on these wastewater treatment plants, the amino acids are converted to N-chloro derivatives which are chloroamino acids.

One of the main products that has been traced in wastewater is cyanide or cyanogen chloride while amino acids are rarely monitored and hence may form disinfection by-products (DPBs). Further reaction of the DPBs with chlorine can lead to the formation of cyanogen chloride (CNCl) with glycine as a precursor. (Anita P, Connie Y, 2006).

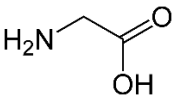
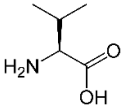
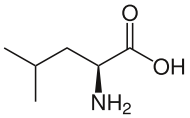
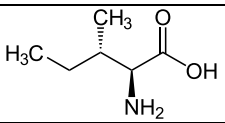
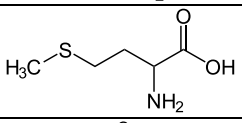
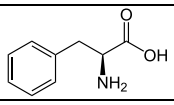
Due to the biogenic amines as precursor formed from amino acids, when they decompose, they are harmful to humans as well as wildlife. (Chongzheng N, Teresa M.O, 2007). Chloramine decomposition seen on GC/MS during identification may be due to it not withstanding the harsh temperature during GC analysis. (Ehud C, Donald J et al,1987).

Some of the previous research studies conducted on Methyl

Chloroformate (MCF, CH_3OCOCl) by multiple reaction monitoring (MRM) derivative profiling, indicate that most amino acids have a retention time ranging from 6-35 min (Silas G.V., Daniel G.D. et al, 2003). Although this range may vary due to how the analyte travels and degrades within the column. Also, the solubility of the sample to be analyzed may affect the retention time. The higher the solubility of the amino acid, the greater retention time.

The aim of these research is to observe how the six amino acids: (glycine, valine, leucine, isoleucine, methionine, and phenylalanine Table 1) act as precursors to chloroamino acids during Gas Chromatography/Mass Spectroscopy (GC/MS) methyl chloroformate derivatization process. At the same time, we will determine the reaction and role played when chlorine is added into the amino acids leading to the formation of aldehydes, nitrile, and N-chloramines.

Table 1. Six Amino Acid Structure, Chemical Formular and Class

Amino Acid	Amino Structure	Chemical formular	class
Glycine		$C_2H_5NO_2$	small
Valine		$C_5H_{11}NO_2$	Non-polar
Leucine		$C_6H_{13}NO_2$	Non- polar
Isoleucine		$C_6H_{13}NO_2$	Non-polar
Methionine		$C_5H_{11}NO_2S$	Non-polar
Phenylalanine		$C_9H_{11}NO_2$	Aromatic

From sample preparation, concentration, (GC/MS) methyl chloroformate derivatization will be used since it is the most suitable due to its identification of different metabolite ata given time as well as detection of changes in quantity that can be observed during analysis. Also, GC analysis with two mass analyzer stages (known as tandem mass spectrometry or GC/MS/MS) will be used since it reduces the involvement of human handling during the derivatization and injection process where reagent is added to the sample. From other research, GC/MS MS1 scan and MRM is mainly used since it is easier to detect and analyze the amino acids analyte while observing the retention time and transition for the analyte internal standard. In the MS1 Scan

mode only the first quadrupole is used to scan a mass spectrum and the collision cell and 2nd MS are idle (i.e., GC/MS). In this research, we will apply the same procedure using GC/MS MS1 scan and MRM procedure for methyl chloroformate derivatives to analyze 6 amino acids. A Triple quadrupole MS instrument in MRM mode lowers the detection limit. In this case, the 1st quadrupole selects a single precursor. In the 2nd quadrupole, nitrogen gas is introduced to collide with ions to produce fragmented product ions, while in the 3rd quadrupole single product ions are selected for detection. Multiple reactions involving different precursor-to-product transitions may be monitored in a single run.

Derivatization can be said to be a procedure that converts compounds of similar chemical structure into products by changing the analytes properties. For amino acids derivatization is the process whereby the active H_2 in OH and NH_2 are replaced with other functional groups. During the ionization process, that occurs between the gas chromatograph and mass analyzer, the energy of the process causes the molecules to fragment into smaller ones. Using chemical ionization (CI), the original molecular ions can be observed. GC/MS derivatization process can also use an internal standard that can be used in the analysis of each analyte.

Type of Derivatization.

The three common derivatization methods include acylation, silylation and alkylation. Silylation is one of the common methods of derivatization used

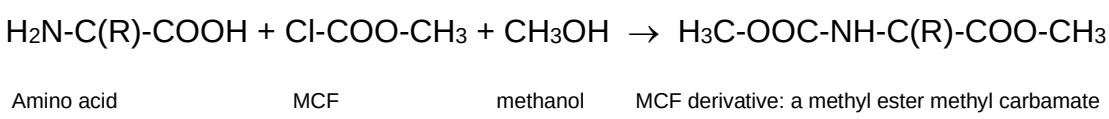
to analyze compounds with hydrogen and amino acids through GC/MS. Its main shortcoming is instability due to its reaction to moisture. (Stina K.L, Hans F.N.Ket al. 2012). Acylation derivatization can be used on polar functional groups such as carbohydrates as well as amino acids. (Restek et al, 2010). Another factor to consider when choosing a method of derivatization is the procedure involved in the preparation and analysis of the sample. The other form of derivatization involves alkyl chloroformate. One of the factors why this procedure is used is that the amino acids can be derivatized directly in aqueous solution without protein removal. The protein can react fast and the derivative derivatized be extracted without organic solvent. (Kaspar H, Dettmer K et al, 2009). Another factor that is considered is that on the sample preparation using methyl chloroformate (MCF) derivatization, the process does not involve heating. Methyl chloroformate derivatization (MCF) is also known to have great susceptibility on amino acids and its formation on anhydrides. (Hans F.N.K, Trygve A et al, 2011). In this research, methyl chloroformate is chosen as a method of derivatization due to reduction in cost of reagent whereby many of them are found within the school laboratory. The other reason for using methyl chloroformate derivatization is due to easier separation of the derivatized products. Although some methods such as pyrolytic decomposition can be used instead of derivatization the nature of the analyte is considered as well while considering the type of method to use. In addition, there would be no change in state of the metabolite analyte under

study during the methyl chloroformate GC/MS derivatization process. (Hans F.N.K, Trygve A et al, 2011). In most cases, derivatization is used due to easier identification during the separation process of different samples or analytes. Another main reason why derivatization of the amino acids is used is to make them nonpolar, so that they can be extracted into an organic solvent (chloroform). The peaks are easily separated from the chloroform solvent peak and the GC chromatogram looks better. Derivatization procedure may be done by two processes pre-column derivatization and post-column derivatization. Pre-column derivatization is used before the separation process while the post-column derivatization is used afterseparation. (Peter K, 2018).

Amino Acid's Reaction with MCF and Other reagents

From the previous research it has been observed that MCF reacts with amino acids forming intermediate R groups of chloroformate before the stable products N (O, S) – methyl methoxycarbonyl esters of amino acids. An alkyl ester that has the R" chain from the chloroformate from the decarboxylation of mixed anhydride induced by pyridine. (Wen P.C, Xiao Y.Y et al, 2010). It is noted that MCF molecules are very reactive and decompose in the presence of water to produce hydrochloric acid (HCl) and ethanol. Addition of pyridine under alkaline conditions causes a nucleophilic attack to occur and chlorine leaves. Chlorine is replaced by oxygen or nitrogen that acts as a base. In the presence of carboxylic acids, the electron pair of methanol oxygen release methyl carbonate molecules leading to the formation of an ester. The amino

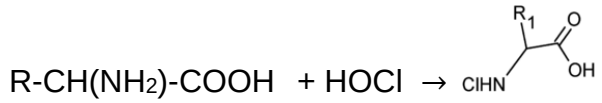
group and methyl carbonate remain connected to nitrogen hence losing its proton to form a carbamate group. (Smart K, Aggio R et al, 2010)



According to previous research done GC/MS methyl chloroformate derivatization can be used on a large scale for industrial metabolite analyte profiling of amino acids. (Silas G.V.B, Daniel G.D et al, 2003). In some cases, during methyl chloroformate derivatization, arginine which is known to be one of the most alkaline amino acids, cannot be easily detected hence GC-MS methyl derivatization can be considered not suitable for its analysis. It has also been observed that for some amino acids such as alanine and glycine, there is low peaks separation, and they cannot be easily identified due to their structural similarity. Amino acids in plants can be easily quantified using GC/MS except histidine that can be barely observed. (Bram V, Kim C, et al 2016).

Amino Acid's Reaction with Chlorine

Hypochlorous acid (HOCl) is used as a disinfectant in water treatment and in presence of amino acids and amine, can lead to formation of N-chloro compounds which are compounds with a N-Cl bond. (Szabo M, Biro V et al, 2020).



