4-3-2017

Molecular Mass of L-Arginine-Based Potentially Carcinogenic Heterocyclic Amines

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HONS 497
Honors Thesis

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April 3, 2017

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Abstract:

Investigation of cancer-inducing molecules in cooked foods has led to the discovery of mutagenic heterocyclic amines (HCAs) in meat. The amino acids creatin(in)e and L-phenylalanine form the precursors for these HCAs. Recent research here at Andrews University have found similar mutagenic HCAs produced from plant-based proteins when L-arginine, rather than creatin(in)e, is substituted in high temperature reactions (simulated cooking) with L-phenylalanine. Our research focused on developing HPLC methods to isolate individual candidates that are then screened for mutagenicity via the Ames test. Mass spectroscopy methods were also developed to identify the molecular structure of these lead mutagenic L-arginine-based HCAs.

Introduction:

Heterocyclic amines (HCAs) are planar, chemical compounds with at least one heterocyclic ring and an amine group. By definition, this means that it has a cyclic structure consisting of at least two different elements, and a functional group with nitrogen respectively. Because smoking, dietary factors, and infection/inflammation are commonly believed to cause cancer, many research groups have begun investigating chemical compounds in human food that may lead to carcinogenesis. Over twenty HCAs have been found in cooked meats and fish where creatin(in)e (found in muscle tissue), amino acids, and sugars are exposed to high temperatures. For example, the known carcinogen PhIP can be synthesized from creatine and the amino acid L-L-phenylalanine in the mechanism demonstrated in **Figure 1**. Such condition can be created in a cooking environment on a grill or over an open flame [Cross et al., 2004]. Many of the carcinogens for humans have also been found strongly mutagenic for prokaryotes, in particular the strain of *Salmonella Typhimurium* (T98), when exposed to HCAs; this test is known as the Ames test. Rodents fed HCAs developed breast, prostate, and colon cancer [Sugimura et al,. 2004].

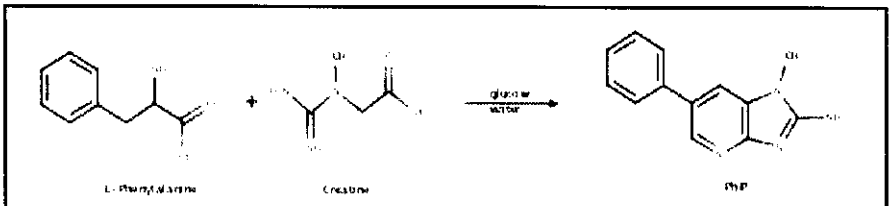


Figure 1. Chemical equation for PhIP synthesis

The majority of research on HCAs has been conducted with meat and fish products, and creatin(in)e has been found to react with other free amino acids and sugars to form mutagenic and carcinogenic HCAs. In the Knize's research lab, however, HCAs were found not in meat but rather in grain-based foods which are absent of creatin(in)e [Knize et al., 1994]. After performing the mutagenicity tests with the Salmonella strain T98, these HCAs also exhibited strong mutagenic characteristics. The research team systematically heated amino acids alone or in binary combinations in conditions similar to those of cooking meat. Under the Ames test, over half of these products were found to be mutagenic with Salmonella strain T98; all 1:1 binary combinations of L-arginine with threonine, valine, cystine, cysteine and tryptophan were also found to be highly mutagenic under the Ames test. The same year, the Knize research group continued investigating and found similarly mutagenic extracts in common grain foods prepared at cooking temperatures of 210°C [Knize et al., 1994]. These extracts from samples exhibited mutagenicity in the Ames test but at slightly lower levels than meat-derived HCAs. L-arginine, an amino acid found abundantly in grains, highly resembles creatin(in)e with the shared guanidyl functional group as well as carboxyl group; this can be seen in **Figure 2**. With this structural similarity, the research team hypothesized that L-arginine is most likely responsible for the mutagencity. Chromatographic separation of food samples with high-performance liquid-chromatography (HPLC) yielded mutagenic compounds, but the samples' unique chromatographic behavior do not match with those of any published HCAs. Information on molecular mass from mass

