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Honors Directed Study

Research Thesis

Determination of Heterocyclic Aromatic Amines at Different Depths of Meat Samples using Gas Chromatography Coupled with Mass Spectrometry

Abstract

Heterocyclic aromatic amines (HAAs) are a class of substances produced naturally when cooking meats at temperatures above 155º Celsius. These compounds are carcinogenic and have been shown to increase the risk of multiple cancers including prostate cancer and leukemia. The impact of cooking time and cooking temperature on the formation of HAAs from various cooking methods and different types of meats has been previously studied, but there is a lack of research investigating the migration of these HAAs in meats during and after cooking. The study intended to quantify the relative concentrations of HAA present at different depths in pan fried Beef Chuck Eye meat samples. The results confirmed that from a "whole meat" comparison, that is without slicing the meat, the common HAA 1, 2-amino-3,8-dimethylimidazo[4,5-*f*] quinoxaline (MeIQx) was found to increase with increasing cooking temperature and time. However, the results of the internal sliced meat trials suggested that layers furthest away from the heat source contained higher concentrations of MeIQx than layers subjected to higher thermal treatment closest to the heat source. These results have potential value for food processing companies or the individual consumer to more effectively incorporate antioxidants into meats and recipes, as these antioxidants can reduce HAA formation.

Introduction

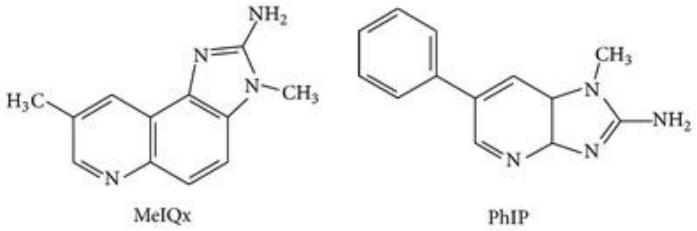
Historically, meat has played an integral part in most cultural diets. Meat not only serves as a primary source of proteins, but also provides fat and micronutrients like iron, magnesium, and zinc[1]. Meat consumption helps provide the essential amino acids for metabolism. The similar structure and composition of animal and human tissue contributes to their functionality in human systems[2].

Cooking meat involves thermal treatments to change the properties of the meat. The cooking process helps to break down tough proteins for easier digestion and processing[1]. Varying levels of heat are used to prepare meat depending on the product desired. Heat treatment methods can run from the mundane, such as pan frying, deep frying, boiling, or grilling, to more complex thermal methodologies like air-roasted meat cylinders[3].

However, large consumption of meats cooked by these high temperature treatments is liked to increases chances of cancers. Since 2014, red meat has been classified by the World Health Organization as a Group 2a carcinogen. This means that limited evidence has demonstrated a positive correlation to red meat consumption and various cancers[5]. Multiple epidemiological studies have indicated a positive correlation between various cancers and high consumption of cooked meat and poultry[4, 6, 7]. This list includes prostate cancer, leukemia, and gastrointestinal tumors[6]. Additionally, processed meat is classified as a Group 1 carcinogen as sufficient evidence has shown a causal relationship between processed meats and colorectal cancer[5]. Evidence suggest this is possibly due to the preservatives and salts within processed meat[3]. The Global Burden of Disease Project estimates that processed meat has caused 34,000 cancer-related deaths per year. Additionally, red meat is predicted to cause 50,000 cancer-related deaths per year if the relationship to cancer is proven causal[5].

Furthermore, the Our World in Data study on the world's meat consumption demonstrates that this issue will only continue to worsen over time. From data collected from 1990 to 2013, the study determined that as countries become richer and larger, their meat consumption per capita increases[8]. Thus as the world's population continues to increase their meat consumption, their chances for cooked meat related cancers also increase.

Figure1: Structure of HAAs MeIQx and PhIP



Heterocyclic aromatic amines (HAAs) produced in cooked meat are believed to be some of the main contributors to the cancer causing properties of red and processed meats. HAAs are potent carcinogenic compounds naturally formed during the cooking of meats and fish[4]. There are currently 25 different HAAs identified. Characterization of HAAs includes molecules that have three fused aromatic rings possessing at least one primary amino group as seen in examples of HAAs in Figure 1[9]. The examples in Figure 1, 2-amino-3,8-dimethylimidazo[4,5-*f*] quinoxaline (MeIQx) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), are the two most commonly found HAAs generated in cooked meats[12].

Studies have predicted that the cause of HAAs' mutagenic effects are through the formation of DNA adducts. They have been showed to work synergistically with polycyclic aromatic hydrocarbons (PAHs) to form these DNA adducts in cell lines[3]. These adducts create

disruptions and other mutations in genes that lead to the formations of tumors[17]. Though literature is not unanimous on the association between human cancers and HAAs, their carcinogenic effects have been demonstrated in animal and cell line studies[3].

The formation mechanism of HAAs has been investigated in some studies. The reactions are not simple since the types of HAAs that form vary greatly. Available mechanistic research predicts that HAA production stems from Maillard browning reactions between hexoses and free amino acids found in the meat[11]. Maillard reactions are what produce the brown pigments and umami flavors associated with cooked meats. They also lead to the production of HAA's in relatively small amounts. The concentrations range from 1 ng/g to 400 ng/g depending on meat type, cooking time, temperature, and style of cooking used[3, 9]. These reactions occur at high temperatures above 155^{o} C. Additionally, the reactions utilize compounds naturally found in the meats including threonine, creatinine, water, and sugars as reactants for HAA production[11]. Studies have shown that different kinds of meat generate different amounts of HAAs depending on the amount of precursors in the uncooked samples such as creatinine and glucose[16]. Other studies have demonstrated that a majority of these precursors are located in the meat juices of the meats rather than throughout their internal matrices[19]. Research has found that white meats like poultry generate lower amounts of HAAs compared to beef, pork, and veal due to the fact that they have lower levels of creatinine and glucose as well as higher pH levels[16].

Since HAA's are present at low concentrations in complex matrix of meat tissues, sensitive and selective methods are necessary to analyze the compounds in food samples. Chromatography is necessary in the studies in order to separate the HAA's from other compounds in the tissue matrix and quantify their relative amounts. Research into the development of purification and extraction of heterocyclic amines has taken place in order to

analyze HAA's using either liquid chromatography (LC) involving ultraviolet diode array detectors or LC coupled with mass spectrometry (MS). LC techniques have been used most frequently in as the sample preparation is less complex[10]. Mass spectrometry is useful in order to confirm the identity of HAA's. Other studies have used gas chromatography due to its relative simplicity and high sensitivity when coupled with mass spectrometry[10]. An initial derivatization step of the amines to convert them to more volatile, non-polar molecules is necessary for successful analysis[11].

Current HAA research has looked into ways to reduce the HAA concentration in cooked meats. Antioxidants are primary additives in the preparation of meats for the reduction of HAA's in the cooked products. Due to banning of many synthetic antioxidants like butylated hydroxytoluene and butylated hydroxyanisole, research into natural antioxidants like Rose (*Rosa rugosa)* extract, has been the focus of some HAA research[9]. The Rose plant produces a polyphenolic compound commonly used in the manufacture of wines, teas, and juices[9]. These polyphenolic compounds may reduce the formation of HAAs in cooked meat.

Additionally, research has also focused on determining the effects of multiple cooking methods on HAA production in various meats. Studies have demonstrated higher HAA production at higher temperatures when pan frying, air roasting, electric grilling, stone grilling, and wire grilling[3, 8, 9, 13]. Alternative cooking methods like sous-vide have been studied. The method involves specialized vacuum packaging materials for delicately controlled heating of the meat at temperatures around 100^{o} C[14]. Alternative cooking like this decreases the amount of HAA's formed due to their decreased cooking temperatures below 155^{o} C..

These past studies have only observed HAA concentration of a whole meat sample. This study intends to investigate the dynamics of how HAA concentration may be distributed

throughout the different layers of a cooked meat sample. Without current analysis into whether HAAs migrate through meat matrices, there are possibly regions within deeper regions of whole meats that aren't being treated with antioxidants. While some studies have observed HAAs at different layers of meats, they have only determined HAA concentrations in the colored crusted regions up to the evaporation fronts which mark the un-crusted areas of cooked meats[3]. This proposed study intends to detect and quantify the concentrations of HAAs produced throughout the layers of pan fried meats using GC-MS purification and detection methods.

Methodology

Prior to cooked meat trials, preliminary testing was needed to determine if pre-purchased, isolated HAAs could be detected in the first place. This study focused on 2-amino-3,8-dimethylimidazo[4,5-*f*] quinoxaline (MeIQx) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP). Gas chromatography (GC) coupled with mass spectrometry (MS) was used due to its relative simplicity, separation efficiency, as well as high sensitivity and specificity compared to other detection techniques[12]. The GC was used to separate and identify the isolated HAAs. However, gas chromatography is only possible with less polar compounds since largely polar compounds, like HAA's, will usually adsorb to the GC column and elute as broad tailing peaks[10]. Thus, HAA's must undergo derivatization before analysis. MS was used to confirm whether a peak from the GC was in fact HAAs based off of its characteristic fragmentation pattern.

After determining a methodology that could be used to successfully detect PhIP and MeIQx, the next step was to analyze whether the two compounds could still be detected following solid phase extraction (SPE). SPE was used as a clean-up step before GC-MS analysis

to filter out any common meat matrix interferences, such as phospholipids, fats, salts, and proteins. SPE is similar to gas chromatography in that it separates analytes by their varying affinities to either a mobile phase running through the column or the stationary phase of the column itself resulting in different elution rates. The same GC-MS detection method was applied to stock HAA solutions run through the SPE step. If the HAA's were unable to be eluted for the SPE column during the clean-up of meat samples, they would not be detected when the resulting eluents were run through GC-MS.

