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Prep-HPLC Method Development to Isolate Potentially-Carcinogenic, Arginine-Based Heterocyclic Amines

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Abstract

Heterocyclic amines (HCAs) are carcinogenic molecules generated from the reaction of creatin(in)e and amino acids at high cooking temperatures in meat. Previous research has shown that replacing creatine with arginine leads to new, uncharacterized HCA molecules. This research entails implementing and optimizing the preparative HPLC analysis of extracts from multiple burnings of arginine with phenylalanine. Isolated fractions from the prep-HPLC analysis were further analyzed using the Ames test to identify mutagenic compounds. Prep-HPLC provides quality control to the burning process along with isolation of larger quantities of materials for further characterization and molecular structure identification.

Introduction

The hazardous smoke generated from cooking meats and fish inspired researchers to dwell upon the question of whether these cooked meats and fish could potentially be carcinogenic. Over twenty heterocyclic amines (HCAs) were found in cooked meats and fish and have been linked to increased risk for multiple types of cancers [Alaejos and Afonso, 2011]. In addition, the World Health Organization (WHO) also confirmed the link between red, processed meat and cancer due to the presence of HCAs (Bouvard et. al, 2015).

HCAs are formed from the reaction between free amino acids, creatin(in)e, and high temperatures. They are planar, aromatic hydrocarbon containing at least one heterocyclic ring and an amine functional group. The flat structures of HCAs allow them to interfere with DNA to form adducts [Schut et al., 1999]. These adducts prevent DNA from being able to properly replicate which will eventually lead to mutations. Luckily, our body has many repair mechanisms that work to undue these mutations; however, in the rare case that these HCA-induced mutations bypass the repair systems, tumors can form and eventually lead to cancer.

The Ames test is used with a strain of *Salmonella typhimurium* TA98 to test the mutagenicity of HCAs [Sugimura et al., 2004]. The Ames test uses bacteria to test whether a certain compound is mutagenic or not. If the compound is mutagenic then it will be a fit candidate for further investigation as a potential carcinogenic agent, because the more mutagenic a compound is, the more potentially carcinogenic it can be. Further testing in animal models (mice, dogs, monkeys, etc.) verify the link between mutagenicity and carcinogenicity.

The most abundant and common HCA proven to be carcinogenic in muscle meats is PhIP (2-amino- 1-methyl-6-phenylimidazo(4,5-b)pyridine), as seen in Figure 1. PhIP is formed from the reaction of L-Phenylalanine, creatin(in)e, and glucose, which serves as a catalyst, under cooking conditions at very low yields. The highest concentration of PhIP was found in grilled chicken with the lowest concentrations in pork [Atwood et al., 2002]. In one particular study PhIP was administered in rodent diets, causing tumor growth to be seen in the small intestine, colon, mammary glands, and prostate [Atwood et al., 2002]. It was observed that PhIP exhibited a dose-dependent relationship. With those exposed to a higher concentration of PhIP to develop breast, kidney, lung, or bladder cancer.

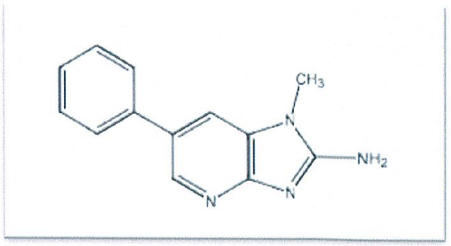

Figure 1. Structure of PhIP

Research has established that muscle meats exposed to heat processes stimulate the production of HCAs, like PhIP; however, no research had been done to show connection between non-creatine products and HCA formation until 1993. The Knize research lab in 1993 focused on the characterization and isolation of potential mutagenic activity in cooked grain food. One of the major components in HCA formation is the presence of creatine, but creatine is absent in vegetarian based products [Knize et al., 1993]. The Knize burnt amino acids in binary sequences which they then separated into individual compounds using high performance liquid-chromatography (HPLC). Although elution behaviors were similar, the chromatographs obtained did not match with any recognizable, established HCA [Knize et al., 1993]. The Ames test revealed however that mutagenic activity was indeed present in