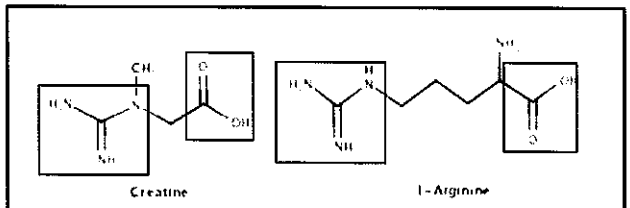


Figure 2. Comparison of creatine and L-L-arginine where blue boxes contain the guanidyl functional group and red boxes contain the carboxyl functional group

spectrometry and functional groups nuclear magnetic resonance (NMR) is lacking but would be crucial in the determination of the chemical structure of these novel HCAs. Unidentifiable with any HCAs previous found in meat products, the results suggest new compounds responsible for the mutagenic activity and leave scientists searching for additional information on these molecules.

As of now, no additional papers following Knize's 1994 publications have been found to continue the identification of the novel HCAs. As a part of the Hayes's research group, the group has built off of the background from the Knize 1994 paper on mutagenic compounds from grain proteins – or in this case soy-based proteins. The focus of the team is primarily on the identification and characterization of these HCAs. The work of Tyler Pender for his Honors Thesis initiated the project by investigating the role of L-arginine in the formation of HCAs by simulating meat and soy-protein system models (Pender, 2013). The meat model used creatin(in)e and L-phenylalanine as the HCA precursor while the soy model used L-arginine and L-phenylalanine. He successfully manufactured micrograms of mutagenic compounds in both meat and soy simulations and confirmed the difference in HCA products. In another Honors Thesis project, Zachery Reichert furthered with the HCA procedure established by Pender by developing a purification process with preparatory HPLC in order to collect larger quantity of our L-arginine-based HCA (Reichert, 2015). He developed a procedure to ultimately collect approximately 500 micrograms. With such a purification method in place, he had sufficient mutagenic compounds to be analyzed with NMR and the Ames test, and potentially also GC-MS.

From this background, the goal of this project is the determination of the molecular mass of these purified compounds with GC-MS. As stated by Knize in the 1994 publication, "...examination of products by mass spectrometry and nuclear magnetic resonance spectrometry for isotope effects would unequivocally confirm the role of L-arginine in the formation of mutagenic products" [Knize et al., 1994]. In order to accomplish this, a search for a compatible mass spectrometer instrument along with corresponding methodology was performed. Once effective instrument and methodology had been established, procurement of pure samples of the HCA of interest was run and mutagenic properties tested for. With pure, mutagenic HCA samples, mass spectrometer analysis can be performed to determine the molecular weight of potentially-carcinogenic, L-arginine-based heterocyclic amines.

Materials and Methods:

Method development and compatibility evaluation of gas chromatography-mass spectrometer

The first instrument to be assessed was the gas chromatography-mass spectrometer (GC-MS). Without a published procedure with the HCA of interest, a methodology must be developed with a test subject similar to that of the unknown HCA, in which case the known carcinogenic HCA 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine (PhIP) was selected. PhIP is one of the most abundant HCAs found in cooked meats, and the U.S. Department of Health and Human Services has declared it as "reasonably anticipated to be a human carcinogen" (Report on Carcinogens, 14th ed). PhIP, the product of creatin(in)e and L-phenylalanine, shares the same precursor amino acid L-phenylalanine as the HCA of interest. This commonality suggests PhIP's similarity in structure and polarity to said HCA, lending it to be a logical candidate for methodology development.

For initial runs, samples were created by dissolving 3 mg of PhIP into 100 mL of methanol. The sample was run through the Agilent 7890A GC System with a (5%-Phenyl)-methylpolysiloxane 25 μm × 200 μm × 0.33 μm column with a splitless, 1 μL injection with the hydrogen carrier gas flow rate of 1 mL/minute. Sample analysis was performed with the attached Agilent 5975C Inert XL MSD with Triple-Axis Detector. In order to bypass detection of solvent, the MS detector will be turned on after a 3 minute delay.

