Analysis of Biogenic Amines by GC/FID and GC/MS

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(Abstract)

Biogenic amines (particularly histamine in the presence of putrescine and cadaverine) can lead to scombroid food poisoning if levels become too high. Low levels of these biogenic amines occur naturally, but high levels (FDA sets 50 ppm of histamine in fish as the maximum allowable level) can lead to scombroid poisoning.

A number of analytical methods have been employed to analyze biogenic amines, especially High Performance Liquid Chromatography (HPLC). Amines in general are difficult to analyze by Gas Chromatography (GC) due to their lack of volatility and their interaction with the GC column often leading to significant tailing and poor reproducibility. GC methods could be simpler and cheaper than HPLC equipment in some cases. Biogenic amines need to be derivatized before both GC and HPLC analyses. The objective of this research was to develop a relatively fast, reproducible method to derivatize and quantitate biogenic amines in fish at trace levels using GC/FID. The derivatizing reagent used in the experiments was propyl chloroformate, useful for aqueous samples. A method for GC/MS was also developed and used to confirm the identity of six derivatized biogenic amines. To our knowledge no reference spectra for these derivatives has been published.

It was concluded that best results are obtained using a Cold-On-Column (C.O.C.) inlet with a short column (15 meters), thick film stationary phase (ZB-5, 1.00μm d_f), and with recommendations to cut 40 cm from the inlet end of the column every 25 injections when using C.O.C. Duplicate samples of Atlantic Salmon were analyzed on days 0, 3, and 5. Levels of histamine were below 50 ppm for days 0 and 3, but day 5 showed average levels of 160 pm (cadaverine), 1000 ppm (histamine), and 350 ppm (tyramine). Good precision of six amine stardards at 50 ppm was shown: heptylamine 5.2%,

putrescine 5.6%, cadaverine 5.0%, histamine 9.9%, tyramine 5.1%, and spermidine 6.2% RSD.

Acknowledgements

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I am truly grateful for all the many friends I have made here at Virginia Tech who have been such a blessing to me. I owe a tremendous thanks to Kari Urias and Dr. Jennifer Smith; I would have perished long ago without you both. Also, I would like to thank past and present members of the McNair group for your encouragement, especially Iris Dadone, Amy Kinkennon, Kevin Schug, Brent Cunningham, Dr. Robert Boggeus, and Jeff Cark.

Lastly, I would like to acknowledge and thank Phenomenex who supplied the GC columns I used for this project.

Dedication

“Make voyages, attempt them. There’s nothing else.”

-Tennessee Williams

Dedicated to my parents John and Joan Nakovich and my brother John Nakovich. Thank you for believing in me to help me see that if I work hard enough there are no limits to what I can do, and for loving me enough to let me go.

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Chapter 1-Introduction and Background

Gas Chromatography and Amine Analysis

Gas chromatography (GC) has been one of the most versatile and widely applicable techniques leading the field of analytical chemistry over the last forty years[1]. The popularity of GC stems from its fast, simple, relatively inexpensive, and reproducible nature[1]. As a method used to separate volatile and semi-volatile organic compounds, GC can be utilized in numerous analyses including petrochemical[1], environmental, pharmaceutical, and food science, to name a few.

In GC the analyte is carried through the column by a mobile phase composed of an inert gas such as helium or hydrogen[1]. As the vaporized analyte travels through the column it interacts with the liquid stationary phase[1]. Depending on the analytes' solubility for the stationary phase, they will separate and elute, from the column[1]. Upon elution the analytes enter a detector, which produces an electrical signal[1]. This signal is sent to a data system that generates an image, called a chromatogram, displaying the analyte peaks[1]. Certain analytes, specifically nitrogen containing compounds (i.e. aliphatic primary, secondary, and tertiary amines), can be difficult to detect using GC because there is significant adsorption of the basic amines on the often acidic column as well as decomposition of the analyte[2]. It would be advantageous to use GC in many applications that analyze amine-containing compounds if a reproducible, reliable method could be developed. An application of particular interest concerning amine analysis is the detection and quantification of biogenic amines. Typically, the decarboxylation of amino acids produces biogenic amines[3] as shown in Figure 1.

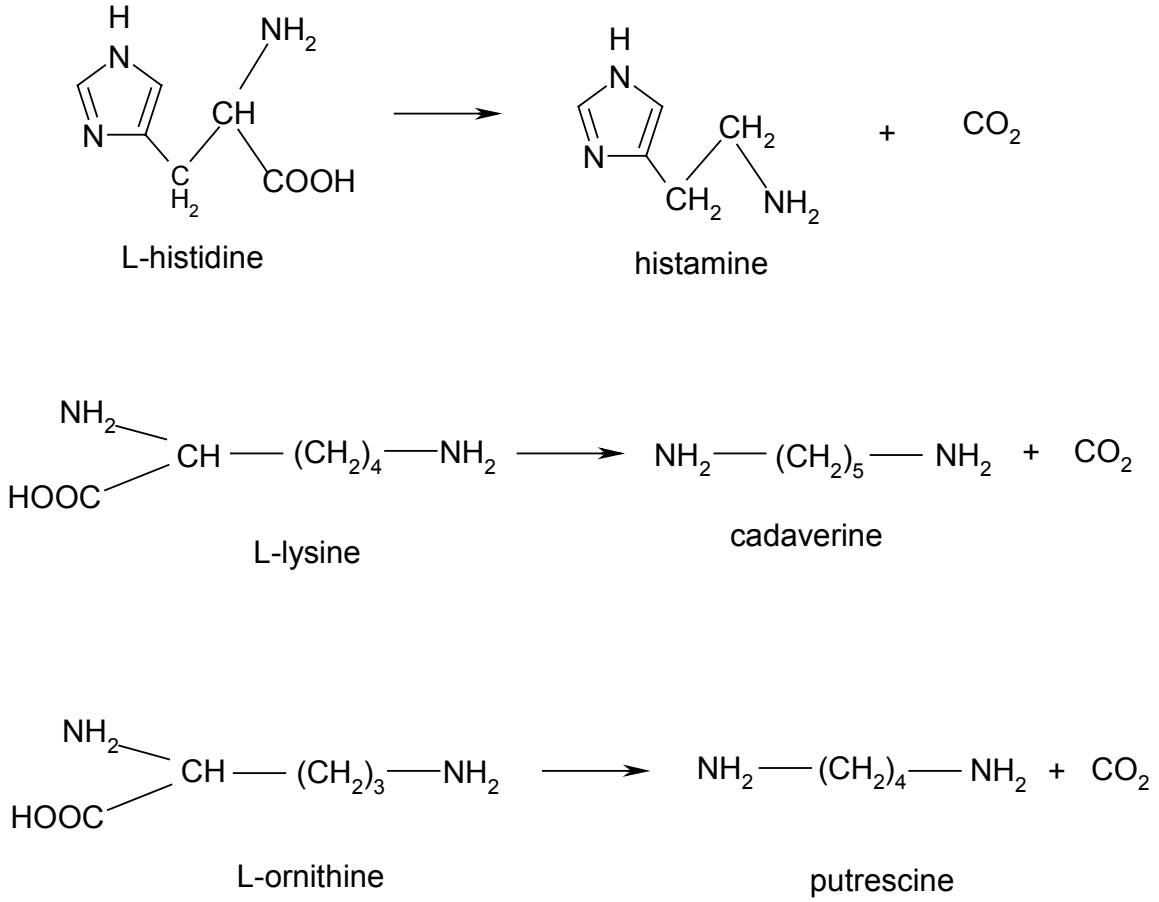


Figure 1. Generation of histamine, putrescine, and cadaverine from their corresponding decarboxylated amino acids.

The formation of biogenic amines can be facilitated by the production of bacteria[4] in foods like cheese, wine, meat, and especially fish[5,6]. In fact, biogenic amines, namely histamine, are used as markers for the degradation of fish[7]. The analysis of biogenic amines is important because the degradation of fish can lead to Scombroid fish poisoning[8], if decomposition is not detected before the fish is sold in markets or prepared in restaurants.

Derivatization of Amines

Although historically amines present difficulties in GC analysis, it is possible to detect them if they are derivatized prior to investigation[2]. There are several advantages

of derivatizing amines in that it enables them to become volatile enough for GC analysis, improves peak shape by reducing tailing, increases sensitivity and selectivity, and enhances overall separation[2]. A number of methods can be employed to derivatize amines such as acylation, silylation, and carbamate formation for example, as Kataoka explains in a comprehensive review[2].

Primary and secondary amines can be derivatized by acylation reactions[2] where an acyl group is added to the amine[2]. This can be achieved via the donation of an acyl group, like an acid anhydride, acyl chloride, acyl imidazole, or an acyl amide[2] (see Figure 2).

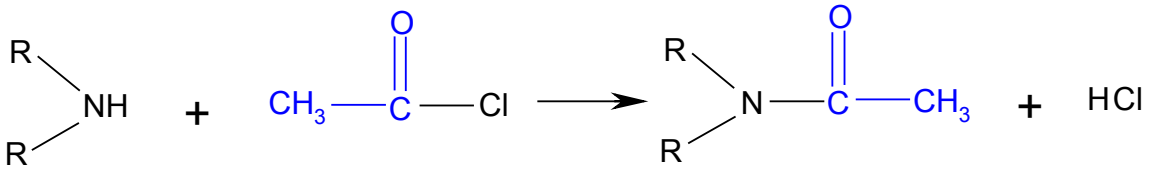
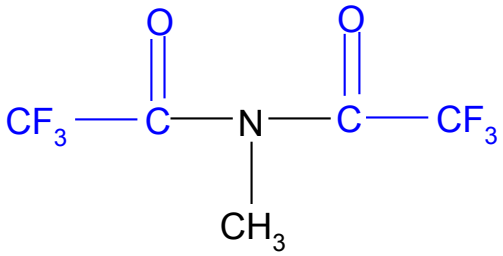


Figure 2. Acylation of a secondary amine by an acyl chloride[2].

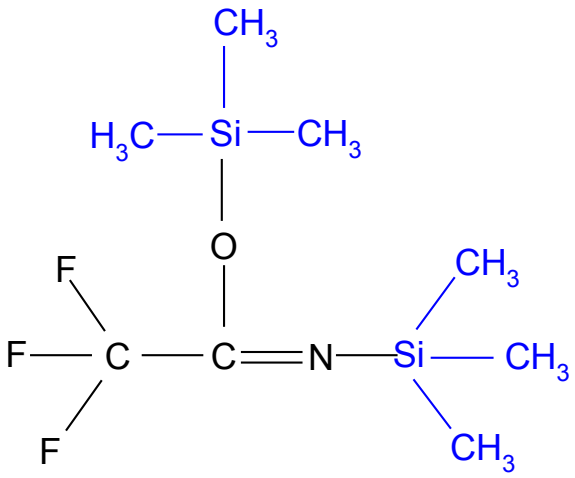
Advantages of using acylation as a derivatization technique for amines are that the reaction occurs readily with mild conditions, and not only amines but other active substituents like phenols, thiols, and hydroxyls can become derivatized[2]. One particularly beneficial acylating reagent is an acylimidazole called N-methyl-bis(trifluoroacetamide) (MBTFA)[2] shown in Strucutre 1.



Structure 1. *N*-Methyl-bis[trifluoroacetamide][9](MBTFA).

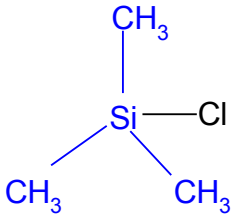
When using MBTFA it is not necessary to remove acid by-products that may destroy the column, as it is when using acid anhydrides and acyl chlorides in acylation reactions[2]. Additionally, MBTFA can be used in conjunction with other derivatizing processes, such as trimethylsilylation, to N-selectively acylate hydroxyamino molecules[10].

A particularly useful reagent of choice for derivatizing amines by silylation is N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA)[11] shown in Structure 2.



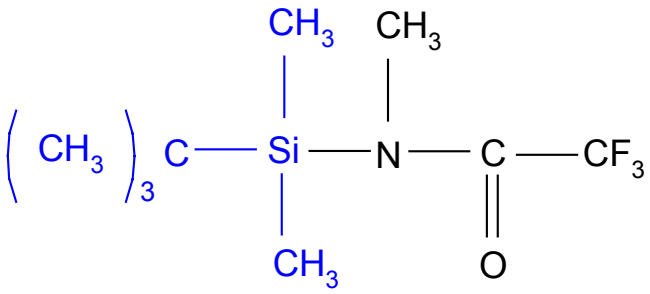
Structure 2. N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA)[9].

As is true with other silylating reagents, BSTFA derivatizes hydroxyl and carboxyl groups under moisture free conditions[2]. Primary amines are more readily derivatized than secondary amines[2]. Trimethylchlorosilane (TMCS), as shown in Structure 3, is an effective catalyst added to silylation reactions to facillitate the amino group derivatization[2]. It should be emphasized that silylation reactions are most effective under anhydrous reaction conditions, and therefore aqueous samples are not recommended for such reactions[2]. It is possible that silyl groups may be added to either or both protons in a primary amine upon derivatization so that mono- and/or di- N-trimethylsilyl (TMS) derivatives may form[2].



Structure 3. Trimethylchlorosilane (TMCS)[9].

Another useful reagent for silylation reactions with amines is N-methyl-N-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA)[2] as shown in Structure 4. The advantage of using MTBSTFA is that it generates derivatives that are orders of magnitude more stable to hydrolysis than TMS derivatives[2]. This is attributed to the protection of the silyl group from moisture by the bulky *tert*-butyl group of the derivative[2]. Generally, *t*-BDMS derivatives are one to two orders of magnitude more sensitive for GC analysis than corresponding TMS derivatives[12].

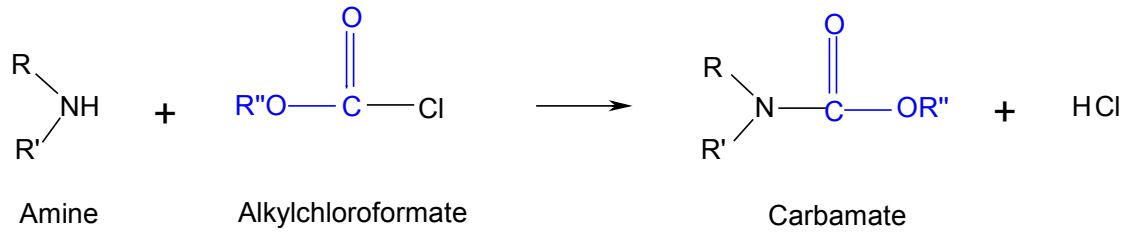


Structure 4. N-methyl-N-(*tert*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA)[2].

Carbamate Derivatives of Amines

One of the most attractive methods used to derivatize amines for GC analysis is through carbamate formation[2]. Primary, secondary, and tertiary amines as well as phenol, thiol, and imidazole groups are derivatized with alkyl chloroformate reagents[2].

In the alkyl chloroformate reaction, an alkyl group is added to the nitrogen of the amine as shown in Figure 3. An advantage of preparing carbamate derivatives is that the technique is not sensitive to moisture so aqueous samples can be derivatized, as demonstrated by Ugland, et al.[13] It is also possible to derivatize tertiary amines using alkyl chloroformate derivatives (see Figure 4).



R"=methyl-, ethyl-, propyl-, butyl-, iso-butyl-, pentafluorobenzyl group

Figure 3. Formation of carbamate derivatives from amines[2].

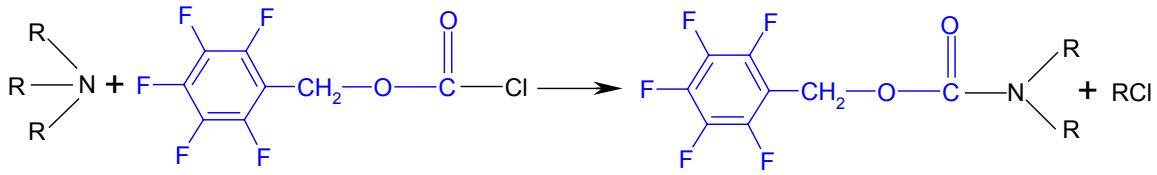


Figure 4. Formation of carbamate derivatives from tertiary amines[2].

The use of chloroformates as derivatizing reagents for amines has been investigated for over thirty years. One of the earlier applications that utilized chloroformate derivatization was demonstrated by Westley and Halpern[14]. In 1968 they successfully derivatized asymmetric amino and hydroxyl groups with menthyl chloroformate and analyzed them by GC[14]. The procedures used by Westley and Halpern called for reaction times of over three hours[14].

Chloroformate reagents can be especially useful in derivatizing problematic tertiary amines[2]. A study conducted in 1976 focused on using

trichloroethylchloroformate to derivatize the tertiary amine pethidine, a pain reliever[15]. In fact trichloroethylchloroformate was found to be a more effective derivatizing reagent than pentafluorobenzyl chloroformate because it reacted three times faster with pethidine[16]. The total derivatization time took around twenty minutes[15]. The detection limits of pethidine in this method were 100 ppb using an electron capture detector[15].

Further experiments have been conducted using various chloroformates in the derivatization of tertiary amines. Pentafluorobenzyl chloroformate has been used to derivatize tertiary amines as was mentioned above[17]. Specific applications where derivatization of tertiary methyl amines is of significance are found in the analysis of drugs containing methylamine[17]. Examples of these medications are imipramine and trimipramine[18], tri-cyclic antidepresants, and diphenhydramine and Recipavrin®, antihistamines[19]. In 1978 the work of Sternson and Cooper showed that tertiary amino compounds not containing the pyridine substituent group reacted well with chloroformate reagents[17]. The time for the reaction was 50 minutes[17]. A gas chromatograph with an electron capture detector was used for analysis, as well as a GC coupled with a mass spectrometer (GC-MS) for further confirmation of the carbamate derivatives[17].

Pentafluorobenzyl chloroformate is not effective for derivatizing dimethyl amine alkane compounds containing a pyridine ring[17]. According to Sternson and Cooper there may be a ring opening reaction involving the pyridine ring and the chloroformate reagent such that derivatization of tertiary methylamines is hindered[17]. A brown tary mass is what resulted in their experiment. It was determined that less than 10% of the dimethylaminoalkane analyte was derivatized to the corresponding carbamate, and the reaction was not reproducible[17].

There are other chloroformate derivatization studies that have been performed using amines as well. For example, in 1992 Hušek et al. were interested in determining whether or not amines and aminoalcohols would react as carboxylic acids do when derivatized with chloroformates[20]. In previous experiments, carboxylic acids had been successfully derivatized into esters using chloroformates[21]. Hušek's earlier work also showed that it was possible to derivatize amino and hydroxy acids with chloroformates[22].

Methylchloroformate (MCF) and ethylchloroformate (ECF) were the reagents of choice for Hušek et al. in the amine and aminoalcohol experiments[20]. There were two

different methods employed in the studies[20]. The first used MCF or ECF to derivatize the amines and chloroform as the organic liquid extractant[20]. The second method incorporated a mixture of acetonitrile-water-methanol-pyridine before the addition of either MCF or ECF and then lastly adding chloroform for the organic liquid extraction[20]. It should also be noted that both methods used sodium bicarbonate in the derivatization of amines and aminoalcohols, however in the second method the sodium bicarbonate was added last while in the first method it was added first to the amine sample[20].

As the results of the Hušek study indicated, both methods were successful in the derivatization of the analytes of interest[20]. There were some differences in the derivatives of spermine and spermidine in that reaction yields were acceptable with the use of only method one[20]. Method two was the preferred technique for derivatization of the acidic analytes[20]. An interesting note is that carboxylic acid groups next to alcoholic groups can be esterified[20]. On side chains of the compounds of interest the primary amines react more readily than the secondary amines, which require a longer reaction time[20]. Esterification of the phenolic groups in the molecules was also shown to occur[20]. In general, the reaction time for the derivatization was around ten minutes[20].