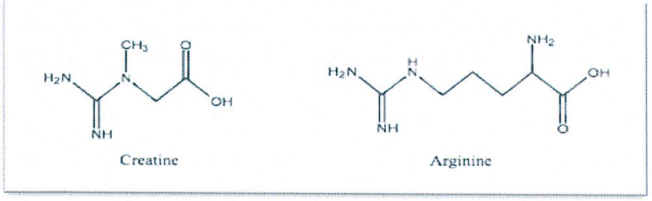

Figure 2. Structures of Creatine and Arginine

the samples, especially with those containing arginine. The levels were still generally lower than those levels found in muscle meat products with creatine though [Knize et al., 1993]. The reason for this may be because arginine and creatin(in)e are similar in their functional group configuration. It can be seen in Figure 2 that both creatine and arginine share guanidyl and carboxyl groups. Similar structures made the research team hypothesize then that the two compounds could potentially react the same, with arginine working as a substitute for creatine in HCA formation. Furthermore, in their discussions section, the Knize team stated that Nuclear Magnetic Resonance (NMR) and Mass Spectrometry (MS) would help to confirm the role of arginine in the formation of HCAs. Since 1993, no further work has been done into studying HCA formation in non-creatine products.

Thus, the goal of the present study is to identify the formation of the arginine-based HCA and characterize it amongst the other creatine-based HCAs using preparatory-HPLC (prep-HPLC). The entire HCA project process can be seen in Figure 3. First and foremost, phenylalanine and arginine were burnt together by previous Hayes Group researchers, Kyle Gordon and Skyler Schell, and underwent multiple extractions and purification techniques such as: liquid extraction with dichloromethane and solid phase extraction with a silica-gel column. The samples then underwent separation with prep-HPLC. Previous scholar Zachery Reichert, in 2015, developed a purification, isolation, and separation process with the prep-HPLC machine in which he was able to identify PhIP at a wavelength of 315 nm. However, further development of Reichert's protocol was needed in order to effectively be able to isolate the arginine-based HCA fraction for mutagenicity testing with the Ames test as well as NMR and MS analysis for compound identification and characterization. Advanced optimization of the prep-HPLC

protocol will be the sole focus for this present study.

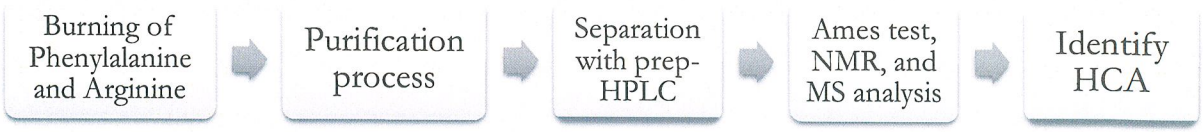

Figure 3. HCA process flowchart.

Materials and Methods

Column Cleaning Procedure

Since different compounds are constantly being run through the column, some residues may get stuck in-between the beads; thus, a proper cleaning procedure had to be developed in order to ensure that the HPLC column was fully functional for separations. The column used for this study was the Phenomenex Luna C18 with measurements 150 x 30.00 mm. The solvents used as the mobile phase for the column runs had to first be filtered and degassed, if they were not HPLC-grade solvents, in order to prevent bacterial build-up in the column and air bubble presence. First, the column was reversed in order to push out the residuals that may be clogging the exit hole of the column. The first solvent system used was 500 mL of 100% isopropyl alcohol at a flow rate of 10 mL/min to remove medium polarity compounds. Afterwards, 500 mL of an 80:20 mixture of isopropyl alcohol to dichloromethane was run through the reversed flow column at 10 mL/min to remove nonpolar adhered compounds. The column was then reversed so that the flow returned back to normal. The column was flushed again with 500 mL of isopropyl alcohol in the normal flow direction at a flow rate of 10 mL/min. Finally, the column was cleaned with 750-1000 mL of 50:50 acetonitrile (ACN)/methanol (MeOH) solution at a flow rate of 15-20 mL/min. The column was left overnight in the 50:50 solution. Once the cleaning was completed, the column was flushed with another 500 mL of the solvent system to be used for the

sample runs. The HPLC back pressure was constantly recorded for each solvent cleaning to help quantify the performance and behavior of the column before, during, and after cleaning.

