Amino acid effects on heterocyclic amine formation in the Maillard reaction model systems and pan-fried beef patties

by

Ziyi Linghu

B.S., South-Central University for Nationalities, Wuhan, China, 2010
M.S., University of Minnesota-Twin Cities, St. Paul, MN, 2013

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Food Science

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2019

ABSTRACT

Heterocyclic amines (HCAs) are a group of highly mutagenic and potentially carcinogenic compounds that typically form on cooked meat through the Maillard reaction. While many physical factors, such as heating temperatures/times and cooking methods, and using food additives with antioxidant properties can affect HCA formed in the Maillard reaction, modifying HCA precursor profiles, such as types and concentrations of amino acids before heating, is a relatively new discovery and could effectively minimize dietary HCA formation in cooked meat.

Simple liquid Maillard model systems were first conducted to evaluate the effect of eight essential and two non-essential amino acids (AAs) on 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP) formation. Each AA was individually added into model systems (glucose: creatinine: phenylalanine) and mixtures were heated at 180 °C for 1 h. Tryptophan, lysine, leucine, and proline exhibited the most pronounced inhibitory effects (> 87% reduction) on PhIP formation, followed by methionine, valine, isoleucine, threonine, and phenylalanine at the highest molar ratio; while aspartic acid increased PhIP formation. The inhibition mechanism for this reaction was investigated in phenylacetaldehyde-AA model systems (180 °C /1 h), which showed that formation of phenylacetaldehyde-AA adducts may be responsible for the reduction in PhIP formed during Maillard reaction.

A simple, fast, and efficient method based on Enhanced Matrix Removal of Lipids (EMR-Lipid) was then developed for identifying trace-level HCAs in cooked meats that ranged from high-protein (beef and chicken) to high-fat (pork bacon) matrices. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) with selective reaction monitoring mode was used for qualitative and quantitative analysis. Good linearity of standard curves was obtained in both pure

solvents and post-spiked meat extracts between 0.5 and 50.0 ng/mL. Satisfactory recoveries of four HCAs were achieved in a range of 65 to 111% in beef, 71 to 106% in bacon, and 42 to 77% in chicken. For the first time, this work demonstrates that EMR-Lipid coupled with LC-MS/MS may be a promising option for polar HCA extraction and detection in fatty meat products.

To further study the inhibitory effect of selected amino acids on HCA formation in cooked meat, tryptophan, lysine, leucine, and proline at 0.05%, 0.20%, and 0.50% (w/w) were investigated in pan-fried beef patties (230 °C/15 min). Tryptophan at 0.50% showed the most reduction on total HCAs (93% inhibition), followed by 0.50% lysine (84% inhibition), while leucine and proline at 0.50% were less effective. Several key flavor compounds of fried beef, such as aldehydes and various pyrazines, were affected by added AAs at 0.50%. Surface application of most AAs had no significant effect on cooking loss, pH, or color change in fried beef patties; however, adding lysine increased pH and surface redness. Overall, the results of this study suggest that adding amino acids to ground beef patties could effectively mitigate mutagenic HCA formation during cooking.

Amino acid effects on heterocyclic amine formation in the Maillard reaction model systems and pan-fried beef patties

by

Ziyi Linghu

B.S., South-Central University for Nationalities, Wuhan, China, 2010
M.S., University of Minnesota-Twin Cities, St. Paul, MN, 2013

A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Food Science

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2019

Approved by:

Major Professor
J. Scott Smith

ABSTRACT

Heterocyclic amines (HCAs) are a group of highly mutagenic and potentially carcinogenic compounds that typically form on cooked meat through the Maillard reaction. While many physical factors, such as heating temperatures/times and cooking methods, and using food additives with antioxidant properties can affect HCA formed in the Maillard reaction, modifying HCA precursor profiles, such as types and concentrations of amino acids before heating, is a relatively new discovery and could effectively minimize dietary HCA formation in cooked meat.

Simple liquid Maillard model systems were first conducted to evaluate the effect of eight essential and two non-essential amino acids (AAs) on 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP) formation. Each AA was individually added into model systems (glucose: creatinine: phenylalanine) and mixtures were heated at 180 °C for 1 h. Tryptophan, lysine, leucine, and proline exhibited the most pronounced inhibitory effects (> 87% reduction) on PhIP formation, followed by methionine, valine, isoleucine, threonine, and phenylalanine at the highest molar ratio; while aspartic acid increased PhIP formation. The inhibition mechanism for this reaction was investigated in phenylacetaldehyde-AA model systems (180 °C /1 h), which showed that formation of phenylacetaldehyde-AA adducts may be responsible for the reduction in PhIP formed during Maillard reaction.

A simple, fast, and efficient method based on Enhanced Matrix Removal of Lipids (EMR-Lipid) was then developed for identifying trace-level HCAs in cooked meats that ranged from high-protein (beef and chicken) to high-fat (pork bacon) matrices. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) with selective reaction monitoring mode was used for qualitative and quantitative analysis. Good linearity of standard curves was obtained in both pure

solvents and post-spiked meat extracts between 0.5 and 50.0 ng/mL. Satisfactory recoveries of four HCAs were achieved in a range of 65 to 111% in beef, 71 to 106% in bacon, and 42 to 77% in chicken. For the first time, this work demonstrates that EMR-Lipid coupled with LC-MS/MS may be a promising option for polar HCA extraction and detection in fatty meat products.

To further study the inhibitory effect of selected amino acids on HCA formation in cooked meat, tryptophan, lysine, leucine, and proline at 0.05%, 0.20%, and 0.50% (w/w) were investigated in pan-fried beef patties (230 °C/15 min). Tryptophan at 0.5% showed the most reduction on total HCAs (93% inhibition), followed by 0.50% lysine (84% inhibition), while leucine and proline at 0.50% were less effective. Several key flavor compounds of fried beef, such as aldehydes and various pyrazines, were affected by added AAs at 0.50%. Surface application of most AAs had no significant effect on cooking loss, pH, or color change in fried beef patties; however, adding lysine increased pH and surface redness. Overall, the results of this study suggest that adding amino acids to ground beef patties could effectively mitigate mutagenic HCA formation during cooking.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

I would like to first express my deep gratitude to my major advisor Dr. J. Scott Smith, for his valuable guidance, support, and mentoring throughout my doctoral studies at K-State. I am truly grateful for all the incredible opportunities he provided for me to broaden my knowledge in the field of food chemistry and toxiciology. Also, special thanks to Dr. Smith's family, Kathy, Robbie, and Katie Smith for their kindness and friendship all these years. I am greatly thankful to Dr. Faris Hussian for being on my committee, giving me incredible guidance and suggestions during my doctoral research studies, and in particular for his continuous help in my research design and lab instrumental training, without which it would have been very difficult to complete my dissertation and research articles for this project. I would also like to thank Dr. Weiqun Wang and Dr. Jayendra Amamcharla for serving on my committee and providing their valuable input, guidance, and support during this research. Special thanks to Dr. Duy H. Hua for serving as my outside chair.

I would like to thank my lab mates, fellow undergraduate and graduate students at the Food Science Institute for their incredible friendships, kind support, and motivation during this long journey. Special thanks and appreciations are also extended to other faculties and staff at K-State for their valuable input, accessibility, and guidance during my doctoral studies.

Last but not least, my sincere thanks and appreciation to my parents, Ruikun Zong and Jia Linghu, for their endless love, support, listening, and continued encouragement that help me reach this milestone. Thanks for always being there for me!

DEDICATION

I would like to dedciaate this dissertation to my beloved my parents, Ruikun Zong and Jia Linghu, and grandparents, Shijun Linghu and Yanran Huang.

CHAPTER 1 - REVIEW OF LITERATURE

CHARACTERISTICS OF HETEROCYCLIC AMINES

The study of potential carcinogenic and mutagenic compounds in foods began in 1939 when a Swedish scientist, E.M.P. Widmark, found that meat extracts of roasted horse caused cancer when applied to mouse skin (Widmark, 1939). Several later research groups isolated a group of carcinogenic compounds from grilled meat products and identified them as polycyclic aromatic hydrocarbons (PAH) (Fretheim, 1983). Japanese scientists tested the smoke condensates collected during charcoal broiling of fish and beefsteak for mutagens using the Ames/Salmonella test (Ames, Mccann, & Yamasaki, 1975), reporting extremely high mutagenicity, which could not be justified by PAHs alone, and indicating that other highly mutagenic compounds may also be in cooked meat products (Nagao, Honda, Seino, Yahagi, & Sugimura, 1977). These food-derived mutagens were later identified and characterized as heterocyclic amines (HCAs) (Anastassiades, Lehotay, Štajnbaher, & Schenck, 2003).

To date, more than 25 HCAs have been isolated and characterized in different protein-rich foods like meat and fish (Herraiz, 2004; Knize, Cunningham, Griffin, Jones, & Felton, 1994; Murray, Lynch, Knize, & Gooderham, 1993; Thiébaud, Knize, Kuzmicky, Hsieh, & Felton, 1995). Table 1 gives the chemical names and abbreviations of these HCAs. All HCAs have molecular weights in the range of 160-250 with boiling points below 200-300°C (Gibis, 2016), and depending on the presence of different numbers of amine groups or nitrogen atoms, HCAs have been reported to have pKa values in the range of 3.5-8.6 (Alaejos & Afonso, 2011). The chemical structure of all HCAs includes at least two condensed aromatics and one heterocyclic structure usually with one exocyclic amino group excluding β-carbolines (harman and norharman) (Cheng, Chen, & Wang, 2006).

Table 1. Abbreviations, chemical names, molecular weights, and properties of polar and nonpolar HCAs (Gibis, 2016).

Abbreviations and Chemical Names			Molecular Weights (g/mol) and Properties [a]
Quinolines	IQ	2-Amino-3-methylimidazo[4,5-f]quinoline	192.2, Polar, p*Ka* 5.86
	MeIQ	2-Amino-3,4-dimethylimidazo[4,5-*f*]quinoline	212.3, Polar, p*Ka* 6.22
Quinoxalines	IQx	2-Amino-3-methylimidazo[4,5-*f*]quinoxaline	199.3, Polar, p*Ka* 1.96
	MeIQx	2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline	213.3, Polar, p*Ka* 2.20
	4,8-DiMeIQx	2-Amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline	227.3, Polar, p*Ka* 2.56
	7,8-DiMeIQx	2-Amino-3,7,8-trimethylimidazo[4,5-*f*]quinoxaline	227.3, Polar, p*Ka* 2.49
	4,7,8-TriMeIQx	2-Amino-3,4,7,8 tetraimethylimidazo[4,5-*f*]quinoxaline	241.3, Polar, p*Ka* 2.85
Pyridines	PhIP	2-Amino-1-methyl-6-phenyl-imidazo[4,5-b]pyridine	224.3, Polar, p*Ka* 7.72
	DMIP	Dimethylimidazopyridine	162.2, Polar, p*Ka* 8.16
	TMIP	Trimethylimidazopyridine	176.2, Polar, p*Ka* 8.66
α-amino-carbolines	AαC	2-Amino-9H-pyrido[2,3-b]indol	183.2, Nonpolar, p*Ka* 6.79
	MeAαC	2-Amino-3-methyl-9H-pyrido[2,3-b]indol	197.2, Nonpolar, p*Ka* 7.08
β-amino-carbolines	Norharman	1-methyl-9H-pyrido[4,3-b]indole	182.2, Nonpolar, p*Ka* 8.62
	Harman	9H-pyrido[4,3-b]indole	168.2, Nonpolar, p*Ka* 7.85
γ-amino-carbolines	Trp-P-1	3-Amino-1,4-dimethyl-5H-pyrido[4,3-b]indole	211.3, Nonpolar, p*Ka* 10.88
	Trp-P-2	3-Amino-1-methyl-5H-pyrido[4,3-b]indole	197.2, Nonpolar, p*Ka* 10.59

δ-amino-carbolines	Glu-P-1	2-Amino-6-methyldipyrido[1,2-a:3’2’-d]imidazole	198.3, Nonpolar, p*Ka* 6.33
	Glu-P-2	2-Amino-dipyrido[1,2-a:3’2’-d]imidazole	184.3, Nonpolar, p*Ka* 5.80

[a] Most basic (temperature 25°C)

Given their chemical properties and formation mechanisms, HCAs are generally classified into polar and nonpolar HCAs (Alaejos & Afonso, 2011). Polar HCAs (known as aminoimidazo-azarenes) contain a 2-amino-imidazole ring with an N-methyl group and one pyrazine or pyridine fused with an aromatic ring structure (Figure 1). Generally, all polar HCAs can be divided into 3 subgroups: imidazo-quinolones-type (IQ) (e.g., IQ, MeIQ), imidazo-quinoxalines-type (IQx) (e.g., IQx, MeIQx), and imidazopyridine-type (e.g., PhIP, DMIP, and TMIP) (Cheng et al., 2006). They normally form at temperatures between 100-300°C from the reaction of free amino acids, creatine/creatinine, and reducing sugars, so polar HCAs are also known as thermic HCAs. In addition, IQ [4,5-b], 7-MeIgQx, IgQx, 6.7-DiMeQIgQx, and 7,9-DiMeIgQx have been identified in cooked meats (Turesky, Taylor, Schnackenberg, Freeman, & Holland, 2005). TriMeIQx is a newly synthesized substance formed in model systems but not observed in heated foods (Murkovic, 2004).

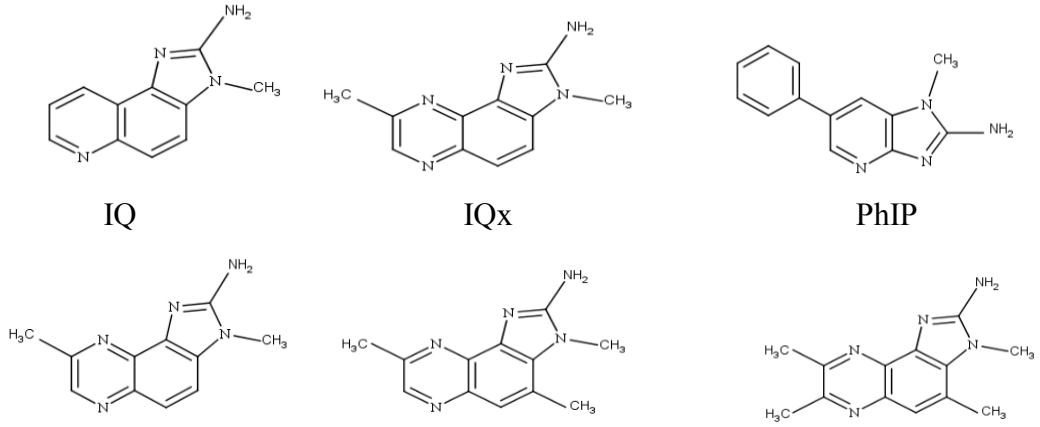


Figure 1. Chemical structure of selected polar HCAs (Murkovic, 2004).

Non-polar HCAs (known as amino-carbolines) are composed of a five-member heterocyclic aromatic ring sandwiched between two six-member aromatic rings, one or more of which can be pyridine (Cheng et al., 2006) (Figure 2). Non-polar HCAs are generally classified into 4 subgroups: an α-subgroup (e.g., AαC and MeAαC); β-subgroup (e.g., norharman, harman); γ-subgroup (e.g., Trp-P-1, Trp-P-2); and δ-subgroup (e.g., Glu-P-1, Glu-P-2). Non-polar HCAs typically form from the pyrolysis of amino acids or proteins at a temperature >300°C, so they are also called pyrolytic HCAs.

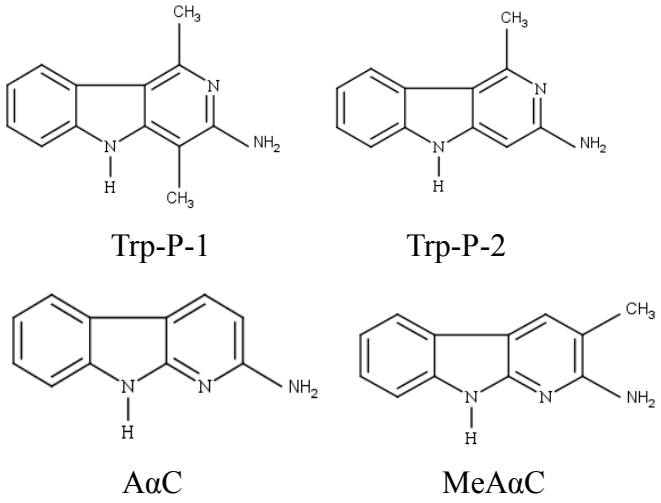


Figure 2. Chemical structure of selected nonpolar HCAs (Murkovic, 2004).

MECHANISMS BY WHICH HETEROCYCLIC AMINES FORM

In the literature, HCAs are reported as mainly produced as byproducts in cooked foods, especially poultry, pork, beef, and fish, heated under normal domestic cooking conditions like pan-frying, grilling, broiling, roasting, and baking (Sinha et al., 1995, 1998, 2000). Because non-polar HCAs like β-carbolines (Harman and Norharman) tested non-mutagenic in the Ames/Salmonella mutagenicity test, due to lacking exocyclic amino groups (Cheng et al., 2006), formation of polar HCAs has received much more attention in the literature and has been well

investigated in heated model systems incorporating common components of muscle tissues, such as sugars, free amino acids, dipeptides, and creatinine. HCAs most likely formed through the non-enzymatic heat-induced browning reaction known as the Maillard reaction (Murkovic, 2004; Pais & Knize, 2000; Sinha et al., 1998). The Maillard reaction is one of the most important chemical reactions between reducing sugars and amino acids and proteins, leading to a distinct flavor and color in thermally-processed foods (Martins, Jongen, & van Boekel, 2000). However, the Maillard reaction produces more than these desirable attributes in foods; it is also responsible for generating mutagenic compounds like HCAs in cooked meats.