The final stage in the study was determination of HAAs in cooked meat samples using GC-MS following their clean-up with SPE and derivatization. In order to determine the concentrations of HAAs at different depths of meat samples, cooked meat samples were sliced into layers and GC-MS was used to determine the amount of HAAs in each layer.

Derivatization and GC-MS Identification of Isolated HAAs

MeIQx and PhIP were purchased from Toronto Research Chemicals and stock solutions were prepared and diluted in acetonitrile (ACN). The HAA's were derivatized with *N*,*N*-dimethylformamide-di-*tert*-butylacetal (DMF-DtBA) due to its advantages of reacting in a single step, easily evaporated excess reagent, and high stability[10, 11, 12]. The solvent was anhydrous methanol since the most important condition to achieve suitable yields is avoiding moisture[10]. For preliminary HAA determination, 1 mL of MeIQx and PhIP stock solutions 1 μg/mL were air dried at 40^{o} Celsius for 25 minutes to produce residues of HAAs at bottoms of their individual vials. 200 μL DMF-DtBA in 300 μL methanol was added to the residues in the vials. The mixture of HAA's and DMF-DtBA in methanol was heated at 100^{o} Celsius for 15 minutes with the cap of the vial off. Excess liquid was dried with the air drier. The remaining dried residue

was dissolved in 50 μL ethyl acetate and injected into the GC-MS. GC-MS instrument procedure was run following the study by Barrachina et al. in order to detect the MeIQx and PhIP.

Solid Phase Extraction

This study utilized Oasis® HLB extraction cartridges to concentrate and purify the samples. Preliminary study of SPE analyzed the solutions obtained from the Load, Wash, and two Elution steps. The Load step was the injection of the pre-treated HAA sample into the cartridge. The Wash step used a mobile phase consisting of 5% methanol in DI water. The Elution steps used 90/10 ACN/methanol[15]. Two Elution steps were used to determine if any HAAs were present after the initial elution. . Each step used 1 mL of liquid and flow rates of about 1 mL per minute using a SUPELCO air pressure system. The collected vials from the wash step were dried using a rotary evaporator under vaccum, while the collected elution vials were dried using the air drier. The dried samples were derivatized with DMF-DtBA and analyzed by the same GC-MS method as described previously.

Limit of Detection and Limit of Quantitation

In order to distinguish the peak of interest from the intrinsic noise generated in the gas chromatogram, the limit of detection (LOD) and limit of quantitation (LOQ) were determined. These are thresholds set through statistical analysis of the detection results from the GC-MS methodology used in the study to identify peaks that are significantly different from noise picked up by the detector. The LOD is the lowest amount of analyte in a sample that can be detected but not necessarily quantitated[18]. The LOQ is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy[18] and thus will set the threshold

of determination higher than the LOD. The LOD is calculated as (3 x Standard Deviation) / slope of calibration curve. The LOQ is (10 x SD) / M. The calibration curve was created by determining the peak area from the GC's of MeIQx run at 50, 100, 150, 250, and 750 ng/mL. The standard deviation was determined from results of running MeIQx 150 ng/mL five times.

Cooked Meat Trials

Prior to the layered GC-MS methodology, preliminary trials were run to determine whether HAAs could be detected in cooked meat samples following the SPE, derivatization, and GC-MS methodologies described above. Beef Chuck Eye Steak was used for cooked meat trials. Meat samples were pan fried with no oil on a non-stick iron pan on a hot plate. Due to hot spots that could have formed on the pan, a digital thermometer was used to determine the specific temperature of the specific cooking spot on the pan prior to thermal treatment.

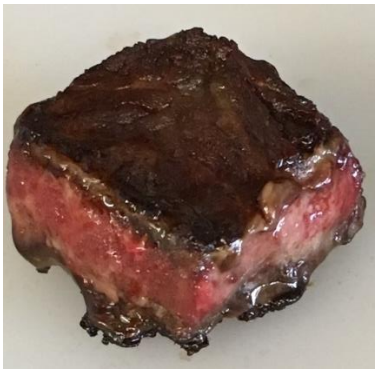
For the first preliminary cooked trial, meat was cooked at 450^{o} Celsius for 35 minutes. 1 gram of the sample was weighed out after cooking. The resulting charred sample, as seen in Figure 2, was homogenized in a methanol solvent using an ultrasonic bath set at 40^{o} Celsius for 20 minutes to ensure formation of HAAs. The resulting solution was processed using SPE and analyzed previously.

The second preliminary cooked trial involved cooking the meat sample at more realistic cooking conditions of 200^{o} Celsius for 10 minutes on two sides of the meat to produce the rare to medium-rare sample seen in Figure 3. The visibly browner ends were cut away from the redder center as seen in Figure 3. The visibly browner ends were collected and weighed out to be 6.1957 grams together. The remaining redder center was weighed out to be 3.6245 grams. The two ends were weighed together and homogenized in ACN in the ultrasonic bath as in Trial 1.

The center portion was prepared similarly. The two samples were run and analyzed with the SPE and GC-MS methodology.

Figure 2: Preliminary Trial 1 cooked at 450º Celsius for 35 minutes

Figure 3: Preliminary Trial 2 cooked at 200º Celsius for 10 minutes on two sides



The final part of the study was the sliced cooked meat trial. A different Beef Chuck Eye Steak was prepared by pan frying as before at 200º Celsius for 10 minutes on just one side as seen in Figure 4. The resulting sample was sliced to 2.5 mm layers resulting in 6 layers that were labeled 1 through 6 from the brownest end that was closest to the pan to the reddest end farthest

away. The resulting layers as seen in Figure 5 were weighed separately and individually homogenized in ACN. The resulting solutions were processed as described previously.

Figure 4: Pre-sliced Meat Cooked at 200° Celsius for 10 minutes on one side

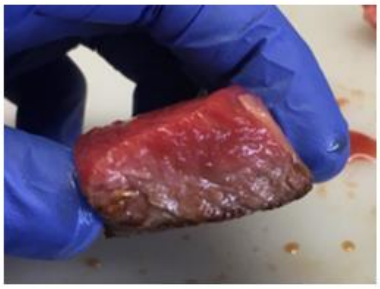
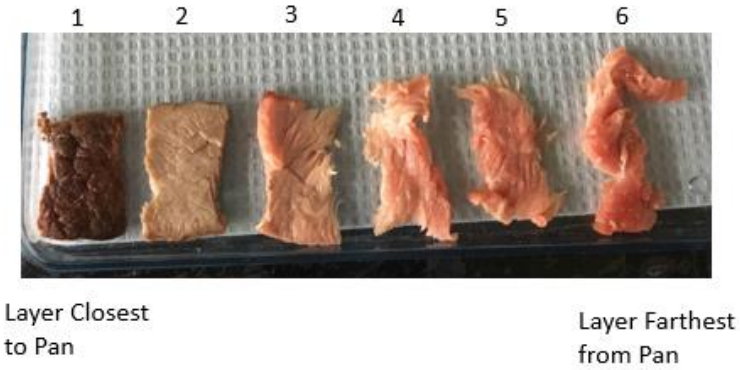


Figure 5: Sliced Cooked Meats 1 through 6



Results

Preliminary Detection Results of GC-MS with Isolated HAAs

Preliminary data showed that the GC-MS methodology was able to detect MeIQx at 1 µg/mL ACN. Figure 6 shows the spectra of MeIQx derivatized with 200 µL DMF-DtBA. MeIQx detection was confirmed by extracted ion chromatograms using detection parameters of quantitative ion peaks and confirmation ion peaks established by Zhang et al. and Barrachina et al. MeIQx peak confirmation was determined by the alignment of elution times of quantitation

peaks at around 13.10 minutes. Table 1 shows relative area of the MeIQx Parent ion (m/z) to be 94.32% and the Quantitation ion (m/z) to be 50.50%. Figure 7 presents the mass spectrum of MeIQx. The molecular ion $[M]^+$ was the base peak at 268 m/z. Fragmentation resulted in ions with smaller masses. The $[M-56]^+$ represented the loss of the -C-N$(CH_3)_2$ group.

PhIP was also detected at 1 μg/mL ACN. Figure 8 shows the extracted chromatogram of PhIP also derivatized with 200 μL DMF-DtBA. In Figure 8, alignment of peaks, also determined from the past literature, at the same elution time 14.86 minutes is indicative of PhIP detection[10, 12]. The mass spectrum of this chromatographic peak confirms that PhIP is detected as seen in Figure 9.

However, Table 1 shows the Parent ion (m/z) has a relative area of only 55.12% and the Quantitation ion peak $[M-15]^+$ with a relative area of 58.97%. The $[M-15]^+$ peak corresponds to loss of a $–CH_3$ group. These result show that the methodology was able to successfully derivatize MeIQx and PhIP at 1 μg/mL with DMF-DtBA and detect them with GC-MS.