Amino acid N-chloroamino acid

Amino acids react differently in the presence of chlorine depending on the chlorine: amino ratio and the structure of the amino acid. Methionine can be reacted completely in the presence of chlorine due to its thiol functional group. Conversely both glycine and proline are observed to be less reactive in the presence of chlorine due to their structure. (Chongzheng N,Teresa M.O, 2007)

Some amino acids like methionine and cysteine are more reactive with chlorine due to the presence of thiol group. When more chlorine acid is added to tyrosine, it can lead to the formation of a phenol ring. The reactive phenol ring side chain does not support the formation of N,N-dichloramine hence producing a different degradation product. Lysine and tyrosine can be said not to lead to the formation of N,N dichloramine due to them having reactive side chain. (How T.Z, Kathryn L.L et al 2016).

Chlorination of valine is noted to be faster and lead to formation of N-monochlorovaline whereby some of its N-monochlorovaline degrades leading to formation of N- chloroisobutyraldimine. N-chloramino acids are formed at a stable condition. It is noted that when amino acids react with chlorine traces of aldehydes are rarely observed in drinking water due to aldehydes being below detection limit of 2μg L^{-1}. Some research has shown that some organic

chloramine formed from amino acids such as phenylalanine and valine result in degraded organic chloramine by-product that retains their structural component of original amino acids precursor. (How T.Z, Kathryn L.L et al, 2016).

Amino Acids Presence in Chlorinated Water

When treated water is chlorinated, some of the amino acids are shown to react leading to formation of odorous aldehyde and N-chloraldimines making it hard forthem to be detected since they are below detection limit. It is worth noting that for N-chloroamino acids to be stable, they are dependent on hydrogen attached to the nitrogen. During the chlorination process some of the chlorinated amino acids that degrade form N-chloramines, nitriles, and aldehydes. (How T.Z, 2016).

Organic chloramines are compounds that have at least one chlorine atom bonded to an amine group. Although little information is known about them, organic chloramines are harmful towards human health and occur as intermediates for other by-product disinfectants. N-chloramino acids are formed from the reaction of amino acids and chlorine and mainly exist in water as free amino or combined amino acids. N-chloroamino acids formed from free amino acids are found in low concentrations in water bodies hence little information is known about their occurrence. (How.T.Z, Ina K et al, 2017)

Some research carried out has shown traces of cyanide by-products in wastewater that contain amino acids. The cyanide by-product formation

occurs when a molar equivalent of sodium hypochlorite is added to wastewater that contains amino acids. High traces of cyanides are observed when the amino acids samples are not preserved. (Luz A.C, 2021)

In water treatment plants, chlorine reacting with amino acids forming chloroamino tends to increase the levels of chlorine hence reactive amino such as methionine that is more reactive cannot be detected in water easily. Monochloramine formed from amino acids in water are analyzed in terms of their precursor reactivity and stability where some amino acids are more stable than the others. With this, N- chlorogylcine is observed to cause high health risks among the amino acids due to its stability. (How T.Z, Kathryn L.L et al, 2016)

Significance of the Research.

The main purpose of this research was to determine how chlorinated by-products of amino acids can be determined alongside unreacted amino acids during the GC/MS methyl derivatization process. By varying the amount of chlorine added to a given amount of amino acid, the observed reduction of the amino acid concentration could be correlated to the amount of chlorination products to determine the order of reactions that occur. Previous research typically looked for the chlorination products without observing the amino acid reactants in the same experiment. This research also investigates the reactivity of a mixture of amino acids instead of single amino acids. The relative reactivity of each could be determined. The reason

the specific six amino acids were chosen is as follows. Except for glycine, the simplest and perhaps most highly studied amino acid, the other amino acids (leucine, isoleucine, methionine, phenylalanine, and valine) are relatively nonpolar and thus are better extracted into the chloroform extract of the derivatization process, thus increasing the sensitivity of their detection. As discussed above, methionine is predicted to react very quickly, Leucine, isoleucine, and valine should form chloroamino acids due to the lower reactivity of the R side chain group. Phenylalanine is predicted to be more reactive but not as reactive as methionine. It is unknown whether mono- or di-chloroamino acids would undergo derivatization or be stable enough to be observed by GCMS with this method.

CHAPTER TWO

METHODOLOGY AND MATERIALS

Gas Chromatography/Mass Spectrometry and Methyl Chloroformate Derivatization.

There were several stages that were involved before the amino acids analysis using the Gas Chromatography-Mass Spectrometry instrument. These involved reagent preparation, amino acids samples preparation, chlorination process and derivatization.

Reagent Preparation

The reagents were chosen from previous published research utilizing simultaneous analysis of amino and non-amino organic acids as methyl chloroformate derivatives using gas chromatography-mass spectrometry.(Silas G.V, Daniel G.D et al, 2003)

Reagents included 2%NaOH, methanol, chloroform, methyl chloroformate, pyridine, 0.05M sodium bicarbonate and anhydrous sodium sulfate. Methanol, chloroform, MCF, pyridine and anhydrous sodium sulfate were reagent-grade and used as purchased.

2% NaOH preparation. 2g of NaOH solid was weighed and dissolved in 100ml volumetric flask using deionized water to make a 2% solution of NaOH.

Buffer preparation. 8.165g potassium dihydrogen phosphate (KH_2PO_4) solid was weighed and dissolved in a 1000ml beaker and deionized water was added to the mark to make a solution 60*mM* buffer. Using a dropper several

droplets of 2% of NaOH were added while at the same time monitoring the pH adjusting it to 7.0.

0.05M sodium bicarbonate preparation. 0.4224g of sodium bicarbonate($NaHCO_3$) solid was weighed and dissolved in 100ml volumetric flask using deionized water to make a 0.05*M* sodium bicarbonate solution.

Sample Preparation and Storage.

The six amino acids to be used were provided as reagent-grade in solid. Several different preparations and concentration of amino acids were measured.

Amino acids preparation. 0.0757g of glycine was weighed and dissolved in 100ml volumetric flask using the 60mM buffer that had a pH of 7.0. Repeating the same process on the other amino acids, 0.1174g of valine, 0.1312g of leucine, 0.1313g of isoleucine, 0.1467g of methionine, and 0.1688g of phenylalanine were each weighed and dissolved in 100ml volumetric flask filling to the mark respectively using the 60*mM* buffer. The concentration of these stock solutions is 10*mM*.

From the already prepared six amino acids, a volume of 10mL of each amino acid was taken and placed in one 100ml volumetric flask where they were all combined and dissolved to the mark using a 60*mM* buffer. Hence each amino acid was 1*mM* and the total concentration of amino acids (ΣAA) in this solution was 6.0*mM*.

After the amino acids samples were prepared, they were all taken and stored at

4°C to prevent degradation.

Chlorination Process.

From the combined amino acid solution, 25ml was pipetted using volumetric pipette and poured into four different reaction beakers, providing 150 μmol total of amino acids (25 μmol each). The four beakers were marked at different reaction molar ratios (RR)of 0.5, 1.0, 2.0 and 3.0 (mol OCl^-: Σ mol AA).

The pH meter was calibrated to a pH of 4.0 and 7.0 to make sure that the reaction between chlorine and the six amino acids was performed at a pH of 7.0. The pH electrode was later placed into the beakers and stirring was started.

A 2.46*M* solution of sodium hypochlorite 14.5% available chlorine was added to the six amino acids in the four beakers to provide chlorine:ΣAA mole ratios of 0.5, 1.0, 2.0, and 3.0 (75, 150, 300, and 450 μmol NaOCl) and reacted for 10minutes. Potassium iodide starch paper turns dark indicating the presence of active chlorine. To stop the reaction by reacting any remaining active chlorine sodium bisulfite was added. Three drops of saturated solution of sodium bisulfite $NaHSO_3$ was added into the beakers and reacted for another additional 5 min to stop the hypochlorite reaction. Potassium iodide was used once again to check whether all the chlorine was gone.

Using a micropipette 1000μl sample were taken from each beaker and placed into different small Thermo Scientific Reacti-vials awaiting derivatization. The process of taking 1000μl samples was done after the

10minutes reaction with hypochlorite and after adding the bisulfite and reacting it for 5 minutes. While this whole process was taking place, the pH was monitored before and after the addition of the bisulfite. The reaction process was conducted at room temperature which was 23 ± 1 °C.

Derivatization Process.

A total of eight 1000 µl samples were taken from the four beakers (2 samples each) one before and one after the addition of the bisulfite to be derivatized in the fume hood and placed into 3mL reaction vial. From the first sample of 0.5 taken without the addition of the bisulfite, 150 µl of 2%NaOH followed by 334 µl of methanol. Within the fume hood the reaction was started when 68 µl of pyridine and 40 µl of MCF were added. Pyridine was the acting catalyst during the derivatization process. Using a vortex mixer, the sample reaction was shaken for 30sec and then another 40 µl of MCF was added and shaken for another addition of 30sec. To separate MCF derivatives or any other relatively nonpolar compounds from the reaction mixture 800 µl of chloroform was added and shaken for 10sec and finally adding 800 µl of 0.05*M* sodium bicarbonate ($NaHCO_3$) and shaking for 10 more seconds. The shaking was done well to get good contact of the aqueous solution with the chloroform for extraction of the relatively nonpolar compounds into the $CHCl_3$. Sodium bicarbonate ($NaHCO_3$) was used to neutralize acidity of the solution. A clear chloroform bottom layer was observed. The top aqueous layer was discarded and the bottom layer that remained was dried using a small amount

of anhydrous sodium sulfate (Na_2SO_4). This was done to remove any remaining water dissolved in the chloroform. Using a dropper, the chloroform extract was transferred to 2ml GC autosampler vials. The process was repeated in all remaining seven samples that had contained bisulfite and without the bisulfite. The derivatized samples were stored at cold temperatures and analyzed the next day.