The goal of the study conducted in this thesis was to develop a rapid, reproducible, trace level quantitative method to derivatize and detect biogenic amines by GC/FID and GC/MS.

Chapter 2-Experimental

Chemicals Used

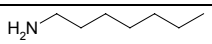
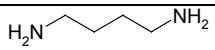
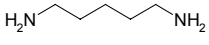
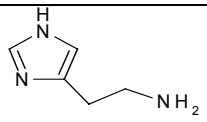
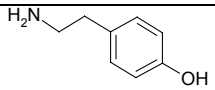
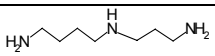
The following biogenic amines were analyzed: heptylamine, putrescine, cadaverine, histamine, tyramine, and spermidine. All standard amine mixtures were prepared using HPLC grade water (Burdick & Jackson, Muskegon, MI) and a Nalgene chemical-resistant propylene 50 mL volumetric flask. The purity of the standards and their manufacturers are listed in Table 1.

Table 1. Six-Amine Standard Solution

Amine	**Purity**	**Supplier**
Heptylamine	99%	Aldrich; Milwaukee, WI
Putrescine	98%	Sigma-Aldrich; St. Louis, MO
Cadaverine	95%	Aldrich; Milwaukee, WI
Histamine	96%	Aldrich; Milwaukee, WI
Tyramine	99%	Aldrich; Milwaukee, WI
Spermidine	99%	Aldrich; Milwaukee, WI

The structures, molecular weight, boiling points, and pKa's for the above amines are shown in Table 2.

Table 2. **Structures of Six Amines**

Amine	Molecular Weight (amu)	Boiling Point (°C)	Structure	pK_a1[23], pK_a2
Heptylamine	115.22	154-156		10.67
Putrescine (1,4-butanediamine)	88.15	158-160[23]		9.35, 10.80
Cadaverine (1,5-pentanediamine)	102.18	178-180		10.05, 10.93
Histamine	111.15	167		6.04, 9.75
Tyramine	137.18	175-181		9.74, 10.52
Spermidine	145.25[23]	128-130		10.51[24]

Chloroformate Derivatization of Amines

Carbamate derivatives of the biogenic amines listed above were created using propyl chloroformate as the derivatizing reagent. The derivatization procedure was based on the methodology of Ugland et al[13]. All of the following procedures were preformed at room temperature. To a 200µL aliquot of a standard amine solution (varying concentrations), 200µL of a 1:4 mixture of chloroform (HPLC grade; Burdick & Jackson, Muskegon, MI) and iso-octane (certified A.C.S.; Fisher Scientific Company, Fair Lawn, NJ) was added to the plastic sample prep vial. To ensure that the solution remained at a constant pH, 50µL of pH 12.2 K_2CO_3-$KHCO_3$ buffer was also added to the mix. Finally, 1µL of propyl chloroformate (98% purity, Aldrich, St. Louis, MO) was added to the solution. The solution was homogenized by a vortex mixer (Barnstead/Thermolyne, Maxi Mix Plus; Dubuque, IA) for 1 minute. Next the sample vial was placed in an IEC Spinette Centrifuge (Needham Heights, MA) for 5 minutes. Prior to GC analysis, 100µL of the top organic layer was diluted by removing and depositing it into a glass sample vial, containing 400µL of the chloroform/iso-octane solvent mixture. One microliter of this solution was injected into the GC.

GC/FID Analysis

A Phenomenex ZB-5 (15m x 0.25mm id x 1.00μm d_f) capillary column (Torrance, CA) was installed in an Hewlett Packard 6890 Gas Chromatograph (Little Falls, DE) equipped with a flame ionization detector. The column was installed into a Cold-On-Column (COC; Hewlett Packard, Palo Alto, CA) inlet suitable for a 0.25mm I.D. column. This column was conditioned at 300°C overnight to ensure it was clean. Blank runs were made before any samples were injected to further ensure that the system was stable and uncontaminated. The GC conditions are listed in Table 3.

Table 3. **GC Conditions**

Temperature Program	50°C (1 min hold) to 280°C (5 min hold) @20°C/min
Carrier Gas	Helium-59cm/sec
Injection Volume	1μL,manual injection
Inlet Setting	Oven Tracking
Detector	FID at 250°C

One microliter of the derivatized amine standard was injected into the GC manually for five replicate injections. It should also be noted that standard solutions of each individual amine were analyzed by GC/FID and GC/MS to confirm the identities of the peaks in the mix based on retention times and spectra. The percent Relative Standard Deviation (%RSD) was calculated using peak areas for all replicate injections.

GC/MS Analysis

An Agilent HP5-MS (15m x 0.25mm id x 0.25μm d_f) column was installed in an HP-6890 GC coupled with an HP-5973 Mass Selective Detector. Before the column was connected to the MSD it was conditioned overnight at 300°C. A 50 ppm derivatized amine standard was injected into the GC/MS for analysis and confirmation of the identity of the derivatives. The conditions used are specified in Table 4. The GC/MS system was

not equipped with a cold-on-column inlet so a hot inlet was used with a 2 mm internal diameter, straight tube liner not containing glass wool.

Table 4. GC/MS Conditions

Temperature Program	50°C (1 min hold) to 280°C (5 min hold) @20°C/min
Carrier Gas	Helium-59cm/sec
Injection Volume	1μL,manual injection
Injection Port Temperature	200°C
Split Ratio	30:1
Transfer Line	280°C

Calibration Curves

A standard amine mix of 2000 ppm was prepared in a 50 mL volumetric Nalgene flask. The 2000 ppm solution was diluted using serial dilution to produce the following concentrations: 500, 250, 100, and 50 ppm. Each of these solutions was derivatized and diluted as specified earlier. Exact concentrations of each component in the standard mix are shown in Table 5. Then the derivatives were analyzed by GC/FID with the conditions described in Table 3. The RSD was calculated and calibration curves were constructed (see Chapter 4) based on these trace concentrations run five times each.

Table 5. Concentration of Amine Standards in Calibration Curves

Amine	Level 1 (ppm)	Level 2 (ppm)	Level 3 (ppm)	Level 4 (ppm)
Heptylamine	78	39	16	7.8
Putrescine	88	44	18	8.8
Cadaverine	87	44	18	8.7
Histamine	91	45	18	9.1
Tyramine	93	47	19	9.3
Spermidine	93	46	19	9.3

Atlantic Salmon Extraction and Derivatization

A fillet of fresh Atlantic Salmon was purchased from Kroger for a five day study. After purchase it was immediately brought into the lab and analyzed as descrbed below. The method is based on part in work done by Antoine et al[25]. It should be noted that the salmon was analyzed in duplicate. Fifteen grams of the salmon were weighed on a Mettler AE 260 Delta Range Scale (Columbus, OH). The fish was then chopped up into very small fragments with a knife and placed into a plastic Nalgene bottle, which contained 50 mL of methanol (Burdick and Jackson, Muskegon, MI) and 50 mL of HPLC grade water. The solution in this bottle was homogenized by vortex mixing for two minutes. The salmon mix was then placed into a 45°C water bath for 45 minutes. Next the extract was cooled to 30°C in a cold water bath. Upon cooling, a portion of the mix was centrifuged in plastic tubes for 20 minutes. Then 200 μL of the supernate was used for derivatization by the same propyl chloroformate derivatization method used on the standard amines, however the sample was not diluted 1:5 after derivatization. Lastly, the derivatized salmon extracts were analyzed by the GC/FID method five times and one time by the GC/MS method. The entire salmon extraction and derivatization process was repeated in duplicate on days three and five. After the initial salmon analysis on day one and until day five, the salmon fillet was stored at 4°C. The resulting data from the salmon study is included and analyzed in Chapter 5-Salmon Analysis Results.

Chapter 3-GC/MS Results of Standards

Introduction

It was necessary to perfom GC/MS analysis on the biogenic amines once they had been derivatized to confirm that the derivatives actually existed. It was not sufficient to use only single standards of each derivatized biogenic amine and retention times for confirmation of peak identity because it would not have ensured that the peak was actually the propyl chloroformate derivative and not a contaminant or degradation product. To my knowledge there is no spectra libraray containing the propyl chloroformate derivatives of the biogenic amines examined in this study. Therefore, it was important to perform a comprehensive analysis of the derivatized amine ion fragments.

Amine Standard Derivatized

The chromatogram of the 50 ppm derivatized amine standard is shown in Figure 5. There are eleven major peaks that appear, as well as a few minor ones that should be examined. Analysis of individual peaks is discussed in depth in this chapter.

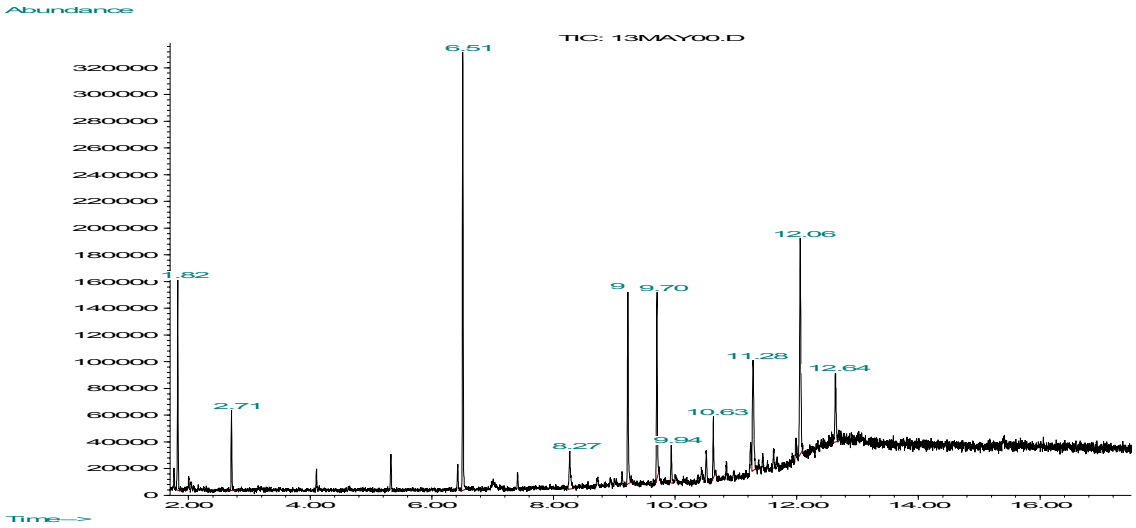


Figure 5. 50 ppm Amine Standard Derivatized (heptylamine 6.51 min, putrescine 9.22 min, cadaverine 9.70 min, histamine 9.94 min, tyramine 10.63 min, spermidine 12.64 min).

Heptylamine Derivative

The chromatogram above has been blown up to show the heptylamine derivative peak at 6.51 minutes (see Figure 6). The peak shape is gaussian with no evidence of fronting or tailing. There is a small peak preceding the heptylamine peak, which may be an impurity.

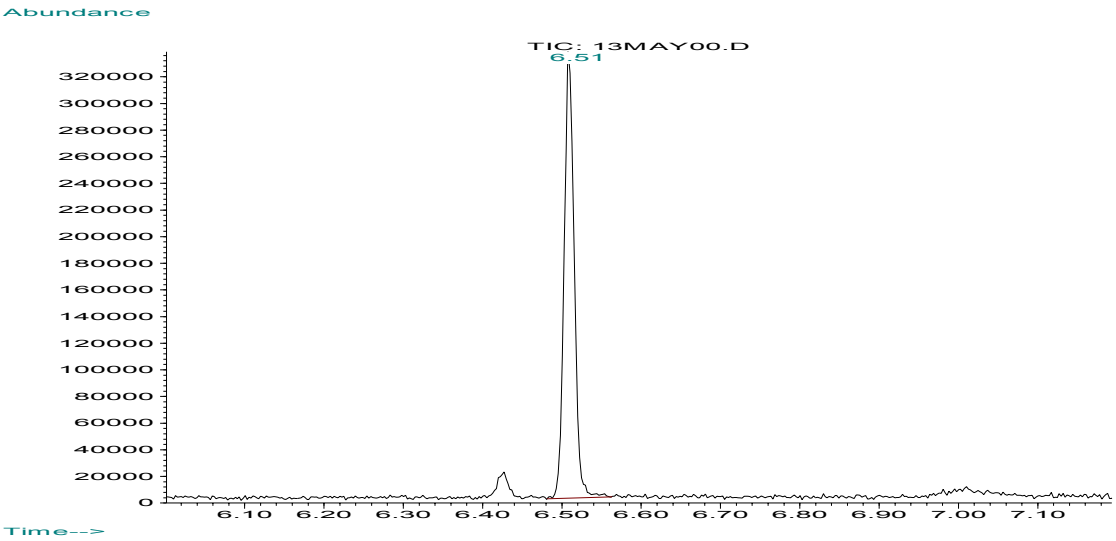


Figure 6. Derivatized heptylamine, 6.51 minutes.

Upon examination of the heptylamine GC/MS spectra it is apparent that heptylamine did in fact become derivatized. As shown in Figure 7 there are several main ions to consider.

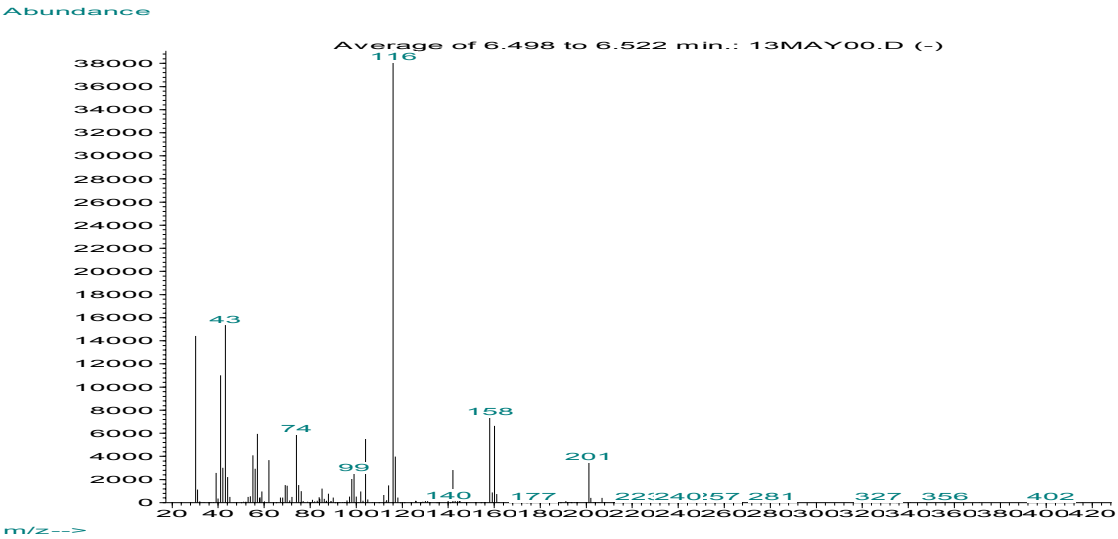
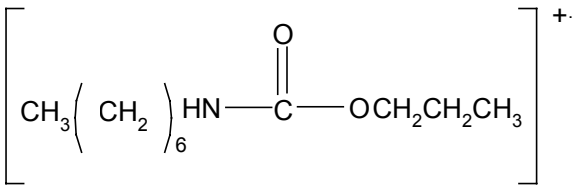
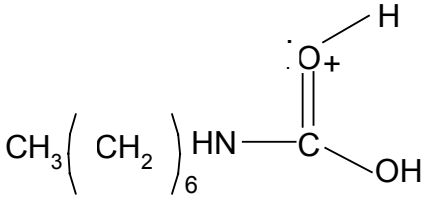
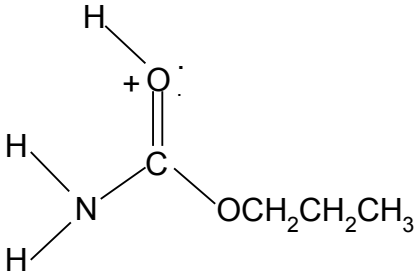
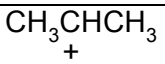


Figure 7. Heptylamine derivative GC/MS spectra.

The fragments and ions for the heptylamine derivative are listed in Table 6.

Table 6. Derivatized Heptylamine-Proposed Fragment Ions

Fragment	Ion (molecular weight)	Suggested Structure
Molecular Ion	201	
Derivative Fragment Ion	160	
Derivative Fragment Ion	104	
Derivatizing Reagent Fragment Ion	43	

Putrescine Derivative

The putrescine peak at 9.22 minutes is shown in Figure 8.

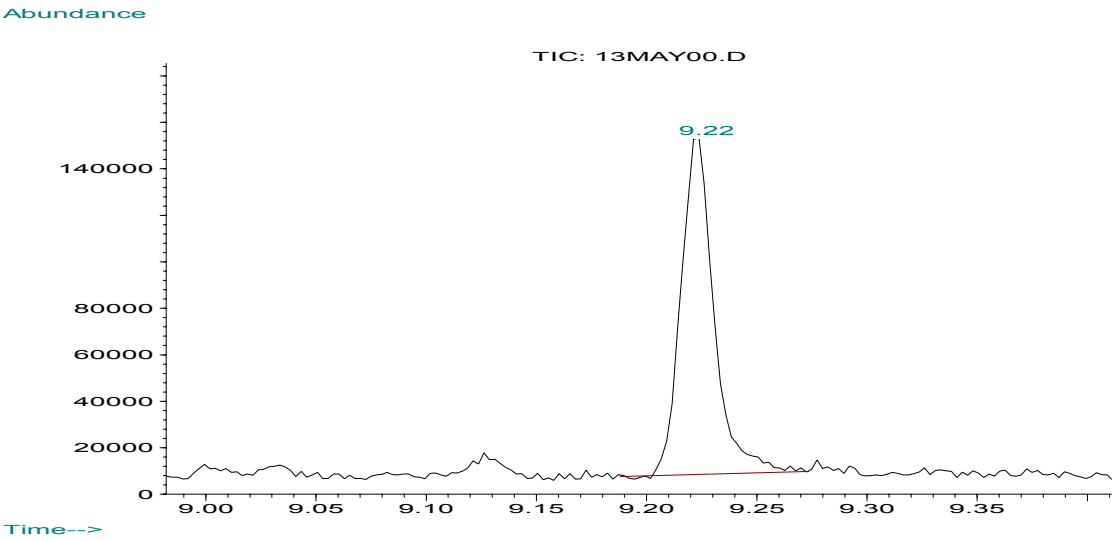


Figure 8. Derivatized Putrescine, 9.22 minutes.

There is minimal tailing and no fronting on the analyte peak. There does not appear to be evidence of degradation products. The spectra for putrescine contains fragments as proof that the derivative was formed (see Figure 9).