A test mixture was then created with equal mass ratios (~1.0 mg) of naphthalene, acetophenone, toluene, and uracil – four compounds with four different polarities – to test whether the column cleaning procedure was effective. The mobile phase used was a 65:35 ACN/water solution that was flushed through the column previously, with a flow rate of 20 mL/min for 15 minutes. The obtained chromatograph was compared to the chromatograph given by the Phenomenex company. If the two chromatographs had significant overlap, then it was safe to assume that the cleaning procedure was effective in flushing out the impurities in the column. The column had to be properly cleaned or else the backpressure would become substantially high. Once it hits a certain limit, the whole prep-HPLC system would shut down causing a disruption to the prep-HPLC run and data acquisition; therefore, the back pressure had to be closely monitored and the column properly cleaned to rid of the impurities.

Sample Preparation

Previous research students Skyler Schell and Kyle Gordon refluxed 2.0 g of phenylalanine and 2.0 g of arginine at 200°C for two hours with diethyleneglycol. Afterwards, the burnt amino acids were purified using a modified version of the Gibis protocol, which includes multiple extractions: liquid extraction, solid-phase extraction, and HPLC (Gibis, 2009). The yields of the product were very low, near the microgram to milligram range, although beginning with 4.0 grams of reactants. These were the samples that were given to this present study.

The samples were first rotovaped in order to dry down the products to obtain the mass of the products. The samples were then diluted in 10 mL of MeOH. Each sample vial was labeled "RF-HCA-XX," (R for arginine, F for phenylalanine) to be able to classify which samples came from where and which student researcher, as can be seen in Table 1.

RF-HCA	Burnt Sample Description	RF-HCA	Burnt Sample Description	RF-HCA	Burnt Sample Description
03	Schell, 7.8 min, 3600µg	06	Schell burning 1, no prior HPLC, 2300µg	09	Gordon burning 1, no prior HPLC, 6100µg
04	Schell, 16.5 min, 3800µg	07	Schell burning 2, no prior HPLC, 4200µg	10	Gordon burning 2, no prior HPLC, 1200µg
05	Schell, 19.0 min, 2500µg	08	Schell burning 3, no prior HPLC, 3700µg	11	Gordon burning 3, no prior HPLC, 200µg

Table 1. RF-HCA-XX samples from student researchers: Skyler Schell and Kyle Gordon.

Prep-HPLC Analysis

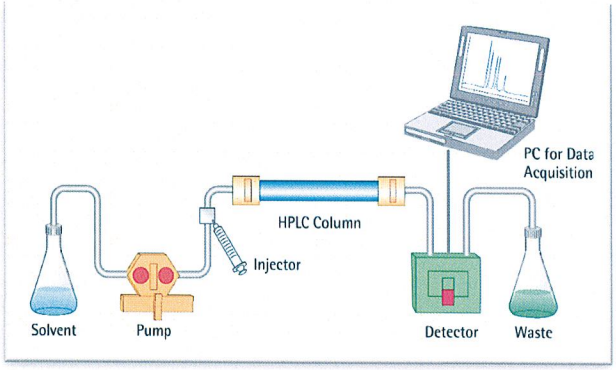

Figure 4. Schematic diagram for HPLC

Each prepared HCA sample was run through prep-HPLC similar to the schematic shown in Figure 4. The prep-HPLC used for this study was the Waters PrepLC 4000 System with Waters 996 Photodiode Array detector. Four parameters were varied when developing the prep-HPLC methodology: solvent system, injection volume, flow rate, and run time. In the literature, the Gibis protocol provided an HPLC method for analysis of both polar and nonpolar HCA. Since the polarity of the specific arginine-based HCA is unknown, the Gibis

paper worked as a good starting point for the development of the prep-HPLC protocol. Adjustments were made in order to optimize separation with our specific prep-HPLC column and with the polarity of our arginine-based HCA.