Sample Control (blank) Preparation and Derivatization.

A sample control was prepared without sodium hypochlorite (NaOCl) or sodium bisulfite ($NaHSO_3$). 1000mL was taken from the six combined amino acid solution that was prepared and derivatized. Similar steps as the chlorinated samples earlier were followed during the process. This sample control served as a one-point calibration solution to calculate the approximate concentration of the amino acids remaining after the reaction.

GC/MS Instrument Analysis.

Figure 3. GC/MS Triple Quad System Instrument Analysis. (Vision, 2019)

An Agilent GC-MS Triple Quad system (Figure 3) was used for analysis. It consists of a Model 7890A GC, Model 7000B MS and Model 7693 autosampler utilizing a Model G4514A injector. A Restek Rtx-5MS GC column was used (30meter long 0.25mm inner diameter, 0.25 µm 5% diphenyl 95% dimethyl polysiloxane film). Helium was used as the carrier gas at a flow rate of 0.9mL/min. Sample volumes of 1µL were injected with split ratio of 20:1. The injector temperature was 290°C and the column temperature program was 40°C for 2min then increased at a rate of 30°C/min to a temperature of 320°C and heled for 4min. The MS transfer line was set to 280°C. Chemical ionization with methane was performed for mass spectrometry analysis and detection.

In this research, a triple quadrupole instrument allows for tandem MS/MS experiments to be performed. Multiple reaction monitoring (MRM) was used due to its ability to lower the detection limit as well as good detection for the sensitive analysis. This was done in the first experiments conducted without chlorination to determine if the MCF derivatization method described would produce a linear calibration graph for the amino acids and whether an internal standard would be required. 16 amino acids were initially tested with the published ions transition. (Hans F.N.K, Trygve A et al, 2011). The MRM transition for the six amino acids chosen for further study are given in the results section.

To investigate chlorination reaction of products while simultaneously monitoring the amino acids concentration, conventional GC/MS total ion chromatograms were obtained to observe the mass spectra of the eluting peaks.

A mass range of 50-300Da was scanned with the first MS in the triple quad (MS1 Scans). In both types of experiment the solvent delay time was set at 4 minutes, and the total run time was 15 minutes.

The derivatized amino acid's reaction product solution were each injected into the GC-MS starting with the chlorine:ΣAA ratio (RR) 0.5, 1.0, 2.0 and later 3.0. with each ratio being performed twice, once with the addition of bisulfite and the without the bisulfite. The retention times, peak areas, height, and mass spectra were observed, recorded and analyzed.

CHAPTER THREE

RESULTS

Gas Chromatography/Mass Spectrometry Results and Calibration.

For all the reactions, the pH remained stable whereby the pH increases with additional of NaOCl were 0.1 pH or less. The reaction pH was 7.1± 0.1.

GC/MS/MS MRM Results

The results on GC/MS analysis showed each of the six amino acids having different retention time as shown in Table 2 below. These results were obtained with Multiple Reaction Monitoring (MRM) tandem MS/MS as compared to total ion chromatograms obtained with ordinary GC/MS (MS1 Scans shown below), the baseline is observed to be very close to zero and the drastically reduced noise level yields a very high signal-to-noise ratio as shown in Figure 4. The relative size of the peaks is related to the polarity of the R groups of the amino acids. The glycine (R=H) derivative is the most polar of the six and thus does not extract as well into chloroform, yielding the smallest peak, whereas the leucine isomers are the largest peaks due to the nonpolar butyl R group (C_4H_9). Initially sixteen amino acids were tested (data not shown) however ten of them had smaller peaks areas due to the presence of a polar functional group within the R group of the amino acids and thus high detection limits.

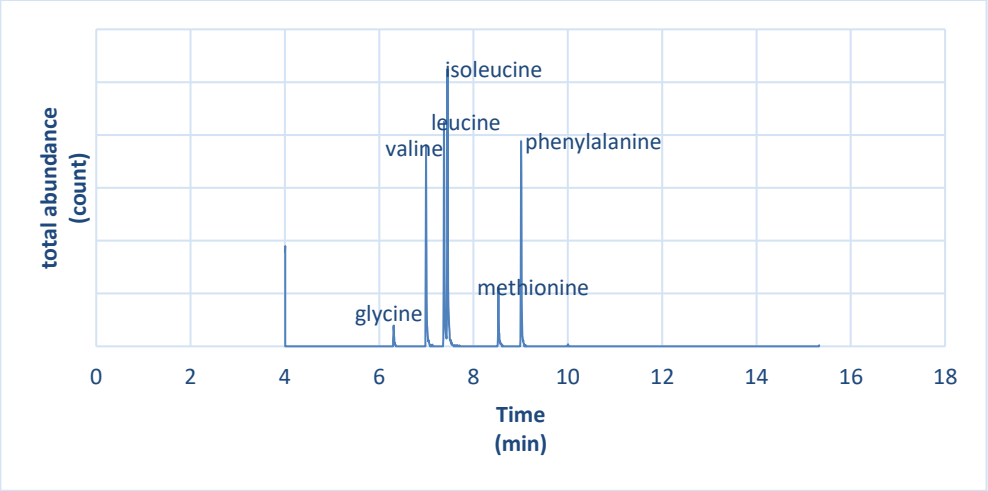


Figure 4. GC/MS/MS MRM Chromatogram of Six Amino Acids

The MS precursor and product ions chosen for MRM (transition) are given in table 2 below for the six chosen amino acids for this research. The molecular ion mass-to-charge ratio (m/z) corresponding to the unfragmented, derivatized amino acids was observed an (M+1) peak as commonly observed with chemical ionization (CI) mass spectrometry due to proton transfer from the ionized methane reagent gas. The chosen precursor and product ions of the MRM transitions were taken from the literature and were confirmed appropriate. (Hans F.N.K, Trygve A et al, 2011). The precursor ion is often the base peak in the mass spectrum (the largest peak) and the product on is the larger-signal ions after fragmentation with collision gas(nitrogen, N_2). The collision energies for fragmentation to the product ion are also reported below.

Table 2. Gas Chromatography Retention Times for the Amino Acid Derivatives and the -Mass Spectroscopy MS-MS Ion Transitions Monitored in Multiple Reaction Monitoring (MRM) Mode.

Amino Acids	MRM Transition	Retention Time	Collision Energy	
	Precursor-Product ion Transition (m/z, Da)	(Min)	(CE)	Molecular Weight of MCF Derivatives
Glycine	116-44	6.306	15	147.07
Valine	130-71	7.00	20	189.15
Leucine	144-43	7.377	15	203.17
Isoleucine	144-69	7.448	25	203.17
Methionine	169-61	8.532	10	221.21
phenylalanine	178-128	9.011	20	237.19

In this research, we observed leucine and iso-leucine to have retention times very close to each other and therefore are poor resolution peaks. Resolution is used to determine quantitation between adjacent peaks with different retention time t in a chromatogram.

In both leucine and iso-leucine the largest base peak and the second largest peak were seen. In this case, from the GC/MS analysis, leucine was observed

to have a retention time of 7.37 minutes with baseline width of 0.051 and isoleucine had a retention time of 7.44 minutes with baseline width of 0.056. The resolution between leucine and isoleucine peak was calculated to be 1.308 as presented in the resolution peak equation below:

$$\frac{2\Delta tr}{w_B + w_A}$$

R = 2(7.44 - 7.37) / 0.056 + 0.051

R = 1.308

Amino Signal Calibration and Concentration

Chromatography peak areas were correlated to concentration to establish whether the relationship was proportional and reproducible as well as whether or not an internal standard was needed. The results for phenylalanine over two ranges of concentration are shown below in Figure 5 and 6.

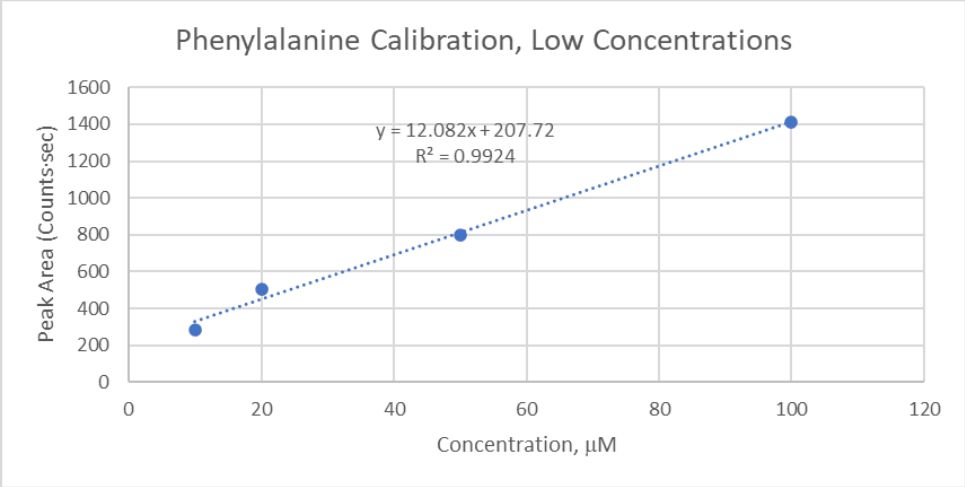


Figure 5. Phenylalanine Calibration Curve at Low Concentrations.