Although the mechanisms through which HCAs form has not been well elucidated, a few formation pathways have been postulated and described in the literature (Jägerstad, Skog, Arvidsson, & Solyakov, 1998; Weisburger, 2002; Yaylayan, 2003). In general, free amino acids and reducing sugars acting as reactants in meat are initially dehydrated during heating and form vinyl-pyridines or vinyl-pyrazines after Strecker degradation. Following aldol condensation reaction with creain(in)e, amino-imidazo forms; amino-imidazo is part of PhIP, IQ-, and IQx-type HCAs (Murkovic, 2004; Pearson, Chen, Gray, & Aust, 1992). To be specific, the amino group ($-NH_2$) of amino acids begins to react with the carbonyl group (-CHO) of reducing sugars to form Schiff's base, and after the Amadori rearrangement, glycol-aldehyde alkylimine (enol type) forms, which can be either condensed with –CHO to form dialkypyrazine radicals or further oxidized to form glyoxal monoalkylimine and then condensed with glyoxal to yield pyridine radicals (see Figure 3). Cyclization with creatinine and Strecker aldehydes (i.e., HCHO or CH_3CHO) follows, and IQ- and IQx-type HCAs form as final products in the Maillard reaction. Since dialkypyrazine radicals form much faster than pyridine radicals, this explains why IQx-type HCAs form in larger quantities in meat and fish than IQ-type HCAs. Jägerstad,

Skog, Grivas, and Olsson (1991) indicated that this reaction mechanism of HCAs depends highly on type of meat, precursor concentrations and types, cooking temperatures, and time.

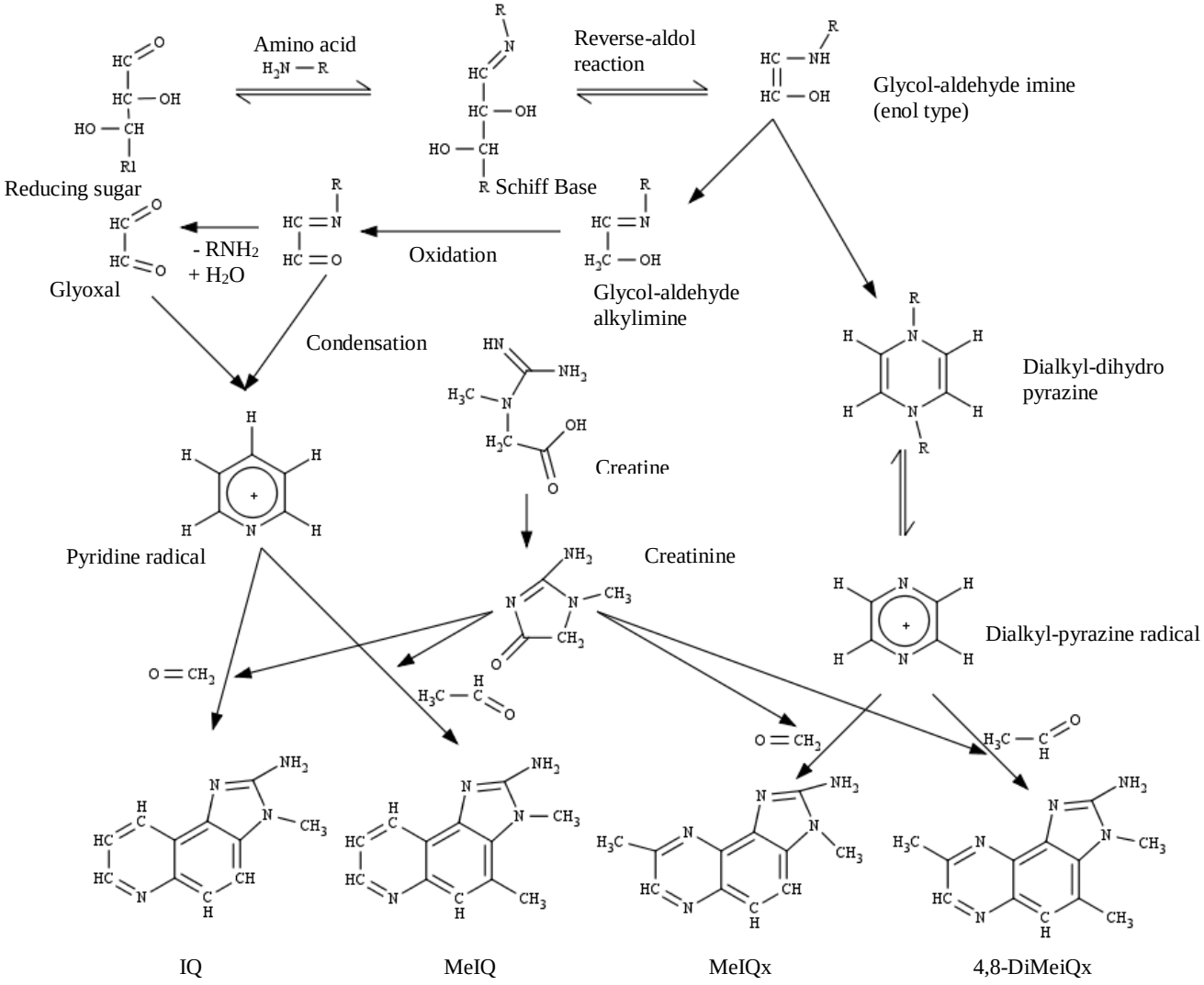


Figure 3. Postulated free-radicals mediated pathway for the formation of IQ- and IQx-type HCAs formation (Adapted from Vitaglione & Fogliano, 2004).

Several studies have also reported an alternative route for HCA formation in which a direct condensation reaction occurs between creatinine and Strecker aldehydes of amino acids (Murkovic, 2004). In the Maillard reaction, the Strecker degradation is part of the oxidative

decarboxylation of amino acids, which converts an α-amino acid into an aldehyde-containing side chain product (Strecker, 1861; Yaylayan, 2003). Vitaglione and Fogliano (2004) and Zochling (2002) demonstrated that PhIP still forms in model systems when phenylalanine is replaced by its Strecker aldehyde, phenylacetaldehyde, in the presence of creatinine. Figure 4 shows phenylacetaldehyde forms first through thermal degradation of its parent amino acid in the model system. It then undergoes an aldol-condensation with creatinine followed by dehydration. PhIP then forms when Schiff's base combines with an amino group of phenylalanine or creatinine. Strecker aldehydes may be important to the formation of HCAs; phenylacetaldehyde is an indispensable precursor for PhIP.

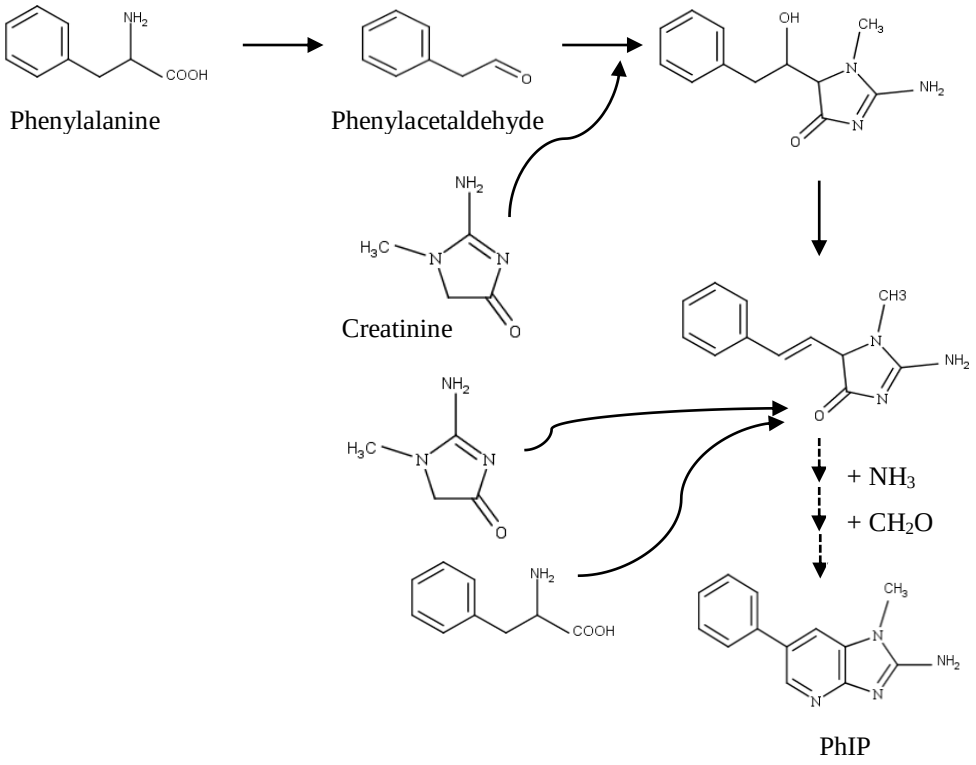


Figure 4. Formation of PhIP with phenylacetaldehyde as intermediate compound (Adapted from Vitaglione & Fogliano, 2004).

MAILLARD MODEL SYSTEMS

The complexity of meat matrices usually induces many concurrent chemical reactions when meat is cooked at high temperatures, which make the study of HCAs in meat challenging (Zöchling & Murkovic, 2002). Therefore, the simple and defined Maillard model systems are often used to simulate the HCA chemical reaction to help in explaining the effects of different chemical and physical variables on HCA formation (Johansson & Jägerstad, 1996; Pais, Salmon, Knize, & Felton, 1999). To date, many different chemical model systems have been developed to mimic aspects of target meat samples where the major precursors are typically a mixture of amino acids, sugars, and creatinine/creatine, prepared in a molar ratio similar to those found in meat products, but usually at higher concentrations (~20 times) to facilitate HCA formation and detection (Arvidsson, Boekel, Skog, & Jagerstad, 1997; Pais et al., 1999; Skog, Johansson, & Jägerstad, 1998). The typical heating temperatures applied to model systems range from 100 to 300°C while times range from 0.5 to 120 min. PhIP, IQ-type, and IQx-type HCAs are the most studied (Arvidsson et al., 1997; Gibis, 2016).

Model systems can use either wet-base or dry-base. In a wet-heating model system, diethylene glycol and water are often used as the reaction medium to dissolve and suspend pure precursors and get the best heat transfer during heating (Messner & Murkovic, 2004). Skog and Jägerstad (1990) reported that the HCAs formed excessively in model systems when sugar is half-molar concentration of amino acids and creatinine/creatine. Dennis, Karim, and Smith (2015) confirmed that the optimal molar concentration of precursors (Creatine: Threonine: Glucose) produced was 0.8:0.8:0.4 mmol in diethyl glycol-15% water. At this concentration, the highest levels of HCAs formed. Although dry-heating model systems can also be used for HCA research (Pais et al., 1999), aqueous heating favors formation of more IQx, MeIQx, and

DiMeIQx than dry heating, indicating that wet-heating model systems can represent HCA formation in meat during cooking (Skog, Solyakov, & Jägerstad, 2000). In addition, model systems based on lyophilized meat, meat juice, and homogenized freeze-dried fresh meat have also been used to study the formation of certain HCAs, providing complicated but more relevant results than using pure compounds in chemical model systems (Jägerstad et al., 1998; Messner & Murkovic, 2004; Skog et al., 2000). Overall, using Maillard model systems can help in evaluating how HCA formation changes with differences in meat compositions (e.g., types and ratios of precursors; food additives) under heating (Bordas, Moyano, Puignou, & Galceran, 2004). Moreover, results from model systems should be considered as preliminary results that provide good agreement with real meat systems.

INFLUENCES ON FORMATION OF HETEROCYCLIC AMINES

Cooking temperature and time

Cooking temperature and time are important physical influences on HCA formation in all kinds of meat products. Much of the research has demonstrated that HCAs form in cooked foods as a function of temperature and time (Arvidsson et al., 1997). In general, HCA formation increases dramatically when cooking temperature is >150°C (Jackson & Hargraves, 1995; Johansson & Jägerstad, 1994). Knize et al. (1994) reported that increasing cooking temperature from 150°C to 230°C and cooking time from 2 min to 11 min enhanced the amounts of HCAs (i.e., MeIQx, PhIP, DiMeIQx, and IQ) that formed in fried beef patties, but no non-polar HCAs were detected. Bordas et al. (2004) confirmed these results and observed that when cooking temperatures were lower than 150°C, fewer MeIQx, PhIP, and 4,8-DiMeIQx formed in a model system even when cooking times were an hour, but their concentration increased dramatically

when the cooking temperature increased 200°C even with a cooking time of 30 min. These results clearly suggest that certain polar HCAs form in foods because of heating parameters. Arvidsson et al. (1997) also studied the formation kinetics of polar HCAs in model systems and concluded that the formation of HCAs fits a first-order reaction model (Figure 5). To be specific, at temperatures < 200°C, HCAs form rapidly, reaching a plateau within 5-10 min of heating, but with a cooking temperature of 225°C, PhIP and IQx production was not detected while MeIQx and 7,8-DiMeIQx began to decrease immediately after peaking. These results reveal that polar HCAs may form and degrade simultaneously at all temperatures, but this phenomenon is more significant for PhIP and IQx-type HCAs once the temperature > 200°C. Ahn and Grün (2005) also demonstrated that PhIP formation was temperature-sensitive with the highest activation enthalpy, indicating that PhIP was more susceptible to temperature change and the least stable HCA at high temperatures, whereas other HCAs like 4,8-DiMeIQx, IQx, and MeIQx were less sensitive. In the same study, MeIQx and PhIP formed at the highest rate constant (k) at all tested temperatures, while IQ and MeIQ had the lowest k values, which explains why MeIQx and PhIP are often the most abundant HCAs in cooked foods even when cooked at low temperatures.

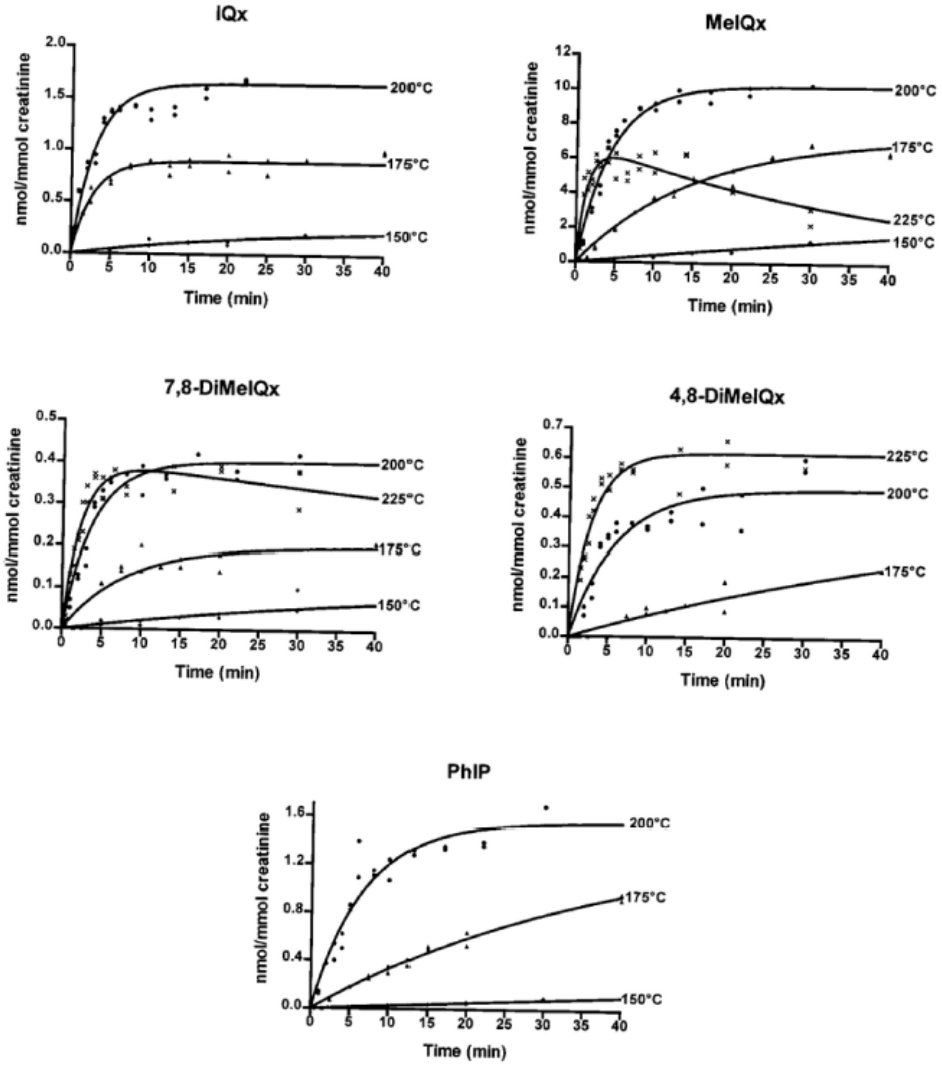


Figure 5. Formation of certain polar HCAs at 150, 170, 200, and 225°C at varying cooking times (Arvidsson et al., 1997).

Cooking methods

Cooking method may also influence HCA formation in cooked meat products. Direct-heating methods like pan-frying, grilling, barbecuing, and oven-broiling reportedly produce the most HCAs in meat and fish products cooked at high temperatures (> 150°C) for a short time (4-20 min) (Chen & Chiu, 1998). On the other hand, oven roasting, baking, boiling, and steaming,

which are indirect-cooking methods, use medium-high or low-high cooking temperatures (Chen & Chiu, 1998). Sinha et al. (1998) found that grilling or barbecuing induced more HCAs to form in meat than other cooking methods because the heat was more efficiently transferred from the grill/metal grates to the surface of the meat. Puangsombat, Gadgil, Houser, Hunt, and Smith (2012) also reported that the pan-frying beef products produced a significantly more total HCAs than broiling; baking produced still fewer HCAs. Furthermore, the mutagenic activity in pan-fried chicken fillets and hamburgers was also significantly higher than in those prepared using a convection oven, while boiling and steaming generated still less mutagenicity, probably because steam can maintain a lower surface temperature of meat (Skog, Eneroth, & Svanberg, 2003). Moreover, doneness of meat, even cooked by different methods, is related to HCA formation. For example, Sinha et al. (1998) reported that regardless of cooking method (i.e., grilling/BBQ, pan-frying, oven-broiling), very well-done steak had the most total HCAs, followed by "well-done" steaks, medium and raw products, suggesting that doneness level also influences the overall mutagenicity and HCA content in cooked meats.