With PhIP's polar properties, addition of a derivatization step was also tested to make PhIP more conducive for gas-phase chromatographic separation. Derivatization is a process in which a compound is chemically altered to render it compatible to transport in the gas phase for analysis. The procedure outlined by Barceló-Barrachina found that N,N-dimethylformamide di-

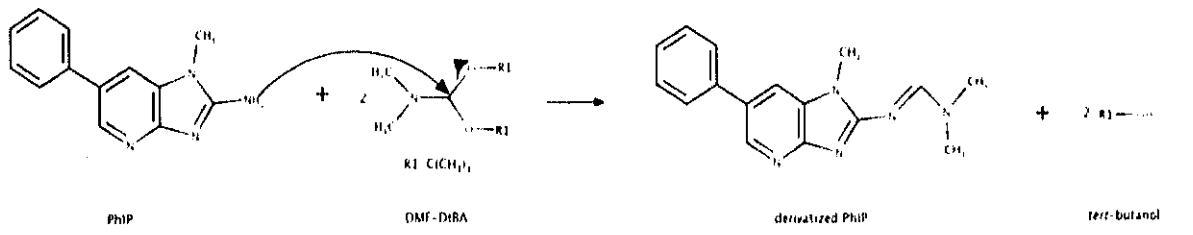


Figure 3. Mechanism for derivatizing PhIP with DMF-DtBA

tert-butylacetal (DMF-DtBA) was most efficient and effective and had greatest yields when used at a 2 μL to 1 ng ratio of derivatizing agent to HCA [Barceló-Barrachina et al., 2005]. This derivatization reaction is shown in **Figure 3**. To ensure the presence of derivatized PhIP, 20 μL of PhIP solution (containing 200 ng PhIP) and 400 μL of DMF-DtBA were placed in a dry 1 mL Agilent auto sampler vial. The solution was vortexed and then placed in an oil bath at 100°C for 10 minutes. After the vial cooled to room temperature, its contents were dried under a stream of nitrogen. At least 200 μL of anhydrous methanol would then be used to rinse the walls of the vial before the contents have dried to completion to concentrate residues. 60 μL of ethyl acetate would then be used to dissolve residues. Because of the small amount of materials, the sample would be placed inside a vial sleeve when ready for GC-MS analysis.

Using the same GC-MS instrument, the sample was injected 280°C, and the pressure in the column at 7.9438 psi. Beginning the oven temperature at 70°C (held for 1 minute), the temperature would then be increased to 240°C (held for 1 minute) at a rate of 25°/minute and again increased to 300°C (held for 5 minutes) at a rate of 10°C/minute.

Methodology development and compatibility analysis of electrospray ionization-mass spectrometer

Another instrument, the electrospray ionization-mass spectrometer (ESI-MS), was also evaluated for its effectiveness in determining the molecular mass of PhIP. For sample preparation, a 1 μg/10 mL of methanol was created. The sample was analyzed by direct infusion using constant flow of a 9:1 acetonitrile:water solvent on a Brunker micrOTOF II mass spectrometer using ESI method positive mode.

Methodology development and analysis with preparatory high performance-liquid chromatography

To find the HCA of interest for mass spectrometry analysis, burned amino acid sample fractions were run through a preparatory high performance-liquid chromatography (prep-HPLC) coupled with UV-vis absorbance spectroscopy. A Waters PrepLC 4000 system with a PDA (photodiode array) detector and a 150 x 30 mm Phenomenex Luna 5 C18 column with a 5 µm particle size packing diameter accomplished the separation. The solvent system utilized consisted of a 1:1 acetonitrile:water which was run at a flow rate of 10 mL/minute. The UV-vis absorbance was set at 315 nm and 256 nm for detection. Samples used for analysis were gained from past student research after initial separation had been performed on the burned binary combination of L-arginine and L-phenylalanine [Reichert, 2016]. All fractions were rotovaped to eliminate solvent, mass obtained, and redissolved in methanol to create 1 µg/milliliter, 10 µg/mL, and 100 µg/mL solutions. All solutions were run through the prep-HPLC with an injection amount of 100 µL.