The Gibis protocol utilized a gradient mobile phase with a phosphate buffer starting a pH of 3.0 then 4.0, and finishing with 100% ACN solution (Gibis, 2009). The protocol had a total run time of 17 minutes with a flow rate of 1.1 mL/min as they used an analytical HPLC. At the beginning of the development for the prep-HPLC method, the flow rate and injection volume were first increased as a prep-HPLC column is much bigger than the column used for analytical-HPLC. The larger volume of prep-HPLC works to this study's advantage in being able to collect samples closer to the 1 mg mark that will be needed for further analysis with MS and NMR. In addition, the buffer solution was taken out for the mobile phase used as buffers contain salts which can potentially add weight and provide misleading date or interfering information in follow up analysis (eg. GC-MS analysis). Creating a prep-HPLC methodology without the use of buffer salts will improve the speed and quality of fraction collection overall.

This study's prep-HPLC protocol consisted of a mobile phase of 65:35 ACN/water, an injection volume of 500 µL, a flow rate of 20 mL/min, and a total run time of 15 min. The entire 2 to 8-minute fraction was collected as one solution for each RF-HCA-XX sample from the "waste" line of the prep-HPLC. Each sample was run through prep-HPLC three times in order to assure enough sample for latter analysis. The fractions were then dissolved in 1 mL MeOH and given to the Ames test for mutagenicity evaluation.

Results

Once the compounds eluted from the prep-HPLC column, they were analyzed with UV-Vis absorbance spectroscopy which measures the amount of light a compound absorbs at varying wavelengths. The spectrum for PhIP can be seen in Figure 5 with two peaks seen at 221.3 and 315.0 nm. Since many compounds potentially absorb light at around 221 nm, that peak will be disregarded for our HCA detection purposes. Therefore, all the analysis will be done at the 315 nm wavelength. The prep-HPLC chromatograph obtained for PhIP can be seen in Figure 6. There retention time for PhIP can be said to be about 4.00 min as seen with the peak seen in Figure 6, which became the 'target time' for similar HCAs. The two graphs in Figures 5 and 6 show that the prep-HPLC method developed is compatible in separating HCAs; therefore, it should hypothetically then separate our arginine-based HCA.

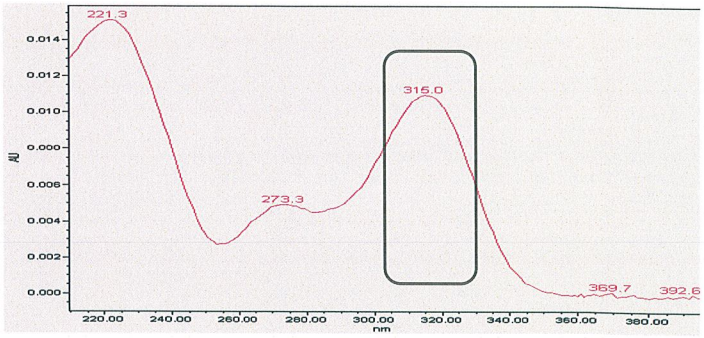

Figure 5. UV-Vis spectrum for PhIP

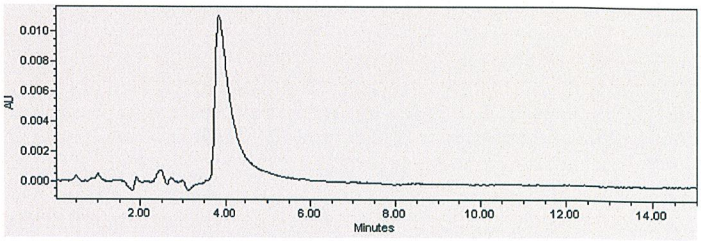

Figure 6. Chromatograph for PhIP

Both chromatographs seen in Figures 7 and 8 were taken at 315.0 nm. Figure 7 shows the typical RF-HCA chromatograph obtained for the old prep-HPLC method which used a mobile phase mixture of 50:50 ACN/water, a flow rate of 10 mL/min, an injection volume of 500 µL, and a total run time of 30 min; whereas, Figure 8 is a representative chromatograph obtained for