Amino acids

Free amino acids contribute heavily to HCA formation in the Maillard reaction. Liao, Xu, and Zhou (2009) confirmed that free amino acids were involved in the formation of HCAs in cooked pork floss; they found a positive correlation between HCA formation and the reduction of certain amino acids like threonine (r=0.91), serine (r=0.91), glutamic acid (r=0.94), isoleucine (r=0.92), tyrosine (r=0.91), and phenylalanine (r=0.92). Overvik, Kleman, Berg, and Gustafsson (1989) reported that adding serine, threonine, alanine, phenylalanine, leucine, or tyrosine to raw pork enhanced PhIP, MeIQx, and TMP formation during cooking, and threonine, at a level of 1%

(w/w), increased the total mutagenicity by 43 times. To further explain how each free amino acid affected the formation of HCAs, the characteristics of single and/or a mixture of amino acid species have been evaluated; glycine, serine, proline, phenylalanine, alanine, threonine, leucine, and tyrosine were all reported to be precursors of certain HCAs. Recently, Zamora, Alcón, and Hidalgo (2013) studied how 20 amino acids affected PhIP formation in model systems containing creatinine and phenylalanine as precursors, finding that adding cysteine, methionine, tyrosine, serine, threonine, proline, tryptophan, aspartic acid, and asparagine significantly increased PhIP formation upon heating. They later demonstrated that active carbonyl compounds like α-keto acids, which are produced by thermal degradation of amino acids, are the efficient inducers of HCA formation by converting phenylalanine into phenylacetaldehyde and producing PhIP. These findings indicate that PhIP could be generated over an alternative reaction pathway in the presence of different amino acids. Furthermore, increasing concentrations of amino acids enhanced HCA production in model systems with an impact even more significant than glucose and creatinine (Bordas et al., 2004). For example, Skog et al. (2000) found that increasing tryptophan content by 5 times greatly enhanced the formation of IQx and β-carbolines in a meat juice model system, and a mixture of amino acids (glycine, alanine, and phenylalanine), increased 50 times more than native amounts, caused more PhIP and MeIQx to form in both wet- and dry-based model systems (Bordas et al., 2004).

Sugars

The yields of mutagenic HCAs also were affected by type and concentration of reducing sugars. In general, when sugar content was one-half that of creatinine and amino acids, more total HCAs formed within the system (Cheng et al., 2006). Shin, Park, and Park (2003)

confirmed that fresh meat with a molar concentration of creatinine and sugar at a level of 1:0.7 produced more HCAs during cooking. Skog and Jägerstad (1990) examined several monosaccharides and disaccharides and reported that glucose and fructose had a more pronounced effect on HCA formation than sucrose and lactose in model systems. However, when sugar content was increased to more than or equimolar amounts of other precursors, fewer HCAs were detected, suggesting that sugars at high concentrations inhibited HCA formation, which was probably due to sugars blocking the reaction of creatinine with certain Maillard reaction intermediates (e.g., pyrazine, pyridine), leading to fewer mutagenic HCAs being produced (Skog et al., 1998; Skog & Jägerstad, 1990). In another study, the mutagenicity of fried beef patties was reduced by 34-76% when more than 4% (w/w) glucose and lactose were added (Skog, Knize, Felton, & Jägerstad, 1992). Similarly, glucose and honey (buckwheat, clover, and sage) were also reported to inhibit HCA formation in fresh pork sausage (Abdulkarim & Smith, 1998; Shin et al., 2003).

Fat content

Research indicates that fat content can both inhibit and enhance overall mutagenicity and HCA formation in real meat products and model systems. Knize et al. (1985) observed that increasing fat content from 8% to 15% in fresh beef patties significantly increased total mutagenicity after the patties were fried. Nilsson et al. (1986) confirmed these results, reporting a significant rise in mutagenic activity in lean pork when fat was added to the meat before frying. Solid food products are usually cooked by conduction heat, so adding fat might itself efficiently improve mass and heat transfer between meat samples and the metal surface of a frying pan. However, when fat content was increased to almost 30% in fresh meat patties, mutagenicity was

significantly reduced (Knize et al., 1985). This phenomenon indicates that increasing fat content beyond a certain value may physically dilute the available HCA precursors and therefore, decrease mutagenic activity. In addition, many researchers have demonstrated that lipids from frying oil might chemically contribute to the formation pathway of HCAs during the Maillard reaction. Johansson and Jägerstad (1996) reported that beef burgers fried with rapeseed oil containing a high level of oleic acids generated more MeIQx and DiMeIQx, than burgers cooked with butter, margarine, or sunflower seed oil. Although the role of lipids in HCA formation remains unclear, free radicals generated from oxidized lipids might contribute to the Maillard reaction and enhance the production of Millard intermediates like pyrazines, pyridines, and Strecker aldehydes; this could consequently create more HCA (Barnes, Maher, & Weisburger, 1983). Zamora, Alcón, and Hidalgo (2012), in a comprehensive study, noted that the oxidation products of unsaturated fatty acids like 4-oxo-2-nonenals and α-keto acids could convert phenylalanine into its Strecker aldehyde and thus accelerate PhIP formation.

TOXICITY OF DIETARY HETEROCYCLIC AMINES

Mutagenicity

Food-derived HCAs are well documented as mutagens in many studies that used the Ames/Salmonella reversion assay (James et al., 1986; Nagao et al., 1977). Table 2 lists the mutagenic potencies of all HCAs measured as revertants/μg in the Ames/Salmonella test (Sugimura, Wakabayashi, Nakagama, & Nagao, 2004). All HCAs, other than harman and nonharman, acted as strong mutagens after they were metabolically activated with the S-9 mix in the Ames test, thus inducing frameshift and base pair substitution mutations in Salmonella Typhimurium strains TA98 and TA100. The level of mutagenic potency of each HCA varied in

the test; IQ and MeIQ exhibited the highest mutagenicity, followed by DiMeIQx, MeIQx, Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2, and PhIP. For MeAαC, AαC, and Phe-P-1, however, mutagenicity was extremely low in both TA98 and TA100. Hatch, Knize, and Felton (1991) and Sugimura et al. (2004) indicated that observed differences in mutagenicity among HCAs correlated with their structural parameters: number of fused aromatic rings, number and location of heteroatomas like nitrogen atoms in the non-imidazole ring, methyl substituent on the imidazole ring or ring carbon atoms, which allow active HCA metabolites to easily access and react with nuclear DNA, as well as block any detoxification reaction.

Table 2. Mutagenicity of HCAs in the Ames/Salmonella TA98 and TA100 strains (Sugimura et al., 2004).

	Revertants/µg	
HCAs	**TA98**	**TA100**
IQ	433,000	7,000
MeIQ	661,000	30,000
IQx	75,000	1,500
MeIQx	145,000	14,000
4,8-DiMeIQx	183,000	8,000
7,8-DiMeIQx	163,000	9,900
PhIP	1,800	120
Trp-P-1	19,000	400
Trp-P-2	39,000	1,700
Glu-P-1	49,000	3,200
Glu-P-2	1,900	1,200
Phe-P-1	41	23
AαC	300	20
MeAαC	200	120

Carcinogenicity

Ohgaki et al. (1986) and Ito et al. (1991) identified nine mutagenic HCAs (PhIP, IQ, MeIQ, Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2, MeAαC, and AαC) that were carcinogenic to mice and rats, inducing formation of colon and mammary carcinomas. So far, all mutagenic HCAs other than DiMeIQx were shown carcinogenic in long-term animal trials (Schut & Snyderwine, 1999). Table 3 summarizes the carcinogenicity of HCAs in a wide range of organs/tissues in CDF1 mice and F344 rats of both sexes (Sugimura et al., 2004). Feeding 300 mg of IQ per kg b/w over 55 to 96 weeks caused tumors in most organs of rats and mice. MeIQ, MeIQx, and PhIP also exhibited similar multi-potent carcinogenic effects on tumors. For non-polar HCAs, Glu-P-1, Glu-P-2, AαC, MeAαC, Trp-P-1, and Trp-P-2 were more likely to induce tumors in liver, blood vessels, intestine, Zymbal gland, and clitoral gland when given to mice and rats at extremely high dosages. The liver appears to be the most susceptible organ, followed by the clitoral gland, mammary gland, skin, and lung, which may correlate to the different locations and expressions of HCA metabolic activation enzymes (i.e., P450 and NAT). The locations and expressions caused the differences in the formation of HCA-DNA adducts. For example, mammalian cells were more susceptible to IQ-induced mutations because CYP 1A2 and NAT2 are highly expressed. The literature shows no data available to prove that HCA-induced tumor mechanisms observed in animals also function in humans, but human mammary epithelial cells and lymphoblastoid cells have shown these reactions in vitro (Fan, Schut, & Snyderwine, 1995; Leong-Morgenthaler, Op Het Velt, Jaccaud, & Turesky, 1998). Moreover, Lightfoot, Coxhead, Cupid, Nicholson, and Garner (2000) detected PhIP-DNA adducts in the breast tissue of cancer patients who were given a single oral dose of [14C] PhIP, revealing that mutagenic PhIP could bind to human breast DNA. However, setting a specific threshold value for HCAs for humans is

difficult because the tested dosage of HCAs in animal trials is much higher than the quantity of HCAs consumed in food by humans.

Table 3. Carcinogenicity of HCAs in rats and mice (Sugimura et al., 2004).

HCAs	Species	Strain	Concentration in diet (ppm)	Target organs	Experimental periods (weeks)
IQ	Rats	F344	300	small and large intestine, Zymbal gland, liver, clitoral gland, mammary gland, skin	55-72
	Mice	CDF1	300	liver, forestomach, lung	96
MeIQ	Rats	F344	300	Large intestine, Zymbal gland, skin, oral cavity, mammary gland	40
	Mice	CDF1	400, 100	liver, forestomach, lung	91
MeIQx	Rats	F344	400	liver, zymbal gland, clitoral gland, skin	61
	Mice	CDF1	600	liver, lung	84
PhIP	Rats	F344	400	Large intestine, mammary gland	52
	Mice	CDF1	400	Lymphoid tissue	80
Trp-P-1	Rats	F344	150	Liver	52
	Mice	CDF1	200	Liver	89
Trp-P-2	Mice	CDF1	200	Liver	89
Glu-P-1	Rats	F344	500	Liver, small and large intestines, Zymbal gland, clitoral gland	64
	Mice	CDF1	500	Liver, blood vessels	57
Glu-P-2	Rats	F344	500	Liver, small and large intestines, Zymbal gland, clitoral gland	104
	Mice	CDF1	500	Liver, blood vessels	84
AαC	Mice	CDF1	800	Liver, blood vessels	104
MeA$\boldsymbol{\alpha}$C	Mice	CDF1	800	Liver, blood vessels	84

HCA metabolism

Like most carcinogenic substances, HCAs are pro-mutagens that possess no mutagenic properties in their native forms but only act mutagenically in organisms because metabolic enzymes convert HCAs into their active, DNA-binding metabolites, suggesting that metabolic activation is a critical part of making HCAs mutagenic and carcinogenic (Schut & Snyderwine, 1999; Tang et al., 2007). The metabolic activation pathway of HCAs has been demonstrated in many in vitro studies using liver microsomal from rats, mice, and rabbits (Gooderham et al., 2001; Turesky & Le Marchand, 2011). In general, HCAs begin to metabolize in phase I bioactivation where the cytochrome P450 (CYP) species oxidizes the exocyclic primary amino groups of HCAs, producing genotoxic N-hydroxy-HCA derivatives (e.g., N-hydroxy-PhIP) or forming a ring-hydroxylated product (e.g., 4'-hydroxy-PhIP). The CYP 1A1, 1A2, 3A4, 2C9, and 2A2 can all activate HCAs into N-hydroxy derivatives although CYP1A2 is the most efficient at activating HCAs metabolically (Gooderham et al., 2001). Subsequently, in phase II bioactivation, the primary oxidative metabolites are further catalyzed by phase II enzymes, N-acetyl-transferases (NAT), and generate a variety of N-acetoxy, N-sulfonyloxy, N-propyloxy, and N-phosphatyl ester derivatives like glucuronides (Kaderlik et al., 1994). Those reactive products then spontaneous convert to arylnitrenium ions ($R\text{-}NH^{+}$) under heterolytic cleavage and covalently bind to DNA or proteins, thus forming adducts (Chou, Lang, & Kadlubar, 1995; Minchin et al., 1992; Turesky & Vouros, 2004). Figure 6 shows an example of the routes of MeIQx metabolism (Turesky & Vouros, 2004). Because DNA damage cannot be repaired once changed by HCAs, mutation in genes controlling cell proliferation could be responsible for cancer (Felton et al., 2007; Nagaoka et al., 1992). Recently, HCA-DNA adducts have been detected in a variety of human tissues and organs including liver, breast, prostate, lung, and

kidney, suggesting that human tissues are vulnerable to an attack by these carcinogens (Cheng et al., 2006; Schut & Snyderwine, 1999).

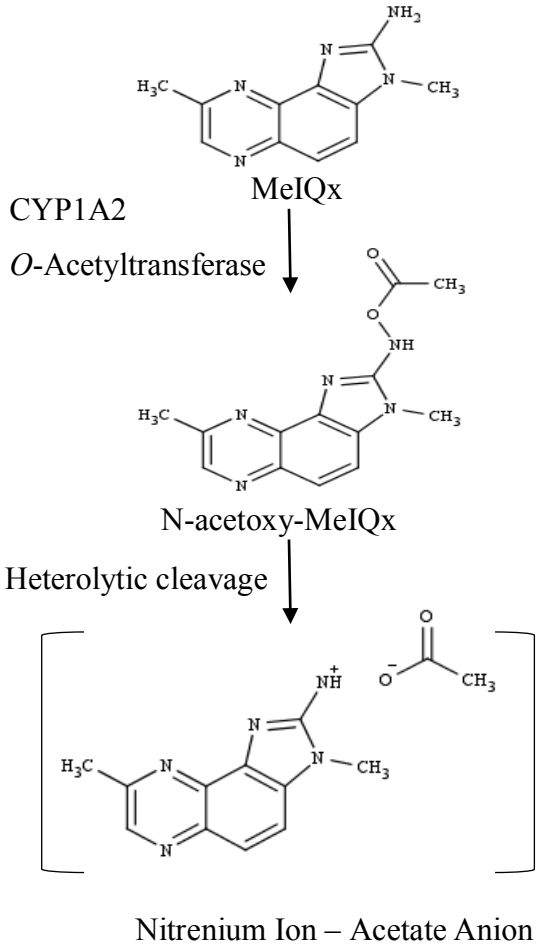


Figure 6. Metabolic activation pathway of MeIQx (Adapted from Turesky & Vouros, 2004).

DIETARY HETEROCYCLIC AMINES AND HUMAN DISEASES

In the past decade, many epidemiological studies have reported the possibility that high exposure to HCAs through the intake of cooked meat is part of the pathogenesis of human cancers, particularly showing that a high intake of well-done meat (particularly red meat) correlated to an increased risk of cancers in humans, particularly colorectal, breast, and pancreatic cancers (Cross et al., 2005; Felton et al., 2007; Zheng & Lee, 2009).

Colorectal cancer

Many epidemiological studies have reported that consuming cooked meat and/or high exposure to HCAs may be a risk factor for colorectal cancer. Nowell et al. (2002) and Butler et al. (2003) both reported a positive association between colon cancer with intake of well-done and pan-fried meats abundant in PhIP, MeIQx, and DiMeIQx. Sinha et al. (2005) reported similar results, finding an increased risk of colorectal adenoma, precursors of colorectal cancer, associated with eating well-done bacon and sausage with high amounts of MeIQx and PhIP. Chiavarini, Bertarelli, Minelli, and Fabiani (2017) revised several meta-analyses, confirming that specific HCAs, especially MeIQx and DiMeIQx, significantly affected colorectal cancer development. Furthermore, the cancer risk may be modified by certain genotypes and phenotypes. Kampman's (1999) early case study reported a 30% higher risk of colorectal cancer associated with food mutagens, which were later found to be modified by NAT2 genotypes. Le Marchand et al. (2001) provided further evidence that phenotypes of NAT2 and CYP1A2 modified the effects of HCAs on the development of colorectal cancer. Nowell et al. (2002) suggested that the CYP2A6 phenotype was a risk factor for colorectal cancer. Furthermore, the UGT1A7 genotype increased the risk of colon cancer in African Americans and White Americans because of the association between dietary HCAs, DiMeIQx, and exposure (Butler et al., 2003). Unlike NAT2 and CYP1A2, the UGT1A7 gene is responsible for encoding a certain enzyme involved in detoxifying HCAs, and therefore, individuals with less UGT1A7 activity were more susceptible to HCAs in the diet.

Breast cancer

Although the precise causes of breast cancer remain unknown, dietary factors like drinking alcohol and including fat in the diet may influence breast cancer development (Kotepui, 2016). Recently, consuming well-done meat with the subsequent exposure to food-derived HCAs have also been implicated in the pathogenesis of breast cancer (Snyderwine, 1994). Zheng et al. (1998) conducted a nested and case-controlled study among 41,836 Iowan women and observed a significant dose-response relationship between breast cancer with eating well-done and very well-done red meat. Sinha et al. (2000) observed a clear-response relationship between breast cancer and high exposure to PhIP in well-done meat, concluding that dietary PhIP intake may be associated with breast cancer development; however, MeIQx and DiMeIQx were not associated with the risk of breast cancer. Similarly, DeBruin, Martos, and Josephy (2001) detected PhIP in breast milk from healthy lactating Canadian women who regularly consumed dietary meats, indicating that dietary PhIP can be directly absorbed during in vivo human digestion, distributed, and eventually transferred across the ductal mammary epithelial cells into breast milk. No PhIP was detected in breast milk from vegetarian donors, and exposure of breast-fed infants to low levels of PhIP may be of concern. In addition, researchers indicated that the rapid and intermediate NAT2 genotypes, which catalyze the metabolic activation of HCAs, may be a risk factor for breast cancer in postmenopausal women who consume high levels of well-done red meat (Deitz et al., 2000). Moreover, research shows that genetic polymorphisms in NAT1, SULT1A1, GSTM, and GSTT1, which are responsible for bioactivation or detoxification of HCAs in human breast tissue, modify breast cancer development induced by exposure to HCAs in high intake of well-done meat (Zheng et al., 1999, 2001, 2002).

Pancreatic cancer

To date, only three epidemiological studies have examined the association between pancreatic cancer and HCA exposure and/or intake of well-done meat. Anderson et al. (2002) conducted a study with 193 cases of pancreatic cancer and 674 controls, reporting that increased intake of grilled and BBQ red meat in the population was positively associated with pancreatic cancer. No statistically significant association was, however, found with intake of fried/broiled red meat and any cooked white meat, suggesting that grilled red meat intake was a risk factor for pancreatic cancer. These findings were replicated by Li et al. (2007), in a hospital-based study with 626 cases and 530 controls; in this study, a high intake of well-done red meat (pork, bacon), grilled and pan-fried chicken, and high daily intake of DiMeIQx were all associated with an increased risk of pancreatic cancer. These data show that the dietary mutagen DiMeIQx can be a predictor for pancreatic cancer. In the NIH-American Association of Retired Persons (NIH-AARP) Diet and Health Study cohort, researchers reported a positive association of pancreatic cancer among men ate high amounts of meat, particularly red meat, as well as meat cooked at high temperatures (including grilled/BBQ and broiled meat); the same association has now been found among women (Stolzenberg-Solomon et al., 2007).

