3-28-2016

Synthesis, Isolation, and Characterization of Potentially Carcinogenic Arginine-Based Heterocyclic Amines

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J. N. Andrews Honors Program
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HONS 497
Honors Thesis

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March 28, 2016

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Abstract

Previous research demonstrates that meat cooked at high temperatures produces heterocyclic amines (HCAs)—a class of carcinogenic molecules—from burned creatin(in)e and amino acids. However, research performed in our lab, as well as in the peer-reviewed literature, suggests that substituting arginine for creatin(in)e may lead to HCA formation. Arginine is structurally similar to creatin(in)e and can be found abundantly in soy-based food products. Therefore, we have burned arginine and phenylalanine to investigate the potential formation of arginine-based HCAs. The present study attempts to isolate and characterize these potential arginine-HCAs.

Introduction

Heterocyclic amines—commonly referred to as HCAs—are chemical compounds that contain at least one heterocyclic ring and an amino group. The term HCA can be used to refer to a broad variety of molecules. However, the literature commonly uses HCA in reference to a specific group of molecules known to exert mutagenic effects in bacterial systems and cause cancer in various mammalian models (Sugimura, 2004). HCA formation has been identified in beef, pork, fish, and poultry cooked using high temperature methods such as pan-frying, grilling, and oven-broiling (Alaejos & Afonso, 2011). The formation of carcinogenic HCAs in foods cooked using common high temperature methods poses a threat to human health. To date, numerous studies have linked increased exposure to HCAs with an increased risk for multiple common cancers (Zheng & Lee, 2009). Additionally, the World Health Organization (WHO) recently published a statement mentioning HCAs as one of multiple factors likely accounting for a link between increased red and processed meat consumption and cancer risk (Bouvard et al., 2015).

Currently, the exact mechanism of HCA formation is unclear. However, the general route of HCA formation appears to be through condensation reactions involving creatin(in)e and amino acids at high temperatures (Puangsombat, 2010). The planar structure of HCAs causes them to act as intercalators when in contact with DNA, slipping between DNA base pairs to form adducts with guanine bases (Schut & Snyderwine, 1999). Over time, the cumulative effect of these intercalating HCAs causes structural modifications in the DNA, leading to errors in DNA transcription, replication, and repair processes. It is this cumulative effect that likely accounts for the carcinogenicity of HCAs.

To date, much of the literature focuses on the formation of HCAs in muscle meat systems containing creatin(in)e and other amino acids with the goal of discovering novel cooking methods that may lead to reduced HCA formation. Alaejos and Afonso recently published a thorough review covering the history and recent advances of this work (2011). However, not all HCA research has centered solely on HCA formation in muscle meat—some work suggests that HCAs can be formed in food products that lack creatin(in)e. For example, Knize et al. (1994) found mutagenic activity after burning binary mixtures of amino acids at high temperatures. In their discussion, they hypothesize that novel heterocyclic amines could account for the mutagenicity observed in the study. In the same year, the Knize group also found mutagenic activity after heating grain products at high temperatures (Knize et al., 1994). The publications by the Knize group suggest the possibility that novel, currently unidentified HCAs may form in the absence of creatin(in)e.

At the high temperatures necessary for the formation of HCAs, it is likely that other molecules could substitute for creatin(in)e in a reaction similar to the HCA formation reaction described above—the most likely candidate being arginine. Like creatin(in)e, arginine contains a guanidyl

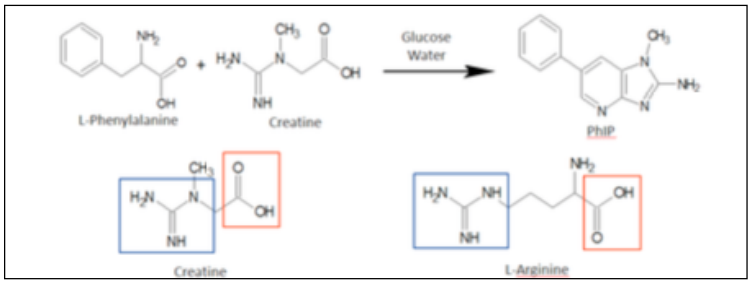

Figure 1. Structures of creatine and arginine with chemical similarities highlighted.

nitrogen group as well as a carboxy group—only small differences can be observed in comparing creatin(in)e and arginine (Figure 1). One highly studied HCA formation reaction involves the amino acid phenylalanine and creatin(in)e, leading to the formation of PhIP, a well known carcinogenic HCA. Soy protein is particularly high in both arginine and phenylalanine (SELFNutritionData, 2016). Therefore, it is likely that the two amino acids could react in a formation reaction similar to that of PhIP, leading to the formation of novel HCAs.