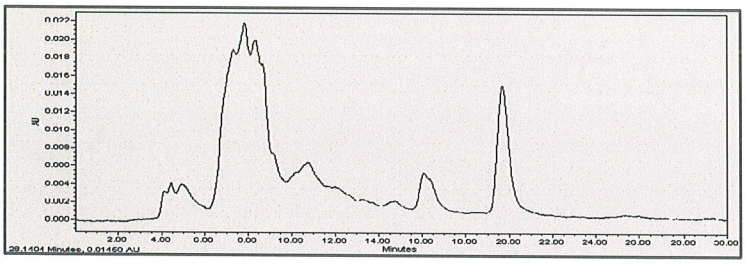

Figure 7. Typical RF-HCA chromatograph at 315 nm for old prep-HPLC method using 50:50 ACN/water

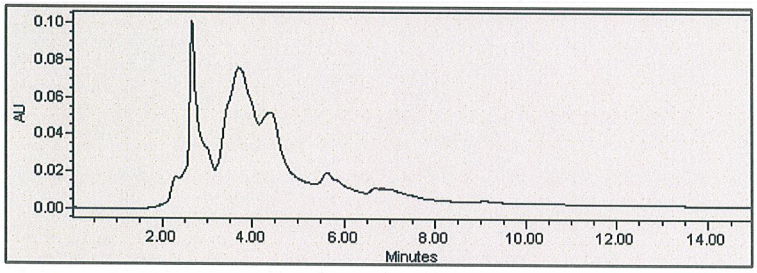

Figure 8. Typical RF-HCA chromatograph at 315 nm for new prep-HPLC method using 65:35 ACN/water

the new prep-HPLC methodology. Comparing the two chromatographs' pros and cons, the old method was advantageous in that it was more 'environmentally friendly' as it used less ACN. However, as seen in Figure 7, the old method had poor peak resolution with lots of peak tailing. In Figure 8, the newer prep-HPLC method provides better resolution in distinguishing between peaks as well as shortening the total run time in half. The disadvantage of the newer method is that it is more organic solvents being used and could be costlier to run as there is a higher proportion of ACN used.

Analysis

Previous work done in our lab by Reichert worked on preparing and characterizing of potentially carcinogenic HCAs; however, optimization of the separation procedure with HPLC fell short and not enough concentrated, purified sample was available for further analysis. Prior to development of a prep-HPLC method, an analytical HPLC was used. The injection volume used was 100 µL which led to only 0.5 µg yield of product for every 20 min run. This proved to be insufficient amounts for latter mutagenic evaluation with the Ames test as well as NMR and MS analysis even if more than 40 runs through analytical-HPLC was completed. With only three runs, each a length of 15 minutes, the current prep-HPLC method is able to produce concentrated

samples in the hundreds to thousands of μg/mL.

When comparing the RF-HCA samples amongst each other, inconsistencies between the chromatographs were seen. Theoretically each burnt sample should contain the same products as they all started with 4.0 grams of phenylalanine and arginine as well as going through the same extraction and purification steps. Thus, the chromatographs should mirror one another. Since that was not the case in the analysis of the RF-HCA chromatographs, as seen in Figures 9a and b. The sample, RF-HCA-08, seen in Figure 9a came from researcher Schell and sample RF-HCA-09 came from another researcher Gordon. The two chromatographs are drastically different from one another and from the representative chromatograph chosen in Figure 8, too. The number of peaks and the retention times of the compounds vary, even though ideally the samples should contain the same products. This led us to believe that the burning procedures may be inconsistent amongst different student researchers. These differences between burnt samples caused there to be great variation when the samples' mutagenic properties were tested with the Ames test.