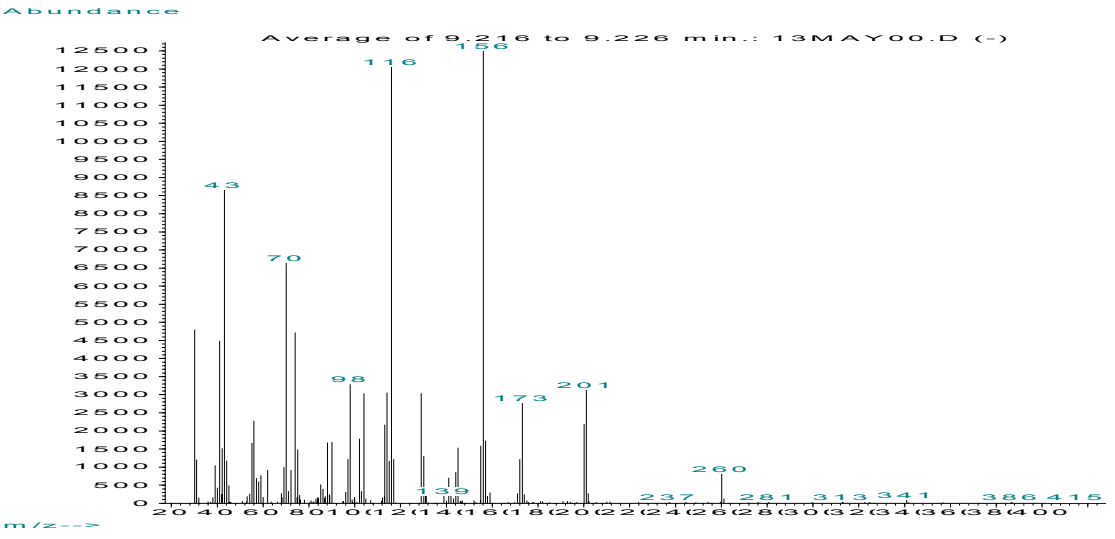
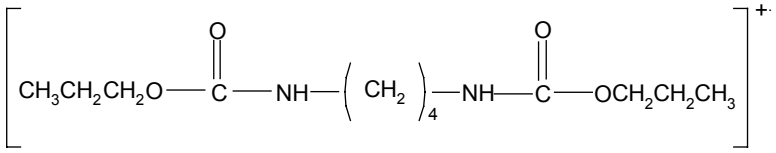
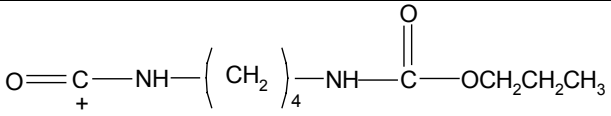
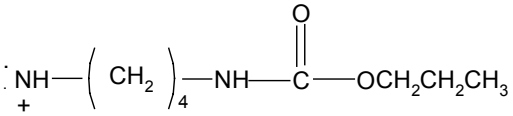
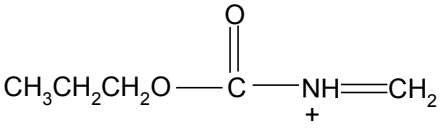
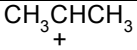


Figure 9. Putrescine derivative GC/MS spectra.

The fragments of the putrescine derivative are located in Table 7. It is clear that the molecular ion is present. The remaining derivative fragments listed are further evidence that the parent compound was broken apart in the GC/MS analysis.

Table 7. Derivatized Putrescine-Proposed Ion Fragments

Fragment	Ion (molecular weight)	Suggested Structure
Molecular Ion	260	
Derivative Fragment Ion	201	
Derivative Fragment Ion	173	
Derivative Fragment Ion	116	
Derivatizing Reagent Fragment Ion	43	

Cadaverine Derivative

The enlarged in cadaverine peak from Figure 5 is shown in Figure 10. There is no fronting and only slight tailing on the analyte peak. Also, there do not appear to be any

degradation or decomposition product peaks from cadaverine. As the GC/MS spectra in Figure 11 shows there are several ions of interest that confirm the prescence of derivatized cadaverine.

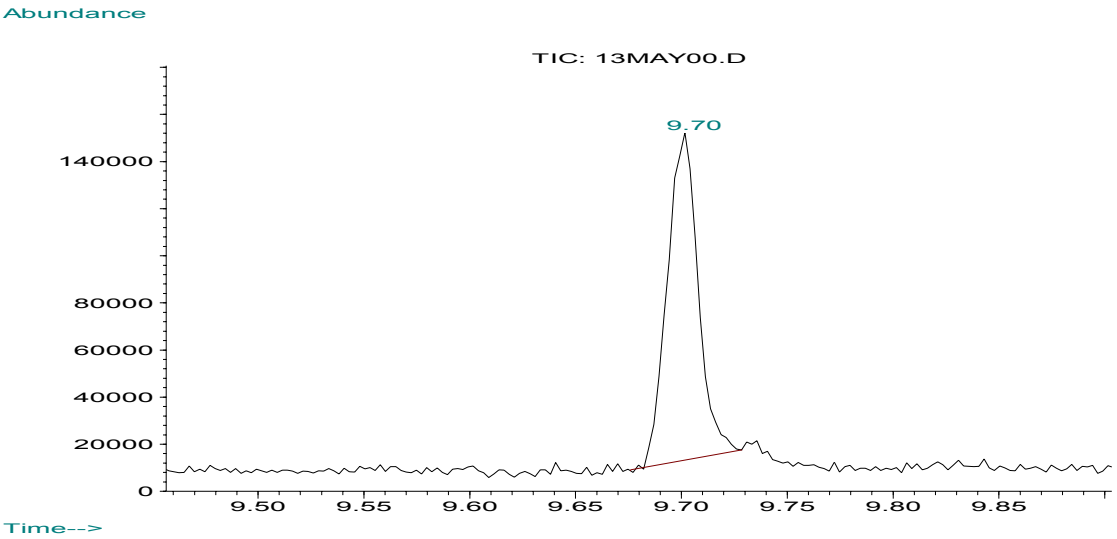


Figure 10. Derivatized Cadaverine, 9.70 minutes.

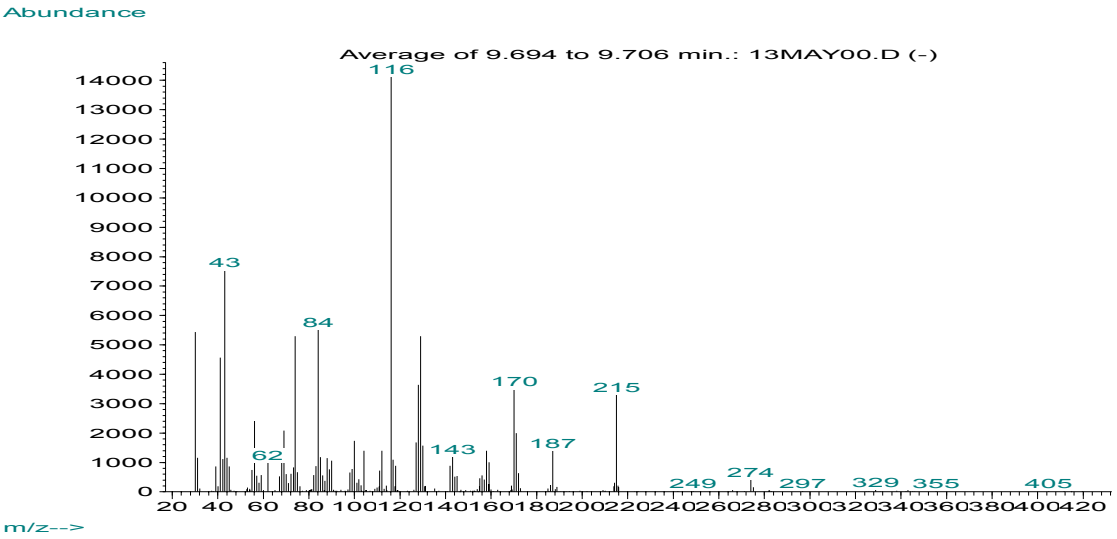
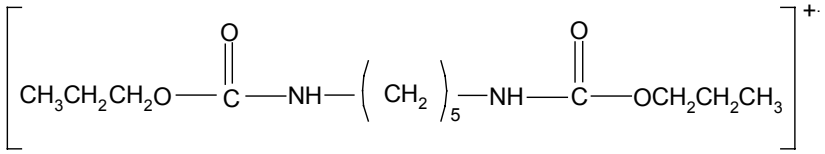
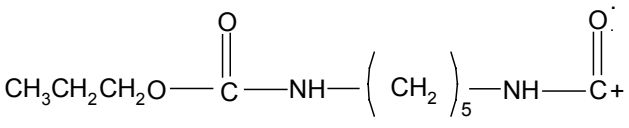
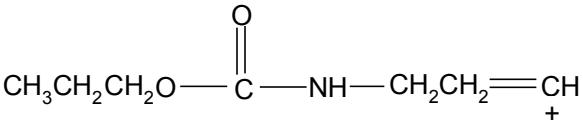
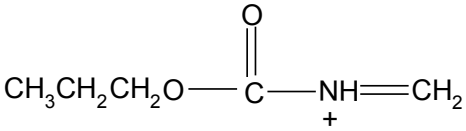
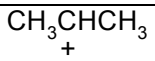


Figure 11. Cadaverine derivative GC/MS spectra.

Table 8 shows the fragment structures of the cadaverine derivative GC/MS spectra.

Table 8. Derivatized Cadaverine-Proposed Ion Fragments

Fragment	Ion (molecular weight)	Suggested Structure
Molecular Ion	274	
Derivative Fragment Ion	215	
Derivative Fragment Ion	143	
Derivative Fragment Ion	116	
Derivatizing Reagent Rragment Ion	43	

Histamine Derivative

The histamine peak from Figure 5 is shown in Figure 12. The peak shape is good with no fronting or tailing.

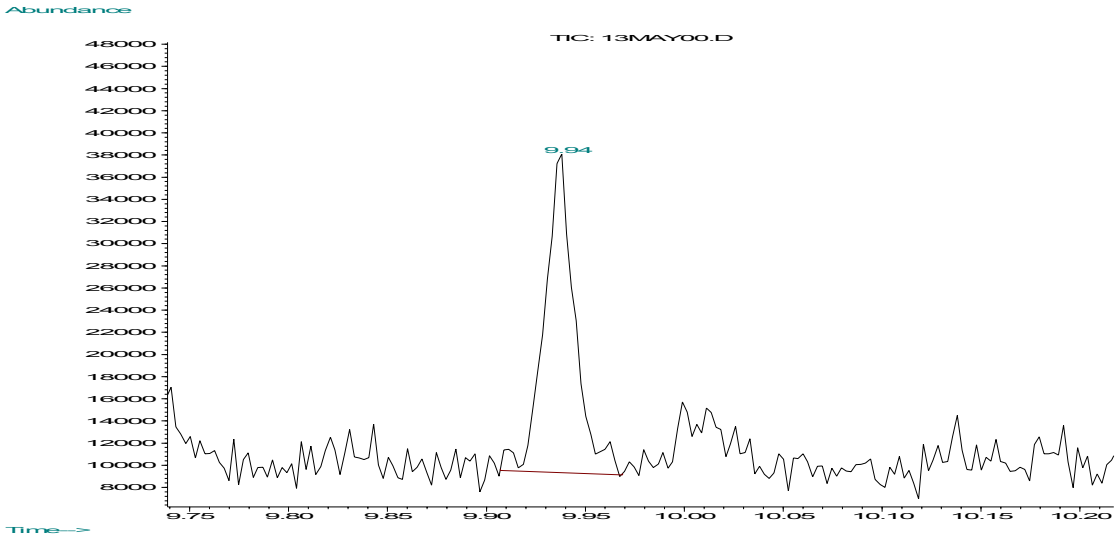


Figure 12. Derivatized Histamine, at 9.94 minutes.

The GC/MS spectra (Figure 13) from the histamine derivative peak is proof that histamine was derivatized. shows the histamine derivative fragments. Histamine is one of the more difficult analytes to derivatize because it contains primary, secondary, and tertiary amines. It is interesting to note that the spectra (Figure 13) provides evidence to confirm that two of the three nitrogens in histamine were derivatized with the propyl chloroformate reagent.

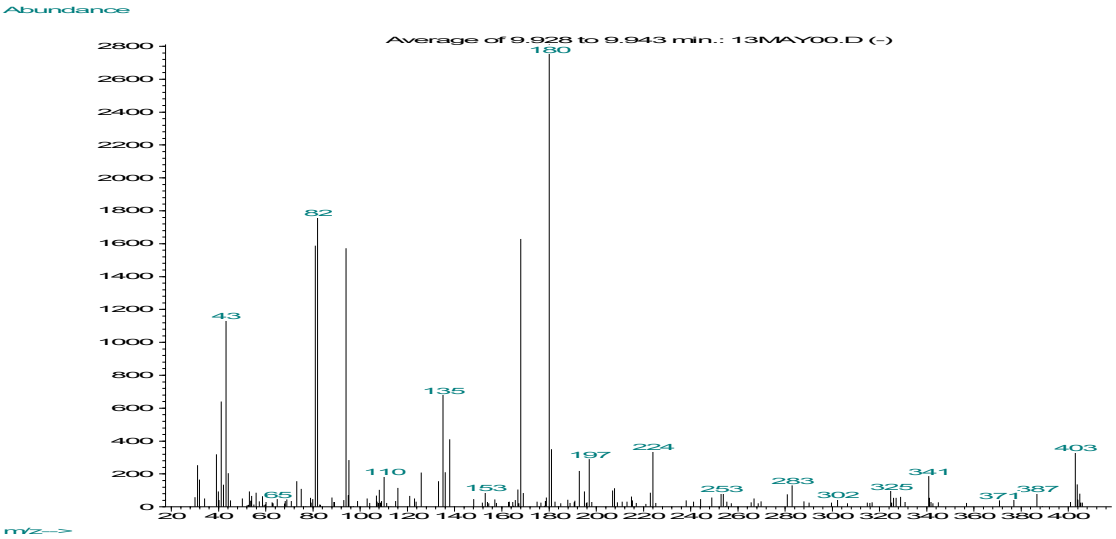
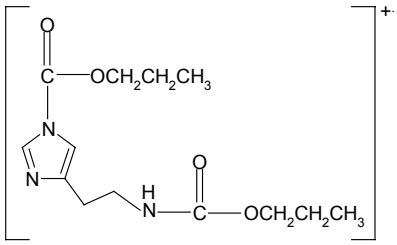
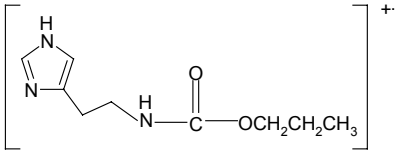
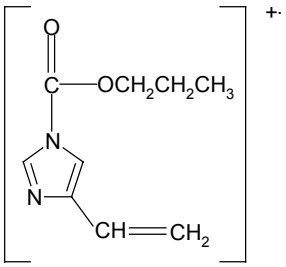
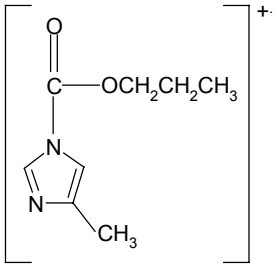
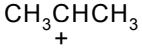


Figure 13. Histamine derivative GC/MS spectra.

Table 9. Derivatized Histamine-Proposed Ion Fragments

Fragment	Ion (molecular weight)	Suggested Structure
Molecular Ion	283	
Derivative Fragment Ion	197	
Derivative Fragment Ion	180	
Derivative Fragment Ion	168	
Derivatizing Reagent Fragment Ion	43	

Tyramine Derivative

The tyramine derivative peak from Figure 5 is shown (Figure 14). There is a small amount of tailing on the peak and no fronting.

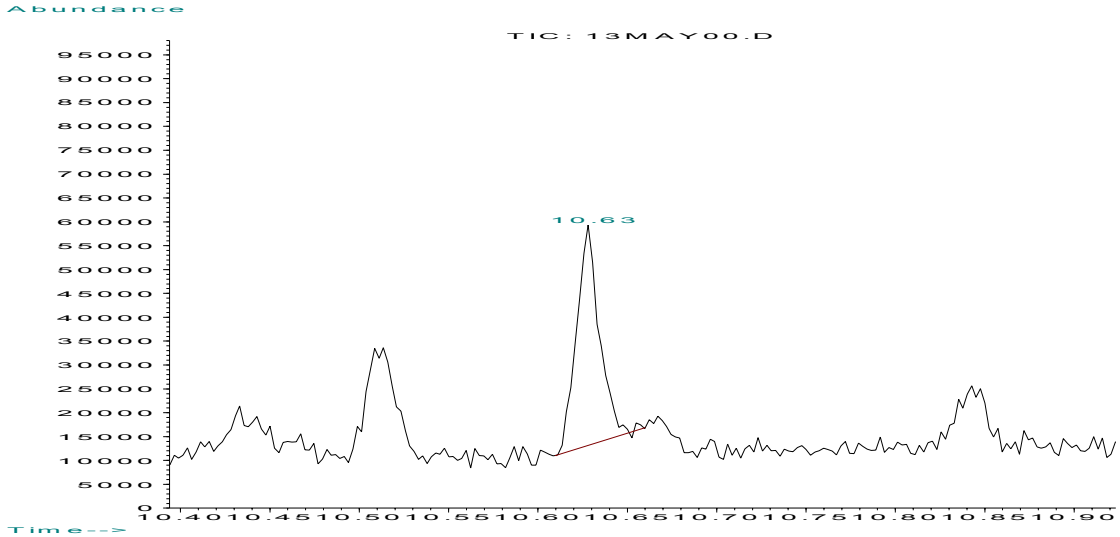


Figure 14. Derivatized Tyramine, 10.63 minutes.

The following GC/MS spectra is sufficient proof to show that tyramine was derivatized (Figure 15). Further analysis of the spectra (Table 10) shows the various fragments of the tyramine derivative.

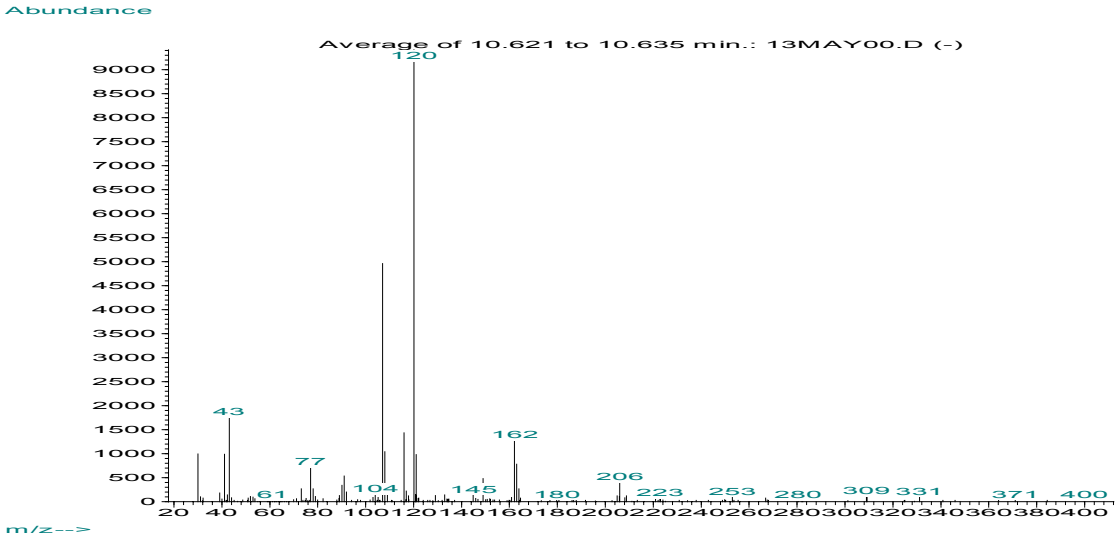
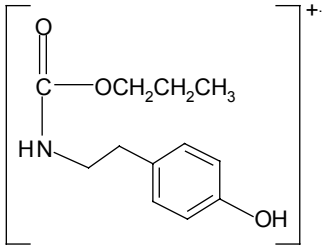
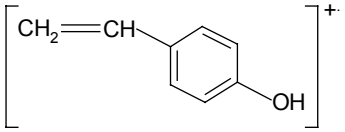
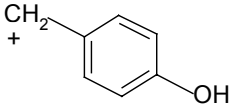
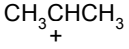


Figure 15. Tyramine derivative GC/MS spectra.