The goal of the present study is to identify whether or not HCAs are formed in the heating of arginine and phenylalanine. Given the cumulative mutagenic and carcinogenic effects of HCAs, the possibility that HCAs could be formed in soy protein based foods has potentially dangerous implications for vegans and vegetarians who cook soy-based meat substitutions at high temperatures. Therefore, identifying potentially dangerous molecules formed in the heating of non-meat food products is essential as it then leads to novel methods of food preparation that discourage the formation of HCAs and other toxic compounds.

Materials and Methods

Formation and purification of potential arginine-based HCAs

In order to identify the potential formation of HCAs in creatin(in)e-free systems, we substituted arginine for creatin(in)e in a reaction similar to the PhIP formation reaction. Specifically, 2.0 g arginine and 2.0 g of L-phenylalanine were added to a 3-neck round bottom flask and refluxed in 40 mL of diethylene glycol at 200°C for 2 hours. After refluxing the amino acids, the resulting reaction mixture was purified using a modified version of a previously published extraction protocol utilizing cation exchange (Gibis, 2009). The purification process includes both liquid extraction and solid phase extraction.

Following the completion of the 2 hour burn process, the reaction mixture was diluted with 150 mL of 0.1 M HCl, and the overall pH of the mixture was then set to pH 2 by gradually adding 5 M HCl. Liquid extraction was then performed, extracting three times with dichloromethane and removing the dichloromethane layer as waste. Next, the aqueous layer was set to pH 9 using 5M NaOH and three more dichloromethane extractions were performed, this time collecting the dichloromethane layer. The collected dichloromethane was then evaporated to dryness and resuspended in ~5 mL ethyl acetate.

To further purify the mixture, a SPE-PRS cation exchange cartridge was preconditioned with 5 mL of ethyl acetate and the product mixture was added to the cartridge. The cartridge was then washed with 12 mL of ethyl acetate and completely dried. Then, 6 mL of 0.01 M HCl followed by 6 mL of a 9:1 HCl (0.1M) – methanol mixture were run through the cartridge. After completely drying the cartridge, a new C18 cartridge was preconditioned with 4 mL of methanol and 4 mL of ammonium chloride and clamped beneath the dried SPE-PRS cartridge. The SPE-PRS was subsequently washed into the C18 with 30 mL of pH 9 0.5 M ammonium chloride followed by 10 mL of a 9:1 mixture of ammonium chloride (pH 9, 0.5 M) – methanol. Next, the SPE-PRS was washed into a glass beaker with 15 mL of a 2:3 solution of 0.1 M HCl – methanol before adding 25 mL of de-ionized water and 500 μL of ammonia. The contents of the beaker were then poured through the C18 cartridge before rinsing with 5 mL of de-ionized water. After

drying the C18 cartridge, 10 mL of a 9:1 mixture of methanol – 25% ammonia was washed through the C18 into a glass vial. The contents of the glass vial were then dried down and resuspended in methanol for HPLC.

HPLC Analysis and Collection

In order to analyze and ultimately collect the components of the purified mixture, HPLC coupled with UV/Vis spectroscopy was used. The HPLC and column used were a Waters PrepLC 4000 system with a PDA detector and a 150 x 30 mm Phenomenex Luna 5 C18 column with particle size 5 μm packing. Figure 2 shows a schematic of a typical HPLC system.