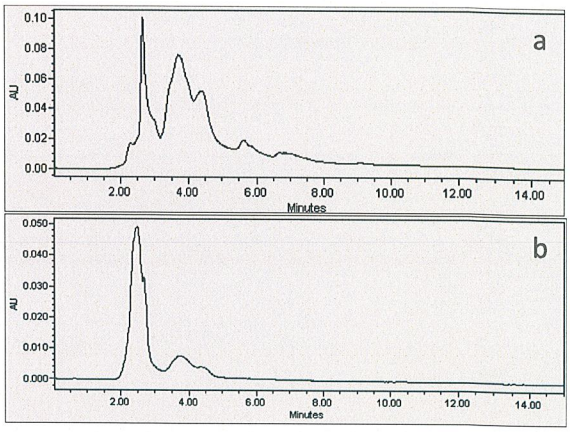

Figure 9. (a) Chromatograph for RF-HCA-08 (b) Chromatograph for RF-HCA-09

The results of the Ames test can be seen in Figure 10. The Ames test is a biological assay that tests whether a certain compound is mutagenic or not by reverting bacteria to only be able to grow in the presence of histidine. The bacteria are placed on a sterile plate that lacks histidine. The compound of interest is then added to the plate. If in fact mutagenic then it'll allow the

bacteria to grow and to multiply even in the absence of histidine due to the formation of mutations. Therefore, the more bacterial colonies seen on the plate, the more mutagenic a compound is. The more mutagenic a compound is, the higher the potential in the compound being carcinogenic. It can be seen that PhIP had an average of 353 bacterial colonies per plate; whereas the highest bacterial count was found in RF-HCA-06 with an average of 180 bacterial colonies per plate. These results coincide with the 1993 paper in which they found mutagenic properties in the arginine-based HCAs but at a still lower concentration than the creatine-based HCA, PhIP. In comparison to the control plate, all of the RF-HCA samples had a higher average bacterial count – something that was harder to prove with the older prep-HPLC methodology since the samples given to the Ames test were not concentrated enough. The varying bacterial colonies per plate for the RF-HCA samples may also be due to the wide array of concentrations between each; therefore, for future studies it may be beneficial to standardize the concentrations given to the Ames test so to better be able to compare the numbers against each other. A significant thing to note in Figure 10 is that MeOH does not interfere with the Ames test even though it is often labeled as poisonous or toxic to humans. This proves that MeOH is a good solvent of choice to be using for sample preparations.

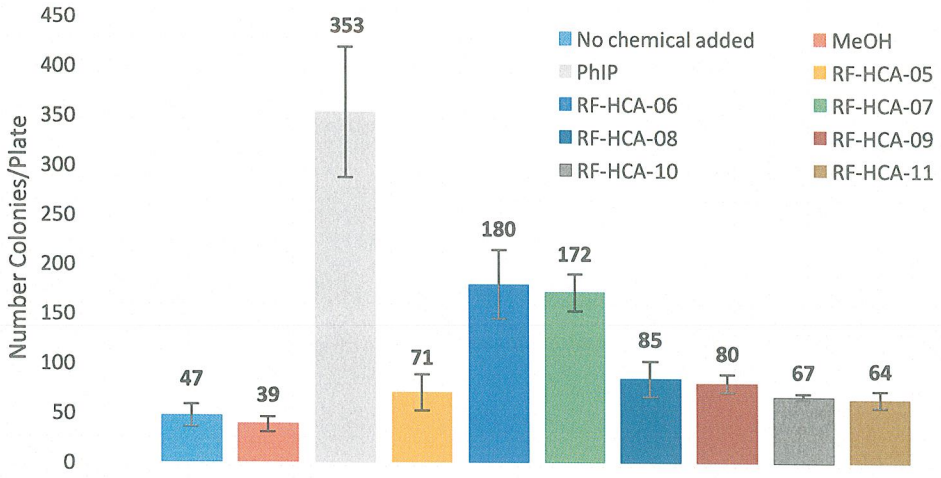

Figure 10. Mutagenicity evaluation results from Ames test.