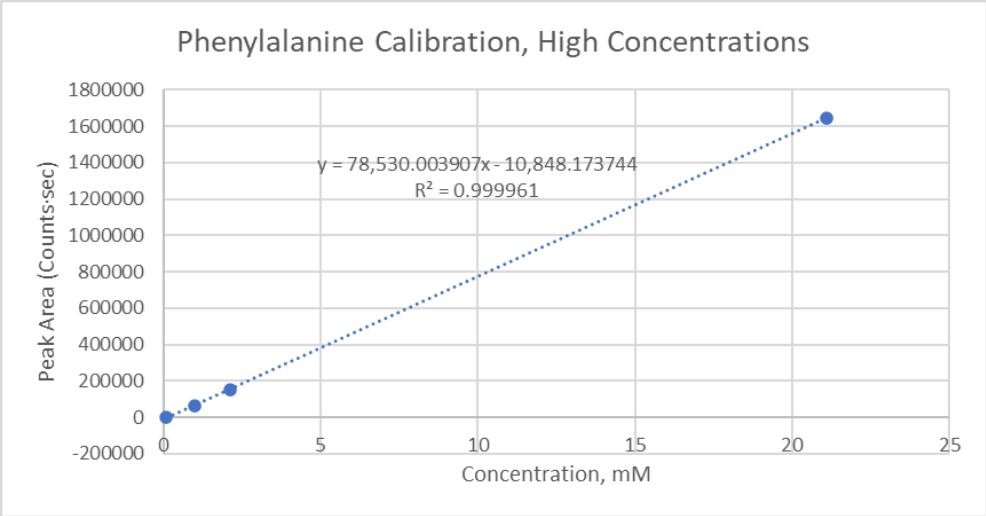


Figure 6. Phenylalanine Calibration Curve at High Concentrations.

Good correlation was observed, showing that the detector response is linear and that the derivatization and extraction procedure was reproducible. It was not the purpose of this study to determine exact concentrations, and to preserve time and reagents, it was decided to conduct one-point calibrations with a single control or chlorine blank for each experiment.

Chlorine Sample Control (Blank) Analysis Results

From the GC/MS analysis (MS1 Scans) done on the derivatized blank or control sample without adding chlorine it was noted that the relative peak area abundances were higher than the MRM scans although the signal-to-noise ratio was lower (see Figures 14-17). This is due to all ions produced by Cl are sent through the first quadrupole to the electron multiplier detector as the m/z range scanned. Table 3 shows the peak areas obtained for the six amino acid mixture with each amino acid at 1 mM and a sum total concentration of ΣAA = 6 *mM*. In the reactivity profiles with chlorine that follow, the amino acid peak areas

observed were divided by these areas to yield normalized areas (and represent the first data point where the chlorine:ΣAA concentration is zero). Since the starting amino acid concentrations were 1 *mM*, the normalized values numerically equal the concentrations in *mM.*

Table 3. Blank Analysis Without the Addition of Sodium Hypochlorite on the Amino Acids

Amino Acids (1mM each)	Retention Time (min)	Peak Area (Count.sec)
Glycine	6.306	98856
Valine	7.00	171398
Leucine	7.377	198254
Isoleucine	7.447	196920
Methionine	8.532	112246
phenylalanine	9.001	225749

Trial Reactivity Profiles of Amino Acids with and/without Bisulfite.

Within the trials done on reactivity of amino acids with sodium hypochlorite and bisulfite, we observed that when bisulfite is added to stop the reaction, the amounts of amino acids detected were greater. At chlorine-to-total AA (Σ AA) reaction ratios 0.5 and 1.0, the amino acids were present in both trials with and without bisulfite however relative units differed (Figure 7 and 8, Table 4 and 5). Amino acids present is lower without the addition of bisulfite.

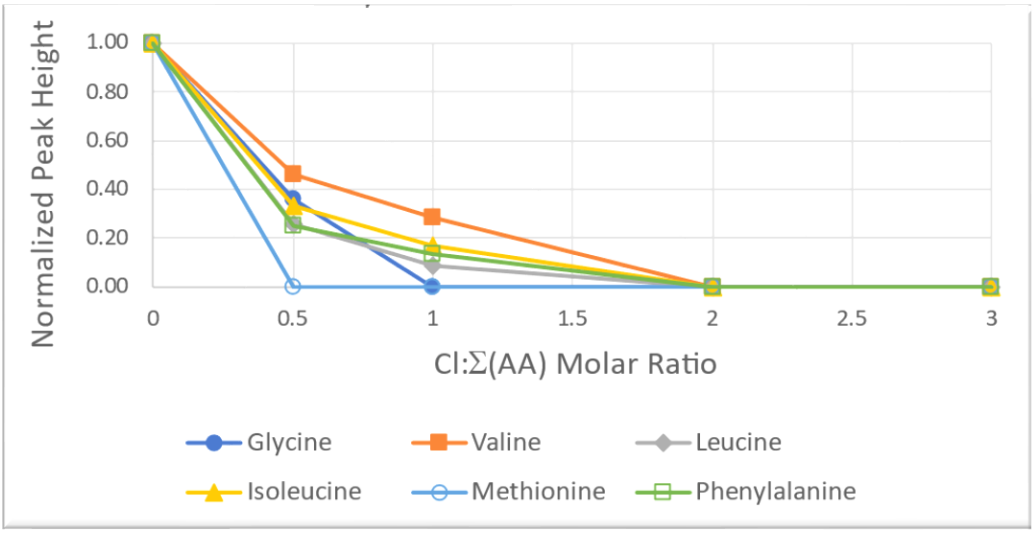


Figure 7. Amino Acids VS Chlorine Dose Without HSO_3

Table 4. Trial Reaction Between Amino Acids and Sodium Hypochlorite Without Bisulfite.

Amino acid	Retention time (min)	Relative abundance			
		0.5	1.0	2.0	3.0
Glycine	6.306	35425	0	0	0
Valine	7.00	78639	48045	0	0
Leucine	7.377	50932	16550	0	0
Isoleucine	7.448	65575	33014	0	0
Methionine	8.532	0	0	0	0
Phenylalanine	9.01	56834	30494	0	0

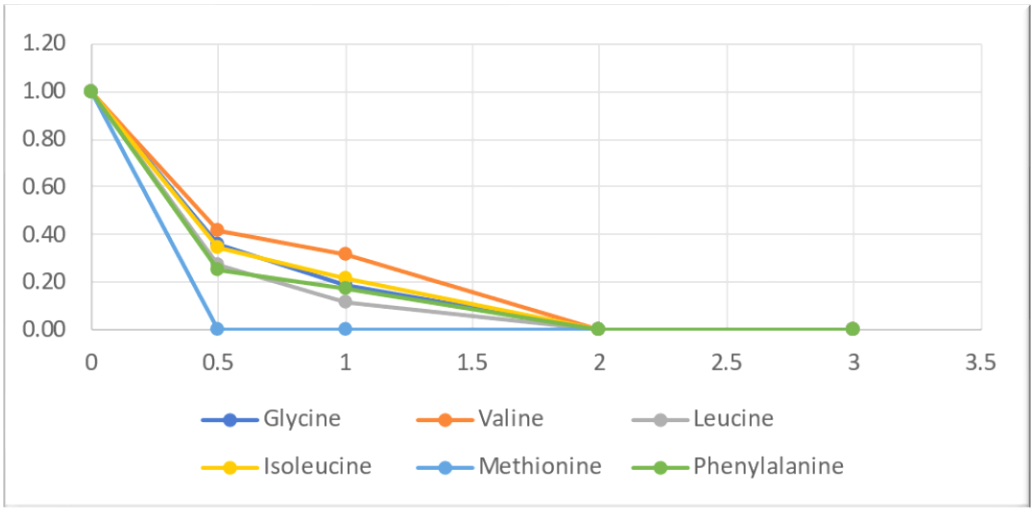


Figure 8. Amino Acids VS Chlorine Dose with HSO_3

Table 5. Trial Reaction Between Amino Acids and Sodium Hypochlorite with Bisulfite.