DIETARY HETEROCYCLIC AMINES IN FOODS

Cooked muscle-rich products are the major dietary source of total HCA intake by humans. Although PhIP and most non-polar HCAs also form in alcoholic drinks (14.1 ± 6.18 and 30.4 ± 16.4 ng/L of PhIP in beer and wine respectively) (Manabe, Suzuki, Wada, & Ueki, 1993), smoked provolone cheese (Naccari et al., 2009), cigarette smoke condensate (Liu, Taylor, Borgerding, Coleman, & Bombick, 2013), and environmental occurrences like cooking fumes,

polluted air, rain, and river water (Kataoka, 1997), their concentrations are much lower. Table 4 summarizes the estimated amounts of major mutagenic HCAs detected in cooked meat and fish products (Alaejos & Afonso, 2011; Knize et al., 1994; Skog et al., 1992; Smith, Ameri, & Gadgil, 2008). Pan-frying produces the most HCAs, followed by grilling/BBQ, and broiling, all reported to produce many more HCAs than oven-roasting or baking because HCAs form more often at high temperatures. Moreover, far more HCAs form in cooked meat than cooked fish, and chicken seems to generate more PhIP (up to 480 ng/g) and MeIQx during cooking than beef and pork. In addition, cooked fish, lamb, and turkey products also contain very low or undetectable amounts of HCAs (Alaejos & Afonso, 2011; Knize et al., 1994). In addition, how much of the HCAs forms in cooked meats can vary by more than 10 to 100-fold, with PhIP the most abundant in almost all cooked foods, with levels ranging from not detected (nd) to as high as 480 ng/g (Knize et al., 1994). MeIQx and 4,8-DiMeIQx are also recognized as principal HCAs forming in cooked products with a concentration ranging from nd to 18.3 ng/g in MeIQx and nd to 29 ng/g in 4,8-DiMeIQx. Other HCAs, such as IQ, MeIQ, IQx, Trp-P-1, Trp-P-2, AαC, and MeAαC, usually occur at a relatively low concentrations ranging from nd to 10.5 ng/g (Knize et al., 1994). In some studies, the non-mutagenic β-carbolines including harman and norharman were also found in cooked meat products up to 30 ng/g (Gross & Grüter, 1992; Smith et al., 2008).

Meat drippings and pan residues after frying different meat products also contain substantial amounts of HCAs and are another dietary source of HCAs. Gross and Grüter (1992) reported that high amounts of HCAs formed in bacon fat drippings and pan scrapings from grilled meat and fish, with PhIP, MeIQx, and DiMeIQx significantly higher (10 to 100 times) than what was found in the corresponding meat products. In addition, gravies made from fat

drippings and grill residues/scrapings also contain comparable or more HCAs than in fried meat products (Janoszka, Błaszczyk, Damasiewicz-Bodzek, & Sajewicz, 2009; Johansson & Jägerstad, 1996; Sinha & Rothman, 1997).

Table 4. Estimated HCA concentration in cooked meat and fish products by muscle type and cooking method (Adapted from Alaejos & Afonso, 2011; Knize et al., 1994; Skog et al., 1992).

		HCA Concentration (ng/g)					
Muscle Type	**Cooking Method**	**PhIP**	**MeIQx**	**DiMeIQx**	**IQ**	**MeIQ**	**IQx**
			Beef				
Beef patty	Pan-fried	nd-33.1	nd-4.8	nd-3.0	nd	nd	nd
	Grilled/BBQ	nd-16.27	nd-5.41	nd-2.35	nd	nd	nd
	Broiled	nd-18.40	nd-1.80	nd-0.11	nd	nd	nd
Beef steak	Pan-fried	nd-33.8	nd-6.50	nd-2.06	0.28	nd	nd
	Grilled	nd-6.99	nd-2.87	nd-1.27	nd-10	nd	nd
Hamburger	Pan-fried	nd-4.9	nd-18.3	nd-29.5	nd-1.5	nd-12.6	nd
	Charbroiled	1.8-290	nd	nd-0.3	nd	nd	nd
			Chicken				
Whole chicken	Fried	8.7-48.55	0.58-2.34	0.78-3.61	0.11	nd	0.07
	Barbecued	78.52-304.71	1.98-7.70	1.4-5.52	0.64	nd	nd
	Oven-broiled	5.6-71.96	0.13-2.81	0.11-1.98	0.07	nd	nd
Chicken breast without skin	Fried	1.7-72	nd	nd-2.3	nd	nd	nd-2.3
	Grilled/BBQ	27-480	nd-0.34	nd-0.28	nd-0.1	nd	nd
	Oven-broiled	6-150	nd	nd	nd	nd	nd
			Pork				
Pork patty	Pan-fried	nd-13.40	nd-5.43	nd-3.30	nd	nd	nd
	Roasted	0.5-2.3	nd	nd	nd	nd	nd
Bacon	Pan-Fried	nd-36.4	nd-5.9	nd-4.51	nd-10.5	nd-2.8	nd-3.11
	Oven-broiled	nd-15.91	nd-2.6	nd-0.53	nd-0.03	nd	nd-0.68
Sausage	Fried	nd-5.83	nd-5.07	nd-0.72	nd-0.07	nd	nd-0.72
	Grilled/BBQ	nd-2.35	nd	nd-2.5	nd-5.1	nd	nd-1.5
Pork chop	Fried	nd-3.27	nd-3.2	nd-7.5	nd-1.1	nd-1.8	nd-0.19
			Fish				
	Fried	nd-17	nd-6.44	nd-3.0	nd	nd	nd
	Grilled/BBQ	nd-50.3	nd-4.00	nd-2.0	nd	nd	nd
	Baked	4.31-5.67	1.27-2.95	0.29-1.66	nd	nd	nd-0.85

HUMAN EXPOSURE TO DIETARY HETEROCYCLIC AMINES

Assessing human exposure to HCAs through consuming cooked foods requires taking into account meat types, frequency and amount of meat consumption, cooking methods, and degree of doneness, which have been as critical factors in many epidemiological studies (Bogen & Keating, 2001). Ushiyama et al. (1991) and Wakabayashi et al. (1993) reported that the estimated total amount of HCAs in the U.S. diet was 9 ng/kg/day, with the average daily intake of PhIP at 100-13,800 ng/person and MeIQx at 200-2,600 μg/person, with PhIP accounting for almost two-thirds of the total intake of HCAs from dietary sources (Bogen & Keating, 2001). Although beef with 40.4 g/day and poultry with 38 g/day are the most frequently consumed meats in the U.S., followed by pork (20.3 g/day) and seafood (11.8 g/day), exposure of humans to dietary HCA was not significantly affected by intrinsic differences in the types of meat (Pouzou, Costard, & Zagmutt, 2018). Pouzou et al. (2018) used meta-regression methods to analyze many of these epidemiological studies, concluding that cooking method, not intrinsic differences among meat species, drove exposure to dietary PhIP and MeIQx in the U.S. population. In the U.S., pan-frying, the most common cooking method, contributed 367.7 ng/day to the total amount of PhIP and MeIQx in the diet, followed by baking (72.4 ng/day), barbecuing (60.9 ng/day), broiling (33.5 ng/day), and roasting (12.8 ng/day). Other cooking methods contributed less than 10 ng/day (Table 5). Although much uncertainty remains in quantifying dietary exposure to HCAs, the cooking method is a significant predictor of the amount of dietary HCAs humans get from food sources. Thus, consumers interested in reducing their daily exposure to HCAs should consider their methods of preparing and cooking meat, not the type of meat itself (Pouzou, Costard, & Zagmutt, 2018; Kerstin Skog, Eneroth, & Svanberg, 2003).

Table 5. Total exposure per day (ng/day) to PhIP, MeIQx and the total amount of PhIP + MeIQx produced from each cooking method (Pouzou, Costard, & Zagmutt, 2018).

	Exposure per day (ng/day)		
Cooking Methods	PhIP	MeIQx	Total PhIP + MeIQx
Fried	317	50.7	367.7
Baked	49.2	23.2	72.4
Barbecued	54.6	6.3	60.9
Broiled	28.8	4.7	33.5
Roasted	9.6	3.2	12.8
Smoked	8.0	1.1	9.1
Deep Fried	2.9	0.6	3.5
Boiled	1.8	1.0	2.8
Microwaved	0.8	0.7	1.5
Dried	0.9	0.4	1.3
Sum	473.6	91.8	565.4

BIOAVAILABILITY OF DIETARY HETEROCYCLIC AMINES IN HUMANS

HCAs are usually present in cooked meats at a low concentration, so human exposure is chronic with HCAs consumed daily over a lifetime. Therefore, understanding how HCAs are absorbed in human subjects is critical, as is how HCAs can be eliminated in human food. Gooderham et al. (1989) measured how much MeIQx was excreted in both mice and humans after they had ingested fried beef, reporting that about 1.8-4.9% of ingested MeIQx remained unmetabolized in human urine; in mice, about 20% was extracted from the urine. Lynch et al. (1992) ran a study where 10 healthy male volunteers ate fried beef patties containing known quantities of MeIQx, DiMeIQx, and PhIP on four separate occasions over 14 months. They found that most ingested HCAs were absorbed within the first 8 h, and only 2.1% of ingested

MeIQx and 1.1% of ingested PhIP were recovered unchanged in the urine within 24 h of the test meal. The native form of DiMeIQx was barely detectable. Reistad et al. (1997) reported similar results, with 1-6% of ingested MeIQx and 0.5-2% of ingested PhIP excreted in the urine in unchanged forms within 24 h of the test meal. Furthermore, Krul et al. (2000) developed a gastrointestinal tract chemical model that mimicked human digestion and found that 50% of ingested HCAs, including PhIP, IQ, MeIQ, and MeIQx, were removed from an in vitro human GI model within 2 h, and after 6 h, 95% of ingested HCAs were eliminated, with most recovered (94%) in jejunal and ileal dialysates. Recently, Kim and Hur (2018) also used chemical model systems to find that almost 52% of HCA intake from pork patties were reduced after stomach digestion and 90% were reduced after large intestine digestion, suggesting that the ingested HCAs degraded in the GI tract and/or by enterobacteria like Lactobacillus sakei, but HCAs were also metabolized by digestive enzymes to form active mutagenic metabolites in stomach, small intestine, and large intestine.

REGULATIONS AND GUIDELINES

In the U.S., no regulations and guidelines have been established to prevent HCA exposure from cooked and commercially processed meat and fish products. However, the International Agency for Research on Cancer (1993) classifies IQ as a probable human carcinogen (Group 2A), and eight HCAs (MeIQ, MeIQx, PhIP, AáC, MeAαC, Trp-P-1, Trp-P-2, and Glu-P-2) as possible human carcinogens (Group 2B). Recently, the National Toxicology Program (NTP) listed four individual HCAs (IQ, MeIQ, MeIQx, and PhIP) in their Report on Carcinogens as reasonably anticipated to be a human carcinogen (NTP 1999 & 2002).

REDUCING FORMATION OF DIETARY HETEROCYCLIC AMINES

Generally, methods to mitigate dietary HCAs in foods fall into two different approaches: modifying cooking methods and adding HCA inhibitors (i.e., antioxidants).

Modifying cooking methods

Pre-cooking treatments like microwaving and marinating can minimize the HCAs that form in foods. Felton, Fultz, Dolbeare, and Knize (1994) reported that pre-treating beef patties in a microwave oven produced significantly fewer HCAs (IQ, DiMeIQx, and PhIP) after they were than fried, up to 3 to 9 times fewer HCAs than untreated beef patties. Jinap et al. (2013) reported similar results for grilled chicken products pre-treated in a microwave oven. The reduction mechanism may be due to HCA precursors (e.g., creatine, sugars, and free amino acids), water, and fat leaching out during the process of microwave pretreatment. In addition, marinating meat, which is a pre-cooking treatment to enhance flavor and improve tenderness/juiciness of meat products, may greatly reduce HCAs in chicken and beef, but results have not been very consistent. For example, Salmon, Knize, and Felton (1997) found that marinating chicken breast with a mixture of brown sugar, olive oil, garlic, mustard, cider, lemon juice, and salt before grilling reduced PhIP formation by 92-99%, although MeIQx showed a 10-fold increase. In another study, barbecued beef steak marinated with teriyaki sauce overnight showed reductions of 67% in PhIP and 60% in MeIQx than unmarinated meats (Nerurkar, Marchand, & Cooney, 2009).

Adding antioxidants

Antioxidants are well known as free-radical scavengers, and their preventive effects on the lipid peroxidation process have been reported in many studies (Balasaheb Nimse & Pal,

2015). In HCA chemical model systems, a variety of synthetic and natural antioxidants have been investigated for effects on HCA formation (Kikugawa, 1999; Pearson et al., 1992; Vitaglione & Fogliano, 2004). Synthetic antioxidants like butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), tert-butylhydroquinone (TBHQ), 1-O-hexyl-2,3,5,-trimethylhydroquinone (HTHQ), and tert-butylhydroquinone (TBHQ) are HCA inhibitors in real meat products as well as model systems (Vitaglione & Fogliano, 2004). Barnes et al. (1983) found that adding 50 mmol of BHA to 100 g beef patties prevented the formation of IQ by 40% during frying. PG and BHA, depending on dosage, prevent IQx-type HCA production in a model system (Kato, Harashima, Moriya, Kikugawa, & Hiramoto, 1996). PhIP formation was reduced by 62.9-99.2% in a model system in the presence of 1,000 ppm of BHA (Moon & Shin, 2013). On the other hand, Pearson et al. (1992) observed that adding BHT appeared to increase mutagenic activity of HCAs, depending on concentration. MeIQx formation was enhanced by TBHQ included in meat at 100 ppm (Johansson & Jägerstad, 1996). Even in real meat systems, Ahn and Grün (2006) noted that using a mixture of BHT and BHA at 0.02% (w/w) significantly inhibited MeIQx formation by 57% and PhIP formation 22% in pan-fried beef patties but had no significant effect on formation of IQ, MeIQ, and DiMeIQx. In recent years, because of increasing concerns about the safety of synthetic antioxidants like BHT and BHA, the most commonly used synthetic antioxidants, which have cytotoxic and carcinogenic properties, using natural antioxidants has attracted more interest among researchers (Bailey, 2005; Saito, Sakagami, & Fujisawa, 2003).

A variety of fruits, vegetables, and Asian/Western spices/herbs, as well as their extracts have been extensively investigated as inhibitors of HCA formation. For example, Oz (2011), Puangsombat et al. (2012), and Zeng et al. (2014) reported that adding turmeric, finger root,

galangal, black pepper, and prickly ash peel to raw beef patties at a level of 1% (w/w) reduced HCAs formation by 29.2% to 100% for pan frying. Adding ethanol extracts of rosemary, oregano, cumin, basil, savory, pomegranate seed, grape seed, apple, elderberry, pineapple, and apple peel at concentrations of 1% (w/w) also reduced HCAs in beef meatballs, chicken meatballs, and beef patties, and suppressed the formation PhIP, MeIQx, DiMeIQx, and IQ particularly during cooking (Cheng et al., 2007; Damašius, Venskutonis, Ferracane, & Fogliano, 2011; Persson, Oroszvári, Tornberg, Sjöholm, & Skog, 2008; Sabally, Sleno, Jauffrit, Iskandar, & Kubow, 2016; Smith et al., 2008). In addition, Cheng et al. (2007) demonstrated that active compounds like proanthocyanidins, phloridzin, and chlorogenic acids were responsible for the inhibitory properties of grape seed extracts. Oguri, Suda, Totsuka, Sugimura, and Wakabayashi (1998) and Weisburger, Dolan, and Pittman (1998), tested the inhibitory activity of pure antioxidants in HCA model systems and reported that compared to the control, up to 75% of PhIP and MeIQx formation was suppressed using lycopene, daidzein, genistein, epigallocatechin gallate (EGCG), quercetin, luteolin, and caffeic acid, which are mostly found in tomatoes, soy products, and black and green tea.

Inhibitory mechanism of antioxidants and HCAs

The mechanism by which antioxidants inhibit HCA formation has been linked to free-radical scavenging through free radical pathways (Maillard free-radical intermediates and pyrazine/pyridine radicals) during the formation of HCAs. However, many recent studies have proposed a non-antioxidant inhibitory mechanism because antioxidant capacity by itself insufficiently accounted for all inhibitory effects (Cheng et al., 2007; Oguri et a., 1998). Antioxidants could react with the reactive carbonyl species (RSC) generated during Strecker

degradation, due to their carbonyl scavenging/trapping functions, thus changing the formation pathways of HCAs (Arvidsson et al., 1997; Kikugawa, 1999; Pearson et al., 1992). Cheng et al. (2008) clearly demonstrated that naringenin could suppress PhIP formation by trapping its key intermediate, phenylacetaldehyde and converting it into different adducts (Figure 7). Two postulated adducts between naringenin and phenylacetaldehyde were identified and characterized by one- and two-dimensional NMR, revealing that C-6 and C-8 are active sites for naringenin in adduct formation.