Figure 6: Extracted Ion Chromatogram of MeIQx derivatives 1 µg/mL using DMF-DtBA as Derivatization Reagent

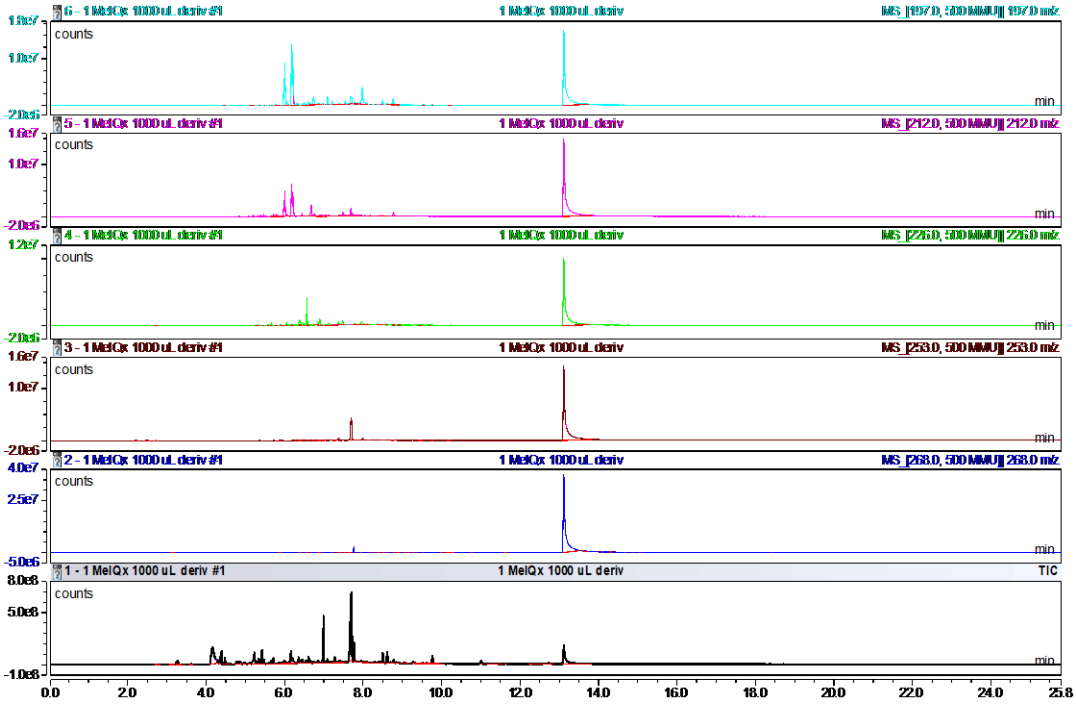


Figure 7: Mass Spectra of MeIQx 1 µg/mL using DMF-DtBA as Derivatization Reagent

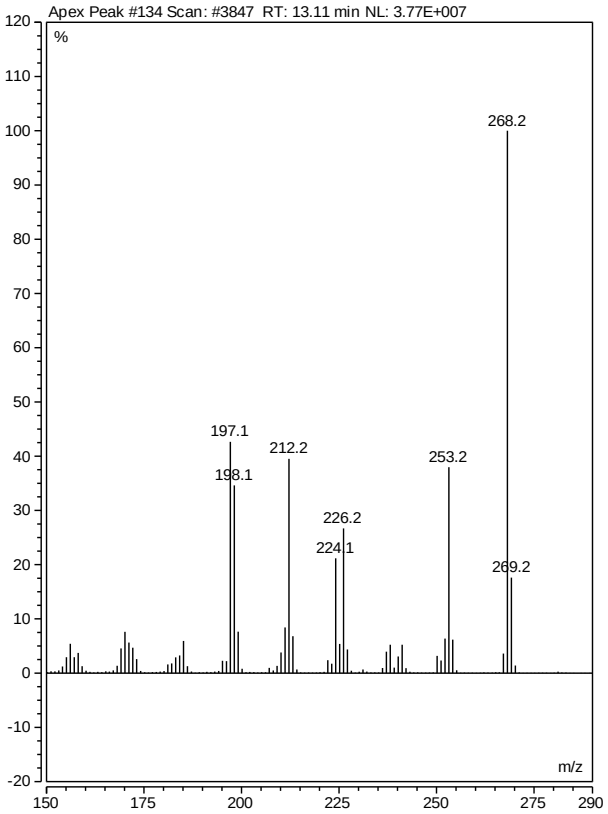


Figure 8: Extracted Ion Chromatogram of PhIP derivatives 200 ng/mL using DMF-DtBA as Derivatization Reagent

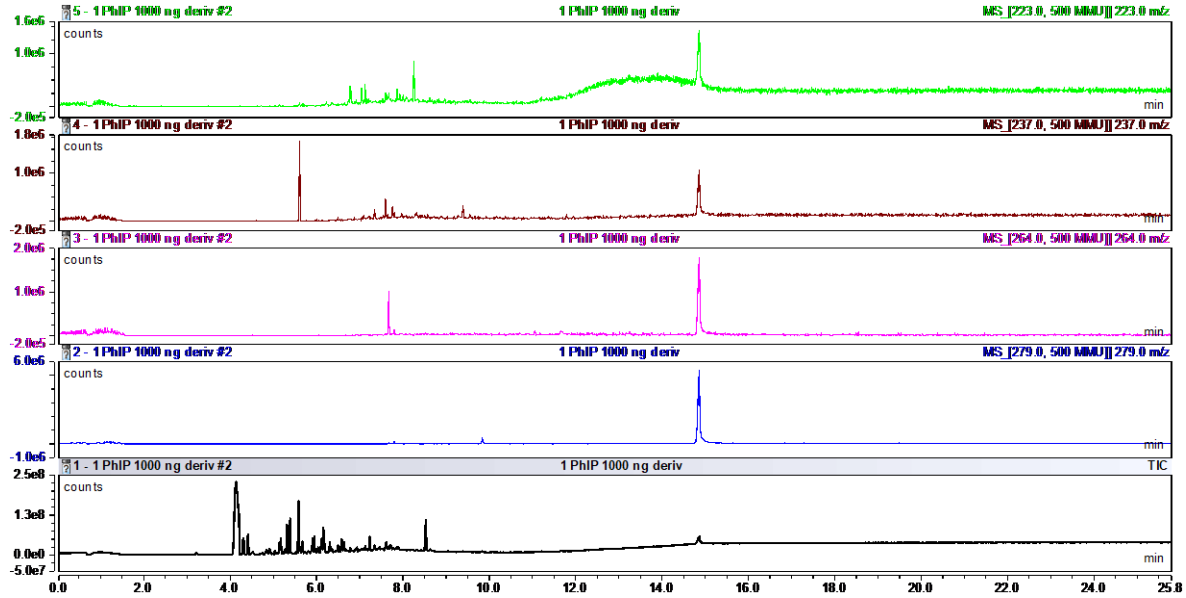


Figure 9: Mass Spectra of PhIP 1 µg/mL using DMF-DtBA as Derivatization Reagent

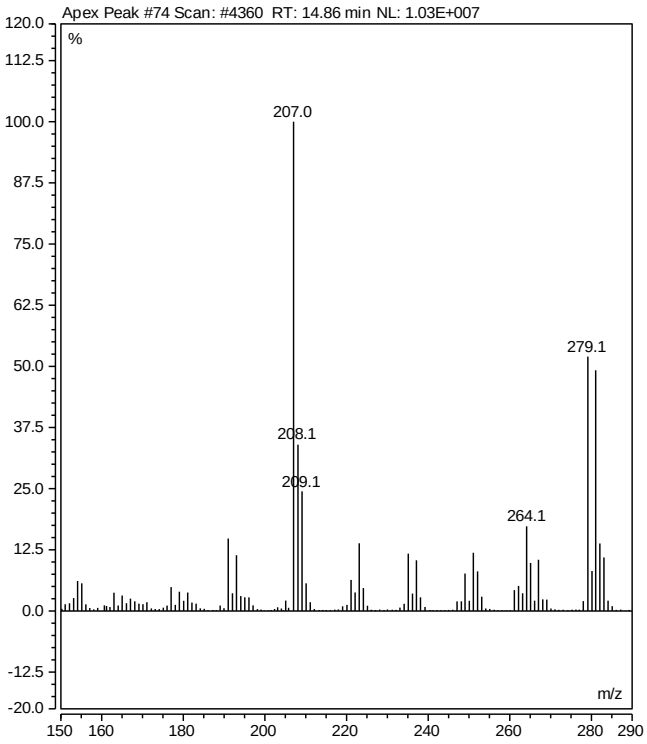


Table 1:

Compounds	**Parent ion, m/z (Relative Area %)**	**Quantitation ion, m/z (Relative Area %)**
MeIQx	268 (94.32)	212 $[M-56]^+$ (50.50)
PhIP	279 (55.12)	264 $[M-15]^+$ (58.97)

Preliminary Solid Phase Extraction (SPE) Results

Using the same determination method as above by looking at the extracted chromatograms and mass spectra, the results showed that MeIQx was able to be detected only in the Wash and Elution 1 steps and not the Load or Elution 2 steps. Figure 10 and Figure 11 show the gas chromatograms of the Wash and Elution 1 steps respectively demonstrating that the MeIQx peaks both come out at 13.10 minutes as seen in the previous data. In Figure 12, PhIP was determined to appear only in the Elution 1 step at 14.60 minutes thus near to the same time as the previous procedure. These results are indicative that MeIQx and PhIP were still able to detected using GC-MS following an SPE clean-up step; for MeIQx in the Wash and Elution 1 steps and for PhIP in the Elution 1 step. All other steps where HAAs were not detected would be discarded in further trials following SPE.