Amino acid	Retention time (min)	Relative abundance			
		0.5	1.0	2.0	3.0
Glycine	6.306	35854	18305	0	0
Valine	7.00	71326	54097	0	0
Leucine	7.377	54107	23317	0	0
Isoleucine	7.448	68406	41931	0	0
Methionine	8.532	0	0	0	0
Phenylalanine	9.011	57089	39791	0	0

Reactivity Profiles of Amino Acids with Sodium Hypochlorite

At a chlorine-to-total AA (Σ AA) relative ratio (RR) of 0.5, significant amount of glycine (36%), valine (42%), leucine (27%), isoleucine (35%), and phenylalanine (25%) peaks were observed to be present after their reaction with sodium hypochlorite. Methionine was not observed since it is more reactive with chlorine. At a ratio of 1.0, valine, leucine, iso-leucine, and phenylalanine were still present with much lower peaks. Glycine was not observed in the absence of bisulfite but present in the presence of bisulfite. At ratios 2.0 and 3.0, none of the amino acid peaks were observed since the hypochlorous ratio was much higher. Individual graphs were plotted from the results of peak observation without bisulfite as indicated below (Figure 9-13)

Glycine was observed to have a peak area of 35853. On further addition of chlorine at ratio 1.0, 2.0 and 3.0, without bisulfite, glycine was observed to have been completely reacted although with the addition of bisulfite the peak could be observed at ratio 1.0. This indicates that glycine continues to react if the reaction is not stopped.

Valine was present at ratio 0.5 with relative peak areas of 71326. At a ratio of 1.0 for chlorine: amino ratio, valine decreased to a relative unit of 54097. Further increase of chlorine to ratios of 2.0 and 3.0 caused valine to have completely react.

For Leucine at a 0.5 chlorine: amino ratio it had a relative peak area of 54106. At ratio 1.0, the relative peak area decreased to 23316. For further increases of chlorine to ratios 2.0 and 3.0, leucine completely reacted.

Isoleucine was present at ratio 0.5 of chlorine: amino with a relative peak area of 68406. At a ratio of 1.0 of the relative area of isoleucine decreased to 41931. Further increase of chlorine to ratios of 2.0 and 3.0 caused the isoleucine to completely react.

Phenylalanine was present at a chlorine: amino ratio of 0.5 with relative peak area of 57089. At a ratio of 1.0 the relative area decreased to 39790. Upon additional increase of chlorine to ratio of 2.0 and 3.0 phenylalanine was completely reacted.

For methionine and glycine, they completely reacted at RR of 1, with little change in the other amino acids if the reaction was allowed to continue.

Due to the presence of thiol ether functional group in methionine, it was the first to react because of its higher reactivity with chlorine as a reducing agent than the other amino acids. Hence, no traces of methionine were found for any chlorine-to-amino ratios. Valine appeared to be the least reactive while the reactivity of the other three amino acids (leucine, isoleucine, and phenylalanine) was similar.

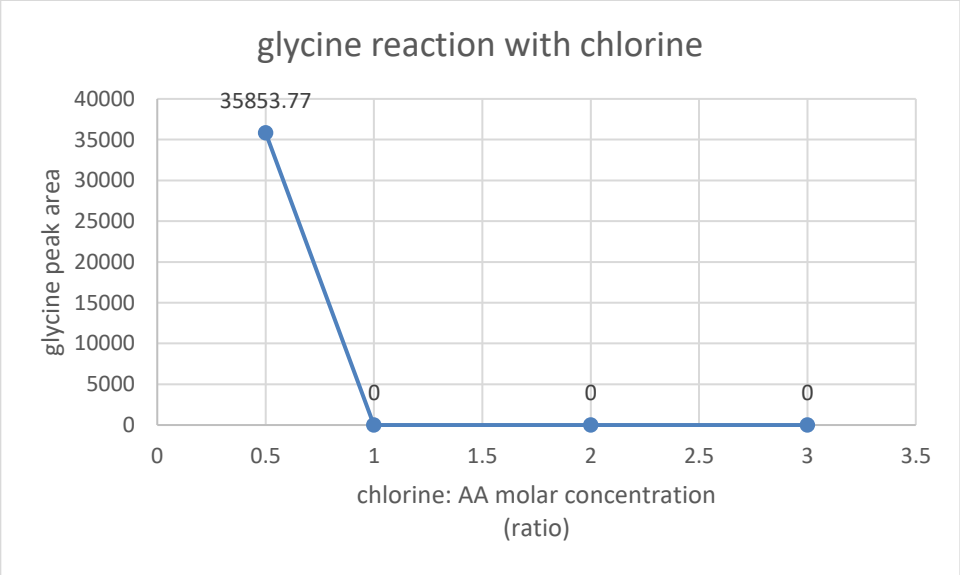


Figure 9. Reaction Between Glycine and Chlorine.

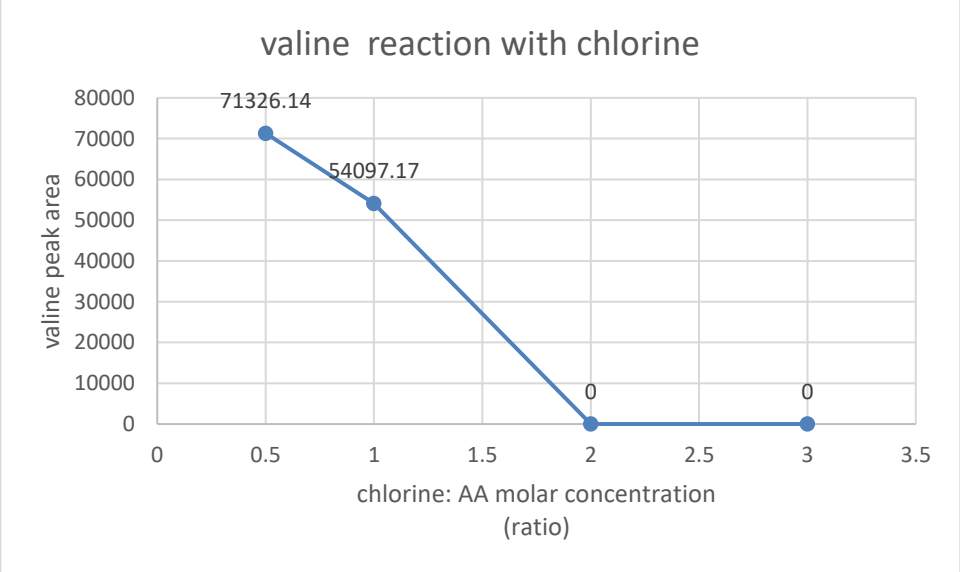


Figure 10. Reaction Between Valine and Chlorine.

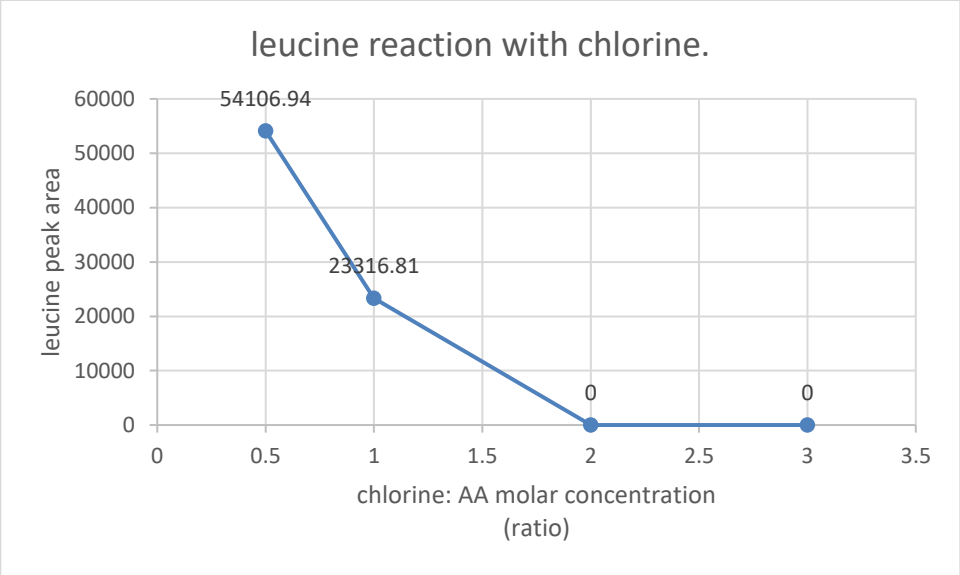


Figure 11. Reaction Between Leucine and Chlorine.