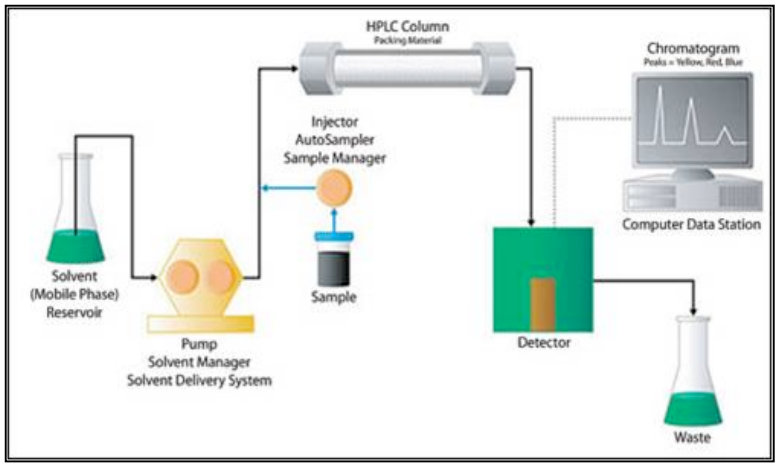

Figure. 2. Schematic of a typical HPLC system.

The HPLC separation method previously used by our lab involves a modified version of the Gibis lab's HPLC method (Gibis, 2009). This method uses a changing mixture of 1% phosphoric acid and acetonitrile with a flow rate of 1.00 mL/min. However, this method was created for use on our Agilent Technologies 1260 analytical HPLC machine. This machine was insufficient for collecting necessary amounts of potential-HCA products, which is why our lab now uses the Waters 4000 system. Given that the Waters 4000 system contains larger diameter tubing, a much larger column, and higher flow rates, a new HPLC separation method was used for analysis and collection in the present study. The new separation method involves a constant 1:1 mixture of distilled water and acetonitrile with a flow rate of 10 mL/min over a 25-minute period. Thus far, this simple, quick, and cheap method has been sufficient for the separation needed by our lab.

In order to collect potential HCA products, we disconnected the tubing leading from the output of the PDA detector and inserted new tubing that was then used to collect drops of HPLC solvent in glass vials as potential HCA products flowed out of the PDA detector. The collected products were then labeled, completely dried with a rotary evaporator, and weighed before structural analysis.

Structural Analysis

Initial structural studies were carried out using the Chemistry/Biochemistry department nuclear magnetic resonance (400 MHz NMR) spectrometer.

Results

Previous work carried out in our lab aimed at the preparation and characterization of potentially carcinogenic HCAs. However, efforts to collect sufficient amounts of product consistently fell short. While it was difficult to identify the exact steps within our methodology that were limiting the amount of product that could be collected, it was determined that our HPLC method involving the Agilent 1260 system was the limiting step. This is due to the fact that only 100 μL could be injected onto the column. With the Agilent 1260 system, we estimated that we were able to collect roughly 0.5 μg of product per 20 minute HPLC run—much too small of a yield for us to carry out effective structural analysis. This leads to the work carried out in the present study, which aims at developing a preparative HPLC method using the Waters 4000 system that will yield higher amounts of product. After developing an efficient, cost-effective preparative method, collecting unknown products, and weighing the products, it was found that the new preparative method allows a 1000-fold increase in collected product in a similar amount of time (Table 1.)

Table 1. Yield of previous and new HPLC protocols. The new preparative HPLC protocol developed in the present study yielded roughly 1000x more product per HPLC run over a similar period of time.

Previous HPLC Isolation Protocol		New HPLC Isolation Protocol	
Mass of product after 1X HPLC isolation	~0.5 μg	**Mass of product after 1X HPLC isolation**	~500 μg
Time per 1 HPLC isolation	20 minutes	**Time per 1 HPLC isolation**	25 minutes

After developing a collection method using the new Waters 4000 HPLC system, two unknown products of interest were identified for further collection and analysis (Figure 3). One large reason behind why these particular unknowns were chosen was due to the fact that these two peaks achieved sufficient separation from the other products present in the injection mixture. While our lab eventually would like to identify all products present in the injection mixture, focusing on two products that can be easily collected will allow quicker identification and characterization of the potential HCAs.