Figure 10: Gas Chromatogram of MeIQx SPE Wash Step Extract

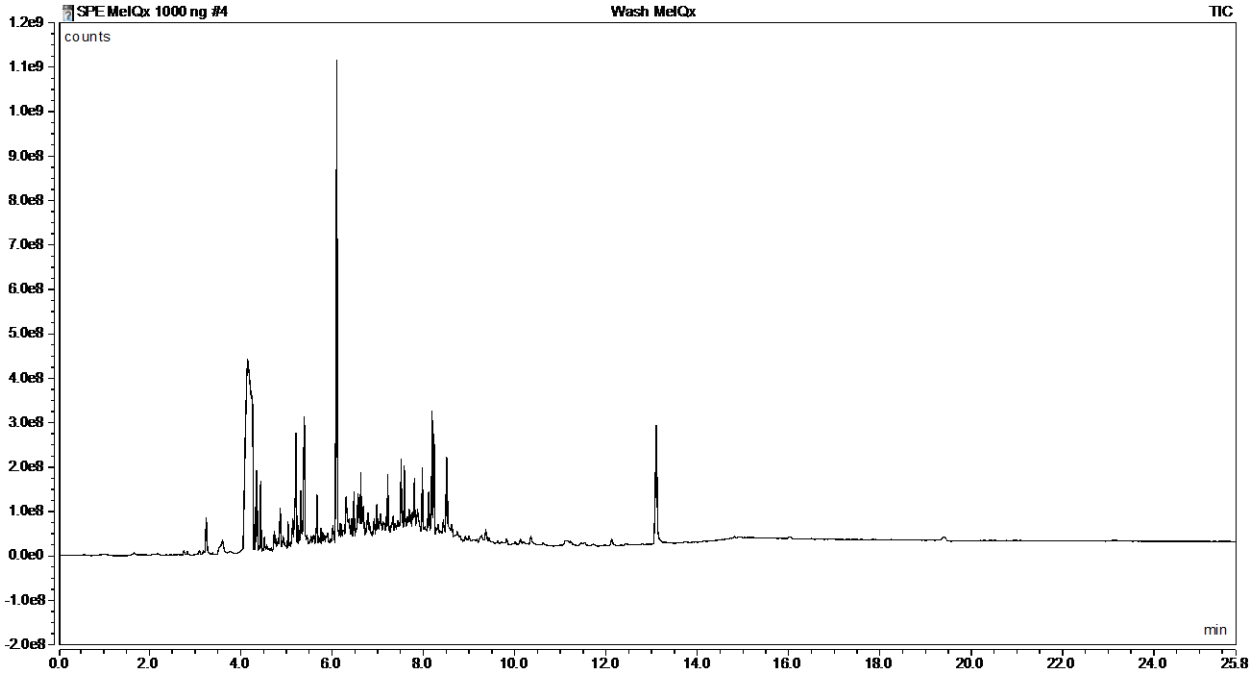


Figure 11: Gas Chromatogram of MeIQx SPE Elution 1 Step Extract

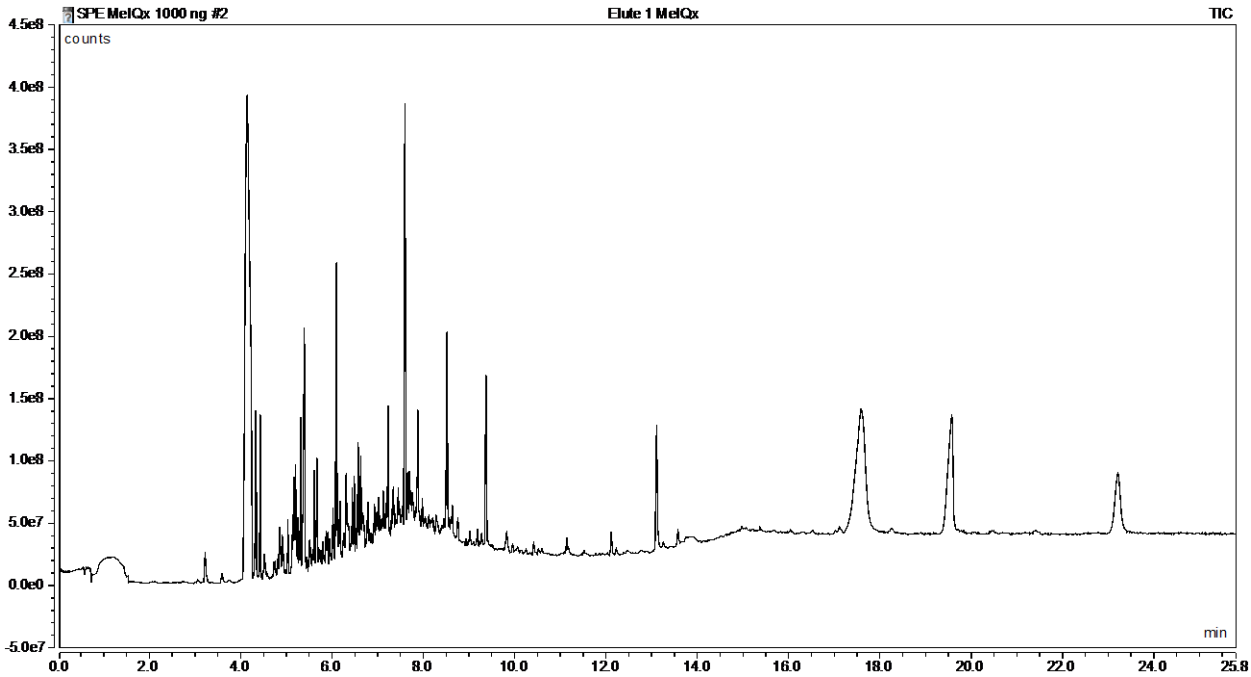
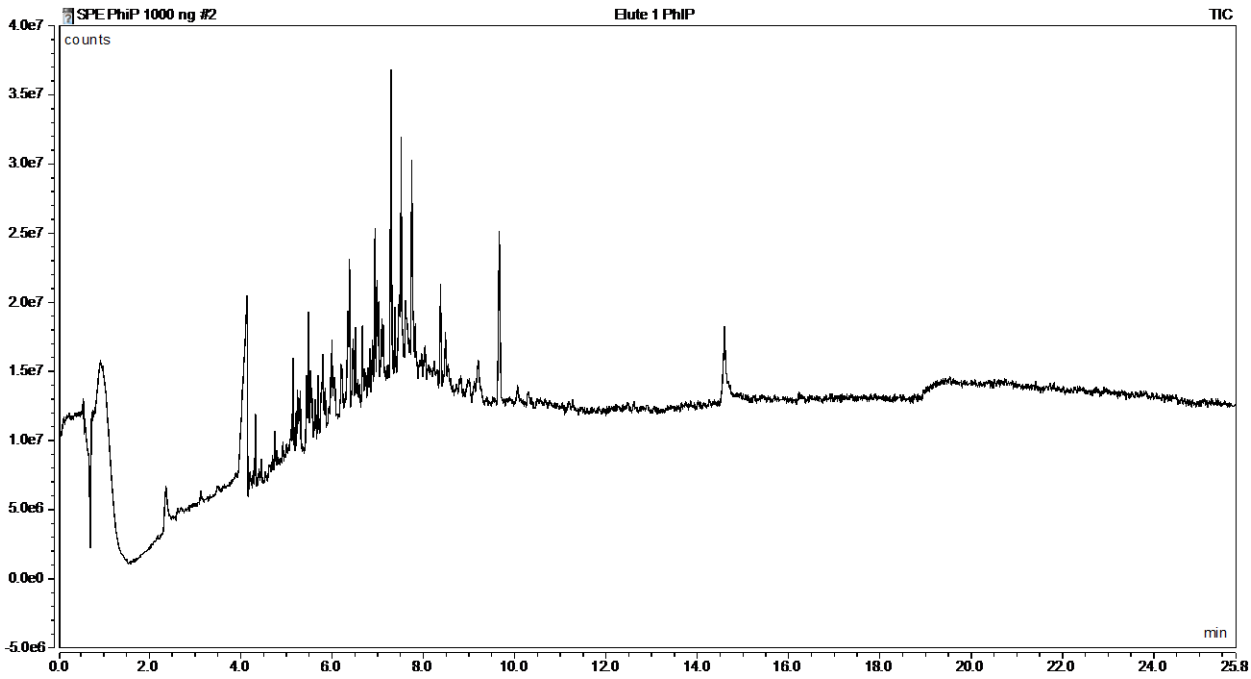