Table 10. Derivatized Tyramine-Proposed Ion Fragments

Fragment	Ion (molecular weight)	Suggested Structure
Molecular Ion	223	
Derivative Fragment Ion	120	
Derivative Fragment Ion	107	
Derivatizing Reagent Fragment	43	

For tyramine only the amine, and not the hydroxyl group, was derivatized.

Spermidine Derivative

The spermidine peak from Figure 5 is shown zoomed in below in Figure 16. The peak has good shape with no fronting or tailing.

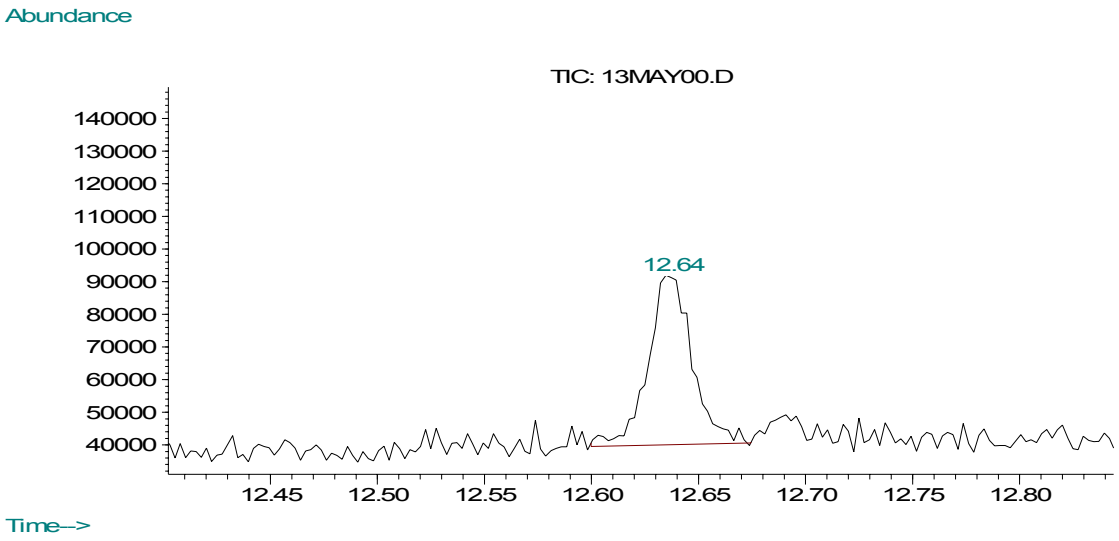


Figure 16. Derivatized Spermidine, 12.64 minutes.

The GC/MS spectra for the spermidine peak contains significant information as proof that spermidine was derivatized (Figure 17). The derivative fragments from the spectra are listed in Table 11.

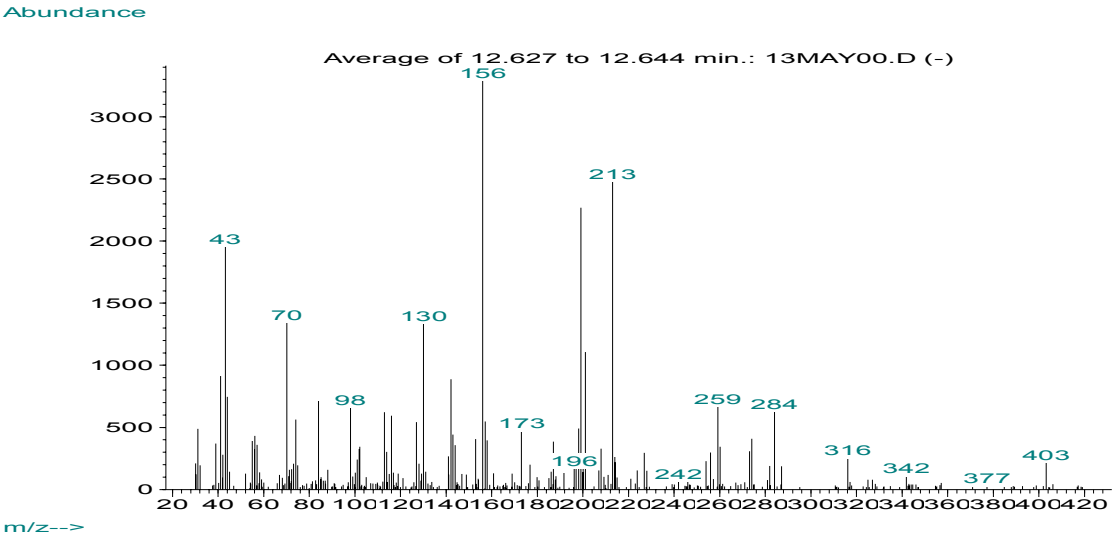
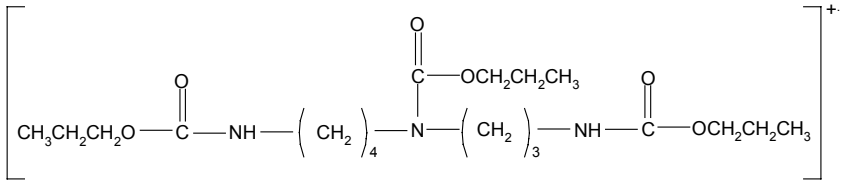
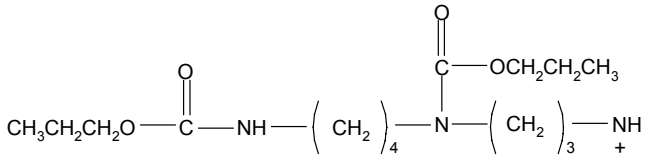
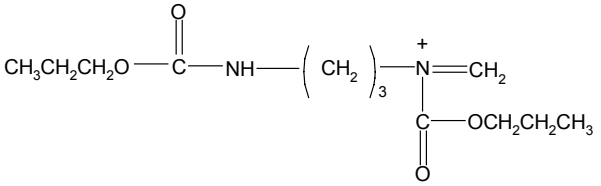
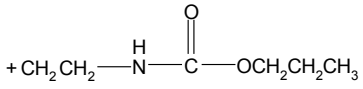
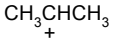


Figure 17. Spermidine derivative GC/MS spectra.

Table 11. Derivatized Spermidine-Proposed Ion Fragments

Fragments	Ion (molecular weight)	Suggested Structure
Molecular Ion	403	
Derivative Fragment Ion	316	
Derivative Fragment Ion	259	
Derivative Fragment Ion	130	
Derivatizing Reagent Fragment Ion	43	

Derivative Blanks

There are several peaks that appear in Figure 5 (retention times 1.82, 2.71, 8.27 minutes) that are not amine derivatives or degradation products. These peaks, as well as

a few smaller ones, appear in Figure 18 of a derivative blank that was run after it was diluted 5:1 in the organic solvent mix iso-octane/chloroform. The blank included the same amounts of reagents and water, but not the amines. The derivative blank was prepared the same as all other derivatives. The peak at 1.87 minutes is a xylene that shows up in most runs and the peak at 2.72 minutes is identified as propyl carbonate. The remaining peaks in Figure 18 are a variety of siloxane peaks most likely from the column or septa that did not show up regularly in other analyses.

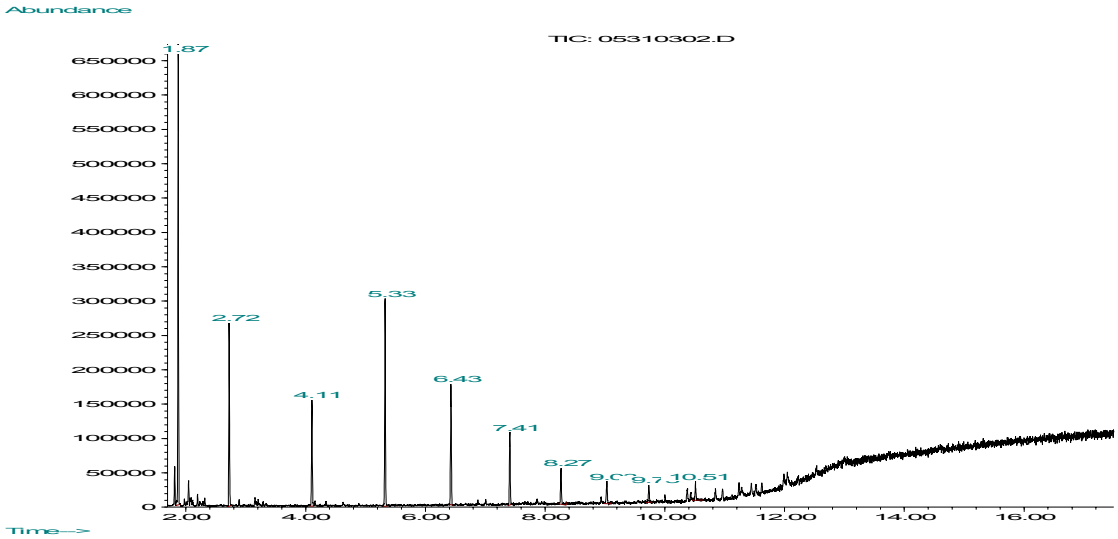


Figure 18. Derivative blank run by GC/MS, diluted 5:1.

One additional derivative blank was run that had been treated in the same way as the derivative above, except that is was not diluted 5:1. The resulting chromatogram is shown in Figure 19. There are several other peaks that appear in Figure 5, which can be explained by this blank derivative run (retention times 1.82, 2.71, 11.28, and 12.06 minutes). These peaks are not amine derivatives or degradation products, however they do appear consistently in almost all derivatives run by either GC/MS or GC/FID (results in Chapter 4-GC/FID Results of Standards).

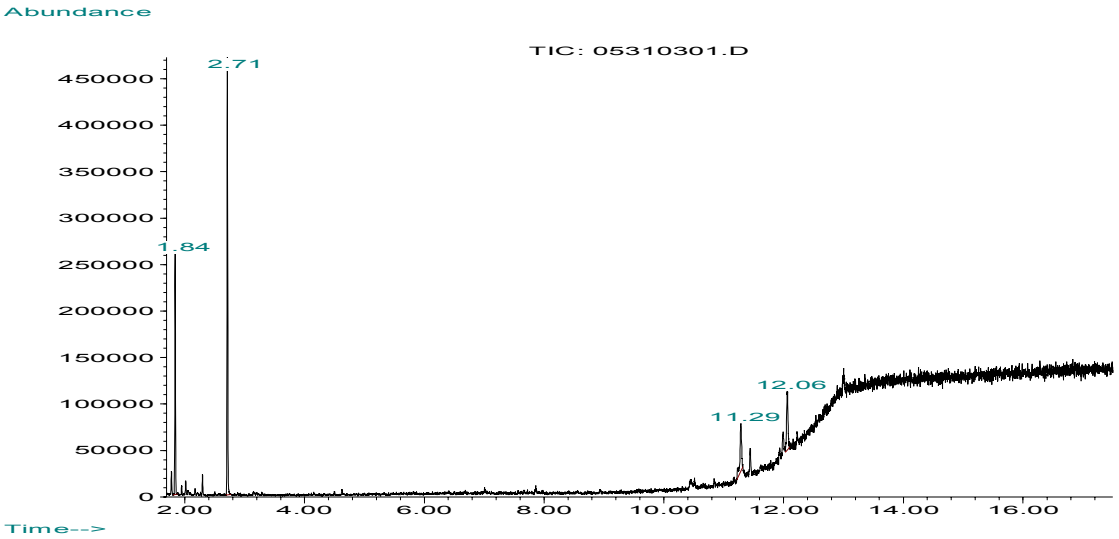


Figure 19. Derivative blank run by GC/MS, not diluted 5:1.

Chapter 4-GC/FID Results of Standards

Calibration Curves

A calibration curve was constructed using the following concentrations: 100, 50, 20, and 10 ppm. The results of the 100 ppm amine standard are listed in Table 12.

Table 12. 100 ppm Amine Standard

Data File	77.7ppm Hep 8.30min	87.7ppm Put 11.11min	87.3ppm Cad 11.61 min	90.7ppm His 11.88 min	93.3ppm Tyr 12.59 min	92.5ppm Spe 16.58 min
06240301.d	818.7	317.1	427.5	82.1	100.7	285.6
06240302.d	883.9	344.8	463.0	85.8	109.7	313.4
06240303.d	943.3	371.0	496.7	89.3	117.4	337.0
06240304.d	963.5	377.8	505.8	87.4	119.8	346.7
06240305.d	1027.8	403.6	539.8	90.5	127.8	371.3
Average	927.4	362.9	486.6	87.0	115.1	330.8
Std Dev	79.6	33.1	42.9	3.3	10.3	32.7
%RSD	8.6	9.1	8.8	3.8	9.0	9.9

The % RSD for the amines is good at less than 10%. The resolution and peak shapes are good with slight fronting on heptylamine, putrescine, and cadaverine (Appendix, Figure 32). There are two peaks at 13.41 and 14.89 minutes that are unknown, but they appear consistently in the runs and are also in the derivative blank run (Appendix, Figure 33). The signal to noise ratios for the 100 ppm standard are all equal to or greater than 20:1. Table 13 lists the results of the 50 ppm standard amine mix. The % RSD's are still below 10% for all of the amines and even below 7% for all amines except histamine.

Table 13. 50 ppm Amine Standard

Data File	38.9ppm Hep 8.30 min	43.9ppm Put 11.11 min	43.7ppm Cad 11.61 min	45.4ppm His 11.88 min	46.7ppm Tyr 12.59 min	46.3ppm Spe 16.58 min
06250324.d	400.8	234.0	304.9	69.7	73.8	199.3
06250325.d	390.6	228.1	296.8	62.8	69.5	195.0
06250326.d	360.1	208.5	274.4	56.7	66.4	177.0
06250327.d	362.7	210.9	276.8	60.5	66.0	177.0
06250328.d	360.2	208.2	274.6	54.2	65.5	175.7
Average	374.9	217.9	285.5	60.8	68.2	184.8
Std Dev	19.4	12.2	14.3	6.0	3.5	11.4
%RSD	5.2	5.6	5.0	9.9	5.1	6.2

The peak shapes and resolution are good (see Appendix, Figure 34). There does not appear to be any fronting or tailing. At 50 ppm the signal to noise ratios for the standard amines are all equal to or greater than 20:1.

A set of data was run at 50 ppm using the same GC/FID conditions as listed in Table 3 except that there was no oven tracking because the inlet used was not COC, but a hot inlet at 200°C. The liner used was a 2 mm inner diameter, straight tube, with no glass wool. The results of the study are listed in Table 14 and a representative chromatogram is shown in the Appendix, Figure 35. The spermidine peak is not detected in this set of runs and the tyramine peak shape is very broad, tailing, and there appears to be a doublet. Overall, the %RSD is at least 9% higher than the 50 ppm sample run COC. Also, the peak areas using the COC inlet are an order of magnitude higher than they are for the hot inlet. The poor results of the hot inlet study may be attributed to thermal degradation of the carbamate derivatives in the inlet. It would have been very difficult and perhaps impossible to use a hot inlet for quantitative analysis with the amine derivatives and still achieve the same low limits of detection seen here (10ppm). Therefore, COC was employed as the inlet of choice for the quantitative portion of the analysis.

Table 14. 50 ppm Amine Standard*, Hot Inlet 200°C

Data File	38.9ppm Hep 8.86 min	43.9ppm Put 11.97 min	43.7ppm Cad 12.52 min	45.4ppm His 12.85 min	46.7ppm Tyr 13.94 min	46.3ppm Spe 16.58 min
06290303.d	16.6	5.9	7.8	NQ	12.5	ND
06290304.d	18.6	6.7	8.6	NQ	7.3	ND
06290305.d	14.2	5.0	6.8	NQ	7.0	ND
06290306.d	22.3	7.8	9.8	NQ	6.3	ND
06290307.d	14.2	4.5	6.1	NQ	5.4	ND
Average	17.2	6.0	7.8		7.7	
Std Dev	3.4	1.3	1.5		2.8	
%RSD	19.8	22.1	18.7		36.1	

*NQ-not quantitatable, ND-not detectable

The 20 ppm standard amine mix results are shown in Table 15. The %RSD for the amines is good in the range from 2.4% to 5.3%. The peak shapes and resolution (see Appendix, Figure 36) are still good with signal to noise ratios greater than or equal to 13:1.

Table 15. 20 ppm Amine Standard

Data File	15.5ppm Hep 8.29min	17.5ppm Put 11.11min	17.5ppm Cad 11.61min	18.1ppm His 11.87min	18.7ppm Tyr 12.59min	18.5ppm Spe 16.57min
06250301.d	158.7	146.2	179.8	45.9	44.1	126.7
06250302.d	149.5	138.2	170.6	40.9	43.1	121.6
06250303.d	150.2	139.1	171.8	44.1	44.9	122.6
06250304.d	157.5	144.7	179.1	41.6	46.3	129.1
06250305.d	150.8	140.8	172.2	40.7	44.3	124.5
Average	153.3	141.8	174.7	42.6	44.5	124.9
Std Dev	4.4	3.5	4.4	2.3	1.2	3.1
%RSD	2.9	2.5	2.5	5.3	2.6	2.4

The last set of data for the calibration curve is the 10 ppm standard, which is shown in Table 16. The %RSD is consistantly good between 3.2% and 5.8% at the 10 ppm level. The peaks are gausssian and there are some extra peaks and drifting baseline toward the end of the run that are amplified at this low concentration (see Appendix, Figure 37). Quantitation is still possible at this level based on a signal to noise ratio of 12:1 or better for all standard amines.

Table 16. 10 ppm Amine Standard

Data File	7.8ppm Hep 8.29min	8.8ppm Put 11.12min	8.7ppm Cad 11.62min	9.1ppm His 11.89min	9.3ppm Tyr 12.61min	9.3ppm Spe 16.61min
06250306.d	70.4	79.1	96.9	28.0	30.6	64.1
06250307.d	67.9	76.2	95.0	25.4	30.1	61.8
06250308.d	62.8	70.3	90.6	28.8	28.1	57.0
06250309.d	68.7	77.3	97.2	27.8	29.9	62.0
06250310.d	68.8	77.7	99.5	29.8	30.1	64.0
Average	67.7	76.1	95.8	28.0	29.8	61.8
Std Dev	2.9	3.4	3.3	1.6	1.0	2.9
%RSD	4.3	4.5	3.5	5.8	3.2	4.7

The calibration curve for heptylamine is shown in Figure 20. The linearity is good with a correlation coefficient of $R^2 = 0.9911$.