A minimum injection amount was to be established since this had not been evaluated previously. 100 µL of a 10µg/mL solution of PhIP was injected as a standard. With a sample of total homogenate of burned L-L-arginine and L-L-phenylalanine, the same concentration and injection volume was applied. A 100 µL injection of a 23 mg/mLsolution was then run through the prep-HPLC followed by a 500 µL injection of the same sample.

Mutagenicity test with Ames Assay

The Ames tests were conducted by undergraduate researcher Rayford Alva ('18). Strains of *Salmonella typhimurium* TA98 activated by rat liver S9 extracts were used for plate seeding. For each plate, the following variables were held constant: bacterial concentrations of 90 million and 130 million cells/mL, S9 fraction of 40 mg/mL protein content, and top agar volumes [Maron et. al, 1983]. With methanol as a negative control, previously prep-HPLC separated HCA fractions and PhIP in methanol were placed onto in 0.1 µg/plate and 1 µg/plate concentrations. All plates were performed in triplicates.

Results:

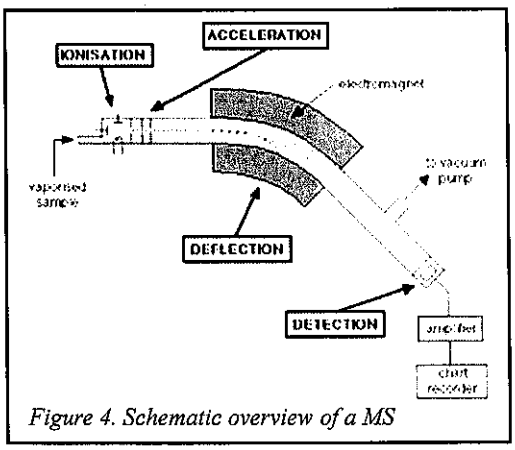


Figure 4. Schematic overview of a MS

In order to determine molecular mass, a mass spectrometer (MS) must be used. The basic principle behind all mass spectrometers involves ionizing (putting a charge on) the molecules, accelerating the ions so that all have the same kinetic energy, deflecting the ions through a magnetic field, and finally detecting each molecules mass. A schematic set up of a MS is shown in **Figure 4** (Clark).The amount of deflection varies depending on the mass of ions and ion charge. While lighter ions are deflected more than heavier ones, ion with a more positive charge (charges 2 or more) will deflect more than those with a positive 1 charge. As the molecules reach the metal detector, electrons flow from the detector to neutralize the charge.

This flow of electrons as well as the location of collision is recorded. These two pieces of information allows for the calculation of a mass per charge ratio (m/z). Since the majority of ions have a positive one charge, the m/z ratio is equivalent to the mass of the charged molecules. The MS, however, must be coupled with another instrument that applies the sample onto the MS. Since the vast majority of analytical instruments deal with a pure analyte, coupling the MS with an instrument with a separatory function is advantageous.

The GC-MS was first chosen as the instrument for evaluation because it combines chromatographic separation with molecular mass analysis. A typical GC-MS set up is shown in **Figure 5** (Hemminger). As with all chromatography techniques, GC involves a mobile phase that carries the sample through the stationary phase that separates out different molecules within the sample. For the GC the mobile phase is hydrogen gas whereas the stationary phase is the nonpolar packing material within the column. After the sample separates and passes through the column, the vaporized molecules that come off the column are analyzed by the MS. The computer is then able to generate two charts essential for analysis: a spectra and chromatogram. The spectra displays the relative intensity of each separated molecule from the column relative to time while the chromatogram displays the relative intensity of each m/z ratio. In this way, the sample is being separated in the column and immediately analyzed by the MS so that both processes occur simultaneously.