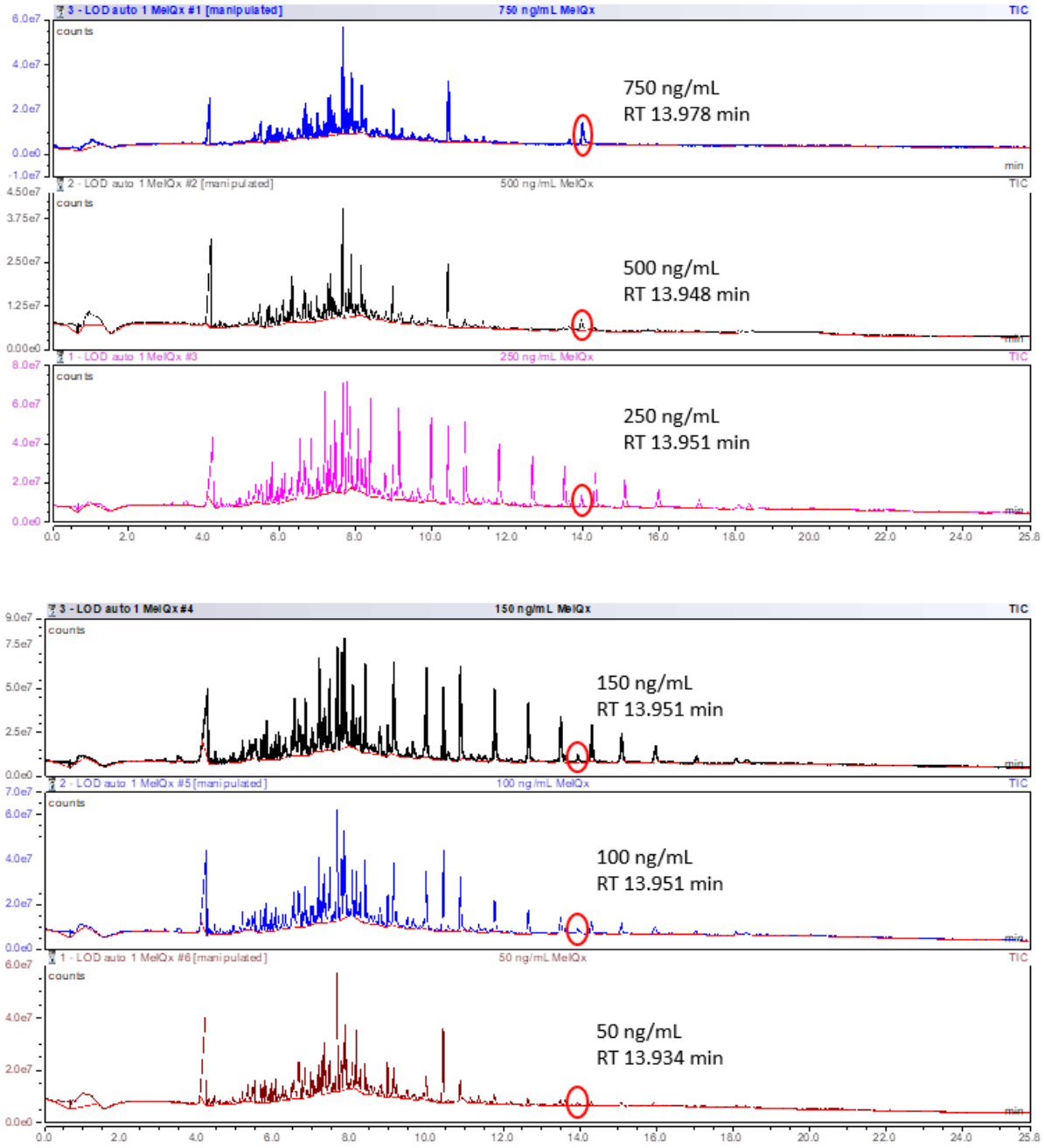
Figure 12: Gas Chromatogram of PhIP SPE Wash Step Extract



Limits of Detection and Quantitation Calculations

The LOD and LOQ for MeIQx was determined by first creating the calibration curve from the quantitative ion (212 m/z) peak areas for concentrations of 50, 100, 150, 250, and 750 ng/mL. Figure 13 shows the gas chromatograms of these concentrations and that the derivatized MeIQx peaks all come out at around the same retention time 13.952 minutes. Since the molecular weight of the derivatized MeIQx does not change while diluting the sample from 750 ng/mL to 50 ng/mL, peaks that come out at about the same time are indicative of the same molecule. The apparent correlation seen by the decrease in peak area from chromatograms as the dilutions decrease from 750 ng/mL down to 50 ng/mL is further evidence that the peaks are associated with MeIQx. These peaks were all confirmed using their associated mass spectra by using quantitative and confirmation ion fragments as described previously.

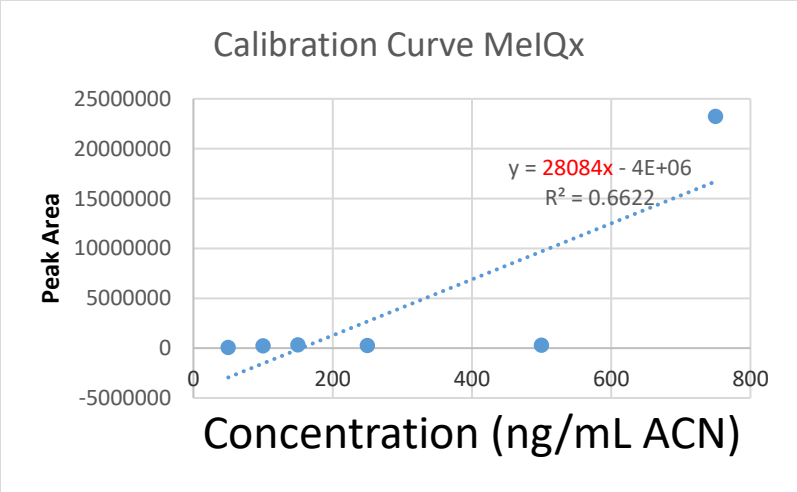
Figure 13: Gas Chromatograms of 50, 100, 150, 250, and 750 ng/mL MeIQx for Calibration Curve



The resulting slope from the generated calibration curve in Figure 14 was 28084. The R^2 value was found to be 0.6622. From running 150 ng/mL MeIQx through the GC-MS method five times, the resulting standard deviation was ± 95374.5127 (14.6%). This relative standard deviation falls into the range seen by Barrachina et al. which was 9.8 to 19.0% for MeIQx

derivatized with DMF-DtBA. The LOD was calculated to be 10 ng/mL and the LOQ was 34 ng/mL. Both of these values were acceptable in comparison to Barrachina et al. values of 10.3 ng g^{-1} LOD and 82.9 ng g^{-1} LOQ. However, Figure 14's low R^2 value coupled with the obvious variation in the data, particularly in the higher concentrations' peak areas for 500 ng/mL and 750 ng/mL, poses difficulty in accepting the accuracy of these results. Possible explanations for the variation in data points on the calibration curve is most likely due to outside factors that interfered with the derivatization of the MeIQx. Barrachina et al. elaborates that the reaction is quite moisture sensitive so excess moisture in the sample could have affected the rates of derivatization for the diluted samples. This would have altered the peak areas and would have affected the resulting calibration curve.

Figure 14: Calibration Curve for MeIQx

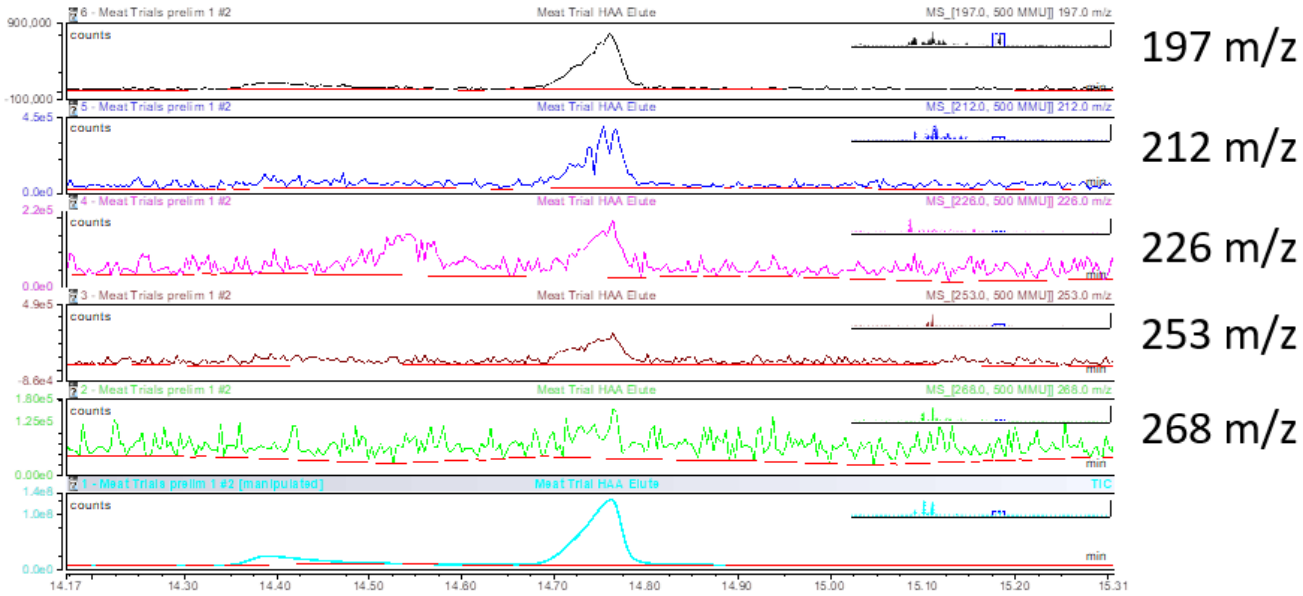


Preliminary Cooked Meat Trials

Trial 1 involved spiking the meat sample with HAAs by thermal treatment at the excessively high temperature of 450° Celsius for 35 minutes. Following SPE and then analysis with GC-MS, MeIQx was able to be detected in both from both the Wash and Elution collected

vials from the charred sample solution seen in Figure 2. This was confirmed using the extracted ion chromatogram for MeIQx using the confirmation ion peaks determined by Barrachina et al. and Zhang et al as seen in Figure 15. Alignment of the confirmation ions at the same retention times was indicative that the MeIQx peak was present for reasons described above. Using the 212 m/z quantitation ion peak area, the calculated concentration of MeIQx for Trial 1 was 27,290.82 counts²/g which was the sum of the concentrations of Trial 1's Wash and Elute step vials as seen in Figure 15.

Figure 15: Preliminary Cooked Meat Trial 1 Extracted Ion Chromatogram of MeIQx derivatives



Furthermore, Trial 2 involved cooking the meat sample at a lower temperature for a shorter amount of time (200° Celsius for 10 minutes) on two opposite sides of the meat. The separate experimental groups were run through SPE and GC-MS. MeIQx was able to be detected in the Wash and Elution steps as described previously. The MeIQx peak was confirmed using the extracted ion chromatograms of the fragmented ion as demonstrated in Figures 16 and 17 with

the same evaluation method as before. The center cut's total MeIQx concentration was 1389.11 counts²/g while the ends' concentration was surprisingly found to be lower at 1142.33 counts²/g. Total concentrations were the sum of the Wash and Elution vials for both conditions.