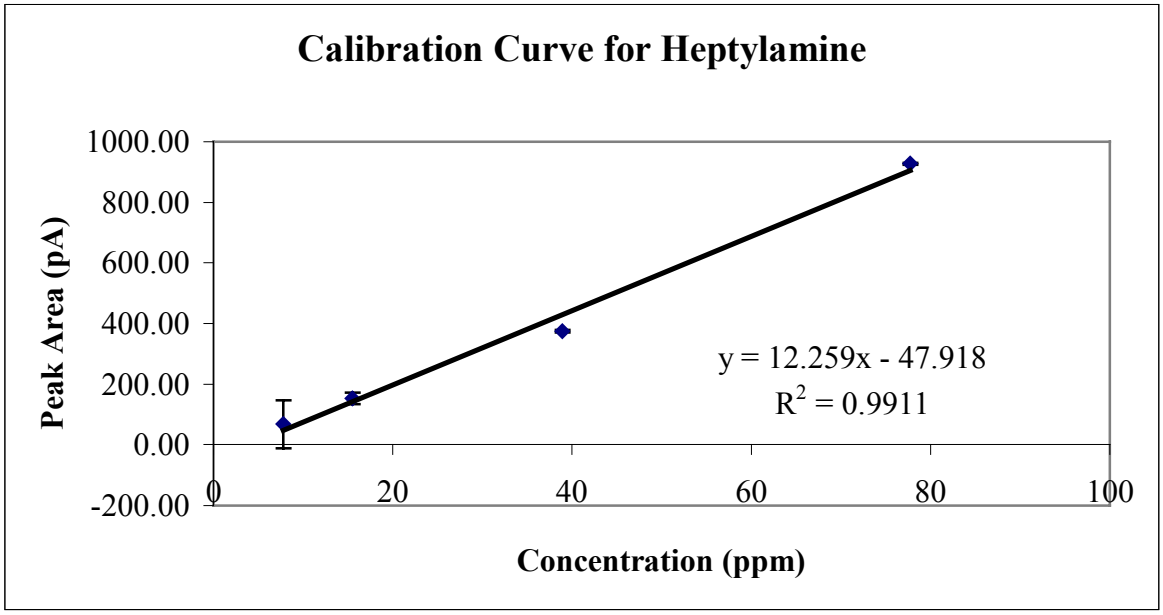


Figure 20. Heptylamine Calibration Curve

Below is the calibration curve for putrescine (Figure 21). The linearity is good with a correlation coefficient of $R^2 = 0.9973$.

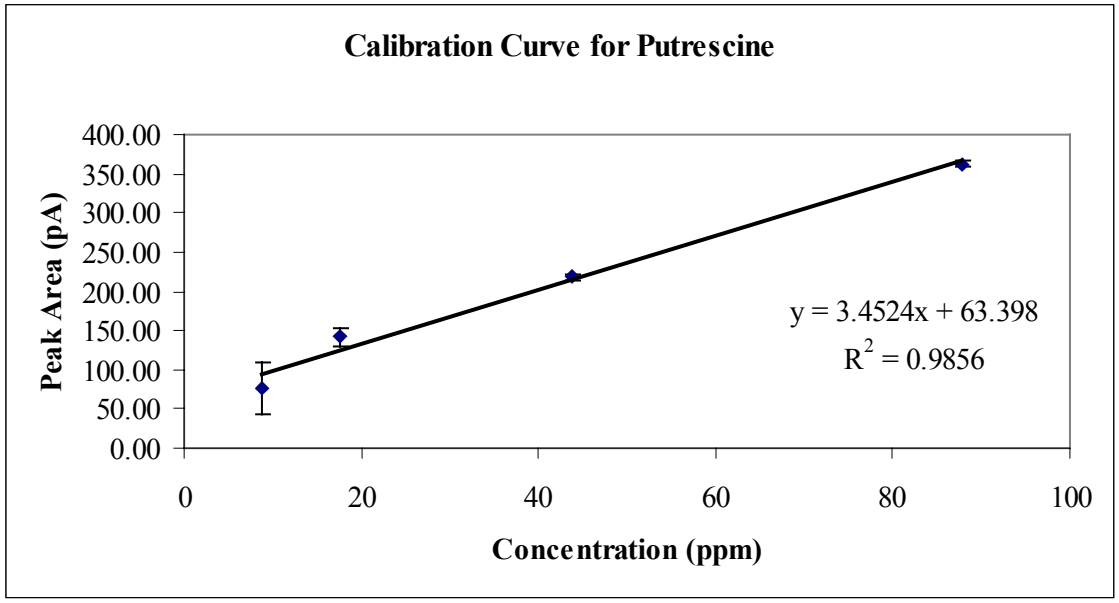


Figure 21. Putrescine Calibration Curve.

Figure 22 shows the calibration curve for cadaverine. The correlation coefficient is very good at $R^2 = 0.9918$.

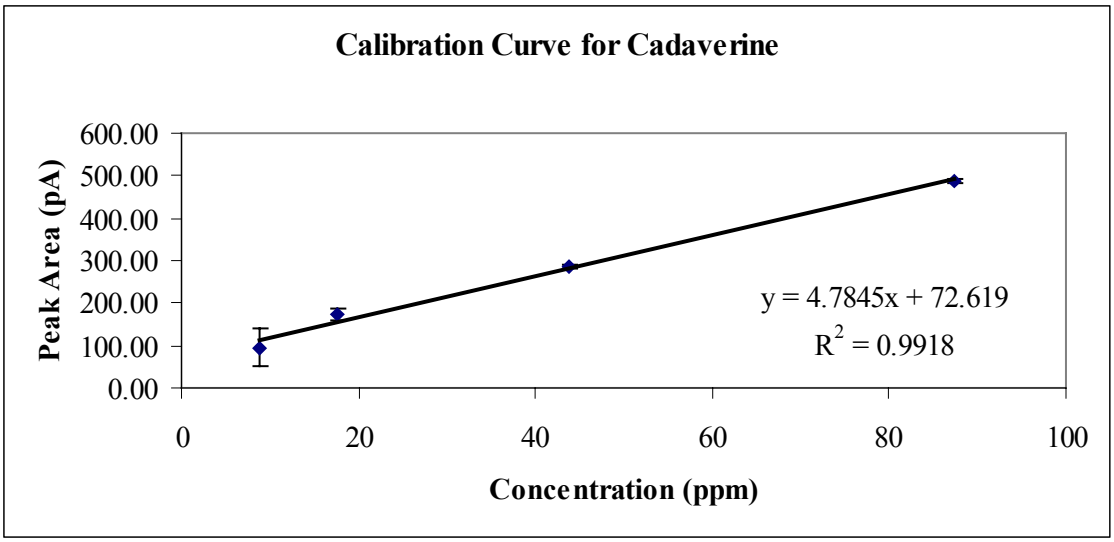


Figure 22. Cadaverine Calibration Curve

The calibration curve for histamine is shown in Figure 23. The linearity is good with a correlation coefficient of $R^2 = 0.9746$.

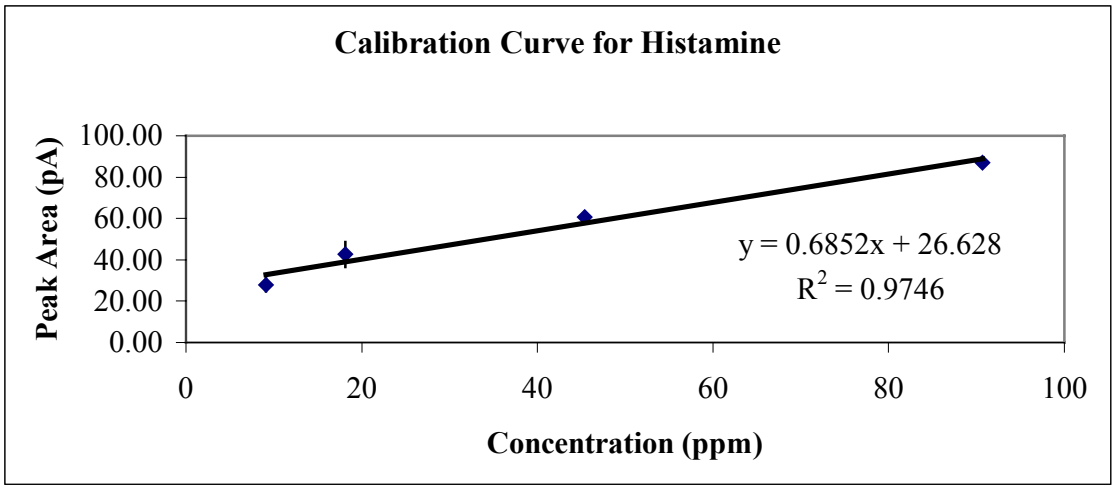


Figure 23. Histamine Calibration Curve.

Figure 24 shows the calibration curve for tyramine. The correlation coefficient is good at $R^2 = 0.9962$.

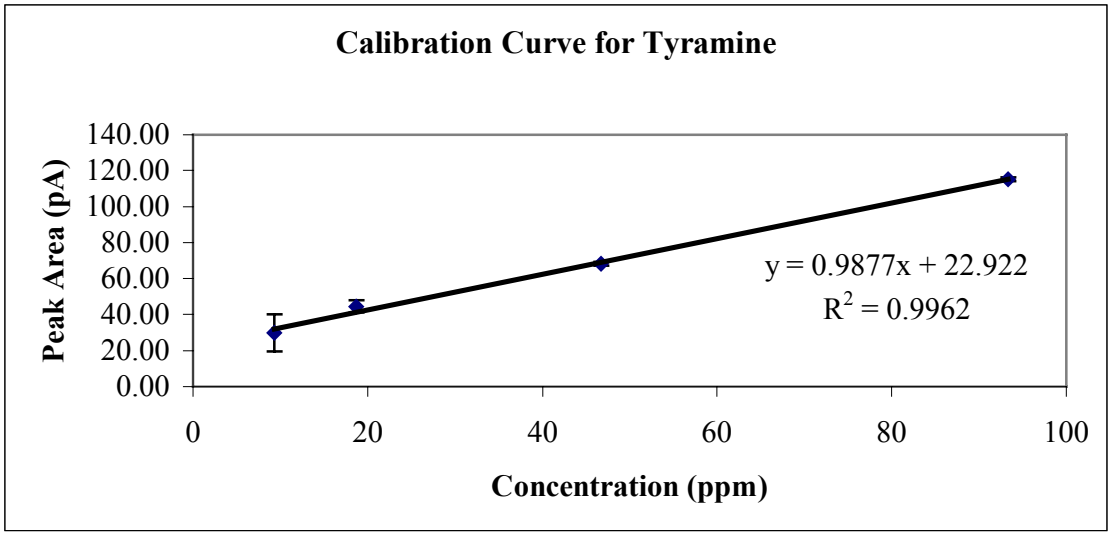


Figure 24. Tyramine Calibration Curve.

The last calibration curve is for spermidine, and it is shown in **Figure 25**. The linearity is good with an $R^2 = 0.9836$.

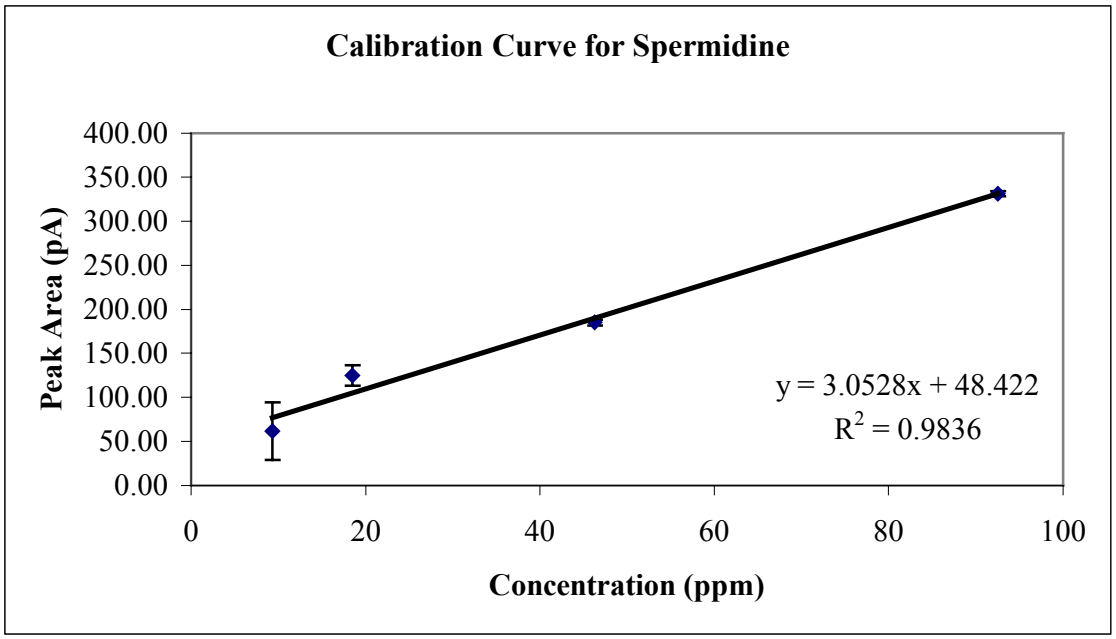


Figure 25. Spermidine Calibration Curve.

Chapter 5-Salmon Analysis Results

Introduction

The guidance level that the FDA has set for the limit of histamine in fish is 50 ppm[26]. It has been shown that people are more likely to become sick from scombroid toxicity with levels of 200 ppm or higher, however if there is 50 ppm histamine in one part of the fish there may be 500 ppm in another part of the fish so the lower limit is used as the acceptable standard[27]. It is significant to also consider the presence of other biogenic amines such as putrescine and cadaverine because it is believed that they contribute to the toxicity of histamine[28]. The results of the salmon study, including levels of biogenic amines detected, are presented in this chapter.

Salmon Results-Day 1

After the fresh salmon was extracted, derivatized, and run by GC/FID it was determined that no biogenic amines were present in either sample replicate 1 (A) or replicate 2 (B). The quantitation limit was based on the lowest level of the calibration curve at 10 pmm, with limits of detection estimated to be around 2-3 ppm for GC/FID. The resulting chromatograms are shown in Figure 26 and Figure 27.

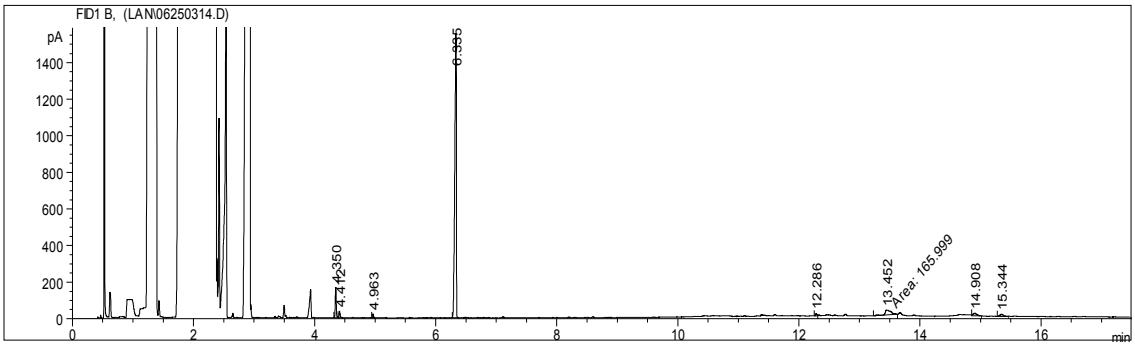


Figure 26. Day 1 Salmon sample A, run 3 out of 5.

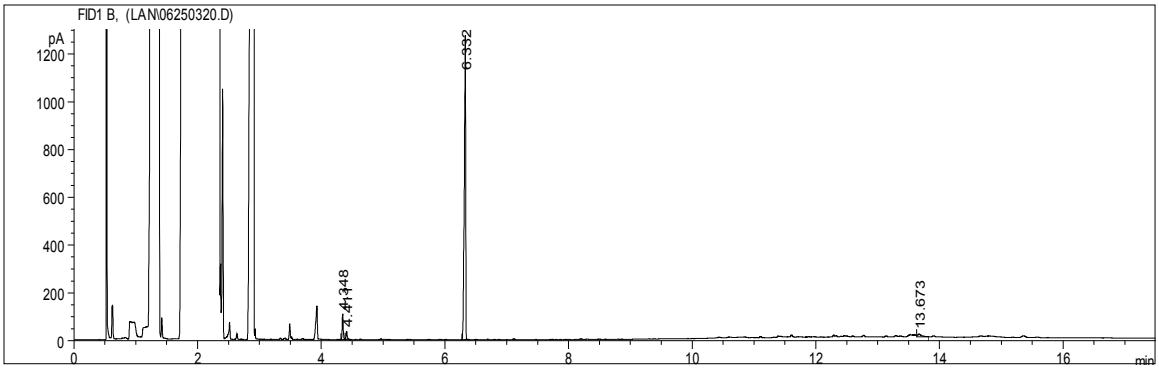


Figure 27. Day 1 Salmon sample B, run 3 out of 5.

There are several small, unknown peaks in the chromatograms, but they do not correspond to the biogenic amines that were run in the standards. GC/MS runs did not show evidence of biogenic amine derivatives for either sample A or B for day one.

Salmon Results-Day 3

After the salmon samples for day three were extracted, derivatized and run by GC/FID there were some trace levels of biogenic amines found, namely cadaverine, histamine, and tyramine. These GC/FID runs are shown in Figure 28 and Figure 29.

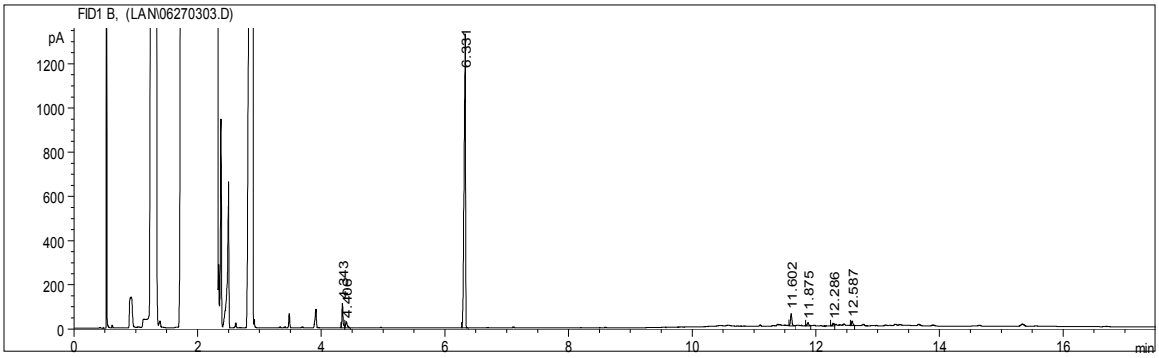


Figure 28. Day 3 Salmon sample A, run 3 out of 5; cadaverine 11.60, histamine 11.88, and tyramine 12.59 minutes.

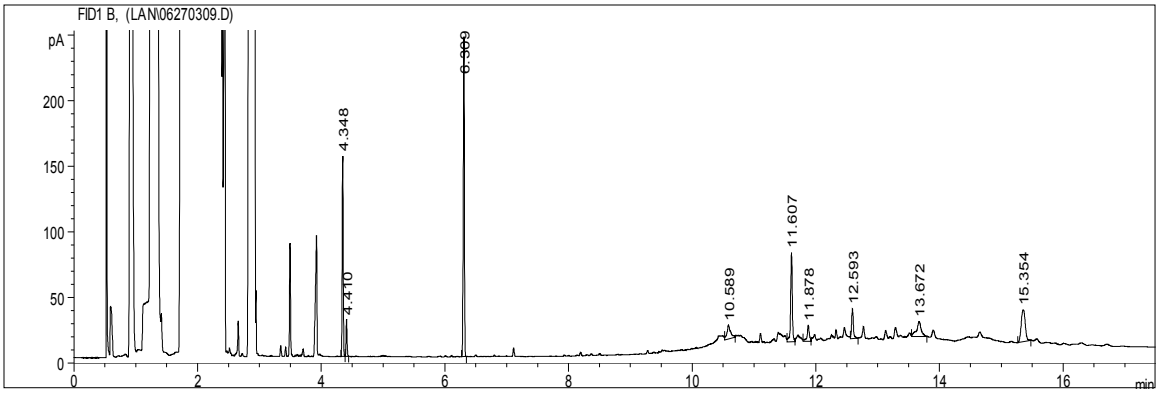


Figure 29. Day 3 Salmon sample B, run 3 out of 5; cadaverine 11.61, histamine 11.88, and tyramine 12.59 minutes.