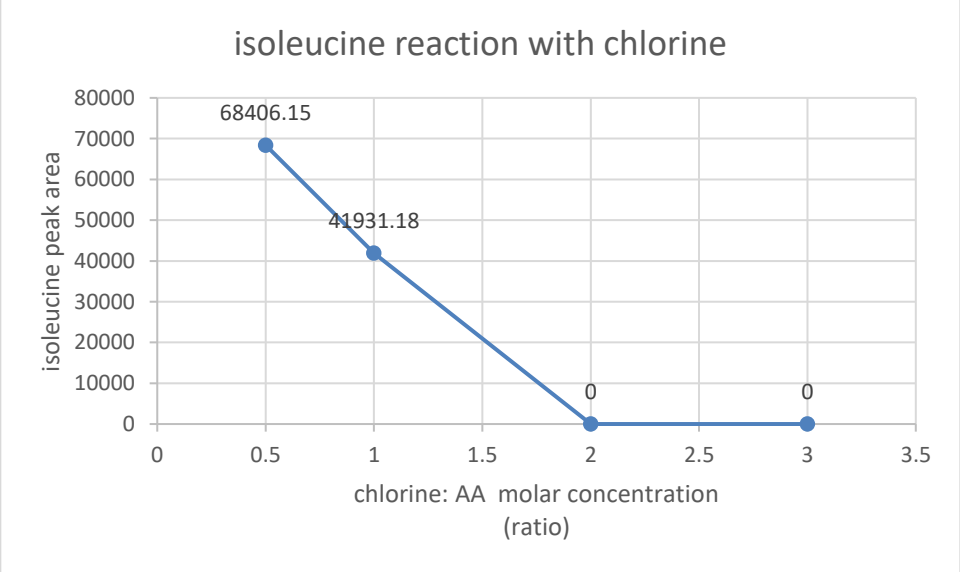


Figure 12. Reaction Between Isoleucine and Chlorine.

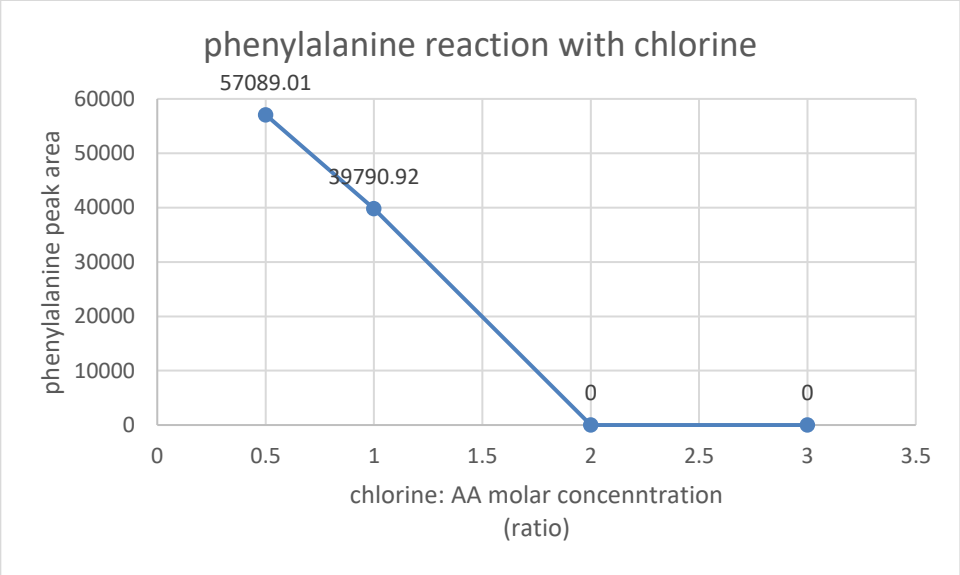


Figure 13. Reaction Between Phenylalanine and Chlorine.

Chromatography and Presence of Other Compounds Detected in Reaction Mixture.

At ratios of 0.5, glycine, valine, leucine, isoleucine, and phenylalanine were observed, and methionine was completely reacted (Figure 14). When the ratio is increased to 1.0, valine, leucine, isoleucine, and phenylalanine, while glycine and methionine were completely reacted (Figure 15). At a ratio of 2.0, none of the amino acids were observed since their reaction was complete (Figure 16). At the highest chlorine: amine ratio of 3.0, none of the amino acids were observed (Figure 17).

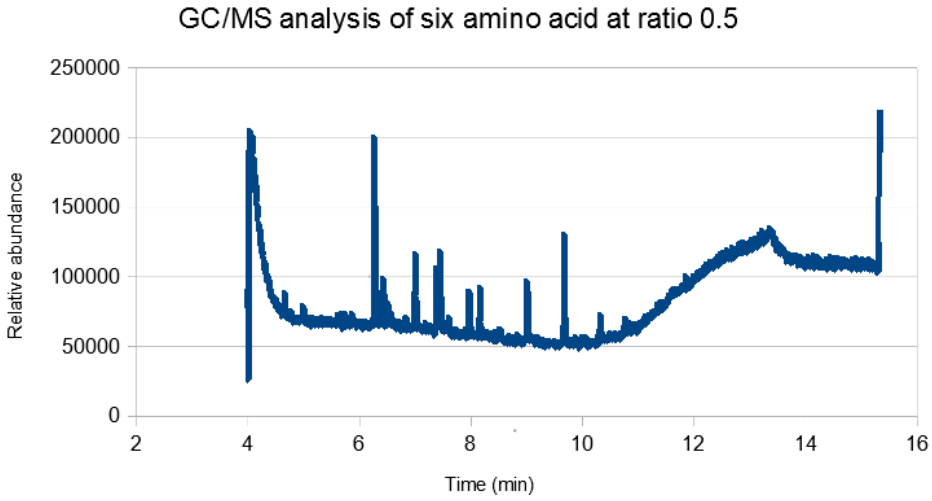


Figure 14. GC/MS Analysis of Six Amino Acids at a Ratio of 0.5.

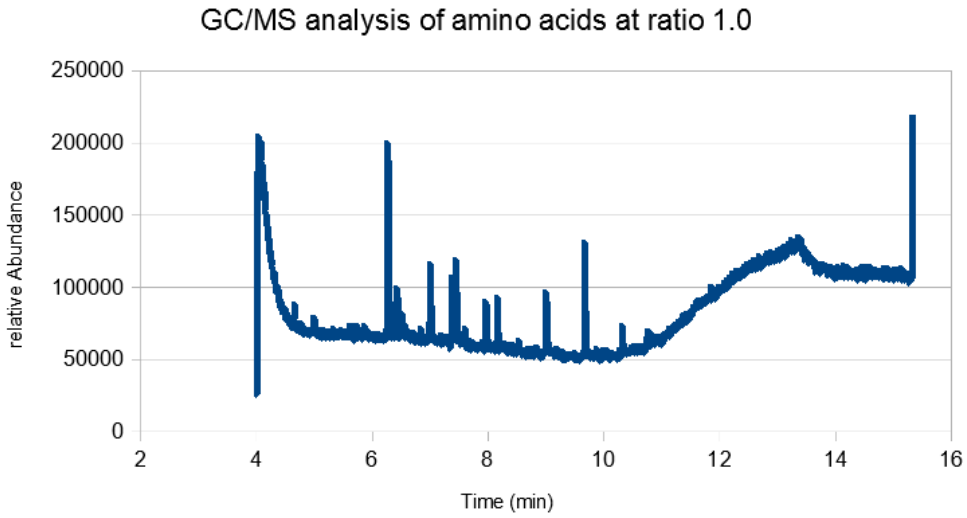


Figure 15. GC/MS Analysis of Six Amino Acids at a Ratio of 1.0

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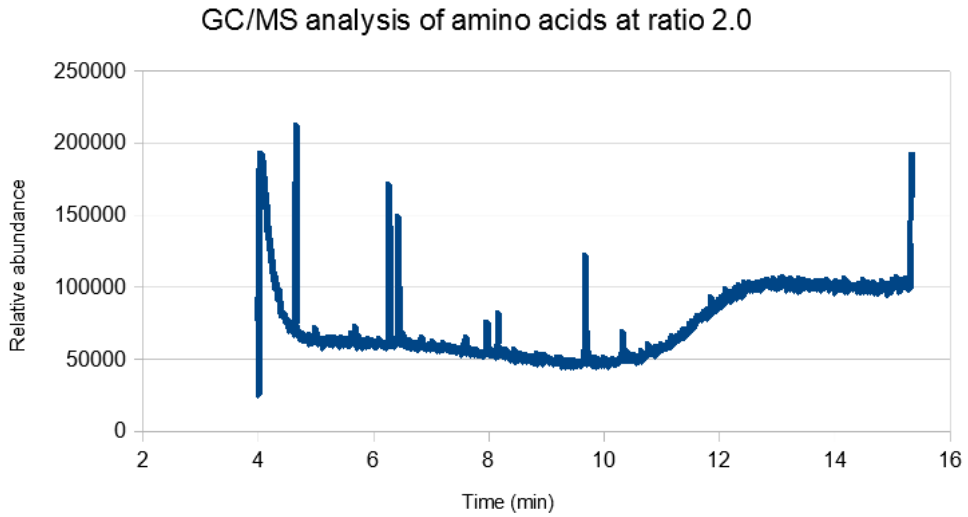