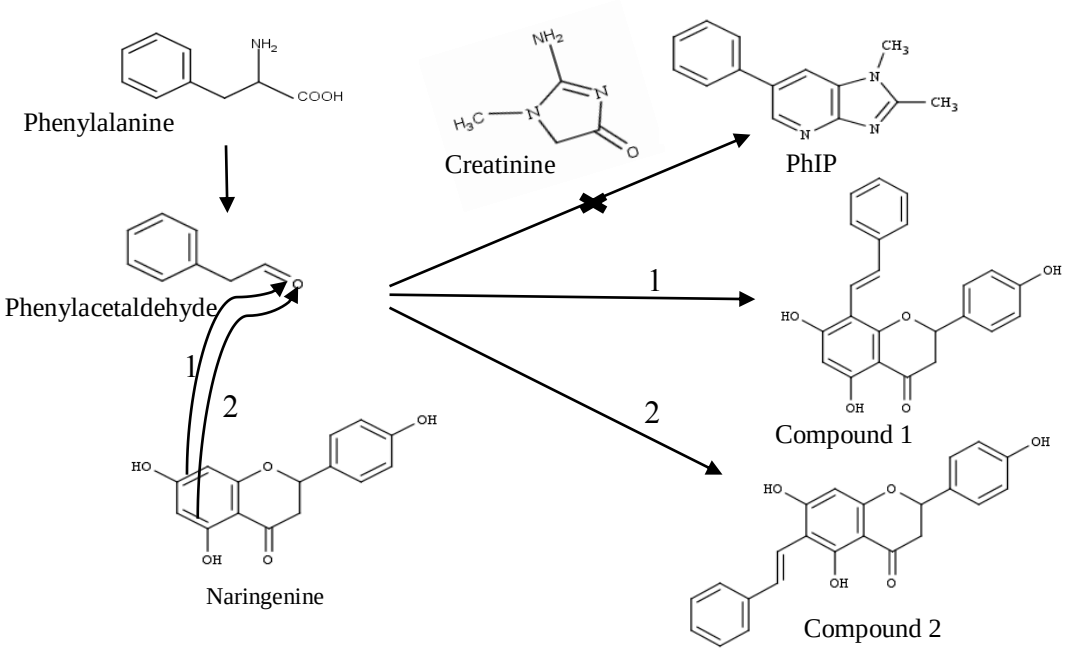


Figure 7. Postulated inhibitory pathways of PhIP with naringenin (Adapted from Cheng et al., 2008).

A subsequent study by Zhu, Zhang, Wang, Chen, and Zheng (2016) confirmed that scavenging/trapping carbonyl compounds was a key mechanism by which flavonoids inhibited PhIP formation. Seven more dietary flavonoids (apigenin, luteolin, kaempferol, genistein, phlorizin, EGCG, and quercetin) also exhibited the ability to trap phenylacetaldehyde in both model and meat systems, and their adducts were characterized by LC-MS. In addition to

aldehyde adducts, Totlani and Peterson (2006) confirmed that epicatechin (EC) formed covalent bonds with dicarbonyl and hydroxycarbonyl sugar fragments (e.g., glyoxal, glycolaldehyde, methylglyoxal, glyceraldehyde) in the Maillard reaction; the structures of EC-methylglyoxal conjugates were identified using NMR. Moreover, in another study, catechin and EGCG trapped reactive imine intermediates (e.g., glyoxal imine and glycoaldehdye imine) via their A-rings, thus influencing the formation of pyrazinium radicals (Bin, Peterson, & Elias, 2012).

Adding other natural ingredients

Adding vitamins, monosaccharides, oligosaccharides, and polysaccharides may also suppress HCA formation. Wong, Cheng, and Wang (2012) found that three water-soluble vitamins (pyridoxiamine, niacin, and ascorbic acid) exhibited the significant inhibition (> 40%) on PhIP and MeIQx formation in both model systems and pan-fried beef patties. Shin et al. (2003) reported that adding fructooligosaccharide, galactooligosaccharde, isomaltooligosacchardie, and inulin at a level of 1.5% (w/w) in fried beef patties could reduce total HCA formation by up to 54%. Directly adding low and medium molecular weight chitosan at a level of 1% (w/w) also reduced up to 100% of the total HCA content in pan-fried beef chops (Oz & Cakmak, 2016). Furthermore, modulating the HCA precursor profile by adding free amino acids also reduced the production of HCAs themselves. Jones and Weisburger (1988a), for example, reported that blending tryptophan into a commercial beef sauce significantly inhibited mutagenic IQ- and IQx- type HCA formation in fried beef patties. In another study, they used a mixture of proline and tryptophan to enhance inhibition of IQx-type HCAs in model systems (Jones & Weisburger, 1988b). However, how adding free amino acids inhibits HCA formation remains unclear although the results of previous studies make exploring the effects of

individual amino acids, especially essential amino acids, an interesting potential study of the formation of HCAs in both model and real meat systems.

METHODS FOR DETECTING AND QUANTIFYING HETEROCYCLIC AMINES

Extraction and clean-up

The complexity of food matrices and the diverse chemical properties of HCAs at the ppb level make analyzing foods for HCAs very difficult and challenging. Traditional extraction and purification for analysis uses a protocol developed by Gross and Grüter (1992), and even now, many researchers still use the Gross method or some modification of the method (Messner & Murkovic, 2004) for standard analysis (Abdulkarim & Smith, 1998; Smith et al., 2008). Liquid-liquid extraction (LLE) combined with solid-phase extraction (SPE) is typically used to clean up sample matrices and for HCA extraction. In general, a meat sample is first homogenized with an alkaline solution (e.g., 1 M NaOH), and after mixing and centrifugation, three-step SPE cartridges using Extrelut diatomaceous earth refill material, propyl sulfonic acid (PRS), and end-capped octadecylsilane (C18) further remove substances that interfere with detecting HCAs. The method also pre-concentrates specific HCA species into diverse fractions without compromising extraction efficiency and detection sensitivity. In some publications, SPE procedures were optimized using a one-step SPE with an Oasis MCX LP cartridge; its mixed mode functions (reversed-phase and cation exchange) proved more selective and concentrated all HCAs into one fraction (Turesky et al., 2005). Other SPE sorbents, like benzene sulfonic acid silica (SCX), LiChrolut EN, and Oasis HLB, were also introduced into the Gross procedures for HCA analysis of meat samples and body fluids (e.g., urine) (Cha, Kim, Jeong, & Na, 2010; Cha et al., 2010; Lee et al., 2015; Shin, Na, Chung, Gorinstein, & Ahn, 2014). However, the Gross method has

several major disadvantages: low extraction efficiency, intensive labor, and use of toxic organic solvents (i.e., dichloromethane and ethyl acetate). Typical recovery of HCAs from meat products ranged from 50% to 80%, with PhIP often exhibiting a relatively lower recovery rate than other HCAs, even using reliable methods (Puangsombat et al., 2012). Martín-Calero, Ayala, González, & Afonso (2007) did develop a fast HCA extraction method using a single-step solid phase microextraction (SPME) coupled with HPLC-FLD. SPME fiber with carbowax-templated resin (CW-TPR) could effectively extract the less polar HCAs (e.g., PhIP, Trp-P-1, and Trp-P-2) from high-fat meat extracts with recovery values > 60% and limits of detection (LODs) ranging from 0.4 to 1.1 ppb. Although this SPME-HPLC method can greatly simplify the traditional SPE, using fewer organic solvents and less extraction time, preparation includes multiple LLE followed by freezing to remove fat before SPME extraction.

Detection and quantification

The analytical techniques for detecting and quantifying HCAs in both model and meat systems mainly use chromatography spectroscopies. A well-known analytical method, high performance liquid chromatography (HPLC) coupled with different detection systems like UV-Vis, diode array detector (DAD) (Martín-Calero et al., 2007), fluorescence detector (FLD) (Ristic, Cichna, & Sontag, 2004), electrochemical detector (ED) (Van Dyck, Rollmann, & De Meester, 1995), and mass spectrometer (MS) (Barceló-Barrachina et al., 2006; Turesky et al., 2005) have been widely used for HCA analysis. Both polar and non-polar HCAs can be separated on HPLC with a series of analytical columns, with the reversed-phase column most commonly used because of its high separation selectivity and sensitivity. These include TSK Gel ODS-80TS, Zorbax SB-C8, “core-shell” Kintex EVO C18, and Luna C18 (Cha et al., 2010;

Linghu, Karim, & Smith, 2017; Puangsombat et al., 2012; Turesky et al., 2005); the UPLC column most commonly used is Acquity BEH C18 (Barceló-Barrachina et al., 2006). The normal-phase column, Asscentis Express PR-Amine, can provide good chromatographic separation (Cha et al., 2010). Identifying polar HCAs can then be achieved using a DAD detector based on characteristic UV spectra at a wavelength of 254 to 263 nm. PhIP and non-polar HCAs are often measured on FLD because of their fluorescence characteristics; FLD has almost 100-400 times higher sensitivity than DAD (Cheng et al., 2006; Gross & Grüter, 1992; Pais & Knize, 2000). Although these detection methods are satisfactory for analyzing HCAs from samples prepared in model systems, because of their low sensitivity, detecting and identifying low abundance HCAs from complex meat matrices is still a challenge. Moreover, large amounts of co-extracted substances present in the meat matrix may cause issues with validation efficiency when using HPLC-FLD/DAD.

Because of these limitations, LC-MS and LC-MS/MS are preferred as more efficient techniques for identifying and quantifying a wide range of HCAs in complex meat matrices. Atmospheric pressure chemical ionization (APCI) and electrospray (ESI) are the most common ionization techniques for coupling LC to MS or tandem MC. As soft ionization techniques, APCI and ESI are appropriate for analyzing ionic compounds with low molecular weights, low to high polarity, and less volatility (Guy, Gremaud, Richoz, & Turesky, 2000; Pais & Knize, 2000). Applying single quadrupole or triple quadrupole (Cooper, Jankhaikhot, & Cuskelly, 2014; Guy, Gremaud, Richoz, & Turesky, 2000; Ni, McNaughton, LeMaster, Sinha, & Turesky, 2008); quadrupole-time of flight (Q-TOF) (Ouyang, Li, Tang, Jin, & Li, 2015); or ion-trap (Toribio, Moyano, Puignou, & Galceran, 2002) in addition to LC-ESI or LC-APCI provide a high degree of sensitivity, selectivity, reduced matrix effects, and better resolution in quantitative analysis of

HCAs (Guy et al., 2000; Toribio et al., 2002). Barceló-Barrachina et al. (2004) evaluated the performance of LC-ESI with different MS analyzers (ion trap, single quadrupole, and triple quadrupole) for HCA analysis and reported that, compared to ion trap and single quadrupole, triple quadrupole, operating in positive ion mode, could detect HCAs at much lower limits of detection (LODs), from 0.02 to 0.1 ppb in meat extracts. Thus, a triple quadrupole MS may be more suitable for detecting low concentrations of HCAs at ppb or even less. Confirmation and quantitation of HCAs require full scan, single ion monitoring (SIM), selected reaction monitoring (SRM), or multiple reaction monitoring (MRM) mode to acquire data (Barceló-Barrachina et al., 2004; Cha et al., 2010). When MS is operated in SIM, many protonated molecular ions $[M+H]^+$ were produced from each HCA and formed as the base peak in mass spectra. Therefore, HCAs can be quantified using their $[M+H]^+$ (Iwasaki et al., 2010). For samples with low concentration levels, SRM and MRM, used as acquisition modes in LC-MS/MS, can improve the sensitivity, selectivity, and reproducibility of HCA analysis. Once subjected to collision-induced dissociation, the molecular ion $[M+H]^+$ of each target HCA generates multiple fragment ions with different mass-to-charge ratios (m/z); thus, ions with high intensity and specificity are selected to establish unique SRM and MRM mass transitions to quantitate and confirm trace-level HCAs from a complex matrix. Guy et al. (2000), for example, demonstrated that LC-APCI-MS/MS in SRM mode could simultaneously measure five mutagenic HCAs with LODs ranging from 0.015 to 0.045 ppb. Cha et al. (2010) reported that LODs of eight different HCAs measured in SIM ranged from 0.16 to 0.31 ppb and in SRM mode ranged from 0.11 to 0.65 ppb, suggesting that SIM was more sensitive than SRM in detecting HCAs although SRM provided a higher degree of selectivity. Moreover, product ion full-scan mode can be used with SRM or MRM for to confirm the identities of multiple HCAs in meat

products (Turesky et al., 2005). In addition, LC-MS and LC-MS/MS produce more accurate quantitative results than HPLC-FLD/DAD in analyzing for HCAs. Turesky et al. (2005) and Ni et al. (2008) demonstrated that using as surrogates stable and isotopically labelled internal standards like 3-[2H3C]-8-MeIQx, 1-[2H3C]-PhIP, compensated accurately in recovering analytes during extraction procedures and giving the most accurate and reproducible quantitative results.

Coupling GC with MS also provides satisfactory separation and detection of HCAs. Because HCAs are non-volatile and polar basic compounds, chemical derivatization treatments (e.g., alkylation, acylation, and silylation) are often required for GC analysis to reduce polarity and improve selectivity, sensitivity, and volatility (Cha, Kim, Jeong, & Na, 2010). Acylation with 3,5-bisTFMBB and DMF-DMA, and silylation with tert-butyldimethylsiyl (TBDMS) are the more popular techniques, producing HCA derivatives with good volatile properties and allowing simultaneous analysis of 10 to 12 different HCAs in one run on GC-EI-MS (Gross & Grüter, 1992; Murray et al., 1993; Richling, Kleinschnitz, & Schreier, 1999; Tsuchiya et al., 1996). However, GC-MS is rarely used routinely to analyze HCAs because the chemical derivatization is limited to certain types of HCAs and problematic for studying PhIP and some non-polar HCAs like Trp-P-1, Trp-P-2, harman, and norharman (Casal, Mendes, Fernandes, Oliveira, & Ferreira, 2004).

Quick, Easy, Cheap, Effective, Rugged, and Safe Method (QuEChERS)

QuEChERS was originally developed by Anastassiades et al. (2003) and featured as a sample preparation method for multi-class pesticide analysis in fruits, vegetables, milk, eggs, avocado, olive oil, flaxseeds, peanuts, and soybeans (Castillo, González, & Miralles, 2011;

Lehotay et al., 2010; Park et al., 2012; Pizzutti, de Kok, Hiemstra, Wickert, & Prestes, 2009; Rejczak & Tuzimski, 2015). QuEChERS is flexible. Based on the types of salt used, it can be generally classified into 3 versions: original unbuffered ($MgSO_4$, NaCl), acetate-buffering ($MgSO_4$, NaAcetate) and citrate-buffering ($MgSO_4$, NaCl, NaCitrate, disodium citrate sesquihydrate). The acetate-buffering QuEChERS method was nominated as AOAC Official Method 2007.01 (Lehotay, Mastovská, & Lightfield, 2005), and the citrate-buffering QuEChERS method was nominated as European Standard EN 15662 (2007). In general, the QuEChERS protocol has two steps: initial salting-out extraction/partitioning by adding anhydrous salts and a dispersive solid-phase extraction (dSPE) (Anastassiades et al., 2003). Salts are often used in the initial extraction step to separate water from organic extraction solvents (i.e., acetone, acetonitrile, or methanol), leading to a high recovery of polar analytes. Subsequently, a mixture of porous sorbents like primary secondary amines (PSA), C18, zirconia-containing sorbents (e.g., Z-Sep, Z-Sep$^+$) and/or graphitized carbon black (GCB) are used in the dSPE clean-up step for removing lipids, organic acids, polar pigments, and sugars (which cause maximum interference with detection) from matrix. Many studies and research papers have compared and evaluated the performance of different QuEChERS methods for extraction efficiency, precision, LODs, and removing co-extracted interference in different types of foods (Cunha et al., 2007; He et al., 2017; Lehotay et al., 2010; Pouech et al., 2012; Rejczak & Tuzimski, 2015). For example, Castillo, González, and Miralles (2011) reported that using QuEChERS (PSA/C18 as dSPE sorbents, $MgSO_4$ as salt), the extraction recoveries of pesticides from poultry, ovine, and swine was 70% to 120%. Compared to the traditional cleanup method, meat extracts treated using QuEChERS had lower fat content and improved chromatogram cleanliness with reduced matrix co-extractives and background noise. Overall, the QuEChERS methodology is a highly

streamlined sample preparation method, and because of its flexibility, simplicity, efficiency, and low solvent consumption, using QuEChERS has expanded from pesticides to other trace-level contaminants in foods. For instance, modified QuEChERS methods have been successful in analyzing acrylamide (Mastovska & Lehotay, 2006), hormones (Pouech et al., 2012), veterinary drugs (Park et al., 2012), polycyclic aromatic hydrocarbons (João Ramalhosa, Paíga, Morais, Delerue-Matos, & Prior Pinto Oliveira, 2009), alkaloids (Krska, Stubbings, Macarthur, & Crews, 2008), and mycotoxin (Pizzutti, de Kok, Hiemstra, Wickert, & Prestes, 2009).

Furthermore, as far as we know, only a few studies have evaluated QuEChERS-based methods for HCA extraction in meat. Hsiao, Chen, and Kao (2017) conducted a full validation experiment for 20 HCAs in fried pork fiber using a modified QuEChERS method (PSA, C18, and MgSO4 as sorbents). Their preliminary results showed that QuEChERS combined with LC-MS/MS-SRM could overcome low LODs (0.003-0.05 ppb) and LOQs (0.01-0.05 ppb) with good extraction precision. The extraction recoveries of HCAs ranged from 59 to 117%, very comparable to the traditional Gross method using tandem SPE. However, They did find a significant matrix effect in QuEChERS treated meat samples because of co-eluting interfering substances, in particular, PhIP, IQ- and IQx- type HCAs, which exhibited the most ion suppression between -8.32% and -61.89%, suggesting that the current commercially available dSPE sorbents are still insufficient to remove large amounts of co-extracted lipids from high-fat sample matrices. They simply lack high selectivity. Although Zr-containing sorbents do provide improved matrix cleanup, certain HCAs appeared to attach to sorbents during extraction, resulting in low recovery of those HCAs (Hsiao et al., 2017; Rajski, Lozano, Uclés, Ferrer, & Fernández-Alba, 2013; Uclés et al., 2015).

QuEChERS Enhanced Matrix Removal –Lipid (EMR- Lipid)

Agilent Bond Elut QuEChERS EMR-Lipid is a promising tool providing maximum lipid removal in sample matrices without compromising extraction efficiency of target analytes in trace amounts (Parrilla Vázquez et al., 2016). EMR-Lipid uses nano-particle materials as dSPE sorbents that, once activated in water or buffer solution, enables developing a combination of size exclusion and hydrophobic interaction with lipid-like functional groups like compounds with bulky ring structures or branches (e.g., vitamin D, pesticides, PAH, and veterinary drugs). Therefore, EMR-Lipid may be the most suitable analytical method for isolating and separating most aliphatic groups containing analytes, especially in complex food matrices (Lucas & Zhao, 2015; Zhao & Lucas, 2015). Many application studies have reported that the EMR-Lipid protocol is as easy to use as QuEChERS, and using EMR-Lipid dSPE for extraction followed by EMR polish salts (NaCl/MgSO4) has been successful for analyzing of trace-level analytes in samples with high lipid content like vegetable oils (Parrilla Vázquez et al., 2016) and avocado (Zhao & Lucas, 2015) by LC-MS/MS or GC-MS/MS. Dias, Cutillas, Lozano, Pizzutti, and Fernández-Alba (2016) pointed out that EMR-Lipid dSPE provided better extraction of multiple pesticides in edible oils with lower RSD% values than dSPE with Z-Sep and PSA. In addition, Han, Matarrita, Sapozhnikova, and Lehotay (2016) reported that co-extractive removal efficiency in the EMR-Lipid protocol was 79% in kale and 83-89% in meats and avocado. Zhao and Lucas (2015) and Lucas and Zhao (2015) compared co-extractives by weight in avocado and bovine liver using different cleanup methods (Table 6). Of the cleanup methods shown in Table 6, EMR-Lipid cleanup could provide dramatically cleaner samples and significantly improved chromatographic background.