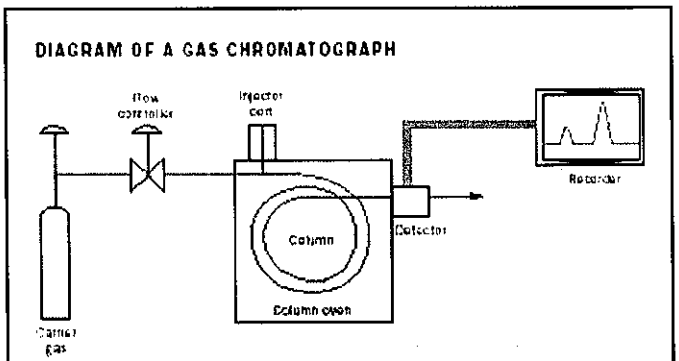


Figure 5. Schematic overview of GC-MS

No peaks, however, could be found for PhIP from the initial runs which lead to the conclusion that PhIP was remaining on the column rather than coming through to the detector. Since PhIP contains the polar guanidyl functional group, the hypothesis was that it was highly likely that there was a large attraction between the guanidyl group with the packing material in the stationary phase or the glass inlet. Despite efforts to prevent PhIP from remaining on the column such as increase the flow rate and increasing injection and column temperatures, no signs of PhIP could be found on the spectra or chromatogram. A different approach needed to be taken.

Addition of a derivatizing agent was determined to a potential solution to the issue. In the reaction with a derivatizing agent, the free nitrogen that contributes to PhIP's polar property would react to gain a nonpolar substituent. Essentially, this addition would negate the attraction the nitrogen would have with the nonpolar lining of the column so that PhIP could successfully pass through. A publication by the Barceló-Carrachina lab concluded the use of N, N-dimethylformamide di-*tert*-butylacetal (DMF-DtBA) to be most effective when analyzing heterocyclic amines. After following the Barceló-Carrachina protocol, a new peak was seen on the GC-MS spectra, but the chromatogram corresponding to that peak did not reveal an m/z ratio of 279 as expected of the derivatized PhIP. GC-MS results are displayed in **Figure 6** in the appendix. After multiple attempts, the conclusion was reached that either the derivatizing agent wasn't sufficient in reducing PhIP's polarity or the sample was getting stuck somewhere else in

the instrument preventing it from reaching the MS. A more direct application of the sample to the MS would be necessary.

One possible instrument that could provide such a feature is the electrospray ionizing-mass spectrometer (ESI-MS). **Figure 7** displays the general set up for ESI-MS. For this instrument, a dissolved, pure sample is injected into a spray nebulizer needle whose high electric field along with the flow of nitrogen gas creates a fine aerosol of charged particles. The charged sample particles then travel through a vacuum that directs the ions into the mass spectrometer for analysis. In **Figure 8** in the appendix, the chromatogram generated by the ESI-MS is shown. Since the MS is in positive mode, the chromatogram shows the m/z H+ number so that the peak indicating the presence of PhIP is 225.224. The strong peak with a m/z H+ value of 225.1123 correctly identifies the molecular mass of PhIP.

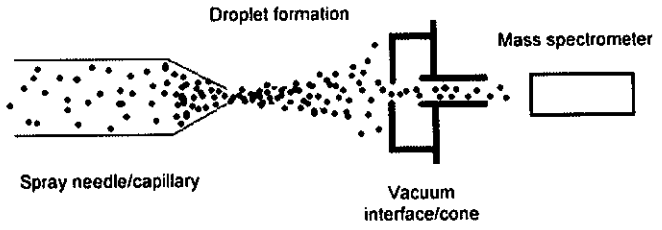


Figure 7. Schematic overview of ESI-MS

With the ESI-MS, the sample doesn't travel through a separatory column but is immediately placed onto the director, making it ideal for analyzing both large and small, polar molecules including HCAs. Because of this set up, the instrument also has very high sensitivity, allowing for analysis of very minute samples. According to the computer software for the ESI-MS, the other peaks on the chromatogram indicate the presence of a cleaning agent and hand cream. These chemical agents are only residues leftover from daily use of the instrument. This property of the ESI-MS provides another benefit to this specific project as the amount of HCA created from each amino acid burning only generates micrograms at most. With other analytical tests that still need to be run, the less HCA sample is consumed by ESI-MS the better. A drawback from utilizing the ESI-MS, however, lies in the fact that it does not contain a separatory column. Whereas a mixture of HCA and burned amino acid sample could be applied onto the GC-MS, separated in the column, and the pure HCA coming off the column evaluated by the MS, the ESI-MS cannot accomplish that. Rather, the separation for a pure HCA sample must occur prior to application with a different instrument. Though a suitable MS-coupled instrument had successfully been found, employment of another separatory instrument would be necessary.