Figure 16: Preliminary Cooked Meat Trial 2 Center Cut Extracted Ion Chromatogram of MeIQx derivatives

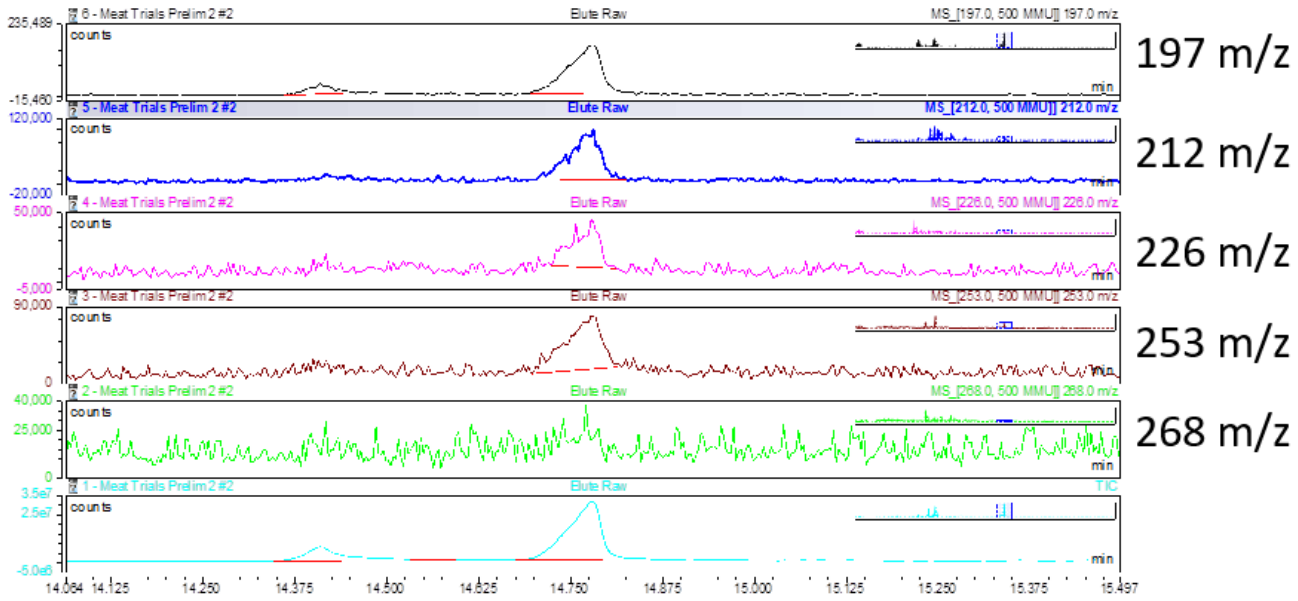
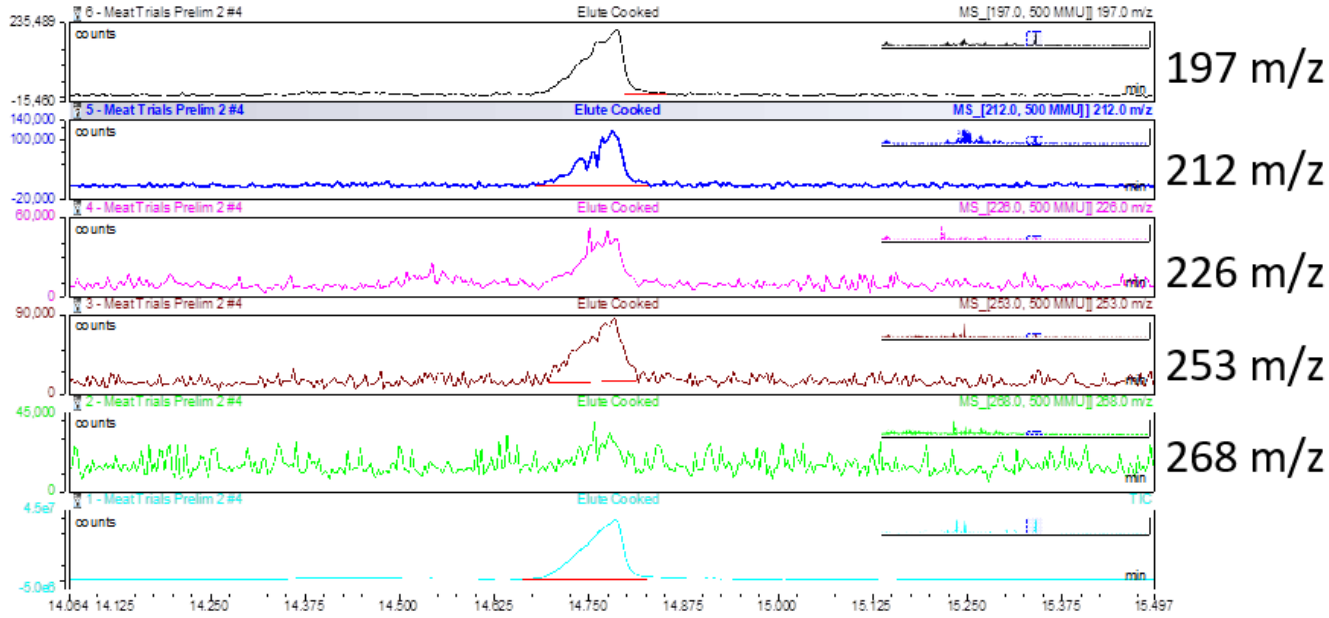
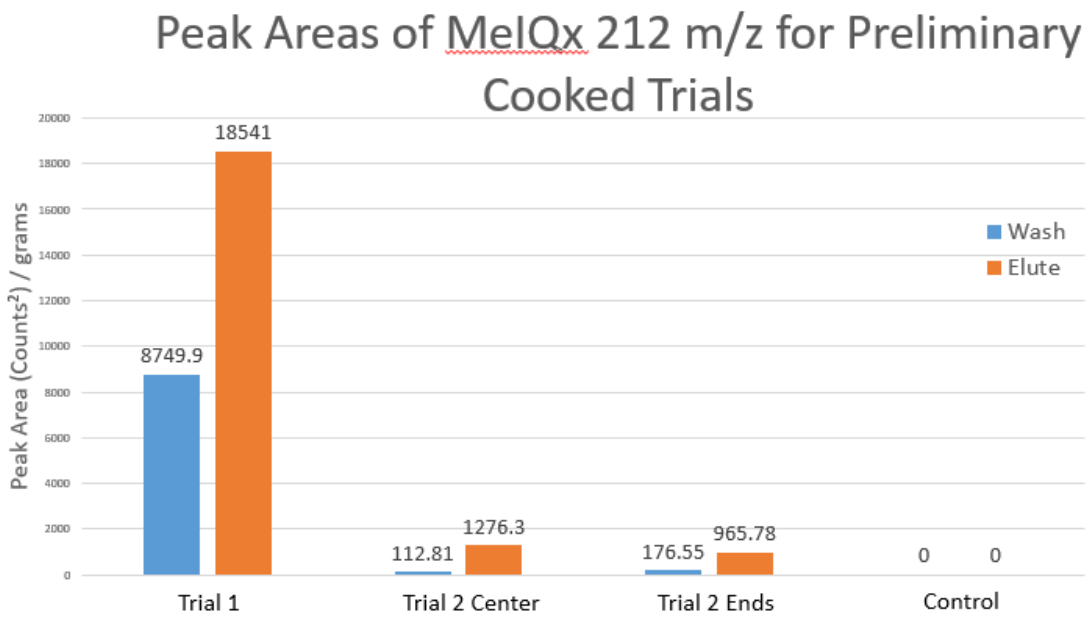


Figure 17: Preliminary Cooked Meat Trial 2 End Cuts Extracted Ion Chromatogram of MeIQx derivative



An uncooked sample of meat was run through the SPE and GC-MS methodology as a negative control. No HAAs were detected in this group. Figure 18 shows a comparison of the Trial 1, Trial 2 Center, Trial 2 Ends, and control group concentrations of MeIQx. For all of the trials, the Wash step concentrations were lower than the concentrations found in the Elution steps. The highest concentration of MeIQx was found in Trial 1 where the meat was cooked longer and at higher temperatures compared to Trial 2 and the control. The Trial 2 Ends had a lower concentration than the Trial 2 Center cut. Additionally, the Trial 2 Center cut had a much higher concentration than the control trial which served as a negative control.

Figure 18: Preliminary Cooked Meat Trials 1 and 2 Quantitation Ion Peak Areas



Sliced Cooked Meat Trial

In order to determine how HAA concentration changed with depth in the meat, a cooked meat sample at 200° Celsius for 10 minutes on one side was sliced into 2.5 mm samples. Figures 4 and 5 show the cooked sample and resulting sliced layers labeled 1 through 6. Labeling followed the gradient of the meat from the visibly brownest in Slice 1 progressing to the visibly reddest in Slice 6. Slice 1 was in contact with the pan during the cooking process, while Slice 6 was furthest away as seen in Figure 4. No MeIQx was able to be detected in slices 1 through 4 as seen in the GC's of Figure 19. MeIQx was only able to be detected in Slices 5 and 6 as seen in the GC's. Figure 20 shows each chromatogram shows MeIQx coming out at about 14.86 minutes which was confirmed using their extracted gas chromatograms evaluation method. The concentration of MeIQx in slice 5 was 5404.98 counts²/g, which was higher than the concentration in Slice 6 at 2485.40 counts²/g. PhIP was not detected in any cooked meat trials.