Table 6. Avocado and bovine liver co-extractives by weight (Lucas & Zhao, 2015; Zhao & Lucas, 2015).

	Pesticides from Avocado		Vet drugs from Bovine liver	
Cleanup	Co-extractives (mg, n=3)	% of matrix co-extractives removed by cleanup	Co-extractives (mg, n=3)	% of matrix co-extractives removed by cleanup
No further cleanup	14.7	--	12.1	--
C18/PSA Cleanup	9.5	35.4	7.8	35.5
Zirconia sorbent Cleanup	7	52.4	6	50.4
EMR-Lipid Cleanup	4.2	71.4	5.3	56.2

REFERENCES

Abdulkarim, B. G., & Smith, J. S. (1998). Heterocyclic Amines in Fresh and Processed Meat Products. *Journal of Agricultural and Food Chemistry*, *46*(11), 4680–4687.

Ahn, J., & Grün, I. U. (2005). Heterocyclic Amines: 1. Kinetics of Formation of Polar and Nonpolar Heterocyclic Amines as a Function of Time and Temperature. *Journal of Food Science*, *70*(2), C173–C179.

Ahn, J., & Grün, I. U. (2006). Heterocyclic Amines: 2. Inhibitory Effects of Natural Extracts on the Formation of Polar and Nonpolar Heterocyclic Amines in Cooked Beef. *Journal of Food Science*, *70*(4), C263–C268.

Alaejos, M. S., & Afonso, A. M. (2011). Factors That Affect the Content of Heterocyclic Aromatic Amines in Foods. *Comprehensive Reviews in Food Science and Food Safety*, *10*(2), 52–108.

Ames, B. N., Mccann, J., & Yamasaki, E. (1975). Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. *Mutation Research*, *31*(6),347-364.

Anastassiades, M., Lehotay, S. J., Štajnbaher, D., & Schenck, F. J. (2003). Fast and Easy Multiresidue Method Employing Acetonitrile Extraction/Partitioning and "Dispersive Solid-Phase Extraction" for the Determination of Pesticide Residues in Produce, 20.

Anderson, K. E., Sinha, R., Kulldorff, M., Gross, M., Lang, N. P., Barber, C., & Kadlubar, F. F. (2002). Meat intake and cooking techniques: associations with pancreatic cancer. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, *506–507*, 225–231.

Arvidsson, P., Boekel, M. A. J. S., Skog, K., & Jagerstad, M. (1997). Kinetics of Formation of Polar Heterocyclic Amines in a Meat Model System. *Journal of Food Science*, *62*(5), 911–916.

Bailey's Industrial Oil and Fat Products, 6th Edition. (2005). Retrieved May 15, 2018, from https://www.wiley.com/en-us/Bailey%27s+Industrial+Oil+and+Fat+Products%2C+6th+Edition-p-9780471384601

Balasaheb Nimse, S., & Pal, D. (2015). Free radicals, natural antioxidants, and their reaction mechanisms. *RSC Advances*, *5*(35), 27986–28006.

Barceló-Barrachina, E., Moyano, E., Galceran, M. T., Lliberia, J. L., Bagó, B., & Cortes, M. A. (2006). Ultra-performance liquid chromatography–tandem mass spectrometry for the analysis of heterocyclic amines in food. *Journal of Chromatography A*, *1125*(2), 195–203.

Barceló-Barrachina, E., Moyano, E., Puignou, L., & Galceran, M. T. (2004). Evaluation of different liquid chromatography-electrospray mass spectrometry systems for the analysis of heterocyclic amines. *Journal of Chromatography. A*, *1023*(1), 67–78.

Barnes, W. S., Maher, J. C., & Weisburger, J. H. (1983). High-pressure liquid chromatographic method for the analysis of 2-amino-3-methylimidazo[4,5-f]quinoline, a mutagen formed during the cooking of food. *Journal of Agricultural and Food Chemistry*, *31*(4), 883–886.

Bin, Q., Peterson, D. G., & Elias, R. J. (2012). Influence of Phenolic Compounds on the Mechanisms of Pyrazinium Radical Generation in the Maillard Reaction. *Journal of Agricultural and Food Chemistry*, *60*(21), 5482–5490.

Bogen, K. T., & Keating, G. A. (2001). U.S. dietary exposures to heterocyclic amines. *Journal of Exposure Analysis and Environmental Epidemiology*, *11*(3), 155–168.

Bordas, M., Moyano, E., Puignou, L., & Galceran, M. T. (2004). Formation and stability of heterocyclic amines in a meat flavour model system. *Journal of Chromatography B*, *802*(1), 11–17.

Butler, L. M., Sinha, R., Millikan, R. C., Martin, C. F., Newman, B., Gammon, M. D., & Sandler, R. S. (2003). Heterocyclic amines, meat intake, and association with colon cancer in a population-based study. *American Journal of Epidemiology*, *157*(5), 434–445.

Casal, S., Mendes, E., Fernandes, J. ., Oliveira, M. B. P. ., & Ferreira, M. (2004). Analysis of heterocyclic aromatic amines in foods by gas chromatography–mass spectrometry as their tert.-butyldimethylsilyl derivatives. *Journal of Chromatography A*, *1040*(1), 105–114.

Castillo, M., González, C., & Miralles, A. (2011). An evaluation method for determination of non-polar pesticide residues in animal fat samples by using dispersive solid-phase extraction clean-up and GC-MS. *Analytical and Bioanalytical Chemistry*, *400*(5), 1315–1328.

Cha, H.-J., Kim, N.-H., Jeong, E.-K., & Na, Y.-C. (2010). Analysis of Heterocyclic Amines in Human Urine Using Multiple Solid-Phase Extraction by Liquid Chromatography/Mass Spectrometry. *Bulletin of the Korean Chemical Society*, *31*(8), 2322–2328.

Chen, B. H., & Chiu, C. P. (1998). Analysis, Formation and Inhibition of Heterocyclic Amines in Foods:An Overview. *Journal of Food and Drug Analysis; Philadelphia*, *6*(4).

Cheng, K.-W., Chen, F., & Wang, M. (2006). Heterocyclic amines: Chemistry and health. *Molecular Nutrition & Food Research*, *50*(12), 1150–1170.

Cheng, K.-W., Wong, C. C., Cho, C. K., Chu, I. K., Sze, K. H., Lo, C., Wang, M. (2008). Trapping of Phenylacetaldehyde as a Key Mechanism Responsible for Naringenin's Inhibitory Activity in Mutagenic 2-Amino-1-methyl-6-phenylimidazo [4,5-b]Pyridine Formation. *Chemical Research in Toxicology*, *21*(10), 2026–2034.

Cheng, K.-W., Wu, Q., Zheng, Z. P., Peng, X., Simon, J. E., Chen, F., & Wang, M. (2007). Inhibitory Effect of Fruit Extracts on the Formation of Heterocyclic Amines. *Journal of Agricultural and Food Chemistry*, *55*(25), 10359–10365.

Chiavarini, M., Bertarelli, G., Minelli, L., & Fabiani, R. (2017). Dietary Intake of Meat Cooking-Related Mutagens (HCAs) and Risk of Colorectal Adenoma and Cancer: A Systematic Review and Meta-Analysis. *Nutrients*, *9*(5), 514.

Chou, H. C., Lang, N. P., & Kadlubar, F. F. (1995). Metabolic activation of N-hydroxy arylamines and N-hydroxy heterocyclic amines by human sulfotransferase(s). *Cancer Research*, *55*(3), 525–529.

Cooper, K. M., Jankhaikhot, N., & Cuskelly, G. (2014). Optimised extraction of heterocyclic aromatic amines from blood using hollow fibre membrane liquid-phase microextraction and triple quadrupole mass spectrometry. *Journal of Chromatography A*, *1358*, 20–28.

Cross, A. J., Peters, U., Kirsh, V. A., Andriole, G. L., Reding, D., Hayes, R. B., & Sinha, R. (2005). A prospective study of meat and meat mutagens and prostate cancer risk. *Cancer Research*, *65*(24), 11779–11784.

Cunha, S. C., Lehotay, S. J., Mastovska, K., Fernandes, J. O., Beatriz, M., & Oliveira, P. P. (2007). Evaluation of the QuEChERS sample preparation approach for the analysis of pesticide residues in olives. *Journal of Separation Science*, *30*(4), 620–632.

Damašius, J., Venskutonis, P. R., Ferracane, R., & Fogliano, V. (2011). Assessment of the influence of some spice extracts on the formation of heterocyclic amines in meat. *Food Chemistry*, *126*(1), 149–156.

DeBruin, L. S., Martos, P. A., & Josephy, P. D. (2001). Detection of PhIP (2-amino-1-methyl-6-phenylimidazo[4,5- *b*]pyridine) in the Milk of Healthy Women. *Chemical Research in Toxicology*, *14*(11), 1523–1528.

Deitz, A. C., Zheng, W., Leff, M. A., Gross, M., Wen, W. Q., Doll, M. A., & Hein, D. W. (2000). N-Acetyltransferase-2 genetic polymorphism, well-done meat intake, and breast

cancer risk among postmenopausal women. *Cancer Epidemiology, Biomarkers & Prevention: A Publication of the American Association for Cancer Research, Cosponsored by the American Society of Preventive Oncology*, *9*(9), 905–910.

Dennis, C., Karim, F., & Smith, J. S. (2015). Evaluation of Maillard Reaction Variables and Their Effect on Heterocyclic Amine Formation in Chemical Model Systems: Evaluation of Maillard reaction variables. *Journal of Food Science*, *80*(2), T472–T478.

Dias, J. V., Cutillas, V., Lozano, A., Pizzutti, I. R., & Fernández-Alba, A. R. (2016). Determination of pesticides in edible oils by liquid chromatography-tandem mass spectrometry employing new generation materials for dispersive solid phase extraction clean-up. *Journal of Chromatography A*, *1462*, 8–18.

Fan, L., Schut, H. A., & Snyderwine, E. G. (1995). Cytotoxicity, DNA adduct formation and DNA repair induced by 2-hydroxyamino-3-methylimidazo[4,5-f]quinoline and 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-b]pyridine in cultured human mammary epithelial cells. *Carcinogenesis*, *16*(4), 775–779.

Felton, J. S., Fultz, E., Dolbeare, F. A., & Knize, M. G. (1994). Effect of microwave pretreatment on heterocyclic aromatic amine mutagens/carcinogens in fried beef patties. *Food and Chemical Toxicology*, *32*(10), 897–903.

Felton, J. S., Knize, M. G., Shen, N. H., Andresen, B. D., Bjeldanes, L. F., & Hatch, F. T. (1986). Identification of the Mutagens in Cooked Beef. *Environmental Health Perspectives*, *67*, 17.

Felton, J. S., Knize, M. G., Wu, R. W., Colvin, M. E., Hatch, F. T., & Malfatti, M. A. (2007). Mutagenic potency of food-derived heterocyclic amines. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, *616*(1), 90–94.

Fretheim, K. (1983). Polycyclic aromatic hydrocarbons in grilled meat products—A review. *Food Chemistry*, *10*(2), 129–139.

Gibis, M. (2016). Heterocyclic Aromatic Amines in Cooked Meat Products: Causes, Formation, Occurrence, and Risk Assessment. *Comprehensive Reviews In Food Science and Food Safety*, *15*(2), 269–302.

Gooderham, N. J., Murray, S., Lynch, A. M., Yadollahi-Farsani, M., Zhao, K., Boobis, A. R., & Davies, D. S. (2001). Food-derived heterocyclic amine mutagens: variable metabolism and significance to humans. *Drug Metabolism and Disposition: The Biological Fate of Chemicals*, *29*(4 Pt 2), 529–534.

Gross, G. A., & Grüter, A. (1992). Quantitation of mutagenic/carcinogenic heterocyclic aromatic amines in food products. *Journal of Chromatography*, *592*(1–2), 271–278.

Guy, P. A., Gremaud, E., Richoz, J., & Turesky, R. J. (2000). Quantitative analysis of mutagenic heterocyclic aromatic amines in cooked meat using liquid chromatography–atmospheric pressure chemical ionisation tandem mass spectrometry. *Journal of Chromatography A*, *883*(1), 89–102.

Han, L., Matarrita, J., Sapozhnikova, Y., & Lehotay, S. J. (2016). Evaluation of a recent product to remove lipids and other matrix co-extractives in the analysis of pesticide residues and environmental contaminants in foods. *Journal of Chromatography. A*, *1449*, 17–29.

Hatch, F. T., Knize, M. G., & Felton, J. S. (1991). Quantitative structure-activity relationships of heterocyclic amine mutagens formed during the cooking of food. *Environmental and Molecular Mutagenesis*, *17*(1), 4–19.

He, Z., Wang, Y., Wang, L., Peng, Y., Wang, W., & Liu, X. (2017). Determination of 255 pesticides in edible vegetable oils using QuEChERS method and gas chromatography tandem mass spectrometry. *Analytical and Bioanalytical Chemistry*, *409*(4), 1017–1030.

Herraiz, T. (2004). Relative exposure to beta-carbolines norharman and harman from foods and tobacco smoke. *Food Additives and Contaminants*, *21*(11), 1041–1050.

Hsiao, H.Y., Chen, B. H., & Kao, T. H. (2017). Analysis of Heterocyclic Amines in Meat by the Quick, Easy, Cheap, Effective, Rugged, and Safe Method Coupled with LC-DAD-MS-MS. *Journal of Agricultural and Food Chemistry*, *65*(42), 9360–9368.

Ito, N., Hasegawa, R., Sano, M., Tamano, S., Esumi, H., Takayama, S., & Sugimura, T. (1991). A new colon and mammary carcinogen in cooked food, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). *Carcinogenesis*, *12*(8), 1503–1506.

Iwasaki, M., Kataoka, H., Ishihara, J., Takachi, R., Hamada, G. S., Sharma, S., & Tsugane, S. (2010). Heterocyclic amines content of meat and fish cooked by Brazilian methods. *Journal of Food Composition and Analysis : An Official Publication of the United Nations University, International Network of Food Data Systems*, *23*(1), 61–69.

Jackson, L. S., & Hargraves, W. A. (1995). Effects of Time and Temperature on the Formation of MeIQx and DiMeIQx in a Model System Containing Threonine, Glucose, and Creatine. *Journal of Agricultural and Food Chemistry*, *43*(6), 1678–1684.

Jägerstad, M., Skog, K., Arvidsson, P., & Solyakov, A. (1998). Chemistry, formation and occurrence of genotoxic heterocyclic amines identified in model systems and cooked foods. *Zeitschrift Fur Lebensmitteluntersuchung Und -Forschung A*, *207*(6), 419–427.

Jägerstad, M., Skog, K., Grivas, S., & Olsson, K. (1991). Formation of heterocyclic amines using model systems. *Mutation Research*, *259*(3–4), 219–233.

Janoszka, B., Błaszczyk, U., Damasiewicz-Bodzek, A., & Sajewicz, M. (2009). Analysis of heterocyclic amines (HAs) in pan-fried pork meat and its gravy by liquid chromatography with diode array detection. *Food Chemistry*, *113*(4), 1188–1196.

Jinap, S., Mohd-Mokhtar, M.S., Farhadian, A., Hasnol, N.D., Jaafar, S.N., & Hajeb, P. (2013). Effects of varying degrees of doneness on the formation of heterocyclic aromatic amines in chicken and beef satay. *Meat Science*, *94*(2), 202-207.

João Ramalhosa, M., Paíga, P., Morais, S., Delerue-Matos, C., & Prior Pinto Oliveira, M. B. (2009). Analysis of polycyclic aromatic hydrocarbons in fish: evaluation of a quick, easy, cheap, effective, rugged, and safe extraction method. *Journal of Separation Science*, *32*(20), 3529–3538.

Johansson, M. A. E., & Jägerstad, M. (1996). Influence of pro- and antioxidants on the formation of mutagenic-carcinogenic heterocyclic amines in a model system. *Food Chemistry*, *56*(1), 69–75.

Johansson, M. A., & Jägerstad, M. (1994). Occurrence of mutagenic/carcinogenic heterocyclic amines in meat and fish products, including pan residues, prepared under domestic conditions. *Carcinogenesis*, *15*(8), 1511–1518.

Jones, C., & Weisburger, J. (1988a). L-Tryptophan inhibits formation of mutagens during cooking of meat and in laboratory models. *Mutation Research*, *206*(3), 343–349.

Jones, C., & Weisburger, J. (1988b). Inhibition of aminoimidazo-quinoxaline-type and aminoimidazol-4-one type mutagen formation in liquid-reflux models by the amino acids L-proline and/or L-tryptophan. *Environmental and Molecular Mutagenesis*, *11*, 509–514.

Kaderlik, K. R., Minchin, R. F., Mulder, G. J., Ilett, K. F., Daugaard-Jenson, M., Teitel, C. H., & Kadlubar, F. F. (1994). Metabolic activation pathway for the formation of DNA adducts of the

carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in rat extrahepatic tissues. *Carcinogenesis*, *15*(8), 1703–1709.

Kampman, E., Slattery, M. L., Bigler, J., Leppert, M., Samowitz, W., Caan, B. J., & Potter, J. D. (1999). Meat consumption, genetic susceptibility, and colon cancer risk: a United States multicenter case-control study. *Cancer Epidemiology, Biomarkers & Prevention: A Publication of the American Association for Cancer Research, Cosponsored by the American Society of Preventive Oncology*, *8*(1), 15–24.

Kataoka, H. (1997). Methods for the determination of mutagenic heterocyclic amines and their applications in environmental analysis. *Journal of Chromatography A*, *774*(1), 121–142.

Kato, T., Harashima, T., Moriya, N., Kikugawa, K., & Hiramoto, K. (1996). Formation of the mutagenic/carcinogenic imidazoquinoxaline-type heterocyclic amines through the unstable free radical Maillard intermediates and its inhibition by phenolic antioxidants. *Carcinogenesis*, *17*(11), 2469–2476.