Levels of histamine were too low to quantitate based on a signal to noise ratio less than 10:1. Cadaverine had a signal to noise ratio greater than 10:1, and based on extrapolation of the calibration curve the average level of cadaverine was found to be 13 ppm (0.62 mg cadaverine/100 mg salmon, 6.2ppm for sample A and 2.0 mg cadaverine/100 mg salmon, 20 ppm for sample B). The percent RSD is very high (73%) at these low levels of cadaverine so the reproducibility of the data is not as good. Tyramine had a signal to noise ratio equal to 20:1 for sample A and 11:1 for sample B and average peak areas did fall within the calibration curve. For sample A there was 5.4 mg tyramine/100 mg salmon (54 ppm) and for sample B there was 6.2 mg tyramine/100 mg salmon (62 ppm). The average, therefore, was 58 ppm tyramine with standard deviation of 6 ppm and %RSD of 9.7%. The data for the replicate injections of samples A and B are listed below in Table 17 and

Table 18, respectively.

Table 17. Day 3, Salmon sample A*

Data File	Hep 8.29min	Put 11.12min	Cad 11.62min	His 11.89min	Tyr 12.61min	Spe 16.61min
06270301.d	ND	ND	68.1	NQ	NQ	ND
06270302.d	ND	ND	104.5	NQ	41.1	ND
06270303.d	ND	ND	82.1	NQ	32.7	ND
06270304.d	ND	ND	106.1	NQ	42.4	ND
06270305.d	ND	ND	93.3	NQ	38.7	ND
Average			90.8		38.7	
Std Dev			16.0		4.3	
%RSD			17.6		11.1	

*ND-not detectable, NQ-not quantitatable.

Table 18. Day 3, Salmon sample B*

Data File	Hep 8.29min	Put 11.12min	Cad 11.62min	His 11.89min	Tyr 12.61min	Spe 16.61min
06270307.d	ND	ND	88.2	NQ	NQ	ND
06270308.d	ND	ND	103.2	NQ	40.5	ND
06270309.d	ND	ND	113.1	NQ	43.9	ND
06270310.d	ND	ND	102.7	NQ	41.0	ND
06270311.d	ND	ND	96.8	NQ	40.1	ND
Average			100.8		41.4	
Std Dev			9.2		1.7	
%RSD			9.1		4.2	

*ND-not detectable, NQ-not quantitatable.

GC/MS analysis was done for both samples A and B of day 3 to confirm the prescence of cadaverine, histamine, and tyramine. These chromatograms and spectra are displayed in the Appendix; Figure 38 through Figure 45.

Salmon Results-Day 5

On the last day of the study four biogenic amines were detected after extraction, derivatization, and GC/FID and GC/MS analysis. Putrescine, cadaverine, histamine, and tyramine were identified. Putrescine was detected with a signal to noise ratio of 11:1 for sample A and 20:1 for sample B, however based on the peak areas being too low the putrescine was not quantifiable. Cadaverine, histamine, and tyramine were quantitatable. The representative chromatograms for the GC/FID runs are shown in

Figure 30 and Figure 31.

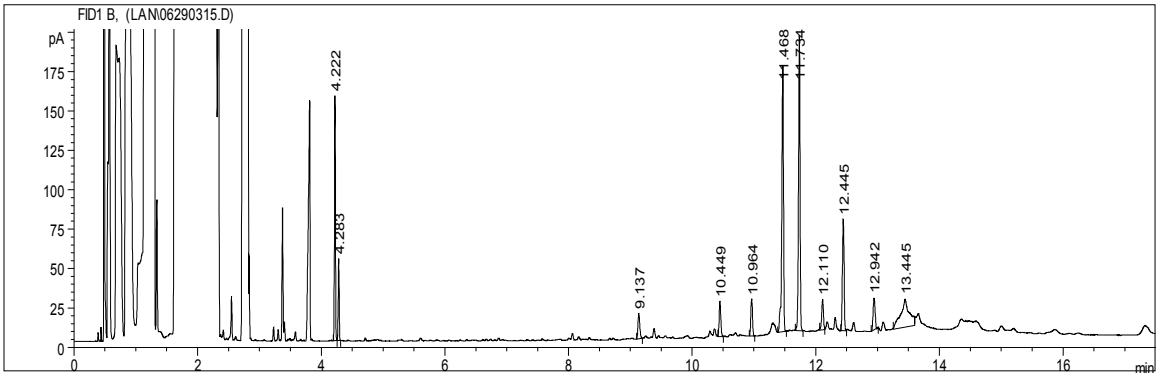


Figure 30. Day 5 Salmon sample A, run 3 out of 5; putrescine 10.96, cadaverine 11.47, histamine 11.73, and tyramine 12.45 minutes.

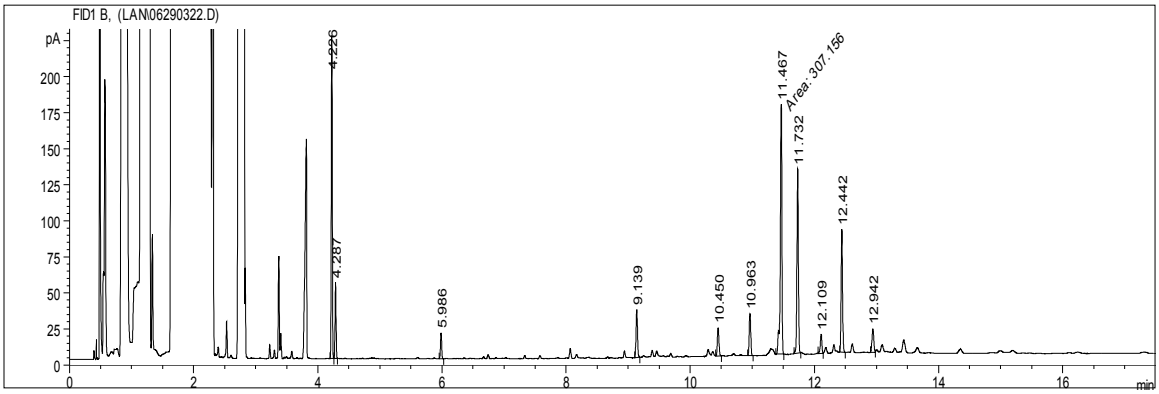


Figure 31. Day 5 Salmon sample B, run 3 out of 5; putrescine 10.96, cadaverine 11.47, histamine 11.73, and tyramine 12.44 minutes.

The peaks shapes are good overall except for a small shoulder on the cadaverine peak. The signal to noise ratios for cadaverine, histamine, and tyramine are all at least 75:1 for both samples A and B. In
Table 19 and Table 21 the %RSD's for the analyte peak areas for day five samples A and B, respectively. All the %RSD's are good at less than 10%.

Table 19. Day 5, Salmon sample A*

Data File	Hep 8.15min	Put 10.96min	Cad 11.46min	His 11.72min	Tyr 12.44min	Spe 16.10min
06290313.d	ND	40.6	298.9	303.9	121.7	ND
06290314.d	ND	42.0	307.3	310.3	122.2	ND
06290315.d	ND	43.9	327.6	324.2	129.1	ND
06290316.d	ND	41.1	307.4	302.6	121.4	ND
06290317.d	ND	43.8	323.0	318.3	127.1	ND
Average		42.3	312.8	311.9	124.3	
Std Dev		1.5	12.0	9.3	3.6	
%RSD		3.6	3.8	3.0	2.9	

*ND-not detectable, NQ-not quantitatable.

Table 20. Day 5, Salmon sample B*

Data File	Hep 8.15min	Put 10.96min	Cad 11.46min	His 11.72min	Tyr 12.44min	Spe 16.10min
06290319.d	ND	53.2	339.0	223.1	149.3	ND
06290320.d	ND	46.8	297.1	198.5	130.4	ND
06290322.d	ND	49.7	317.9	209.4	138.0	ND
06290323.d	ND	48.8	310.8	207.5	135.4	ND
06290324.d	ND	41.3	262.3	177.4	115.5	ND
Average		48.0	305.4	203.2	133.7	
Std Dev		4.4	28.5	16.9	12.3	
%RSD		9.1	9.3	8.3	9.2	

*ND-not detectable, NQ-not quantitatable.

The quantitative data for the day five salmon analysis is listed in Table 21. The %RSD is good for cadaverine and tyramine at less than 6%. Histamine is a bit higher at 33.7%. The histamine level is extremely high and is out of range of the calibration curve so that may contribute to the reproducibility inconsistencies. It is clear from this data that after five days of salmon stored at 4°C, it is not fit to be prepared for human consumption, based on the levels of biogenic amines, without significant risk of scombroid poisoning.

Table 21. Day 5 Salmon-Biogenic Amine Levels

Analyte Peak	Sample A Concentration (ppm)	Sample B Concentration (ppm)	Average Concentration (ppm)	Standard Deviation (ppm)	%RSD
Cadaverine	168	162	165	4	2.6
Histamine	1390	855	1123	379	33.7
Tyramine	342	372	357	22	5.9

GC/MS analysis was performed on the salmon samples from day five. These chromatograms and spectra can be found in the Appendix; Figure 46 through Figure 55.

Chapter 6-Conclusions

GC/FID Analysis

In summary, the propyl chloroformate derivatization method employed for the biogenic amines heptylamine, putrescine, cadaverine, histamine, tyramine, and spermidine, was successful for trace level quantitation. The limits of quantitation are below 10 ppm, where the error bars on the calibration curves are the largest, however Relative Standard Deviation is still low and acceptable at less than 5% at the 10 ppm level for all six amines. The signal to noise ratios at 10 ppm are 12:1 for both histamine and tyramine, therefore limits of detection are estimated to be 2-3 ppm.

The advantages of using this method are that it is a rapid derivatization method taking less than ten minutes and it is not moisture sensitive. Extraction and derivatization from urine and aqueous matrices are possible. The chromatography is very reproducible (~4% RSD) at trace levels (10 ppm). It should be noted that it is best to use a column 15 meters or perhaps shorter, as opposed to a 30 meter column. Additionally, a thicker stationary phase film such as 1.00 μm d_f will lengthen the life of the column. It is also recommended that the column be cut at the inlet end 40 cm every 25 runs so that maximum sensitivity and reproducibility are achieved. It also may be useful to incorporate an internal standard into the method to increase limits of quantitation and improve reproducibility.

GC/MS Analysis

The GC/MS method used to confirm the identities of the biogenic amine derivatives proved to be successful. This method is not, however, recommended for quantitation using a hot split inlet, due to the thermal degradation of the carbamate derivatives. For multiple injections using the hot inlet in the GC/MS method, the reproducibility is not sufficient for good, quantitative analysis. Although for a quick, qualitative confirmation using the hot split inlet, with GC/MS, works well.

Salmon Analysis

The extraction and derivatization process for biogenic amines from the salmon is a relatively fast and reproducible method. The extraction time may be optimized further depending on the type of fish to be analyzed. Fish samples can be troublesome due to the complex matrices, especially those high in fish oils. This method works well on fish samples largely due to the methanol that is 50% of the liquid extraction media. Salmon extraction attempts were made using only water, and it was found that derivatization of the supernate after centrifuging the extract was not possible. This is most likely due to the matrix effects of the fish oils that caused the derivative to form an insoluble clump of white matter. It is believed that the methanol is useful to break up the fish oils so that the derivatization can proceed.

It is not known how addition of methanol to the homogeneous salmon mix affects the efficiency of the derivatization. It is possible that trace levels of water exist in the organic layer of the derivative, due to the solubility of methanol in chloroform and the solubility of water in methanol. This may slightly decrease the overall efficiency in the derivatization of aqueous biogenic amine samples. For the derivatization of the salmon extracts clearly two distinct layers were formed, the top being the organic and the bottom the aqueous layer. In between the two layers was a thin, white, insoluble membrane most likely composed of some residual fat from the salmon, although it did not appear to interfere with the analysis.

Based on the salmon study conducted in this thesis, and considering the 50 ppm histamine safety level set by the FDA[26], it is recommended not to use raw salmon in food preparation after three days, even if it is stored in the refrigerator.

Future Applications

The methods developed in this thesis were applied to fish samples, but may also be applied to other biogenic amine containing samples, especially in aqueous media. The derivatization method used here was based on Ugland's work[13], which incorporated derivatization of urinary amines such as amphetamine and methamphetamine, using

propyl chloroformate as one of the more successful derivatizing reagents in the trials. Ugland also experimented successfully with solid phase microextraction (SPME) directly in the amine/derivatizing solution where the derivatized amines partition to the SPME fiber[13]. A quantitative technique similar to this may be possible for biogenic amine derivatives in aqueous solutions, especially with the availability of an automated headspace system to achieve a more rapid analysis. It can be hypothesized that even greater sensitivity may be obtained with the use of SPME in the derivatization process.

Appendix

GC/FID Data

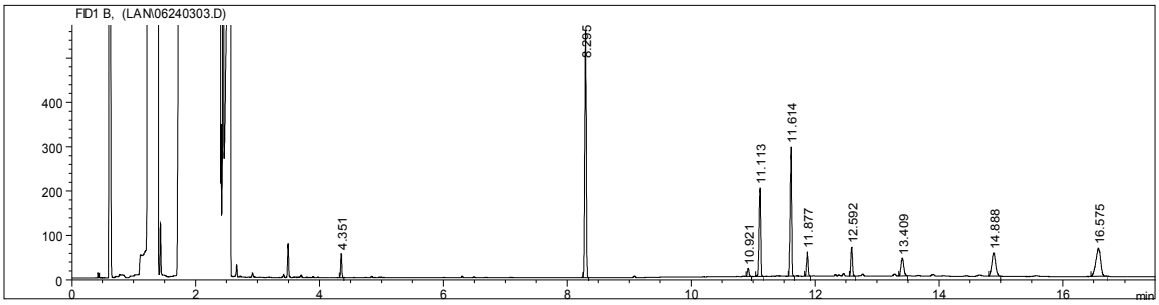


Figure 32. Run 3 of 5, 100 ppm amine mix (heptylamine 8.30, putrescine 11.11, cadaverine 11.61, histamine 11.88, tyramine 12.59, spermidine 16.58 minutes).

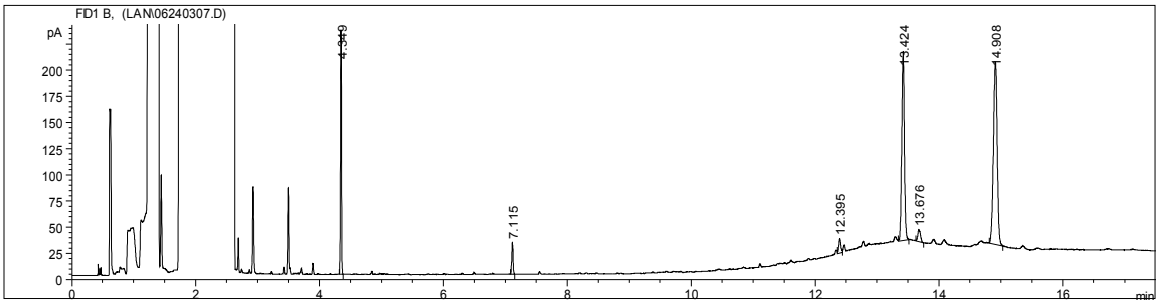


Figure 33. Derivative blank run with unknown peaks at 13.42 and 14.90 minutes.

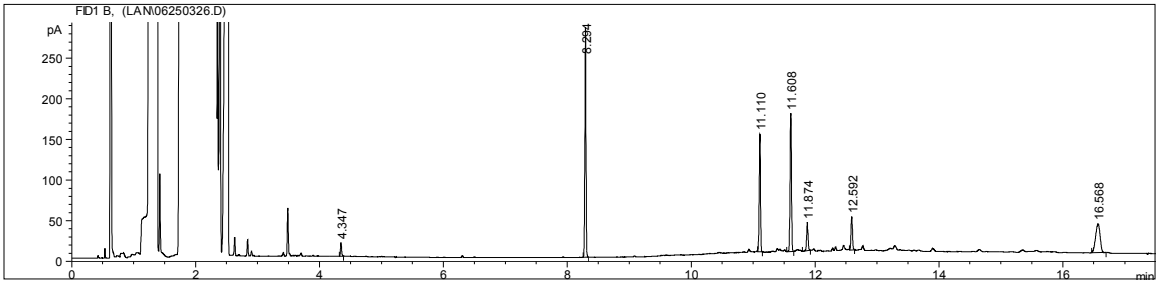


Figure 34. Run 3 of 5, 50 ppm amine mix (heptylamine 8.29, putrescine 11.11, cadaverine 11.61, histamine 11.87, tyramine 12.59, spermidine 16.57 minutes).

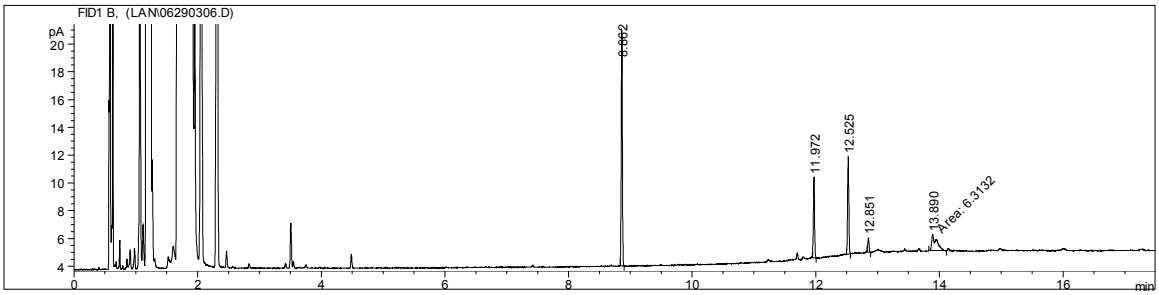


Figure 35. Run 3 of 5, 50 ppm amine mix with hot inlet 200°C (heptylamine 8.86, putrescine 11.97, cadaverine 12.53, histamine 12.85, tyramine 13.89 minutes).

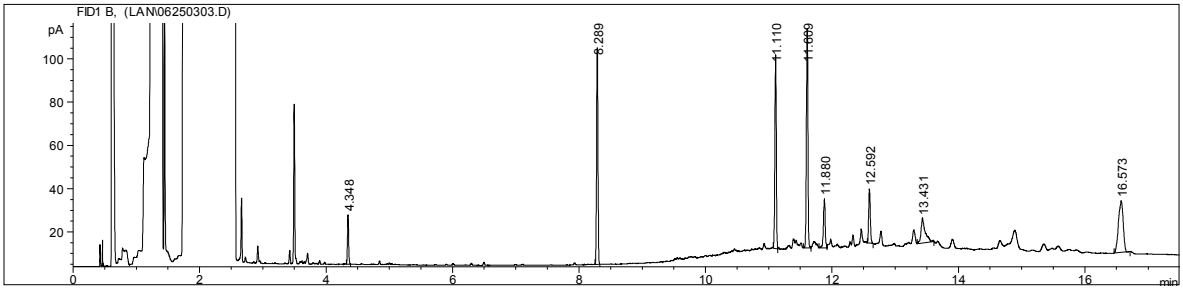


Figure 36. Run 3 of 5, 20 ppm amine mix (heptylamine 8.28, putrescine 11.11, cadaverine 11.60, histamine 11.88, tyramine 12.59, spermidine 16.57 minutes).