Figure 19: Cooked Meat Trial Slices 1, 2, 3, and 4 Gas Chromatograms

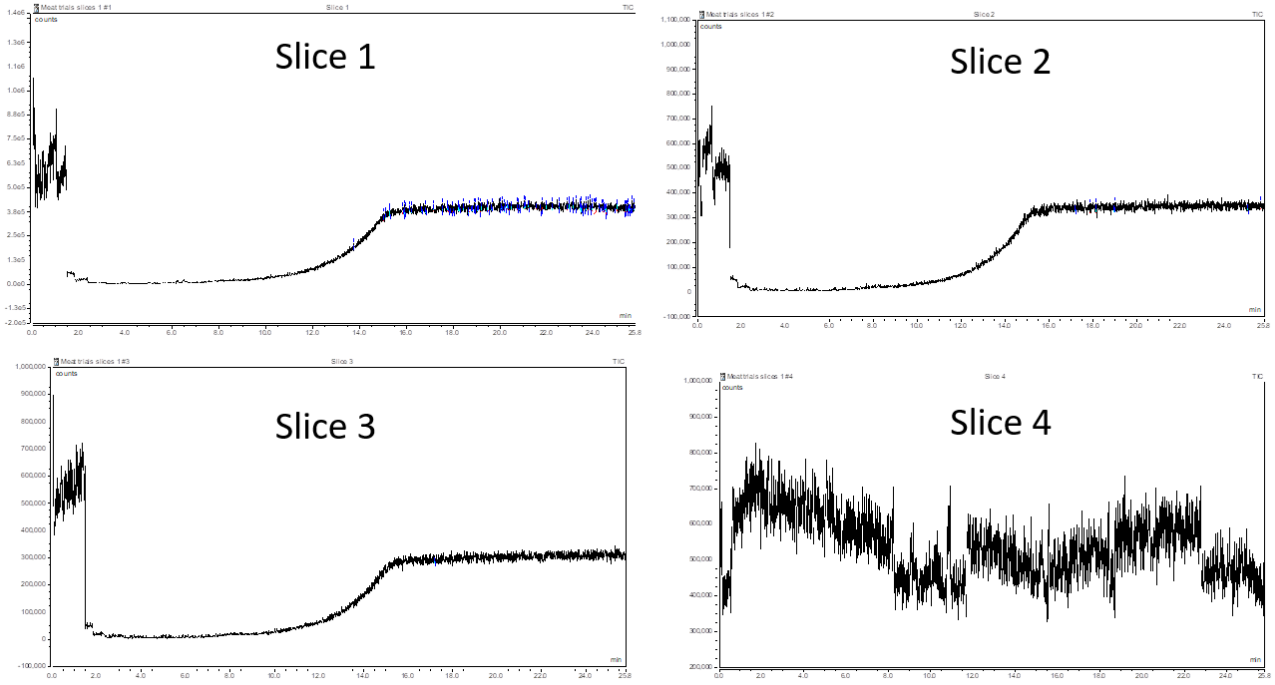
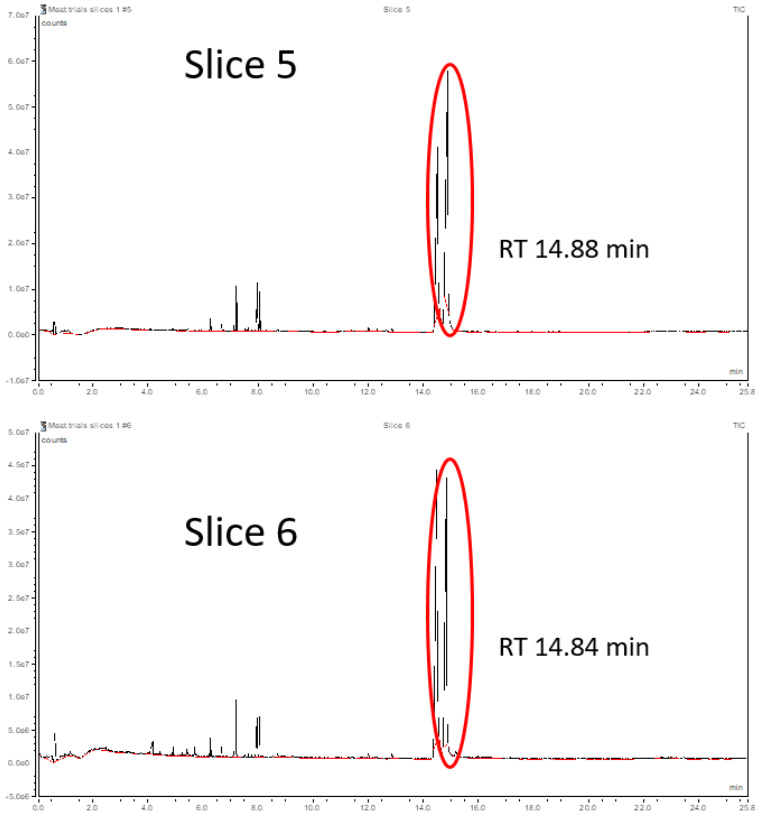


Figure 20: Cooked Meat Trial Slices 5 and 6 Gas Chromatograms



Discussion and Conclusions

The main purpose of the proposed project was to determine the relative concentration of HAA's formed at different depths of meat. The preliminary work focused on adapting extraction and analysis methods for MeIQx and PhIP using SPE, GC-MS, and derivatization procedures. MeIQx and PhIP spectra peaks were able to be confirmed using extracted ion chromatograms and mass spectra. Additionally, these same detection parameters were applied to vials collected after each step of SPE to determine when HAAs are coming out of the extraction cartridge. The results demonstrated MeIQx appears only in the Wash and Elution 1 steps. PhIP was determined to only be present in the Elution 1 step. This data meant that a second elution step was no longer necessary moving forward as the rest of the HAAs are extracted in the first elution. Though the times that the peaks come out in the chromatogram differ from the previous Barrachina et al. results, this is expected as the sizes of the columns differ. However, the fact that PhIP comes out at a later time (14.60 min) compared to MeIQx (13.10 min) is expected and is similar to the past studies. This is due to the higher weight of PhIP (279 amu) compared to MeIQx (268 amu). Additionally, the fact that samples with moisture were unable to yield an HAA peak is confirmed by the findings in Barrachina et al. and Zhang et al.

After determination that stock solutions of isolated MeIQx and PhIP could be detected using SPE and GC-MS methodology, the study shifted to investigating whether the same methodology could be applied to HAAs naturally produced during the cooking processes of meat samples. From Trial 1 to Trial 2, the results were as expected. The cooking temperature and time comparison from Trial 1 to Trial 2 lays out a "whole meat" analysis of the HAA concentration similar to multiple past studies[4, 6, 7]. Two separate cooked meat samples are wholly compared to

one another instead of comparison of internal slices. The Trial 1 concentration was found to be 91.11% higher than the average of Ends and Center cut groups' concentrations from Trial 2. This demonstrated that the higher temperature and longer amount of cooking time produced a higher concentration of MeIQx compared to meat cooked at lower temperatures for a shorter amount of time overall. These results do not demonstrate whether the higher cooking time (difference of 15 minutes between Trial 1 and Trial 2) or the higher temperature (difference of 250° Celsius between Trial 1 and Trial 2) had a higher contribution to the generation of more MeIQx seen in Trial 1. Literature has investigated these two factors but has yet to elucidate which one is more significant[4, 6, 7]. However, the results of this study are in agreement with past results that have shown that cooking temperature and cooking time in coordination together lead to a general increase in HAA generation in meat samples[4, 6, 7]. Additionally, the difference in the solvent used between Trial 1 and Trial 2 could have led to the differences in values observed. Since MeIQx, like all HAAs, is highly polar then it will most likely more readily dissolve in polar solvents. Methanol, the solvent in Trial 1, is more polar than ACN, the solvent in Trial 2. Due to this, Trial 1 may have had overall higher solvation than Trial 2 leading there to be more MeIQx present in solution to begin with.

Interesting results came from comparison of the Center cut group of Trial 2 to the Ends group of Trial 2. This comparison was a preliminary examination of large layers of visually distinct thermally treated in-product meat samples. Visual distinction was determined beforehand to be an appropriate determination of distict regions subjected to thermally different conditions during the cooking process since the browned crusts that form are the result of Maillard reactions that only take place at temperatures above 155º Celsius[3]. As mentioned before, these Maillard reactions are also what generate HAAs. The Trial 1 to Trial 2 comparison showed that meat

cooked at higher temperatures produces more MeIQx. This trend was expected to be observed internally within the meat in Trial 2. The two visibly browner ends seen in Figure 3 should have been subjected to the highest thermal treatment as they were closest in proximity to the heat source (the pan). On the other hand, the center cut should have been subjected to much lower temperatures. Visually, Maillard non-enzymatic browning reactions, which occur at 155° Celsius, did not occur in the Center cut since the region was still visibly red. However, this Center region did result in a higher concentration of MeIQx at 1389.11 counts²/g compared to the concentration of the two cooked Ends regions together was found to be only 1142.33 counts²/g. As a result, these findings contradict the cooking time and temperature for the kinetics of the HAA generation as demonstrated in the comparison of Trial 1 to Trial 2 and in multiple other studies[3, 4, 6, 7]. The negative control, in which an uncooked meat sample was run through the SPE and GC-MS methodology, demonstrated that no HAAs could be detected.