Kikugawa, K. (1999). Involvement of free radicals in the formation of heterocyclic amines and prevention by antioxidants. *Cancer Letters*, *143*(2), 123–126.

Kim, H. S., & Hur, S. J. (2018). Changes in the mutagenicity of heterocyclic amines, nitrite, and N -nitroso compound in pork patties during in vitro human digestion. *LWT*, *92*, 47–53.

Knize, M. G., Andresen, B. D., Healy, S. K., Shen, N. H., Lewis, P. R., Bjeldanes, L. F., & Felton, J. S. (1985). Effects of temperature, patty thickness and fat content on the production of mutagens in fried ground beef. *Food and Chemical Toxicology: An International Journal Published for the British Industrial Biological Research Association*, *23*(12), 1035–1040.

Knize, M. G., Cunningham, P. L., Griffin, E. A., Jones, A. L., & Felton, J. S. (1994). Characterization of mutagenic activity in cooked-grain-food products. *Food and Chemical*

Toxicology: An International Journal Published for the British Industrial Biological Research Association, *32*(1), 15–21.

Kotepui, M. (2016). Diet and risk of breast cancer. *Contemporary Oncology*, *20*(1), 13–19.

Krska, R., Stubbings, G., Macarthur, R., & Crews, C. (2008). Simultaneous determination of six major ergot alkaloids and their epimers in cereals and foodstuffs by LC–MS–MS. *Analytical and Bioanalytical Chemistry*, *391*(2), 563–576.

Krul, C., Luiten-Schuite, A., Baandagger, R., Verhagen, H., Mohn, G., Feron, V., & Havenaar, R. (2000). Application of a dynamic in vitro gastrointestinal tract model to study the availability of food mutagens, using heterocyclic aromatic amines as model compounds. *Food and Chemical Toxicology: An International Journal Published for the British Industrial Biological Research Association*, *38*(9), 783–792.

Le Marchand, L., Hankin, J. H., Wilkens, L. R., Pierce, L. M., Franke, A., Kolonel, L. N., & Donlon, T. (2001). Combined effects of well-done red meat, smoking, and rapid N acetyltransferase 2 and CYP1A2 phenotypes in increasing colorectal cancer risk. *Cancer Epidemiology, Biomarkers & Prevention: A Publication of the American Association for Cancer Research, Cosponsored by the American Society of Preventive Oncology*, *10*(12), 1259–1266.

Lee, K. J., Lee, G. H., Kim, H., Oh, M.-S., Chu, S., Hwang, I. J., & Park, H.-M. (2015). Determination of Heterocyclic Amines and Acrylamide in Agricultural Products with Liquid Chromatography-Tandem Mass Spectrometry. *Toxicological Research*, *31*(3), 255-264.

Lehotay, S. J., Mastovska, K., & Lightfield, A.R. (2005). Use of buffering and other means to improve results of problematic pesticides in a fast and easy method for residue analysis of fruits and vegetables. *Journal of AOAC International*, 88(2), 615-629.

Lehotay, S. J., Son, K. A., Kwon, H., Koesukwiwat, U., Fu, W., Mastovska, K., & Leepipatpiboon, N. (2010). Comparison of QuEChERS sample preparation methods for the analysis of pesticide residues in fruits and vegetables. *Journal of Chromatography A, 1217*(16), 2548–2560.

Leong-Morgenthaler, P. M., Op Het Velt, C., Jaccaud, E., & Turesky, R. J. (1998). Mutagenicity of 2-amino-3-methylimidazo[4,5-f]quinoline in human lymphoblastoid cells. *Carcinogenesis, 19*(10), 1749–1754.

Li, D., Day, R. S., Bondy, M. L., Sinha, R., Nguyen, N. T., Evans, D. B., & Hassan, M. M. (2007). Dietary mutagen exposure and risk of pancreatic cancer. *Cancer Epidemiology, Biomarkers & Prevention: A Publication of the American Association for Cancer Research, Cosponsored by the American Society of Preventive Oncology*, *16*(4), 655–661.

Liao, G., Xu, X., & Zhou, G. (2009). Effects Of Cooked Temperatures And Addition Of Antioxidants On Formation Of Heterocyclic Aromatic Amines In Pork Floss. *Journal of Food Processing and Preservation*, *33*(2), 159–175.

Lightfoot, T. J., Coxhead, J. M., Cupid, B. C., Nicholson, S., & Garner, R. C. (2000). Analysis of DNA adducts by accelerator mass spectrometry in human breast tissue after administration of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine and benzo[a]pyrene. *Mutation Research, 472*(1–2), 119–127.

Linghu, Z., Karim, F., & Smith, J. S. (2017). Amino Acids Inhibitory Effects and Mechanism on 2-Amino-1-Methyl-6-Phenylimidazo [4,5-b]Pyridine (PhIP) Formation in the Maillard Reaction Model Systems: Amino acids inhibit HCA PhIP formation. *Journal of Food Science, 82*(12), 3037–3045.

Liu, S., Taylor, L. T., Borgerding, M. F., Coleman, W. M., & Bombick, B. R. (2013). Trace Analysis of Mutagenic Heterocyclic Aromatic Amines in Cigarette Smoke Condensate and its Base Fractions via Silylation-GC-MS. *Beiträge Zur Tabakforschung International/Contributions to Tobacco Research*, *25*(5), 550–562.

Lucas, D., & Zhao, L. (2015). PAH Analysis in Salmon with Enhanced Matrix Removal, 8.

Lynch, A. M., Knize, M. G., Boobis, A. R., Gooderham, N. J., Davies, D. S., & Murray, S. (1992). Intra- and Interindividual Variability in Systemic Exposure in Humans to 2-Amino-3,8-dimethylimidazo[4,5-/]quinoxaline and 2-Amino-l -methyl- 6-phenylimidazo[4,5-o]pyridine, Carcinogens Present in Cooked Beef. *Cancer Research*, 52, 6216-6223.

Manabe, S., Suzuki, H., Wada, O., & Ueki, A. (1993). Detection of the carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in beer and wine. *Carcinogenesis*, *14*(5), 899–901.

Martín-Calero, A., Ayala, J. H., González, V., & Afonso, A. M. (2007). Determination of less polar heterocyclic amines in meat extracts. *Analytica Chimica Acta*, *582*(2), 259–266.

Martins, S. I. F. ., Jongen, W. M. ., & van Boekel, M. A. J. (2000). A review of Maillard reaction in food and implications to kinetic modelling. *Trends in Food Science & Technology*, *11*(9–10), 364–373.

Mastovska, K., & Lehotay, S. J. (2006). Rapid Sample Preparation Method for LC−MS/MS or GC−MS Analysis of Acrylamide in Various Food Matrices. *Journal of Agricultural and Food Chemistry*, *54*(19), 7001–7008.

Messner, C., & Murkovic, M. (2004). Evaluation of a new model system for studying the formation of heterocyclic amines. *Journal of Chromatography B*, *802*(1), 19–26.

Minchin, R. F., Reeves, P. T., Teitel, C. H., McManus, M. E., Mojarrabi, B., Ilett, K. F., & Kadlubar, F. F. (1992). N-and O-acetylation of aromatic and heterocyclic amine carcinogens by human monomorphic and polymorphic acetyltransferases expressed in COS-1 cells. *Biochemical and Biophysical Research Communications*, *185*(3), 839–844.

Moon, S.-E., & Shin, H.-S. (2013). Inhibition of mutagenic 2-amino-1-methyl-6-phenylimidazo[4,5-b] pyridine (PhIP) formation using various food ingredients in a model systems. *Food Science and Biotechnology*, *22*(2), 323–329.

Murkovic, M. (2004). Formation of heterocyclic aromatic amines in model systems. *Journal of Chromatography B*, *802*(1), 3–10.

Murray, S., Lynch, A. M., Knize, M. G., & Gooderham, M. J. (1993). Quantification of the carcinogens 2-amino-3,8-dimethyl- and 2-amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine in food using a combined assay based on gas chromatography-negative ion mass spectrometry. *Journal of Chromatography*, *616*(2), 211–219.

Naccari, C., Galceran, M. T., Moyano, E., Cristani, M., Siracusa, L., & Trombetta, D. (2009). Presence of heterocyclic aromatic amines (HAs) in smoked "Provola" cheese from Calabria (Italy). *Food and Chemical Toxicology*, *47*(2), 321–327.

Nagao, M., Honda, M., Seino, Y., Yahagi, T., & Sugimura, T. (1977). Mutagenicities of smoke schutcondensates and the charred surface of fish and meat. *Cancer Letters*, *2*(4–5), 221–226.

Nagaoka, H., Wakabayashi, K., Kim, S.-B., Kim, I.-S., Tanaka, Y., Ochiai, M., & Nagao, M. (1992). Adduct Formation at C-8 of Guanine on in vitro Reaction f the Ultimate Form of 2-Amino-l-methyl-6-phenylimidazo[4,5-b]pyridine with 2′-Deoxyguanosine and Its Phosphate Esters. *Japanese Journal of Cancer Research*, *83*(10), 1025–1029.

Nerurkar, P. V., Marchand, L. L., & Cooney, R. V. (2009). Effects of Marinating With Asian Marinades or Western Barbecue Sauce on PhIP and MeIQx Formation in Barbecued Beef. *Nutrition and Cancer*.

Ni, W., McNaughton, L., LeMaster, D. M., Sinha, R., & Turesky, R. J. (2008). Quantitation of 13 Heterocyclic Aromatic Amines in Cooked Beef, Pork, and Chicken by Liquid Chromatography−Electrospray Ionization/Tandem Mass Spectrometry. *Journal of Agricultural and Food Chemistry*, *56*(1), 68–78.

Nilsson, L., Övervik, E., Fredholm, L., Levin, Ö., Nord, C.-E., & Gustafsson, J.-Å. (1986). Influence of frying fat on mutagenic activity in lean pork meat. *Mutation Research/Genetic Toxicology*, *171*(2), 115–121.

Nowell, S., Coles, B., Sinha, R., MacLeod, S., Luke Ratnasinghe, D., Stotts, C., & Lang, N. P. (2002). Analysis of total meat intake and exposure to individual heterocyclic amines in a case-control study of colorectal cancer: contribution of metabolic variation to risk. *Mutation Research*, *506–507*, 175–185.

Oguri, A., Suda, M., Totsuka, Y., Sugimura, T., & Wakabayashi, K. (1998). Inhibitory effects of antioxidants on formation of heterocyclic amines. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, *402*(1), 237–245.

Ohgaki, H., Hasegawa, H., Kato, T., Suenaga, M., Ubukata, M., Sato, S., & Sugimura, T. (1986). Carcinogenicity in Mice and Rats of Heterocyclic Amines in Cooked Foods. *Environmental Health Perspectives*, *67*, 129.

Ouyang, Y., Li, H., Tang, H., Jin, Y., & Li, G. (2015). A reliable and sensitive LCMS-IT-TOF method coupled with accelerated solvent extraction for the identification and quantitation of six

typical heterocyclic aromatic amines in cooked meat products. *Analytical Methods*, *7*(21), 9274–9280.

Overvik, E., Kleman, M., Berg, I., & Gustafsson, J. A. (1989). Influence of creatine, amino acids and water on the formation of the mutagenic heterocyclic amines found in cooked meat. *Carcinogenesis*, *10*(12), 2293–2301.

Oz, F. (2011). Quantitation of heterocyclic aromatic amines in ready to eat meatballs by ultra fast liquid chromatography. *Food Chemistry*, *126*(4), 2010–2016.

Oz, F., & Cakmak, I. H. (2016). The effects of conjugated linoleic acid usage in meatball production on the formation of heterocyclic aromatic amines. *LWT - Food Science and Technology*, *65*, 1031–1037.

Pais, P., & Knize, M. G. (2000). Chromatographic and related techniques for the determination of aromatic heterocyclic amines in foods. *Journal of Chromatography. B, Biomedical Sciences and Applications*, *747*(1–2), 139–169.

Pais, P., Salmon, C. P., Knize, M. G., & Felton, J. S. (1999). Formation of Mutagenic/Carcinogenic Heterocyclic Amines in Dry-Heated Model Systems, Meats, and Meat Drippings. *Journal of Agricultural and Food Chemistry*, *47*(3), 1098–1108.

Park, K. H., Choi, J. H., Abd El-Aty, A. M., Cho, S. K., Park, J. H., Kwon, K. S., & Shim, J. H. (2012). Development of QuEChERS-based extraction and liquid chromatography–tandem mass spectrometry method for quantifying flumethasone residues in beef muscle. *Meat Science*, *92*(4), 749–753.

Parrilla Vázquez, P., Hakme, E., Uclés, S., Cutillas, V., Martínez Galera, M., Mughari, A. R., & Fernández-Alba, A. R. (2016). Large multiresidue analysis of pesticides in edible vegetable oils by using efficient solid-phase extraction sorbents based on quick, easy, cheap, effective, rugged

and safe methodology followed by gas chromatography–tandem mass spectrometry. *Journal of Chromatography A*, *1463*, 20–31.

Pearson, A. M., Chen, C., Gray, J. I., & Aust, S. D. (1992). Mechanism(s) involved in meat mutagen formation and inhibition. *Free Radical Biology and Medicine*, *13*(2), 161–167.

Persson, E., Oroszvári, B. K., Tornberg, E., Sjöholm, I., & Skog, K. (2008). Heterocyclic amine formation during frying of frozen beefburgers. *International Journal of Food Science & Technology*, *43*(1), 62–68.

Pizzutti, I. R., de Kok, A., Hiemstra, M., Wickert, C., & Prestes, O. D. (2009). Method validation and comparison of acetonitrile and acetone extraction for the analysis of 169 pesticides in soya grain by liquid chromatography–tandem mass spectrometry. *Journal of Chromatography A*, *1216*(21), 4539–4552.

Pouech, C., Tournier, M., Quignot, N., Kiss, A., Wiest, L., Lafay, F., & Cren-Olivé, C. (2012). Multi-residue analysis of free and conjugated hormones and endocrine disruptors in rat testis by QuEChERS-based extraction and LC-MS/MS. *Analytical and Bioanalytical Chemistry*, *402*(9), 2777–2788.

Pouzou, J. G., Costard, S., & Zagmutt, F. J. (2018). Probabilistic assessment of dietary exposure to heterocyclic amines and polycyclic aromatic hydrocarbons from consumption of meats and breads in the United States. *Food and Chemical Toxicology*.

Puangsombat, K., Gadgil, P., Houser, T. A., Hunt, M. C., & Smith, J. S. (2012). Occurrence of heterocyclic amines in cooked meat products. *Meat Science*, *90*(3), 739–746.

Rajski, Ł., Lozano, A., Uclés, A., Ferrer, C., & Fernández-Alba, A. R. (2013). Determination of pesticide residues in high oil vegetal commodities by using various multi-residue methods and

clean-ups followed by liquid chromatography tandem mass spectrometry. *Journal of Chromatography. A*, *1304*, 109–120.

Reistad, R., Rossland, O. J., Latva-Kala, K. J., Rasmussen, T., Vikse, R., Becher, G., & Alexander, J. (1997). Heterocyclic aromatic amines in human urine following a fried meat meal. *Food and Chemical Toxicology*, *35*(10), 945–955.

Rejczak, T., & Tuzimski, T. (2015). A review of recent developments and trends in the QuEChERS sample preparation approach. *Open Chemistry*, *13*(1).

Richling, E., Kleinschnitz, M., & Schreier, P. (1999). Analysis of heterocyclic aromatic amines by high resolution gas chromatography-mass spectrometry: a suitable technique for the routine control of food and process flavours. *European Food Research and Technology*, *210*(1), 68–72.

Ristic, A., Cichna, M., & Sontag, G. (2004). Determination of less polar heterocyclic aromatic amines in standardised beef extracts and cooked meat consumed in Austria by liquid chromatography and fluorescence detection. *Journal of Chromatography B*, *802*(1), 87–94.

Sabally, K., Sleno, L., Jauffrit, J. A., Iskandar, M. M., & Kubow, S. (2016). Inhibitory effects of apple peel polyphenol extract on the formation of heterocyclic amines in pan fried beef patties. *Meat Science*, *117*, 57–62.

Saito, M., Sakagami, H., & Fujisawa, S. (2003). Cytotoxicity and apoptosis induction by butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). *Anticancer Research*, *23*(6C), 4693–4701.

Salmon, C. P., Knize, M. G., & Felton, J. S. (1997). Effects of marinating on heterocyclic amine carcinogen formation in grilled chicken. *Food and Chemical Toxicology: An International Journal Published for the British Industrial Biological Research Association*, *35*(5), 433–441.

Schut, H. A. J., & Snyderwine, E. G. (1999). DNA adducts of heterocyclic amine food mutagens: implications for mutagenesis and carcinogenesis. *Carcinogenesis*, *20*(3), 353–368.

Shin, H.-S., Park, H., & Park, D. (2003). Influence of Different Oligosaccharides and Inulin on Heterocyclic Aromatic Amine Formation and Overall Mutagenicity in Fried Ground Beef Patties. *Journal of Agricultural and Food Chemistry*, *51*(23), 6726–6730.

Shin, J. H., Na, Y.C., Chung, J. H., Gorinstein, S., & Ahn, Y. G. (2014). Quantitative analysis of heterocyclic amines in urine by liquid chromatography coupled with tandem mass spectrometry. *Analytical Biochemistry*, *447*, 169–176.

Sinha, R., Knize, M. G., Salmon, C. P., Brown, E. D., Rhodes, D., Felton, J. S., & Rothman, N. (1998). Heterocyclic amine content of pork products cooked by different methods and to varying degrees of doneness. *Food and Chemical Toxicology: An International Journal Published for the British Industrial Biological Research Association*, *36*(4), 289–297.

Sinha, R., Rothman, N., Brown, E. D., Salmon, C. P., Knize, M. G., Swanson, C. A., & Felton, J. S. (1995). High concentrations of the carcinogen 2-amino-1-methyl-6-phenylimidazo- [4,5-b]pyridine (PhIP) occur in chicken but are dependent on the cooking method. *Cancer Research*, *55*(20), 4516–4519.

Sinha, R., Gustafson, D. R., Kulldorff, M., Wen, W.-Q., Cerhan, J. R., & Zheng, W. (2000). 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine, a Carcinogen in High- Temperature-Cooked Meat, and Breast Cancer Risk. *JNCI: Journal of the National Cancer Institute*, *92*(16), 1352–1354.