Figure 16. GC/MS Analysis of Six Amino Acids at Ratio 2.0

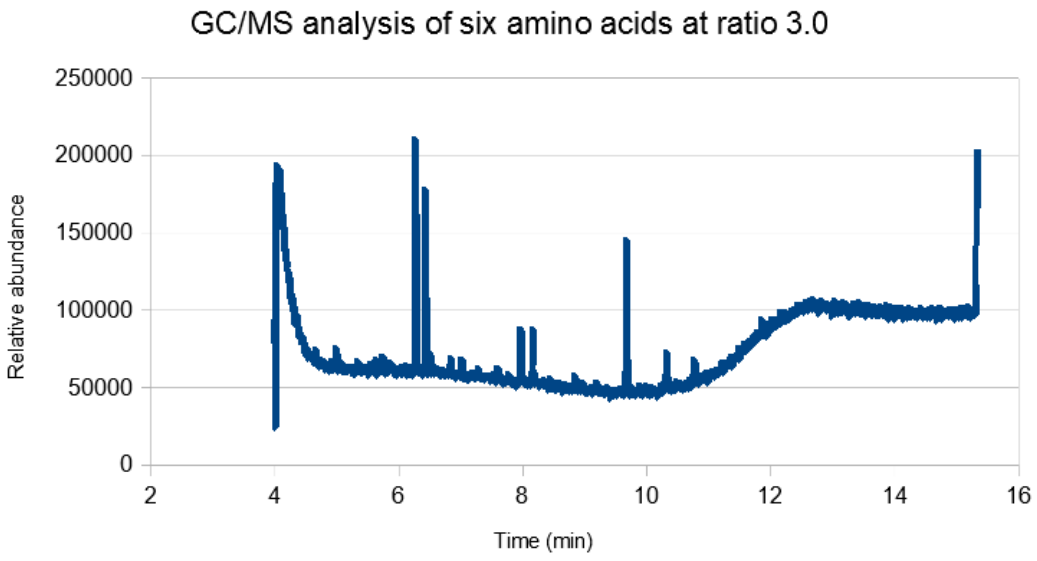

Figure 17. GC/MS Analysis of Six Amino Acids at Ratio 3.0

Mass Spectra and Possible Disinfectant By-Products

From the reaction of the amino acids and sodium hypochlorite, there was formation of two new peaks. Their retention time were observed to be 4.65 and 6.42 minutes respectively Unfortunately, the identities or parent amino acids could not be determined. (see figures below). This is in agreement with previous research that some amino acids do act as precursors.

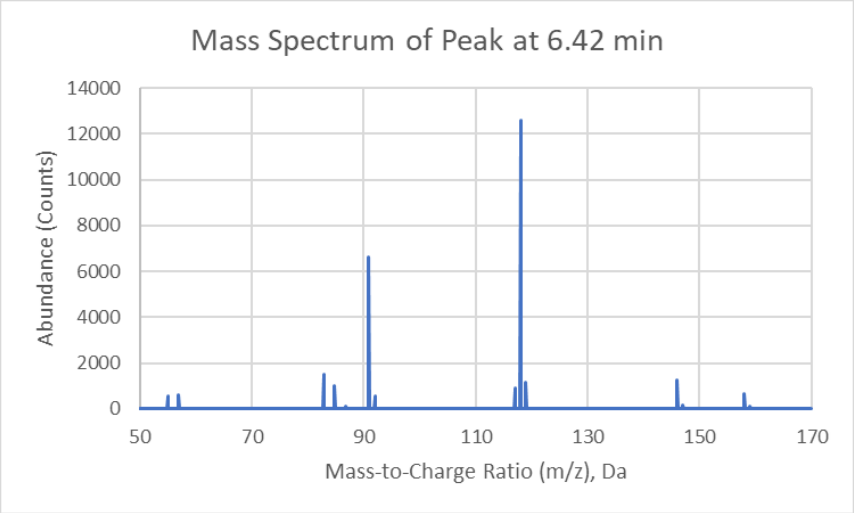

Figure 18. Mass Spectra of Product Peak.

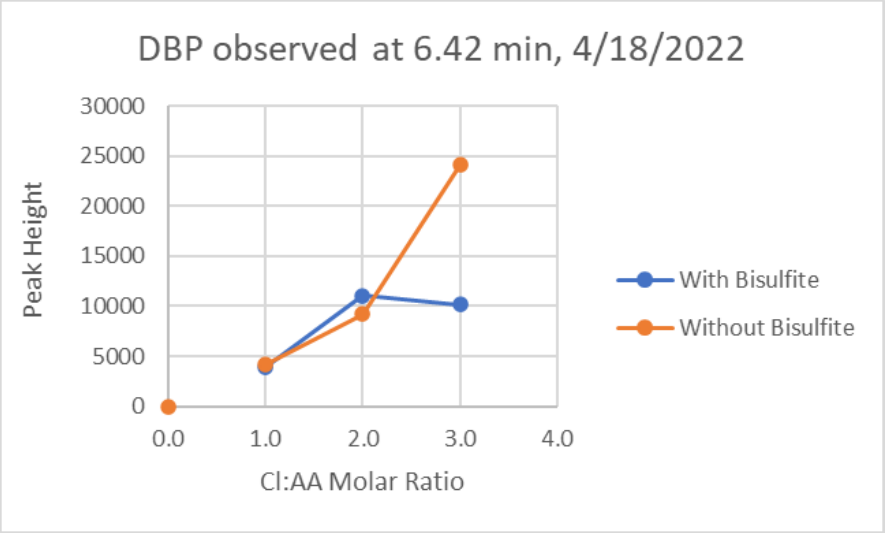

Figure 19. Peak Height for the DBP Measured at 6.42 Minutes as Function of Chlorine-to-Amino Ratio (data from April 18).

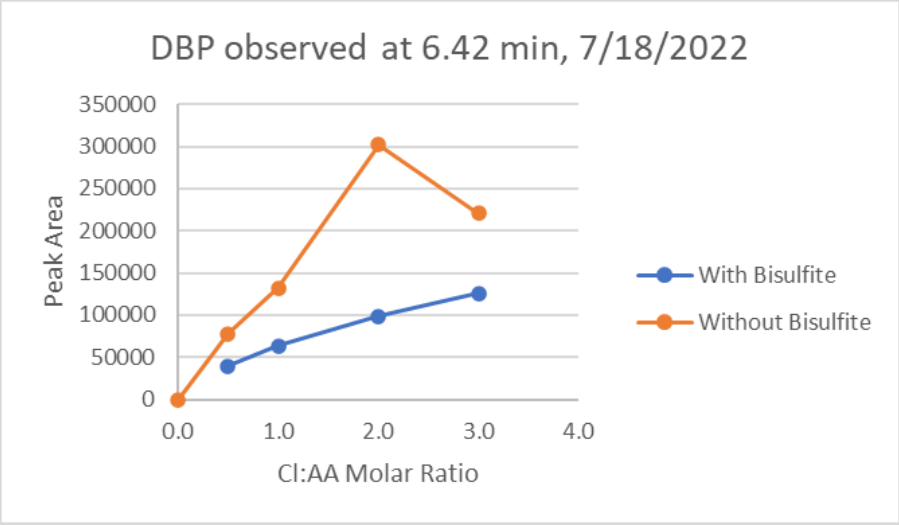

Figure 20. Peak Area for the DBP Measured at 6.42 Minutes as a Function of Chlorine-to-Amino Ratio (data from July 18).

From Figure 18 the base peak at 118Da is proposed as the M+1 peak for phenylacetonitrile (Figure 21, M = nominal mass = 117Da). The second largest peak at 91 Da is consistent with the loss of HCN (M+1-27). Thus, identified peak at 6.42 had a mass spectrum consistent with phenylacetonitrile that has been proposed in literature as a DBP (Ma, X; Deng, J.; et al. 2016.

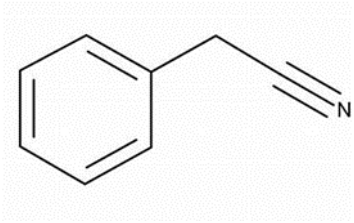

Figure 21. Phenylacetonitrile

Unfortunately, we were unable to identify any of the other peaks that appeared in the reaction extracts. Chlorine is known to have a distinctive isotope signature that is not present in most mass spectra, which suggests that chloroamino acids were not observed in this study. If originally present in the reaction mixture, they may not have reacted with MCF or if they did form, they could have decomposed in the heated GC injector.

An experiment with only glycine present at a concentration of 5*mM* was conducted and an identified product peak at 8.12min appears to increase with chlorine concentration as an unidentified compound (Figure 22 and 23 below). Mass spectra of this peak shows a base peak at 80Da and interestingly mass spectra peaks at 200 and 204 Da that grow with increase in the chlorine-to-amino ratio (Figure 24).