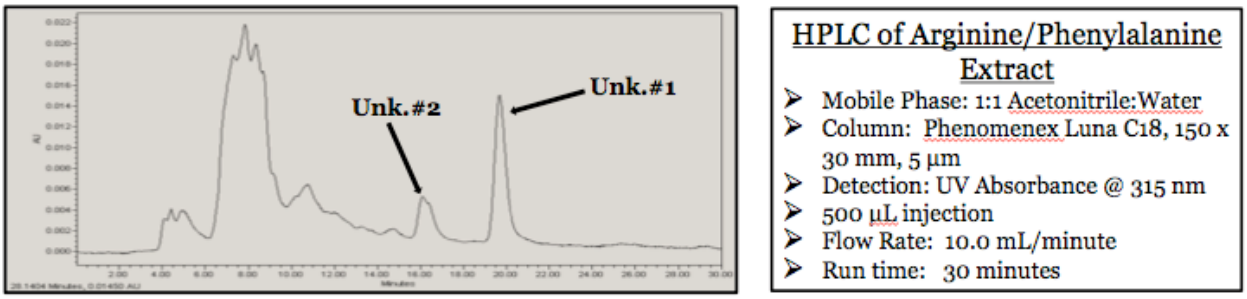

Figure 3. HPLC of Arginine/Phenylalanine Extract. Two unknowns present in the injection mixture were chosen for further collection and analysis.

In addition to the fact that unknown products 1 and 2 separate out as individual peaks, we have further reason to be interested in them given their UV-Vis absorbance values—these values are similar to that of PhIP, a known carcinogenic heterocyclic amine (Figure 4).

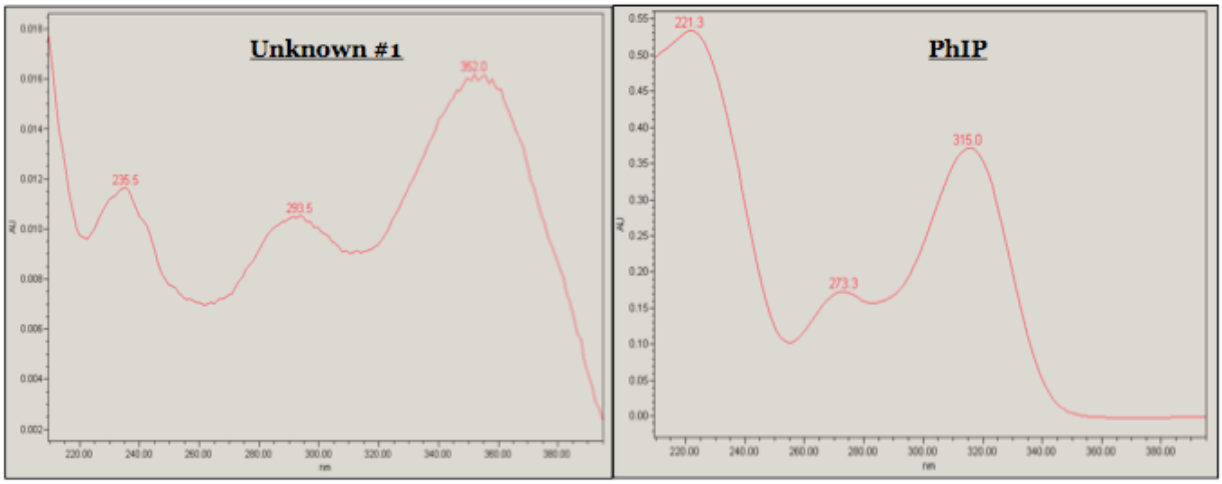

Figure 4. UV-Vis absorbance readings for Unknown #1 and PhIP. While differences between the molecules certainly exist, there is enough similarity to suggest that unknown #1 could be similar in structure to PhIP.