The sliced meat trial was performed to generate a clearer picture, so to speak, of the different concentrations of HAAs at different depths of the meat sample. However, further unexpected results were that no HAAs were able to be detected in the layers that were apparently subjected to the highest thermal treatments in slices 1 through 4. Similar to the crust formation in the Trial 2 Ends group, these slices also appeared visually browner signifying completion of Maillard reactions in these regions of lower numerical value compared to the redder regions of higher numerical value such as Slices 5 and 6. The results found that MeIQx was actually only able to be detected in these layers furthest from the heat source. Slices 5 and 6 had MeIQx concentrations of 5404.98 counts²/g and 2485.40 counts²/g respectively. In evaluating MeIQx concentration, or lack thereof, in Slices 1 through 4 to the concentrations in Slices 5 and 6, these results are comparable to the analysis made between Preliminary Trial 2 Ends to the Preliminary

Trial 2 Center cuts. For both of these comparisons, regions that were supposedly subjected to higher thermal treatments did not generate as much HAAs as regions at lower temperatures. However, when comparing MeIQx concentration from Slice 5 to Slice 6 the results appear as expected such as seen in analysis of Preliminary Trial 1 to Trial 2 in general. Slice 5, which was closer to the heat source and thus should have been subjected to higher temperatures, generated a higher concentration of MeIQx than Slice 6.

There are multiple reasons that could explain why the browner, more thermally treated regions could have generated lower concentrations of MeIQx. First, there could have been an uneven distribution of HAA precursors such as creatinine and glucose throughout the meat matrix. This is unlikely for comparison of the two preliminary trials as they come from the same meat sample. However this may have influenced why in the sliced meat trials the concentrations were so much higher at sum of 7890.38 counts^2/g compared to the sum of MeIQx concentrations for the Center and Ends regions of preliminary Trial 2 at 2531.44 counts^2/g. Past research has shown that the meat juices are the primary reservoir of these precursors within the meat matrices[19]. Since a fresher meat sample was used for the sliced meat trials, the meat matrix most likely retained more of the juices containing the multiple precursors needed to generate HAAs. The older meat used in Trial 2 may have had breakdown in the components of its meat matrix over time leading to lower retention of these precursor containing juices. Additionally, the HAA precursors could have migrated throughout the meat sample quickly. Not only the precursors but the HAAs themselves could have migrated to these distant, less cooked locations of the meat samples during the cooking process or from the manipulation of the samples after thermal treatments for slicing. Another reason is that there could have been internal variations and differences in temperatures throughout the meat samples. This could have created pockets of

regions of higher temperatures that could have generated more Maillard reactions for higher formation of HAAs. Furthermore, MeIQx could possibly form at lower temperatures than previously expected. However, the most likely reason was possible error during the derivatization process before analysis of the HAAs with GC-MS. As mentioned previously, the reaction is moisture sensitive. Residual moisture could have led to incomplete derivatization, preventing the detection of MeIQx and leading to the results seen for Slices 1 through 4 in Figure 19. These eluting chromatograms are consistent with the Barrachina et al. negative results demonstrating improper HAA derivatization.

Further research will look into adjusting methods in order to determine if PhIP can be detected in the Wash step. Addressing why PhIP did not appear in the Wash step like MeIQx, observations while working with PhIP revealed that its Wash vial did not produce a dried residue suggesting that moisture was still present during derivatization. Though the reason is unclear, the undried residue may have had to do with PhIP's larger size at 279 amu to MeIQx's 268 amu causing it to have a higher difficulty. Since water molecules interact with DMF-DtBA at a faster rate due to their smaller size[10], then the PhIP was possibly left underivatized. Furthermore, the lowest limit of detection for the HAAs will be determined for the same procedures except while running MeIQx and PhIP at 800 ng/mL, 600 ng/mL, 400 ng/mL and 200 ng/mL.

Since only one sliced meat trial was performed, more trials are necessary to determine the significance of these findings. Use of molecular sieves during the actual derivatization step could reduce the amount of moisture present and lead to better results. Possible detection of HAAs in Slices 1 through 4 in the future would result in different conclusions. Additionally, quick freezing the meat samples immediately after cooking would help immobilize the HAAs and their precursors in their specific layers.

Future research can look into exploring the amount of precursors such as creatinine or glucose are in a specific layer of the meat sample before, during, and after cooking. Additionally, testing can be done to determine the temperature of specific layers during cooking to distinguish these distinct environments more quantitatively rather than qualitatively overserving their different colors after cooking.

This study coupled with future investigation will give information about the concentration of HAA's at different depths of cooked red meat. The study can be repeated with various experimental variables such as different types of meat, cooking temperatures, cooking times, and cooking styles. Different meats such as poultry generally have lower levels of creatinine than other meats and thus could be an avenue to investigate further. Furthermore, different antioxidants such as *Rosa rogusa* extract and lime juice could be applied and injected into different layers of the meat. Using this layered methodology, HAA concentration levels can be compared with and without treatment. This information can prove useful to meat processing companies or the individual consumer to more effectively apply antioxidants to their meats or explore alternative cooking methods to reduce the formations of HAAs.

References

1.) Shabbir, M. A., Raza, A., Anjum, F. M., Khan, M. R., & Suleria, H. R. (2015). Effect of thermal treatment on meat proteins with special reference to heterocyclic aromatic amines (HAAs). *Critical Reviews In Food Science And Nutrition*, *55*(1), 82-93.

2.) National Health and Medical Research Council. (2006). Nutrient Reference Values for Australia and New Zealand. Department of Health and Ageing, Australian Government, Canberra.

3.) Kondjoyan, A., Chevolleau, S., Portanguen, S., Molina, J., Ikonic, P., Clerjon, S., & Debrauwer, L. (2016). Relation between crust development and heterocyclic aromatic amine formation when air-roasting a meat cylinder. *Food Chemistry*, *213*641-646.

4.) Oz, F., Kızıl, M., & Çelık, T. (2016). Effects of Different Cooking Methods on the Formation of Heterocyclic Aromatic Amines in Goose Meat. *Journal Of Food Processing And Preservation*, *40*(5), 1047-1053.

5.) World Health Organization. (2015). Global Health Observatory Data. WHO 2015.

6.) Bylsma, L. C., & Alexander, D. D. (2015). A review and meta-analysis of prospective studies of red and processed meat, meat cooking methods, heme iron, heterocyclic amines and prostate cancer. *Nutrition Journal* 14, 125.

7.) Yuzer, M. O., & Oz, F (2016). The effects of cooking on wire and stone barbecue at different cooking levels on the formation of heterocyclic aromatic amines and polycyclic aromatic hydrocarbons in beef steak. *Food Chemistry*, *203*59-66.

8.) Hannah Ritchie and Max Roser (2018). Meat and Seafood Production & Consumption. *OurWorldInData.* Retrieved from: 'https://ourworldindata.org/meat-and-seafood-production-consumption'

9.) Jamali, M. A., Zhang, Y., Teng, H., Li, S., Wang, F., & Peng, Z. (2016). Inhibitory Effect of Rosa rugosa Tea Extract on the Formation of Heterocyclic Amines in Meat Patties at Different Temperatures. *Molecules (Basel, Switzerland)*, *21*(2), 173.

10.) Barcelo-Barrachina, E., Santos, F.J., Puignou, L. Galceran, M.T. (2005). Comparison of dimethylformamide dialkylacetal derivatization reagents for the analysis of heterocyclic amines in meat extracts by gas chromatography-mass spectrometry. *Analytica Chima Acta*, Volume 545, Issue 2, 2005, 209-217.

11.) Kataoka, Hiroyuki, Kijima, Koji. (1996). Analysis of Heterocyclic amines as their N-dimethylaminomethylene derivatives by gas chromatography with nitrogen-phosphorus selective detection. *Journal of Chromatography A,* 767 (1997), 187-194.

12.) Zhang, Feng, Chu, Xiaogang, Sun, Li, Zhao, Yansheng, Ling, Yun, Wang, Xiujuan, Yong, Wei, Yang, Minli, Li, Xiuqin. (2008). Determination of trace food-derived hazardous compounds in Chinese cooked foods using solid-phase extraction and gas chromatography coupled to triple quadrupole mass spectrometry. *Journal of Chromatography A*, 1209 (2008) 220-229.

13.) Szterk, A. (2015). Heterocyclic aromatic amines in grilled beef: The influence of free amino acids, nitrogenous bases, nucleosides, protein and glucose on HAAs content. *Journal Of Food Composition & Analysis*, *40*39-46.

14.) Zikirov, E. & Oz, F. (2015). The effects of sous-vide cooking method on the formation of heterocyclic aromatic amines in beef chops. *LWT - Food Science & Technology*, *64*(1), 120-125.

15.) Oasis Sample Extraction Products (2018). Taking the Complexity out of SPE Method Development. *Waters*.

16.) Gibis, M., & Weiss, J. (2015). Impact of Precursors Creatine, Creatinine, and Glucose on the Formation of Heterocyclic Aromatic Amines in Grilled Patties of Various Animal Species. Journal Of Food Science, 80(11), C2430–C2439.

17.) Thorgeirsson, S. S., Davis, C. D., Schut, H. A., Adamson, R. H., & Snyderwine, E. G. (1995). Possible relationship between tissue distribution of DNA adducts and genotoxicity of food-derived heterocyclic amines. *Princess Takamatsu Symposia, 23,* 85–92.

18.) World Health Organization. 2006. WHO Supplementary Training Modules: Validation, Water, Air Handling Systems - Validation (Part 4): Analytical Method Validation.

19.) Arvidsson, P., van Boekel, M. A. J. S., Skog, K., Solyakov, A., & Jägerstad, M. (1999). Formation of heterocylic amines in a meat juice model system. Journal of Food Science, 64(2), 216–221.