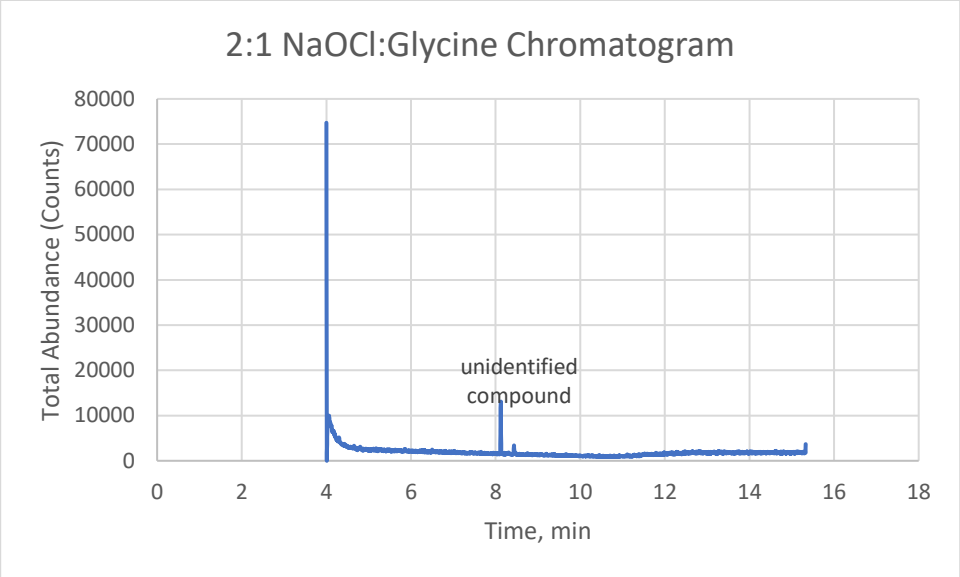

Figure 22. NaOCl: Glycine Chromatogram Showing the Presence of an Unidentified Peak at 8.12minutes

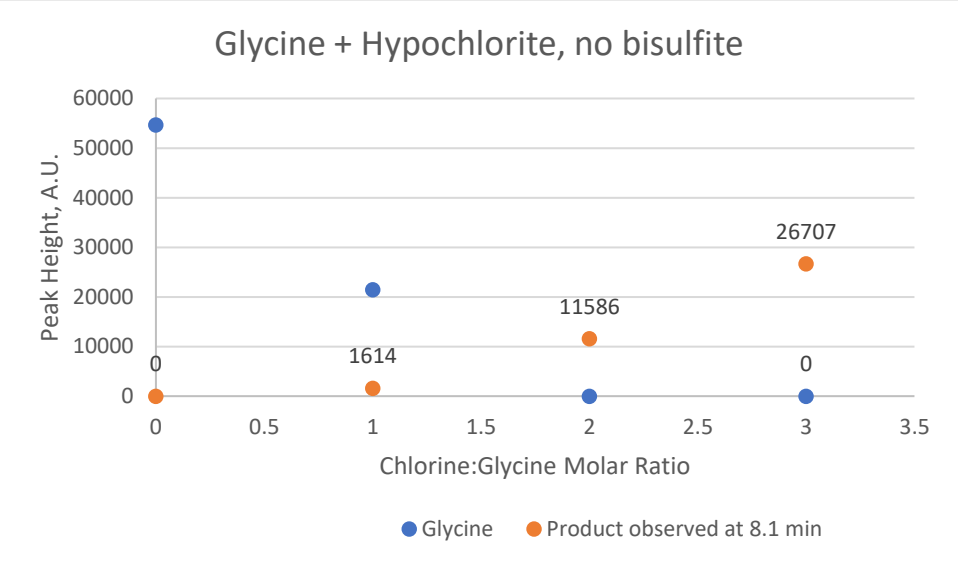

Figure 23. Peak Heights for the Unidentified Compound from the Reaction Between Glycine and Hypochlorite Without Bisulfite.

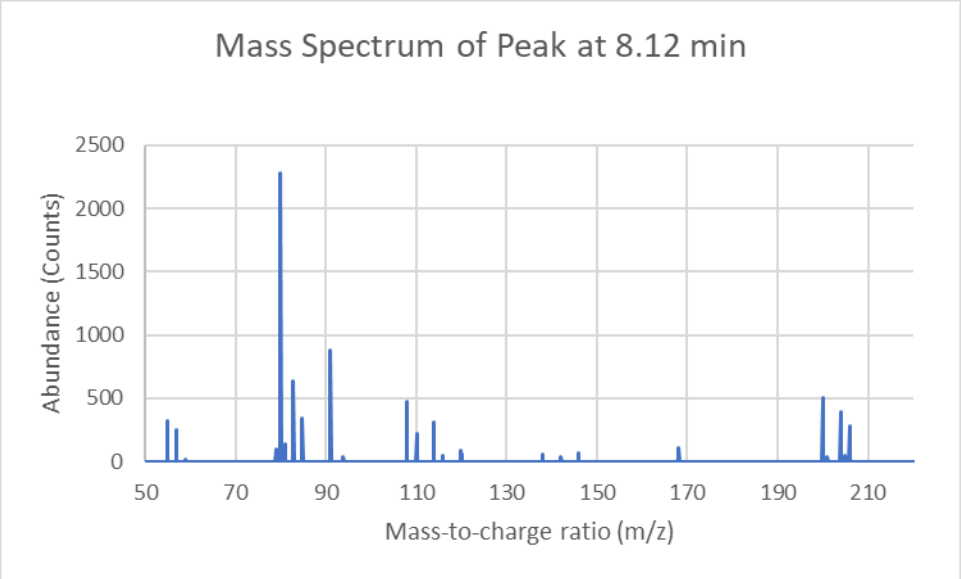

Figure 24. Mass Spectrum of Unidentified Peak Measured at 8.12 Minutes

It was also worth noting that storing the amino acids for many days influenced or affected the yield result of the amino acids while analyzing them using the GC-MS due to degradation. This is seen by a change in their area whereby different results were observed between a one-day storage and a week storage whereby in one day storage the GC-MS analysis were higher as compared to a week storage that were much lower.

CHAPTER FOUR

CONCLUSION

Six amino acids were noted to be highly reactive in the presence of chlorine. The higher the chlorine-to-amino ratio was, the more the amino acids reacted. It can be noted that the addition of bisulfite to stop the reaction between amino acids and sodium hypochlorite leads to a decrease in amino acid removal. Without additional bisulfite, the reaction continues for a short period leading to amino acid concentrations to decrease.

In this research, there were no chloroamino acids identified during the derivatization extract method including no observation of N-monochloroamino acids or N,N-dichloroamino acids. One of the reasons for the lack of observation was because chlorinated amino acids do not undergo derivatization and thus were not extracted in chloroform. The other possible reason is that the chlorinated amino acids were not thermally stable to withstand the injector temperature or the higher GC oven temperature.

Potential DBPs such as phenylacetonitrile were observed after their reaction with chlorine proving previous research done that those amino acids can act as precursor for other DBPs. Some of the common DBPs such as cyanogen chloride, di-or tri-chloroacetonitrile or other nitriles could not be observed implying they may be very reactive with derivatization reagents producing unknown products. Although little could be observed during this research, the study supports other research that shows that chloroamino acids act as an

intermediate on the way to DBPs. However, overall methyl chloroformate derivatization does not appear to be amenable to looking for chloroamino acids or other DBPs in the same experiment with a mixture of amino acids.

Further studies could be done to determine the relationship between precursor mixtures, DBPs and water treatment process.

APPENDIX A

AMINO CALCULATIONS.

Example 1

Glycine stock solution preparation

Step 1

= (0.0757 g) / (75.07 g/mol) = 0.00101 mol

= 0.00101 mol x 1000 mmol/mol = 1.008 mmol

= 1.008 mmol / 0.100 L =10.0 mM stock solution.

Step 2

= (10mM) x (10ml) / 100ml = 1mM

Example 2

Step 1

Valine stock solution preparation

= (0.1174 g) / (117.1g/mol) = 0.001 mol

= 0.001 mol x 1000 mmol/mol = 1.0 mmol

= 1.0 mmol / 0.100 L =10.0 mM stock solution.

Step 2

= (10mM) x (10ml) / 100ml = 1mM

Example 3

Leucine stock solution preparation

= 0.1312 g) / (131.2g/mol) = 0.001 mol

= 0.001 mol x 1000 mmol/mol = 1.0 mmol

= 1.0 mmol / 0.100 L =10.0 mM stock solution.

Step 2

= (10mM) x (10ml) / 100ml = 1mM

Example 4

Isoleucine stock solution preparation

= 0.1313 g) / (131.2 g/mol) = 0.001 mol

= 0.001 mol x 1000 mmol/mol = 1.0 mmol

= 1.0 mmol / 0.100 L =10.0 mM stock solution.

Step 2

= (10mM) x (10ml) / 100ml = 1mM

Example 5

Methionine stock solution preparation

= 0.1467 g) / (147.2 g/mol) = 0.001 mol

= 0.001 mol x 1000 mmol/mol = 1.0 mmol

= 1.0 mmol / 0.100 L =10.0 mM stock solution.

Step 2

= (10mM) x (10ml) / 100ml = 1mM

Example 6

Phenylalanine stock solution preparation

= 0.1688 g) / (165.2g/mol) = 0.001 mol

= 0.001 mol x 1000 mmol/mol = 1.0 mmol

= 1.0 mmol / 0.100 L =10.0mM stock solution.

Step 2

= (10mM) x (10ml) / 100ml = 1mM

Sum Concentration of each of the amino acids to give a total Amino Acids (ΣAA) of 6.0mM.

APPENDIX B

RESOLUTION CALCULATION.

Example 7

= leucine retention time = 7.37

Baseline width = 0.051

= isoleucine retention time = 7.44

Baseline width = 0.056

Resolution peak =

$$\frac{2\Delta tr}{w_B + w_A}$$

R = 2(7.44 - 7.37) / 0.056 + 0.051

R = 1.308

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