We recently began collecting 1H and 13C NMR data for the "unknown #1" sample (Figure 5). While the results ultimately do not provide enough information to propose an exact structure, they do provide clues about structures present in the molecule. For example, numerous peaks can be observed with chemical shifts in the 1.0-2.0 range of the spectrum. The peaks nearest to 1.0 likely indicate the presence of hydrogen atoms bonded to methyl group carbons, due to the fact that the low chemical shift indicates that the hydrogens are bonded to a carbon that is not directly bonded to a more electronegative atom. However, the peaks shifted

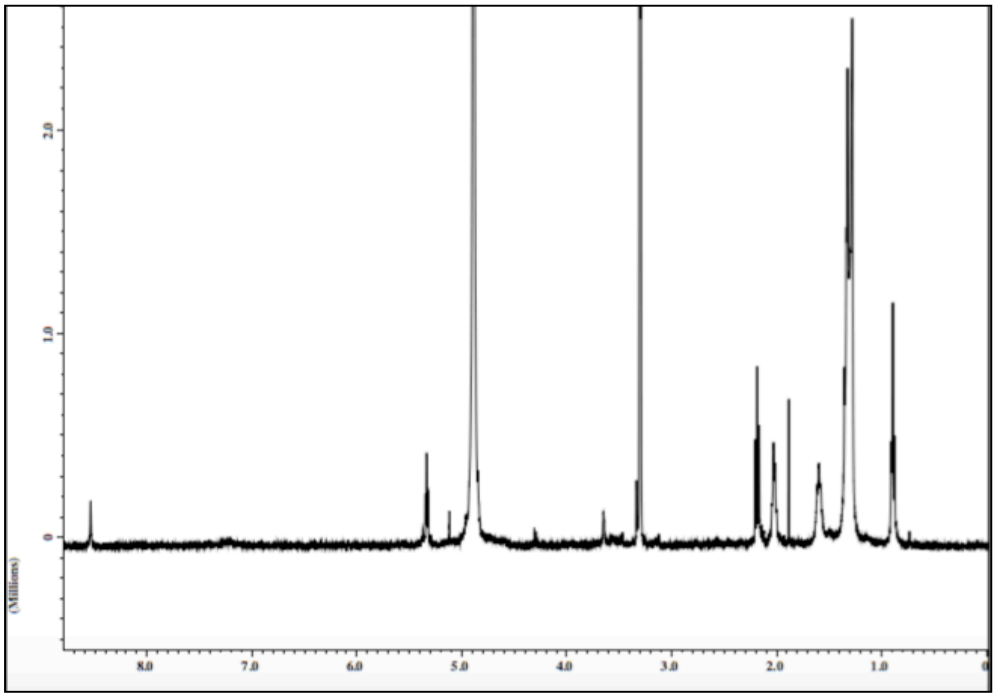

Figure 5. 1H NMR spectrum of unknown #1. Numerous peaks were observed in the 1.0-2.0 range, as well as one peak at about 5.4 and one around 8.5. We are currently unsure of what this suggests in terms of the overall structure of unknown #1. The two large peaks at 3.3 and 4.9 are solvent peaks and are therefore not part of the structure of unknown #1.

nearer to 2.0 indicate hydrogens bonded to a carbon that is nearby a more electronegative atom such as a nitrogen atom, which we would predict to be present in the unknown given the similarity between the unknown and other known nitrogen-containing molecules such as PhIP. The peaks with a chemical shift of 5.4 likely indicate the presence of alkenes, or carbon-carbon double bonds. Interestingly, no abundance of 1H peaks were observed in the aromatic range (7.0-8.0). This is surprising given our prediction that unknown #1 is an HCA-like molecule, as HCAs like PhIP show numerous peaks in the aromatic region (Figure 6). 1H NMR studies were also conducted using deuterated chloroform (Figure 7). The results were nearly identical to those where deuterated methanol was used. However, no peaks were observed past a chemical shift of 5.4. This leads to further questions of whether unknown #1 is an aromatic, heterocyclic molecule. However, mass spectrometry and further NMR studies must be carried out. The peak at 7.2 in Figure 7 results from residual undeuterated hydrogens in the deuterated chloroform solvent and the peak around 1.5 indicates the presence of residual water. In both figures 5 and 6, the prominent peaks with chemical shifts near 3.0 and 5.0 are solvent peaks from the deuterated methanol solvent. The peaks at 3.0 result from residual undeuterated hydrogens connected to the carbon in the deuterated methanol solvent that we used. The peaks at 5.0 indicate the hydrogen signal from trace amounts of water and the O-H bond in methanol. Therefore, these solvent peaks do not indicate anything structurally about our unknown compound as they result from the solvent used for the NMR studies. Further NMR studies must be carried out to elucidate the structure of the unknown as well as its similarity to PhIP. We have not yet been able to run 13C NMR studies due to the fact that the signal for 13C NMR is 99% smaller than for 1H NMR. Thus, more product must be collected in order to run 13C NMR.