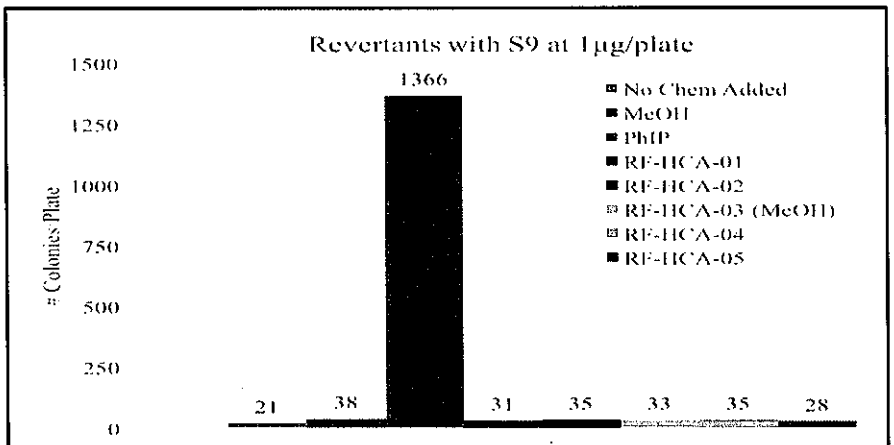
Two methods could be used to accomplish the necessary separation: analytical high performance liquid chromatography (HPLC) and preparatory high performance liquid chromatography (prep-HPLC). Given the relatively high volume of burned amino acid mixture that needed to be separated, the prep-HPLC was chosen as the instrument for use. A methodology had been developed by a previous student research. A minimum injection amount, however, had not been established. Setting the response for PhIP as the standard, the results from varying levels of injection mass are recorded in **Table 1**.

Sample	Injection Volume	Sample Concentration	Amount Injected	Prep-HPLC Response
PhIP	100 µL	10 µg/mL	1 µg	strong peak
HCA Mixture	100 µL	10 µg/mL	1 µg	no peak
HCA Mixture	100 µL	23 mg/mL	2.3 mg	weak peak
HCA Mixture	500 µL	23 mg/mL	11.5 mg	medium peak

Table 1. Results from minimum injection tests with instrument response

Whereas only 1 microgram of PhIP was sufficient to elicit a strong response on the prep-HPLC chromatogram, 11.5 mg of the purified, burned amino acid mixture was necessary to produce a response half that of PhIP. This confirms the small amount of HCA found with each burning process. The sample of purified, burned amino mixture was created burning a 1:1 binary combination of L-arginine and L-phenylalanine at 200°C for 2 hours with diethylene glycol. This total homogenate solution was first purified following the Gibis protocol which called for repeated liquid extractions at pH 2 and pH 9, followed by a SPE-PRS cation exchange chromatography, before being sufficiently eliminated of impurities for possible chemical analysis with prep-HCA [Gibis, 2009]. Judging from the high injection levels necessary for a medium response, the HCA mixture still mainly contains impurities with only trace levels of HCA. In order to find and collect sufficient amounts of relatively pure HCA of interest to use for analytical analysis, the methodology developed by past student research combined with this new information will allow for samples of the same peaks in the prep-HPLC spectra from separate extracted, burned amino acid to be compiled. Further method develop may be necessary before such a step in the research project can continue. Only after purification with prep-HPLC will the samples be relatively pure enough to analyze with ESI-MS

Not only does a relatively pure sample of HCA need to be procured to allow for ESI-MS analysis, but mutagenicity of the compound must also be confirmed. As the discovery of tumor-causing HCAs are the focus, mutagenicity, or the ability to alter DNA sequencing, must be observed to cause such a growth. Performance of the Ames test with the assistance of the Andrews University Biology Department accomplishes this examination. For this test a specific strain of bacteria that cannot survive without histadine, an amino acid most bacteria can produce, are grown in petri dishes. The relatively pure, HCA peak samples ("HCA peaks") separated using the prep-HPLC from a past student researcher's work were applied onto the bacteria plates. To determine the level of mutagenicity, the number of bacterial colonies that have an altered DNA capable of producing histadine again are counted. Shown in **Graph 1** the results from the Ames test are displayed.