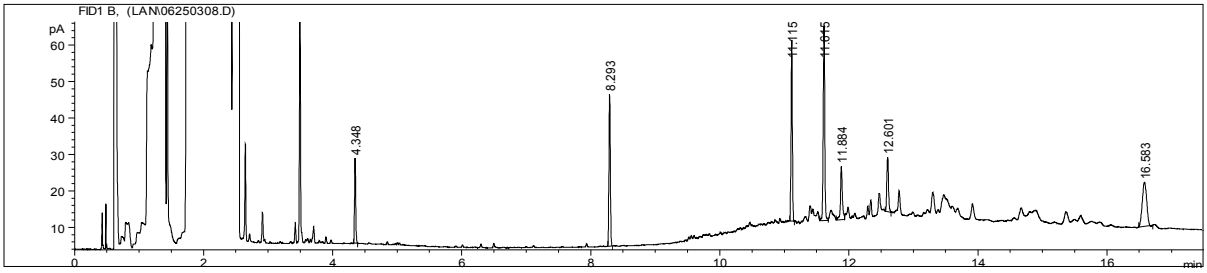


Figure 37. Run 3 of 5, 10 ppm amine mix (8.293 heptylamine, 11.11 putrescine, 11.61 cadaverine, 11.884 histamine, 12.601 tyramine, 16.583 minutes spermidine).

GC/MS Data

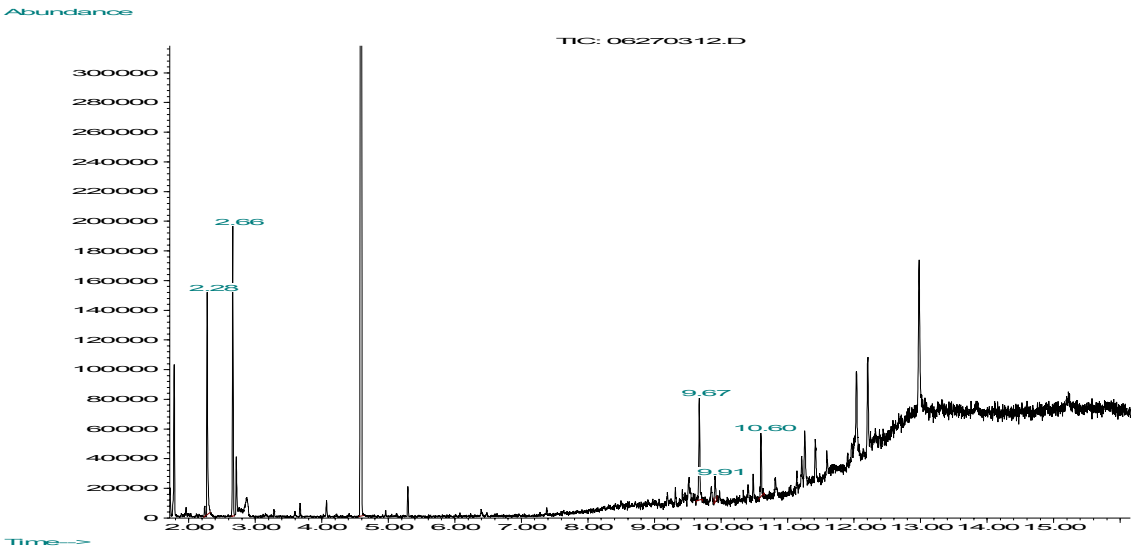


Figure 38. Day 3 Salmon sample A, GC/MS run, cadaverine 9.67, histamine 9.91, and tyramine 10.60 mintues.

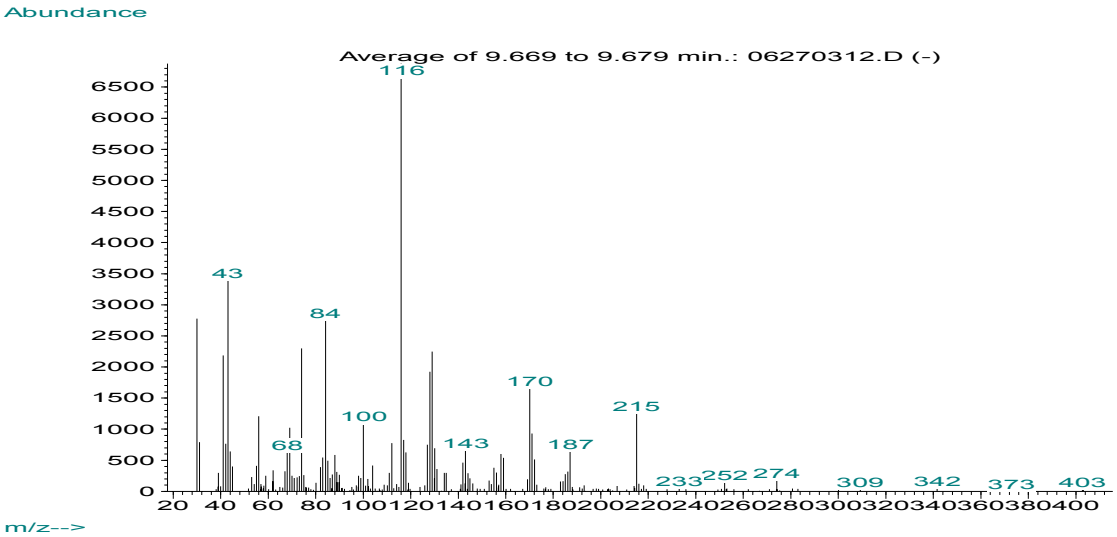


Figure 39. Day 3 Salmon sample A, GC/MS spectra of cadaverine 9.67 minutes.

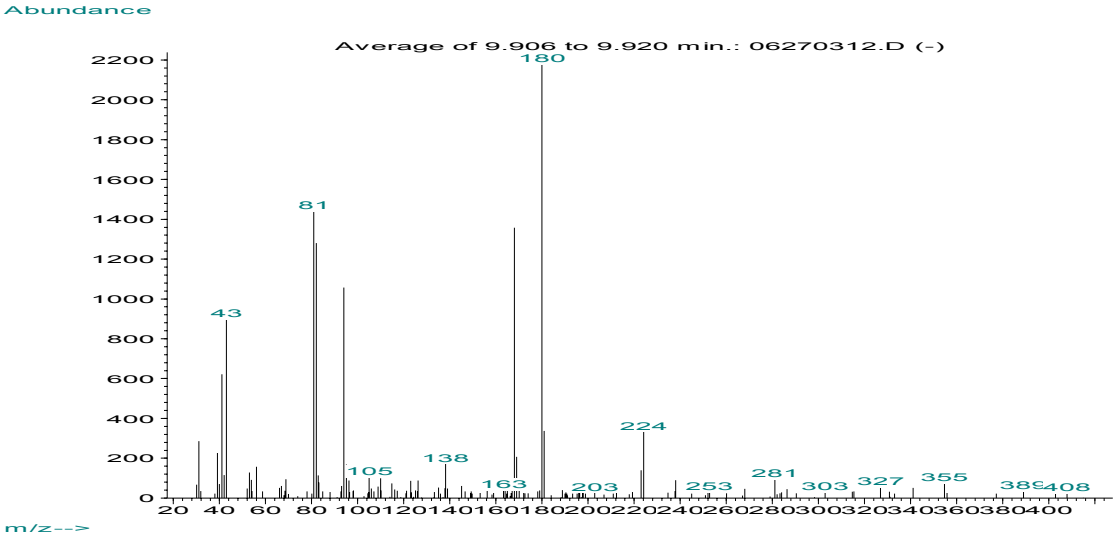


Figure 40. Day 3 Salmon sample A, GC/MS spectra of histamine 9.91 minutes.

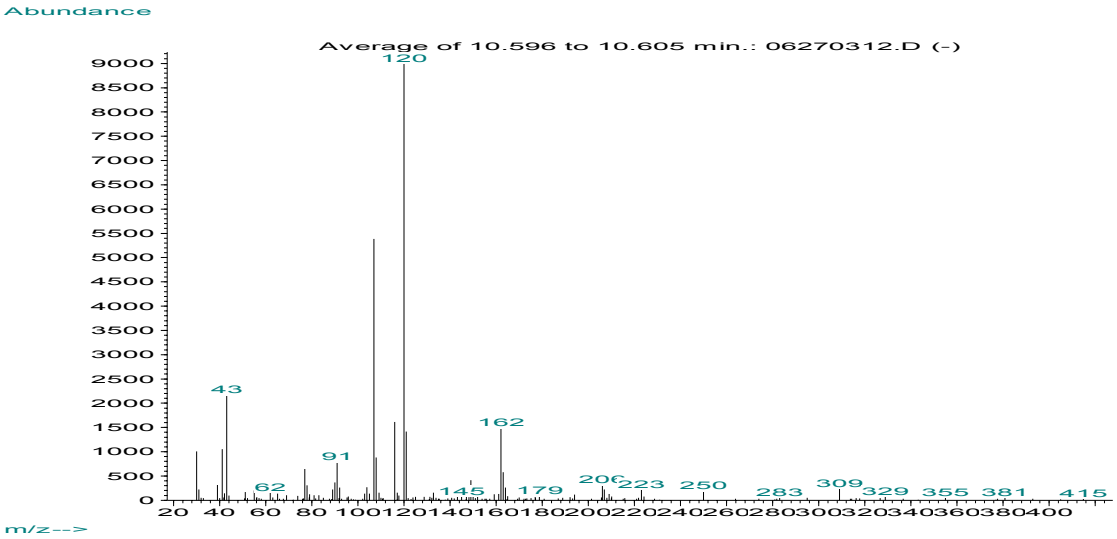


Figure 41. Day 3 Salmon sample A, GC/MS spectra of tyramine 10.60 mintues.

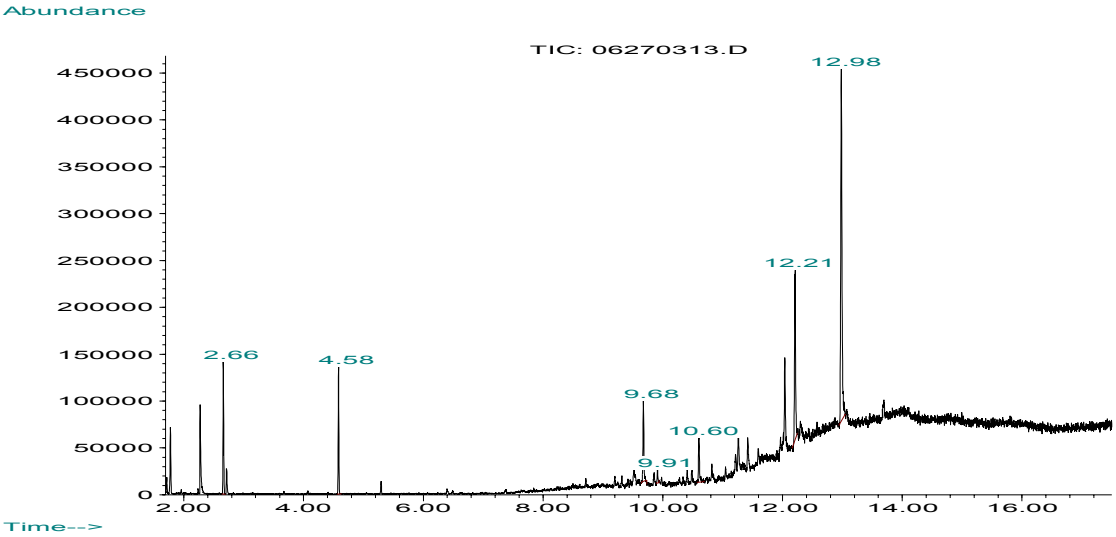


Figure 42. Day 3 Salmon sample B, GC/MS run, cadaverine 9.68, histamine 9.91, tyramine 10.60 minutes.

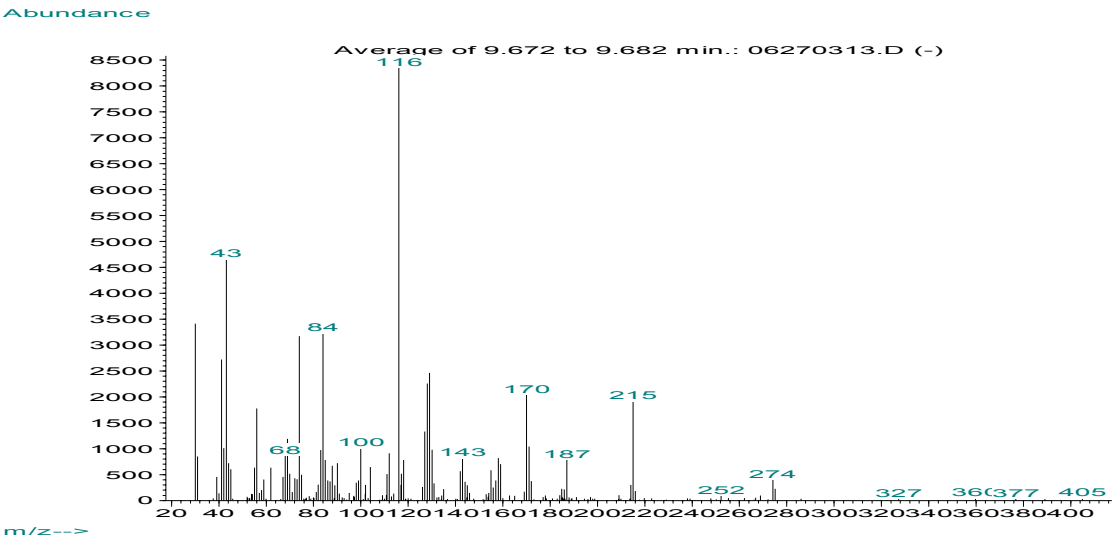


Figure 43. Day 3 Salmon sample B, GC/MS spectra of cadaverine 9.68 minutes.

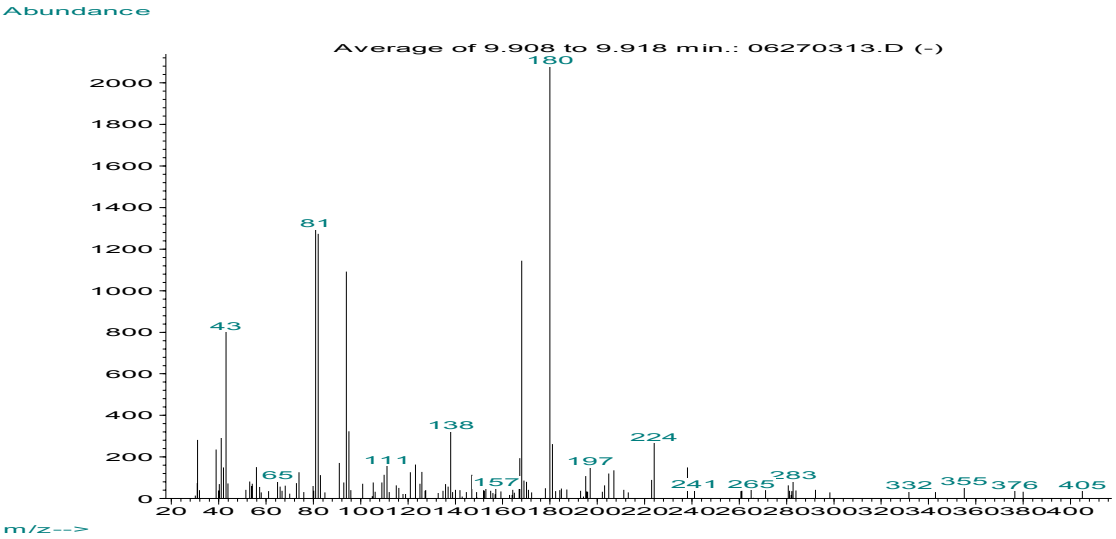


Figure 44. Day 3 Salmon sample B, GC/MS spectra of histamine 9.91minutes.

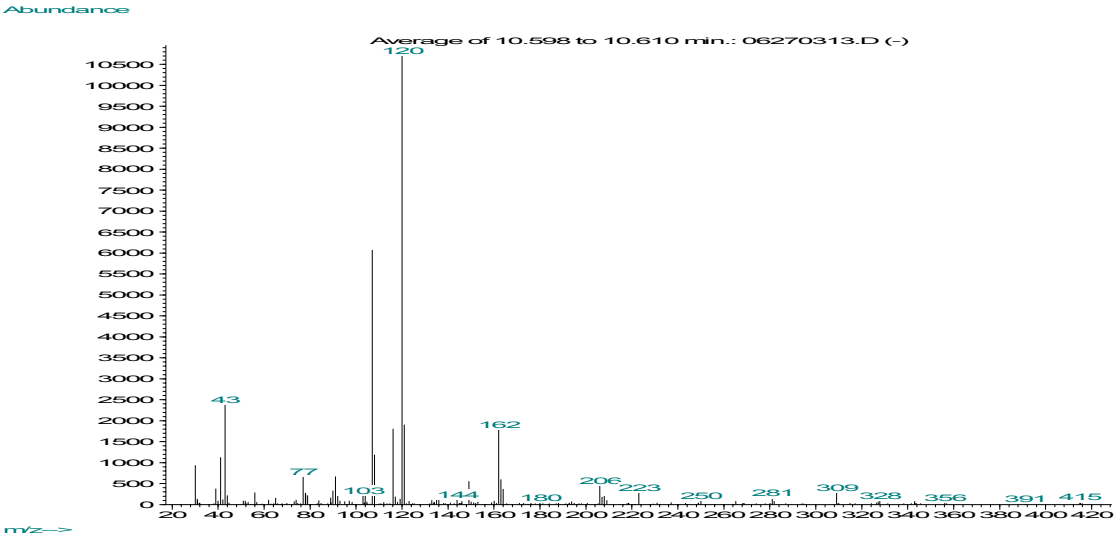


Figure 45. Day 3 Salmon sample B, GC/MS spectra of tyramine 10.60 minutes.

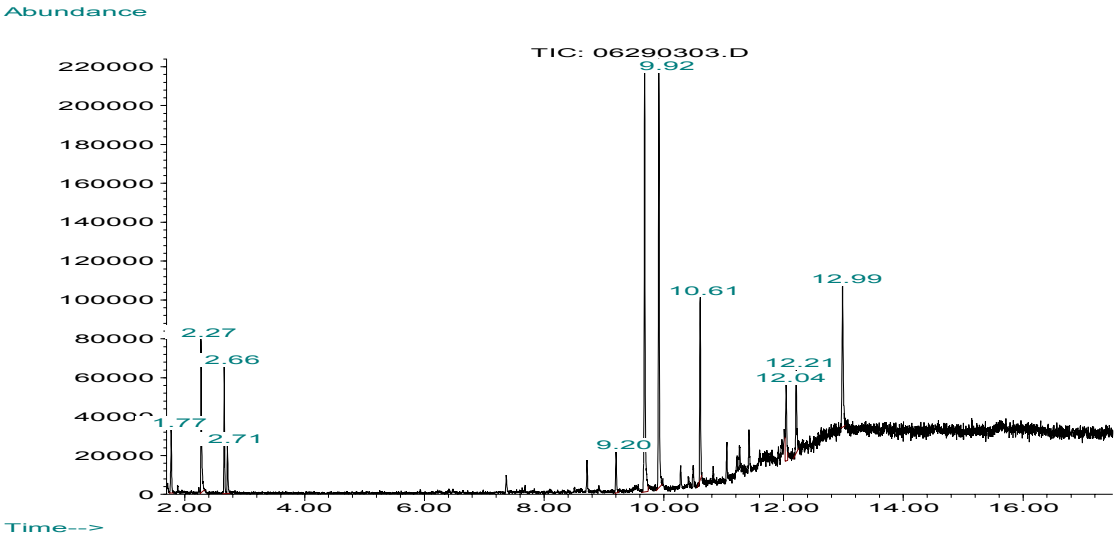


Figure 46. Day 5 Salmon sample A, GC/MS run, putrescine 9.20, cadaverine 9.67, histamine 9.91, tyramine 10.60 minutes.