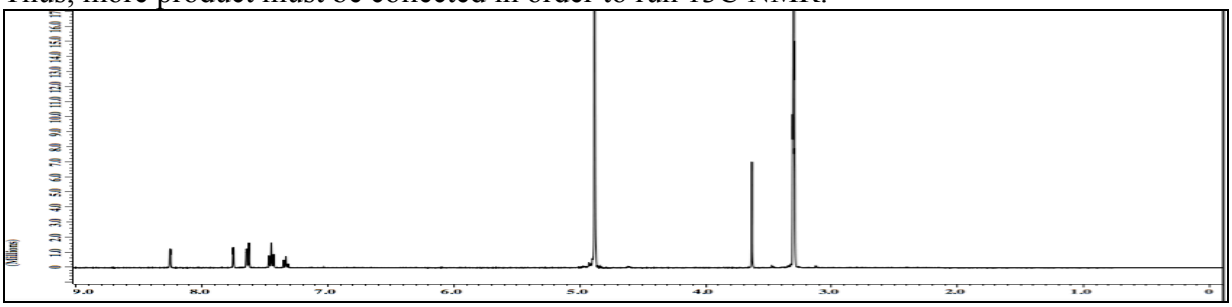

Figure 6. 1H NMR spectrum of PhIP. The peaks observed in the 7.0-8.0 range indicate aromatic hydrogens. The peaks at 3.2 and 4.9 are solvent peaks from deuterated methanol.

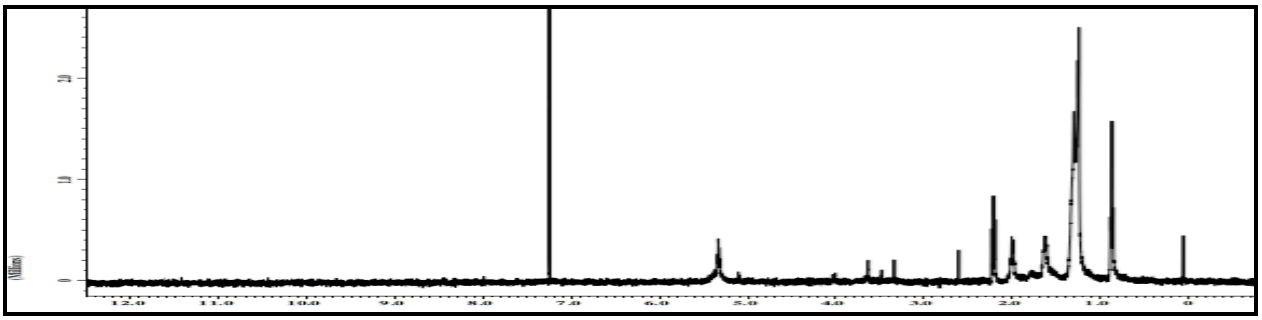

Figure 7. 1H NMR spectrum of Unknown #1 in deuterated chloroform. The peaks observed in deuterated chloroform were nearly identical to Figure 5, with the only difference being the absence of a peak around 8.5. Solvent peaks can be observed at 7.2 and 1.5.

Conclusions

Given the struggles that our lab has experienced with the collection of potential HCA products, the development of a new preparative method for collection will allow the lab to more quickly and efficiently isolate and characterize novel HCAs. Whereas before we would need to undergo 500-1000 twenty minute HPLC runs to collect enough product for NMR analysis, our lab now only needs a few HPLC runs to collect enough for analysis. Furthermore, given that our HPLC method involves a solvent composed of just water and acetonitrile, it is cheap and very fast and easy to prepare. We also believe that we will be able to recover and reuse acetonitrile from the HPLC solvent waste through the use of large-scale rotary evaporation.