Graph 1. Levels of mutagenicity of control groups, PhIP, as well as HCA fractions. Note that PhIP is the only reagent to elicit a strong mutagenic response

PhIP, being a known carcinogen or cancer-causing agent, produced a high number of mutants as expected. The HCA peaks (labeled as RF-HCA-01, 02, …), however, demonstrated similar or even less mutagenicity than the control. These findings contradict those from a past student research performing the same test with the same HCA peaks. While the present test results show little to no mutagenicity in all the HCA peaks, past work showed at least one peak containing mutagenicity levels close to that of PhIP.

There remain two possible explanations for the conflicting results. One potential reason could lie in the fact that the HCA peak samples had been made a year prior. Being organic molecules, degradation is a possibility. Nevertheless, the solutions of PhIP made during the same time were tested with both prep-HPLC and Ames test exhibited the same results as a year prior despite the fact that PhIP is also an organic molecule. The other speculation remains that insufficient levels of HCA were placed upon the bacteria plates to evoke levels of mutagenicity shown in past research. As seen with the determination of minimum injection level for HCA into prep-HPLC, the HCA of interest can only be found in very low levels in a sample of burned amino acids. Even though the test materials placed on the bacterial plates were HCA peak samples rather than the mixture with burned amino acids, the fact still remains that very low doses of HCA actually exist in the burned amino acid samples. If that is the case, more compilation of same peaks from various burned amino acid samples would be necessary to provide sufficient HCA to bring out a strong mutagenic response from the Ames test. Otherwise, the project may redirect its focus towards other binary amino acid combinations in search for similar mutagens.

Conclusions:

Springboarded by the statement made by Knize's lab, the investigation into the molecular mass of L-arginine-based HCAs became the focus of this particular project. Beginning with a search for an effective and suitable mass spectrometer instrument with the test material PhIP, the GC-MS was evaluated for its appropriateness to satisfy the needs of this investigation. Changes in every variable from solvent mixture to temperature, to column type, and sample injection method did not produce satisfactory separation and MS analysis with the GC-MS. Hypothesizing an attraction between the polar heterocyclic rings and the nonpolar column packing material to be the cause for the lack of response, experimentation with the addition of a derivatizing agent was conducted. DMF-DtBA was tested under the Barceló-Barrachina protocol and found ineffective in assisting with PhIP's passage through the column to the detector. An instrument with a more direct application of PhP to the MS needed to be evaluated. When using the ESI-MS, the molecular mass of PhIP was correctly identified. Even though an effective instrument had been found, the ESI-MS requires relatively pure samples of HCA since it lacks a separatory column that could enable it to separate and perform MS analysis simultaneously. Attention was then directed towards the prep-HPLC, an instrument that could separate samples in large amounts. Development of a minimum sample injection amount further refined the previous methodology and paved the way for compilation of HCA peaks. This amassing of pure, identical molecules from various burned amino acid samples enables the progress into another tests for ESI-MS and Ames test. The present mutagenicity tests by the Ames test have shown inconsistent data with past research so further exploration with fresh samples from developed prep-HPLC methodology will be conducted in the future.

Even though the attainment of molecular mass of the potentially carcinogenic, L-arginine-based heterocyclic amines has not been accomplished, a suitable instrument with compatible methodology has been found which will be utilized for future HCA samples. Evidence obtained during this project have yielded results dissimilar to that of past researchers who have replicated data mirroring that of the 1994 Knize lab results. With a successful mass spectrometer instrument and methodology in place, further investigation into potential cancer-causing agents in vegetable-based proteins exposed to high temperatures will continue.

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