Conclusion

The new prep-HPLC method developed improved peak separation and resolution compared to the older methodology carried out by previous researchers. Optimization of the prep-HPLC method allows us to be able to quickly and efficiently isolate and characterize new non-creatine HCAs; whereas samples had to be run 50+ times with HPLC in order to collect enough product for the Ames test as well as NMR and MS analysis. Utilizing a higher ratio of ACN to water allowed for faster separation, cutting the run time in half from 30 minutes to 15 minutes, without the use of any buffer salts that could have potentially interfered with analysis. Although much improvement has been seen with this new prep-HPLC method, more work still needs to be done. Further optimization of the prep-HPLC method is needed in order to produce individual peaks so to be able to confidently isolate the phenylalanine-arginine HCA peak.

The Ames test is still currently being done with RF-HCA samples prepared and so it is hard to definitively say if the results shown in this study are significant or not. If the Ames test results come back significant, then the 2-8 minute fractions will be further separated by

individual peaks. If the Ames test results come back insignificant, then the 1993 paper will be further explored by burning different amino acid pairs, namely arginine with threonine.

In addition, the new prep-HPLC method can be used to monitor for quality control of the whole HCA project, specifically with checking the consistency of burnings. The original flow chart seen in Figure 3 shows a total of five steps whereas the updated flow chart in Figure 11 shows six. Due to the inconsistencies in the burnings seen in the drastically different chromatographs collected from prep-HPLC, the burnt products will be analyzed with prep-HPLC in order to analyze the chromatographs against one another. If the chromatographs do in fact mirror one another, then the phenylalanine-arginine burnt samples will proceed onto the extraction and purification process.

Figure 11. Updated flow chart of for HCA project. Prep-HPLC will be conducted after the burning procedure to test for consistencies in burnings.

Developing an optimal prep-HPLC methodology is crucial to the HCA project overall because it serves as a quality control point in checking the consistencies of the burnings and works as a separation step in order to isolate the specific arginine-based HCA. Once the phenylalanine-arginine HCA is able to isolated and concentrated, future work will be done in analyzing it with NMR and MS so to be able to identify and characterize it in comparison with other established creatine-based HCAs. This step was something that Knize research team in 1993 were unable to perform. If the arginine-based HCA is able to be identified, then it'll confirm the need for further research to be done into vegetarian products as being possible

cancer-causing agents although being absent of creatine – something that's important especially within the Adventist community.

References

Alaejos, M.; Afonso, M. Factors That Affect the Content of Heterocyclic Aromatic Amines in Foods. Comprehensive Reviews in Food Science and Food Safety. **2011**, 10, 52-108.

Atwood, Stanley; Garner, Sanford; Thomas, Ronald. Selected Heterocyclic Amines: PhIP, MeIQ, and MeIQx. National Institute of Environmental Health Sciences. **2002**.

Bouvard, V; Loomis, D.; Guyton, K.; Grosse, Y.; Ghissassi, F.; Benbrahim-Tallaa, L.; Guha, N.; Mattock, H.; Straif, K. Carcinogenicity of Consumption of Red and Processed Meat. *Lancet Oncology*. **2015**, 16, 1599-1600.

Gibis, Monika. Optimized HPLC Method for Analysis of Polar and Nonpolar Heterocyclic Amines in Cooked Meat Products. *Journal of AOAC International*. **2009**, 92, 715-724.

Knize, M. G.; Cunningham, J. R.; Avila, A. L.; Jones, E. A., Jr.; Felton, J. S. Formation of Mutagenic Activity from Amino Acids Heated at Cooking Temperatures. *Fd Chem. Toxic.* **1993**, 32, 55-60.

Knize, M. G.; Cunningham, J. R.; Avila, A. L.; Jones, E. A., Jr.; Felton, J. S. Characterization of Mutagenic Activity in Cooked-Grain-Food Products. *Fd Chem. Toxic*. **1993**, 32, 15-21.

Schut, H.A.J; Snyderwine, E.G. DNA adducts of heterocyclic amine food mutagens: implications for mutagenensis and carcinogenesis. *Carcinogenesis*. **1999**, 20, 353-368.

Sugimura, Takashi; Wakabayashi, Keiji; Nakagama, Hitoshi; Nagao, Minako. Heterocyclic Amines: Mutagens/Carcinogens Produced During Cooking of Meat and Fish. *National Cancer Center.* **2004**, *95*, 290-299.