The successful development of a preparative HPLC collection method has allowed our lab to begin collection of products for analysis via NMR, infrared spectroscopy (IR), and mass spectrometry. Currently, the lab is focused on collecting NMR spectra that are sufficient for molecular characterization. However, while we have collected spectra of one unknown, additional NMR data must be collected in order to make more reliable structural predictions. Additionally, in order to assist our structural identification through the technique of NMR, mass spectra must be collected for each of the unknown molecules. Our lab is currently working on the potential development of a gas chromatography-mass spectrometry method (GC-MS) in order to obtain mass spectra. Dr. David Alonso has additionally offered his assistance in helping us obtain mass spectrometry data for our unknowns.

In addition to the absorbance similarities observed between products of the arginine-phenylalanine extract and PhIP, other preliminary results obtained by members of our lab suggest similarity. The collection of these results involves the use of a microbiological technique called the Ames test (Figure 8). This test uses a strain of *Salmonella* that is unable to synthesize histidine, an amino acid essential to the survival of the bacteria. However, when exposed to mutagenic chemicals, these bacteria revert back to wild type, regaining the ability to synthesize histidine as well as the ability to survive on minimal glucose agar plates. Preliminary Ames test results from our lab indicate that mutagenic products comparable to the mutagenicity of PhIP are formed in the high temperature reaction between arginine and phenylalanine (data not shown). These data provide further evidence suggesting the idea that HCAs or similar molecules can form in the absence of creatin(in)e and provide our lab with further reason to continue our attempts at characterization of the molecules formed from the reaction between arginine and phenylalanine.

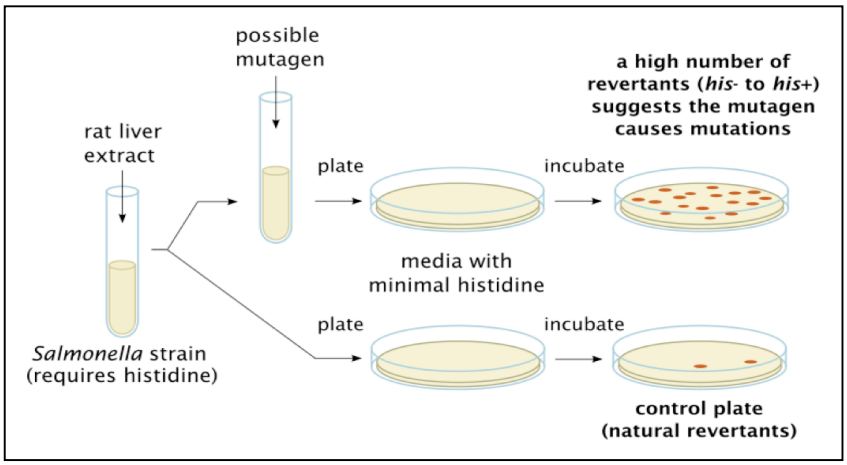

Figure 8. Ames test method.

While the present study has allowed our lab to draw closer to the structural identification and characterization of potential HCAs formed from the high temperature reaction of amino acids, we are still unable to confirm whether or not our unknown molecules are HCAs or another class of mutagens. Future work must aim for reliable structural identification of these unknown molecules and must also attempt to increase and enhance the yield of these unknowns. Although our current preparative HPLC method allows for sufficient collection of compounds for analysis, improving the method will lead to quicker and more efficient collection and characterization in future studies. The ultimate goal of the lab does not end with identification of mutagens formed from the reaction between arginine and phenylalanine, but includes the characterization of any mutagens formed from other reactions between pairs of amino acids. Future work will also include characterization of any mutagenic products formed from the heating of soy protein isolate and other soy protein-based products.

Although our lab has not yet fully characterized any molecule formed from the reaction described in this paper, we have obtained evidence suggesting similarities between the unknown molecules and currently known carcinogenic heterocyclic amines, mirroring the evidence of past studies by Knize (1994). This leads us to the belief that care should be taken in the high temperature cooking of any protein-based food products, not simply products containing high amounts of creatin(in)e.

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