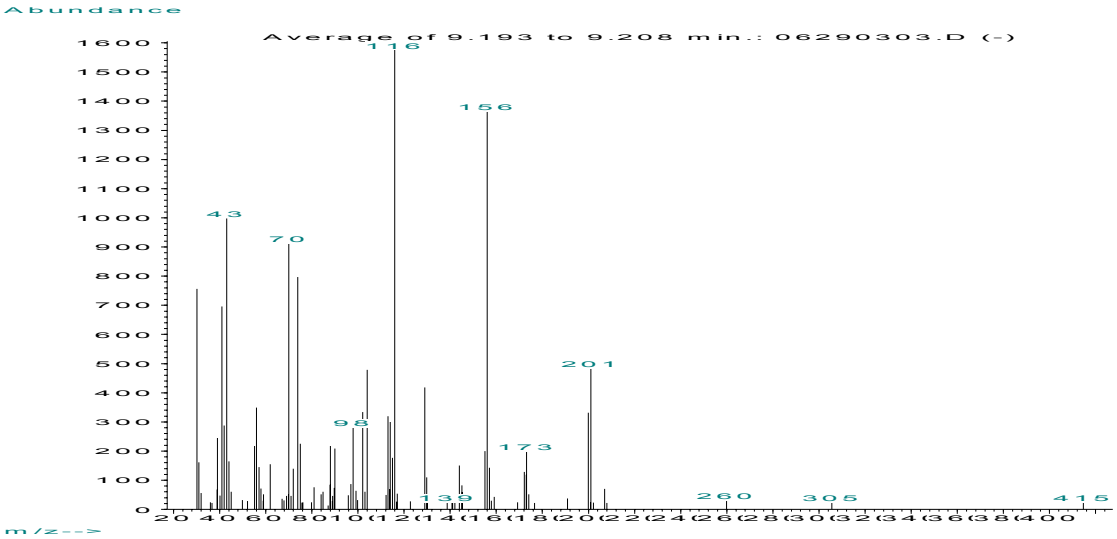


Figure 47. Day 5 Salmon sample A, GC/MS spectra of putrescine 9.20 minutes.

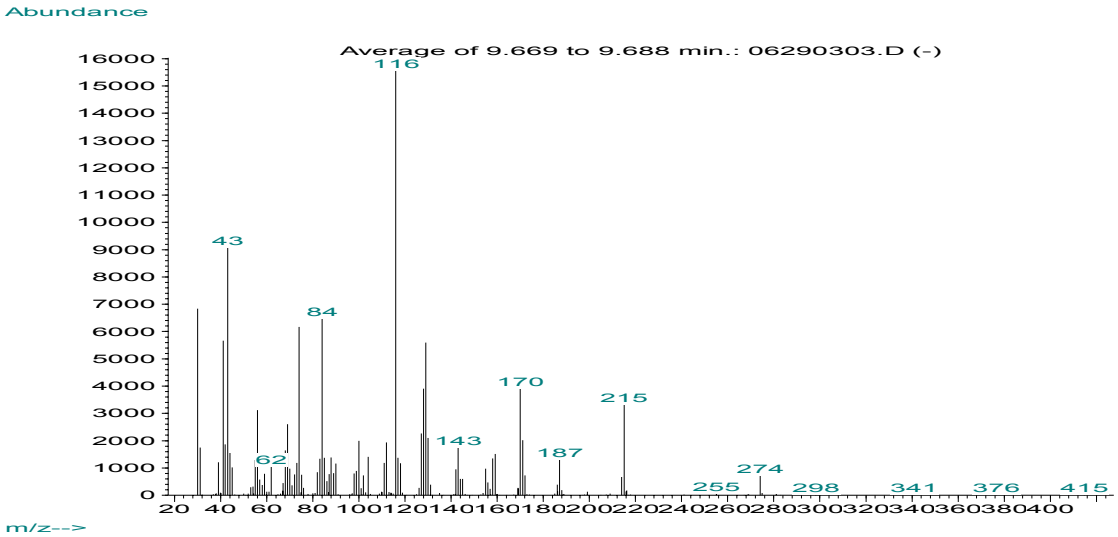


Figure 48. Day 5 Salmon sample A, GC/MS spectra of cadaverine 9.70 minutes.

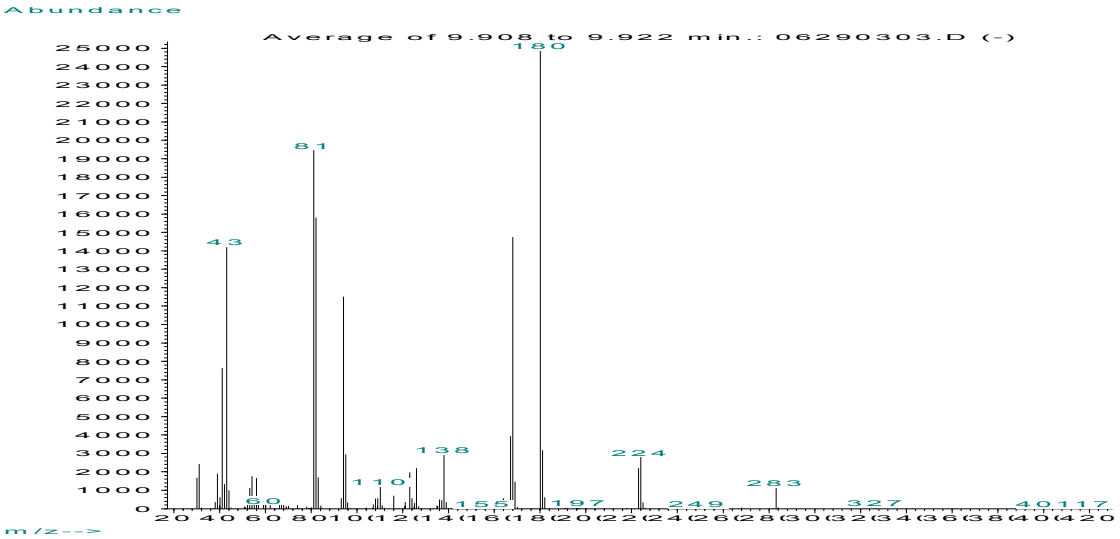


Figure 49. Day 5 Salmon sample A, GC/MS spectra of histamine 9.91 minutes.

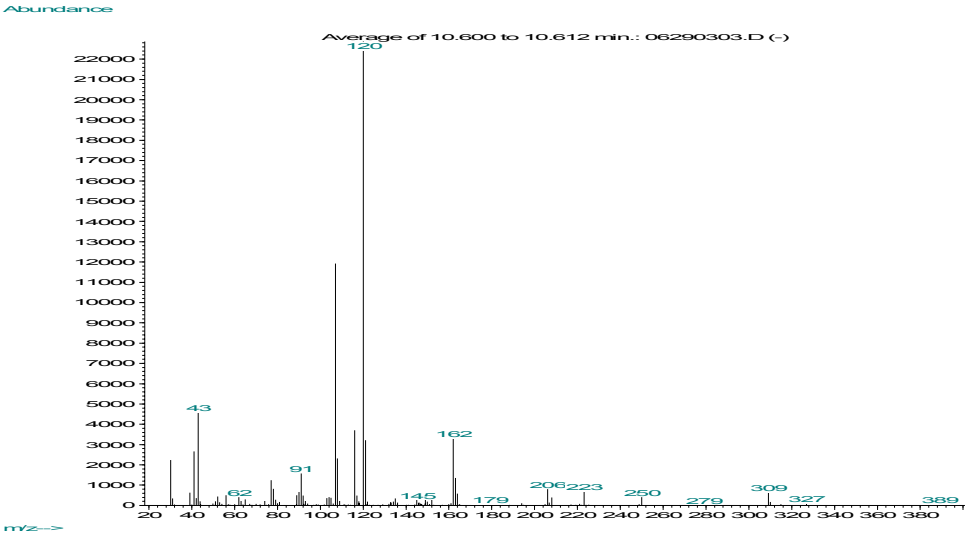


Figure 50. Day 5 Salmon sample A, GC/MS spectra of tyramine 10.60 minutes.

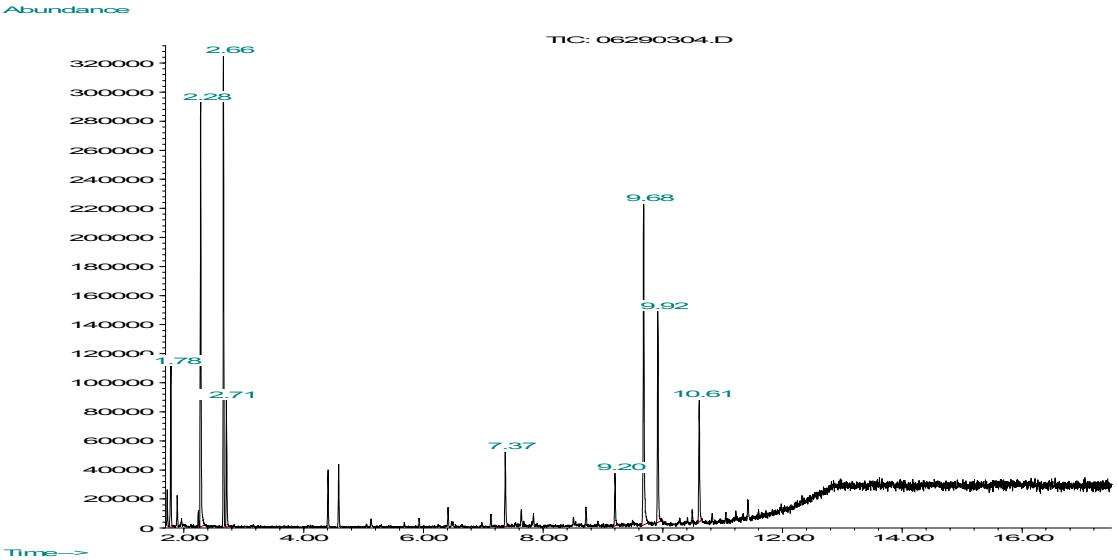


Figure 51. Day 5 Salmon sample B, GC/MS run, putrescine 9.20, cadaverine 9.68, histamine 9.92, tyramine 10.61 minutes.

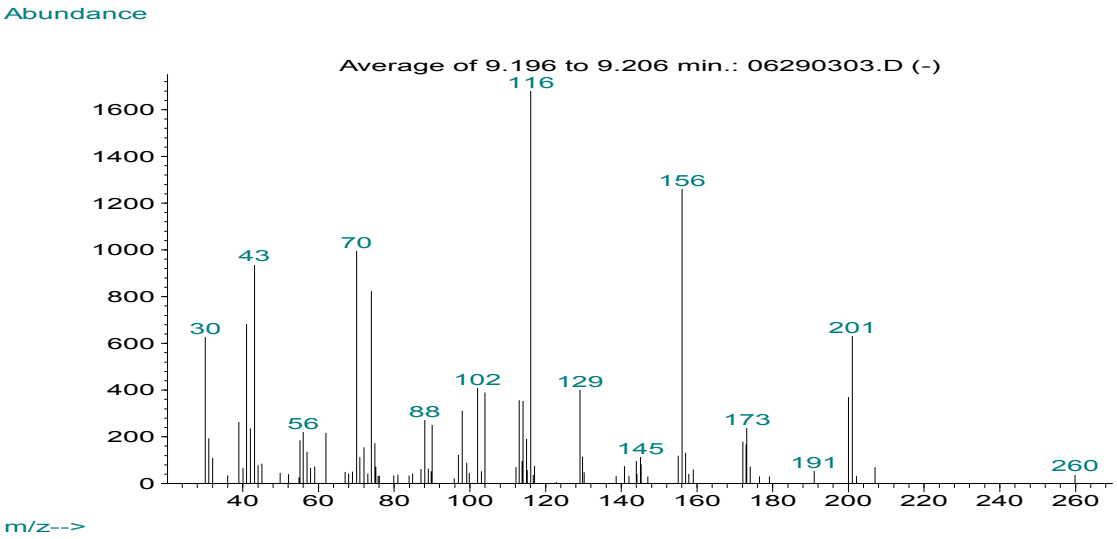


Figure 52. Day 5 Salmon sample B, GC/MS spectra of putrescine 9.20 minutes.

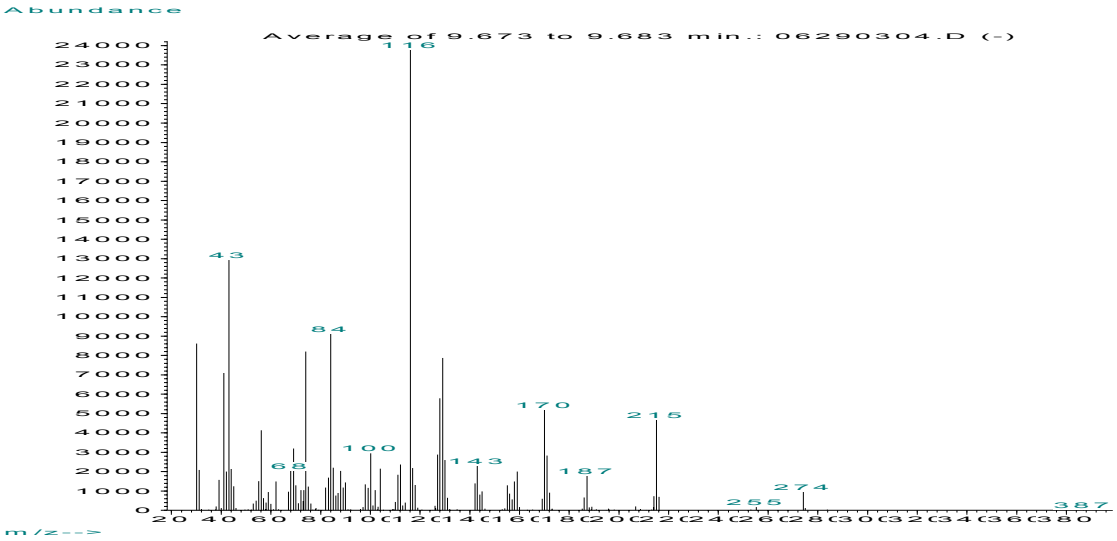


Figure 53. Day 5 Salmon sample B, GC/MS spectra of cadaverine 9.67 minutes.

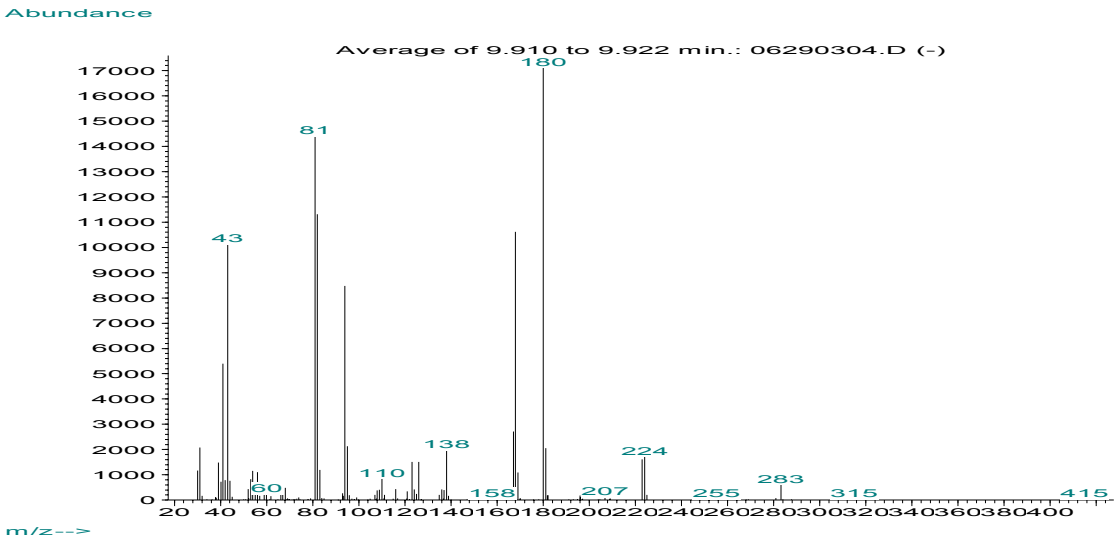


Figure 54. Day 5 Salmon sample B, GC/MS spectra of histamine 9.91 minutes.

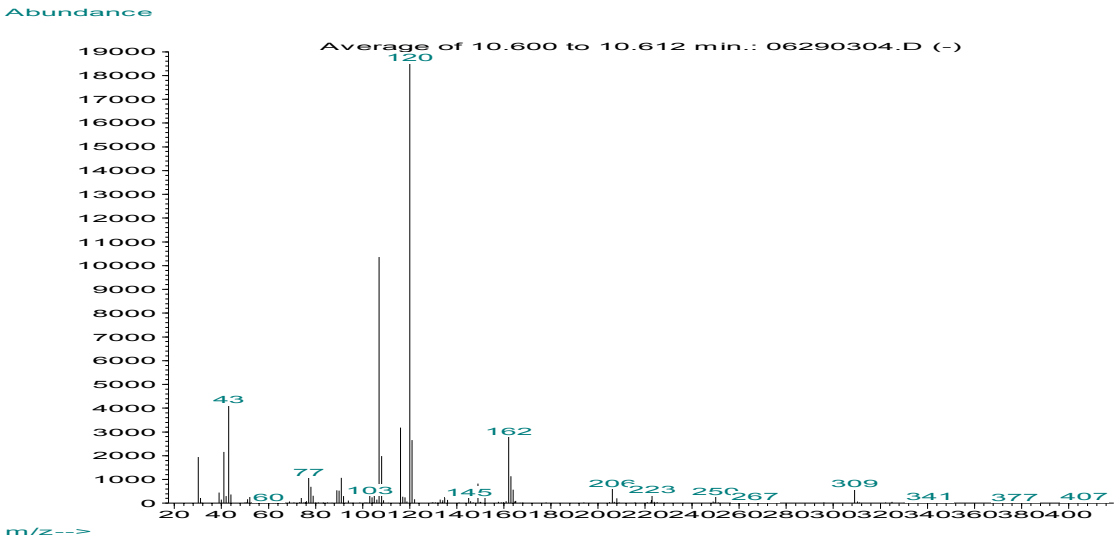


Figure 55. Day 5 Salmon sample B, GC/MS spectra of tyramine 10.61 minutes.

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Vita

Laura Nakovich was born to John and Joan Nakovich on November 13, 1977 in Silver Spring, MD. She grew up in Herndon, VA with her parents and brother John. After high school she attended Virginia Tech where she received a Bachelor's of Science degree in Biochemistry in May of 2000. She did undergraduate research in Dr. McNair's lab for two semesters and one summer. In the fall of 2000 she began work on her Master's degree in Analytical Chemistry under the advisorship of Dr. Harold McNair. She will finish her Master's degree in September of 2003 and plans to pursue an industry career in the field of analytical chemistry.