Sinha, R., Peters, U., Cross, A. J., Kulldorff, M., Weissfeld, J. L., Pinsky, P. F., & Prostate, Lung, Colorectal, and Ovarian Cancer Project Team. (2005). Meat, Meat Cooking Methods and Preservation, and Risk for Colorectal Adenoma. *Cancer Research*, *65*(17), 8034–8041.

Sinha, R., & Rothman, N. (1997). Exposure assessment of heterocyclic amines (HCAs) in epidemiologic studies. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, *376*(1), 195–202.

Skog, K. I., Johansson, M. A., & Jägerstad, M. I. (1998). Carcinogenic heterocyclic amines in model systems and cooked foods: a review on formation, occurrence and intake. *Food and Chemical Toxicology: An International Journal Published for the British Industrial Biological Research Association*, *36*(9–10), 879–896.

Skog, K., & Jägerstad, M. (1990). Effects of monosaccharides and disaccharides on the formation of food mutagens in model systems. *Mutation Research*, *230*(2), 263–272.

Skog, K., Knize, M. G., Felton, J. S., & Jägerstad, M. (1992). Formation of new heterocyclic amine mutagens by heating creatinine, alanine, threonine and glucose. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, *268*(2), 191–197.

Skog, K., Solyakov, A., & Jägerstad, M. (2000). Effects of heating conditions and additives on the formation of heterocyclic amines with reference to amino-carbolines in a meat juice model system. *Food Chemistry*, *68*(3), 299–308.

Skog, K., Eneroth, A., & Svanberg, M. (2003). Effects of different cooking methods on the formation of food mutagens in meat. *International Journal of Food Science and Technology*, *38*(3), 313–323.

Smith, J. S., Ameri, F., & Gadgil, P. (2008). Effect of Marinades on the Formation of Heterocyclic Amines in Grilled Beef Steaks. *Journal of Food Science*, *73*(6), T100–T105.

Snyderwine, E. G. (1994). Some perspectives on the nutritional aspects of breast cancer research. Food-derived heterocyclic amines as etiologic agents in human mammary cancer. *Cancer*, *74*(3 Suppl), 1070–1077.

Stolzenberg-Solomon, R. Z., Cross, A. J., Silverman, D. T., Schairer, C., Thompson, F. E., Kipnis, V., & Sinha, R. (2007). Meat and meat-mutagen intake and pancreatic cancer risk in the NIH-AARP cohort. *Cancer Epidemiology, Biomarkers & Prevention: A Publication of the American Association for Cancer Research, Cosponsored by the American Society of Preventive Oncology*, *16*(12), 2664–2675.

Strecker, A. (1861). Untersuchungen über die chemischen Beziehungen zwischen Guanin, Xanthin, Theobromin, Caffeïn und Kreatinin. *Justus Liebigs Annalen Der Chemie*, *118*(2), 151–177.

Sugimura, T., Wakabayashi, K., Nakagama, H., & Nagao, M. (2004). Heterocyclic amines: Mutagens/carcinogens produced during cooking of meat and fish. *Cancer Science*, *95*(4), 290–299.

Tang, D., Liu, J. J., Rundle, A., Neslund-Dudas, C., Savera, A. T., Bock, C. H., & Rybicki, B. A. (2007). Grilled Meat Consumption and PhIP-DNA Adducts in Prostate Carcinogenesis. *Cancer Epidemiology Biomarkers & Prevention*, *16*(4), 803–808.

Thiébaud, H. P., Knize, M. G., Kuzmicky, P. A., Hsieh, D. P., & Felton, J. S. (1995). Airborne mutagens produced by frying beef, pork and a soy-based food. *Food and Chemical Toxicology: An International Journal Published for the British Industrial Biological Research Association*, *33*(10), 821–828.

Toribio, F., Moyano, E., Puignou, L., & Galceran, M. (2002). Ion-trap tandem mass spectrometry for the determination of heterocyclic amines in food. *Journal of Chromatography A*, *948*(1–2), 267–281.

Totlani, V. M., & Peterson, D. G. (2006). Epicatechin Carbonyl-Trapping Reactions in Aqueous Maillard Systems: Identification and Structural Elucidation. *Journal of Agricultural and Food Chemistry*, *54*(19), 7311–7318.

Tsuchiya, H., Sato, M., Hayashi, H., Kato, H., Kureshiro, H., & Hayashi, T. (1996). Simultaneous determination of tetrahydro-β-carbolines and β-carbolines. *Chromatographia*, *43*(7–8), 419–425.

Turesky, R. J., & Le Marchand, L. (2011). Metabolism and biomarkers of heterocyclic aromatic amines in molecular epidemiology studies: lessons learned from aromatic amines. *Chemical Research in Toxicology*, *24*(8), 1169–1214.

Turesky, R. J., Taylor, J., Schnackenberg, L., Freeman, J. P., & Holland, R. D. (2005). Quantitation of Carcinogenic Heterocyclic Aromatic Amines and Detection of Novel Heterocyclic Aromatic Amines in Cooked Meats and Grill Scrapings by HPLC/ESI-MS. *Journal of Agricultural and Food Chemistry*, *53*(8), 3248–3258.

Turesky, R. J., & Vouros, P. (2004). Formation and analysis of heterocyclic aromatic amine–DNA adducts in vitro and in vivo. *Journal of Chromatography B*, *802*(1), 155–166.

Uclés, A., Herrera López, S., Dolores Hernando, M., Rosal, R., Ferrer, C., & Fernández-Alba, A. R. (2015). Application of zirconium dioxide nanoparticle sorbent for the clean-up step in post-harvest pesticide residue analysis. *Talanta*, *144*, 51–61.

Van Dyck, M. M. C., Rollmann, B., & De Meester, C. (1995). Quantitative estimation of heterocyclic aromatic amines by ion-exchange chromatography and electrochemical detection. *Journal of Chromatography A*, *697*(1), 377–382.

Vitaglione, P., & Fogliano, V. (2004). Use of antioxidants to minimize the human health risk associated to mutagenic/carcinogenic heterocyclic amines in food. *Journal of Chromatography B*, *802*(1), 189–199.

Weisburger, J. H. (2002). Comments on the history and importance of aromatic and heterocyclic amines in public health. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, *506*, 9–20.

Weisburger, J. H., Dolan, L., & Pittman, B. (1998). Inhibition of PhIP mutagenicity by caffeine, lycopene, daidzein, and genistein. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, *416*(1), 125–128.

Widmark, E. M. P. (1939). Presence of Cancer-producing Substances in Roasted Food. *Nature*, *143*(3632), 984.

Yaylayan, V. A. (2003). Recent Advances in the Chemistry of Strecker Degradation and Amadori Rearrangement: Implications to Aroma and Color Formation. *Food Science and Technology Research*, *9*(1), 1–6. https://doi.org/10.3136/fstr.9.1

Zamora, R., Alcón, E., & Hidalgo, F. J. (2012). Effect of lipid oxidation products on the formation of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in model systems. *Food Chemistry*, *135*(4), 2569–2574.

Zamora, R., Alcón, E., & Hidalgo, F. J. (2013). Effect of amino acids on the formation of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in creatinine/phenylalanine and creatinine/phenylalanine/4-oxo-2-nonenal reaction mixtures. *Food Chemistry*, *141*(4), 4240–4245.

Zeng, M., He, Z., Zheng, Z., Qin, F., Tao, G., Zhang, S., & Chen, J. (2014). Effect of Six Chinese Spices on Heterocyclic Amine Profiles in Roast Beef Patties by Ultra Performance Liquid Chromatography-Tandem Mass Spectrometry and Principal

Zhao, L., & Lucas, D. (2015). Multiresidue Analysis of Pesticides in Avocado with Agilent Bond Elut EMR—Lipid by LC/MS/MS, 12.

Zheng, W., Deitz, A. C., Campbell, D. R., Wen, W. Q., Cerhan, J. R., Sellers, T. A., & Hein, D. W. (1999). N-acetyltransferase 1 genetic polymorphism, cigarette smoking, well-done meat intake, and breast cancer risk. *Cancer Epidemiology, Biomarkers & Prevention: A Publication of the American Association for Cancer Research, Cosponsored by the American Society of Preventive Oncology*, *8*(3), 233–239.

Zheng, W., Xie, D., Cerhan, J. R., Sellers, T. A., Wen, W., & Folsom, A. R. (2001). Sulfotransferase 1A1 polymorphism, endogenous estrogen exposure, well-done meat intake, and breast cancer risk. *Cancer Epidemiology, Biomarkers & Prevention: A Publication of the American Association for Cancer Research, Cosponsored by the American Society of Preventive Oncology*, *10*(2), 89–94.

Zheng, W., Gustafson, D. R., Moore, D., Hong, C.-P., Anderson, K. E., Kushi, L. H., & Cerhan, J. R. (1998). Well-Done Meat Intake and the Risk of Breast Cancer. *JNCI: Journal of the National Cancer Institute*, *90*(22), 1724–1729.

Zheng, W., & Lee, S.-A. (2009). Well-Done Meat Intake, Heterocyclic Amine Exposure, and Cancer Risk. *Nutrition and Cancer*, *61*(4), 437–446.

Zheng, W., Wen, W.-Q., Gustafson, D. R., Gross, M., Cerhan, J. R., & Folsom, A. R. (2002). GSTM1 and GSTT1 polymorphisms and postmenopausal breast cancer risk. *Breast Cancer Research and Treatment*, *74*(1), 9–16.

Zhu, Q., Zhang, S., Wang, M., Chen, J., & Zheng, Z.-P. (2016). Inhibitory effects of selected dietary flavonoids on the formation of total heterocyclic amines and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in roast beef patties and in chemical models. *Food & Function*, *7*(2), 1057–1066.

Zochling, S. (2002). Formation of the heterocyclic aromatic amine PhIP: identification of precursors and intermediates. *Food Chemistry*, *79*(1), 125–134.

Zöchling, S., & Murkovic, M. (2002). Formation of the heterocyclic aromatic amine PhIP: identification of precursors and intermediates. *Food Chemistry*, *79*(1), 125–134.

CHAPTER 2-AMINO ACIDS INHIBITORY EFFECTS AND MECHANISM ON 2-AMINO-1-METHYL-6-PHENYLIMIDAZO [4,5-B]PYRIDINE (PHIP) FORMATION IN THE MAILLARD REACTION MODEL SYSTEMS [1,2]

ABSTRACT

This study was to investigate the inhibitory effects of amino acids (AAs) on the formation of 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP) and to evaluate the inhibition mechanism of PhIP in Maillard model systems. Different AAs were individually added into model systems heat-treated at 180 °C/1 h. The PhIP, phenylacetaldehyde (PheAce), and pyrazines derivatives were determined using HPLC and GC-MS. AAs significantly reduced ($p < 0.05$) PhIP levels in a dose-dependent response, ranking as: Trp = Lys > Pro > Leu > Met > Val > Ile > Thr > Phe > Asp, at the highest molar ratio. The PheAce content was gradually reduced with increasing AAs levels, suggesting that AAs may inhibit PhIP formation through scavenging the available PheAce. A correlation between PhIP inhibition and PheAce-scavenging activity of AAs was observed when PheAce and AAs were heated. The variety and quantity of pyrazines formed are highly depending on the type of AAs.

1-Presented in part at IFT Annual Meeting 2016. Toxicology and Safety Evaluation Division.
2-Reproduced with permission from: Linghu, Z., Karim, F., & Smith, J. S. 2017. Amino acids inhibitory effects and mechanism on 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP) formation in the Maillard reaction model systems. J. Food Sci. 82(12):3037-3045.

INTRODUCTION

Heterocyclic amines (HCAs) are a class of highly mutagenic and carcinogenic substances formed through the Maillard browning reaction (Alaejos & Afonso, 2011). More than 25 HCAs have been isolated from cooked meats and other muscle-rich foods during frying, baking, roasting, grilling, and broiling at temperatures from 100 to 300 °C (Alaejos & Afonso, 2011). Among these, 2-amino-1-methyl-6-phenylimidzo [4,5-b]-pyridine (PhIP) is the most abundant dietary HCAs present in cooked meats (Puangsombat, Gadgil, Houser, Hunt, & Smith, 2012), with an estimated daily intake from 0.1 to 13.8 μg per day per person (Wakabayashi et al., 1993). Several animal experiments have indicated that PhIP is a powerful mutagen and carcinogen in mice, rats, and monkeys in a wide variety of organs/tissues, specifically in lung, small/large intestine, liver, mammary gland, colon, prostate, and lymphoid tissue (Sugimura, Wakabayashi, Nakagama, & Nagao, 2004). Epidemiological studies also suggested that high dietary consumption of PhIP-rich meats can increase the risk of colon and breast cancer (DeBruin, Martos, & Josephy, 2001, Gerhardsson, Hagman, Peters, Steineck, & Overvik, 1991). Therefore, the International Agency for Research on Cancer (IARC) considered PhIP as a possible human carcinogen (group 2B) (IARC, 1993). Moreover, the National Toxicology Program of The U.S. Department of Health and Human Services (2011) has listed PhIP as reasonably anticipated to be human carcinogens.

Processed foods contain PhIP possess health risks to human and it is recommended to reduce excessive exposure to PhIP through cooked/processed meat and fish by adding effective inhibitors into food products before or during the heating process. Several literatures had pointed out adding synthetic antioxidants such as, butylated hydroxyanisole (BHA), and butylated hydroxytoluene (BHT) (Vitaglione & Fogliano, 2004), herbs/cooking spices (Tsen, Ameri, &

Smith, 2006), natural tea, or their natural extracts/antioxidants (Alaejos & Afonso, 2011) into meats or marinade (Smith, Ameri, & Gadgil, 2008) prior or during cooking can effectively reduce PhIP formation. Contrary, several studies have found that natural herbs/spices/antioxidants may promote PhIP formation in cooked foods. For example, Zeng et al. (2014) reported that adding 1% cumin, chili, or black pepper into roasted beef patties significantly increased PhIP formation by 52, 33, or 45%. A mixture of basil (0.5%), coriander (0.2%), and sweet grass (0.2-0.5%) extracts was also reported to increase the concentration of PhIP in cooked beef samples (Damašius, Venskutonisa, Ferracaneb, & Foglianob, 2011). These controversial findings may be attributed to the pro-oxidant activity of many antioxidants in higher concentrations (Zöchling, Murkovic, & Pfannhauser, 2002). In addition, the safety concern regarding using synthetic antioxidants, and concern of the particular aroma, taste, and color attributes of herbs/cooking spices, as well as the high cost of pure natural antioxidants, may limit the acceptance and usage of those ingredients in foods.

Amino acids (AAs) and their derivatives, like, L-Trp, and L-Lys, as food additives have been widely utilized in commercial foods to increase the nutritive value (Belitz, Grosch, & Schieberle, 2004). In the Maillard reaction, free AAs are considered to be indispensable precursors and they significantly affect the variety and quantity of HCAs formation (Zamora Alcón & Hidalgo, 2013; Murkovic, 2004). Heating of different single AAs along with other precursors (hexose, creatine/creatinine) has been widely used in model systems to study in vitro HCAs formation. For example, MeIQ and 4,8-DiMeIQx can both be formed from threonine (Jackson & Hargraves, 1995); while PhIP is only produced from phenylalanine (Felton, Knize, Shen, Andresen, & Bjeldanes, 1986). Also, some studies reported that adding certain types of AAs into meats or model systems are able to either enhance or inhibit mutagens formation.

Ashoor et al. (1980) found that adding proline to ground beef patties before frying promoted mutagens formation. Overvik et al. (1989) found that adding 1% of threonine, serine, proline, and alanine into beef patties greatly enhanced the mutagenicity in the crust and pan-residue. Zamora et al. (2013) tested 20 AAs and found cysteine, serine, aspartic acid, threonine, tryptophan, tyrosine, proline, and methionine were able to significantly induce PhIP formation in a model system (phenylalanine + creatinine) after heating at 200 ℃ for 1 h. Contrary, Jones et al. (1988a) reported that tryptophan blended into a commercial sauce significantly inhibited mutagens formation (IQ and IQx type) in beef patties during the cooking, especially when tryptophan was spread directly on the top of the patties compared to mixing with the other ingredients before frying. Accordingly, mixtures of proline and tryptophan were also observed to complement each other's activity and enhanced inhibition of IQx-type mutagens formation (MeIQx and 7,8-DiMeIQx) in a reflux model system (Jones & Weisburger, 1988b). However, the exact mechanism behind the inhibitory effect of AAs is still not fully understood and there has not been much research conducted on the values of AAs as potential inhibitors against PhIP. Kataoka et al. (2012) found that AAs could reduce PhIP through forming PhIP-AA adducts in a dry heat treatment, however, a recent study performed by Zamora, Alcón, and Hidalgo (2013) suggested that reactive carbonyl compounds generated from AAs significantly affected the PhIP formation. Thus, the mechanism other than PhIP-AA adducts formation could be responsible for PhIP inhibition. In view of PhIP formation mechanism, it has been well reported that PhIP is formed from a direct condensation reaction of creatinine with an intermediate Maillard product phenylacetaldehyde (PheAce), rather than with the free radical intermediates (pyrazine or pyridine cation radicals), followed by cyclization and dehydration (Alaejos & Afonso, 2011;

Murkovic, 2004). Therefore, scavenging of PheAce may prevent PhIP formation during Maillard reaction, leading to an inhibitory effect.

To our best knowledge, this is the first time to study the role of AAs on the formation of PhIP in liquid model systems containing glucose, creatinine, and phenylalanine as essential precursors. We hypothesized that inhibition of PhIP was related to the reaction activity between PheAce and AAs, and this interaction could be considered as an alternative PhIP inhibition mechanism, "PheAce-scavenging mechanism", explaining the inhibitory effects of AAs reported in previous researches. Therefore, the aims of this study were to evaluate the effects of 8 essential and 2 non-essential AAs on PhIP formation in Maillard model systems and to elucidate the involved inhibition mechanism. The contribution of each AAs to the browning formation and Maillard-type volatile compounds formation, concentrated on pyrazines derivatives, were also investigated.

MATERIALS AND METHODS

Chemicals and reagents

HCAs standards 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), 2-amino-3-methylimidazo[4,5-f]quinoxaline (IQx), 2-amino-3-methylimidazo[4,5-f]quinolone (IQ), 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx), and 2-amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline (4,8-DiMeIQx) were purchased from Toronto Research Chemicals, Inc. (Ontario, Canada). L-tryptophan (98%), L-lysine (99%), L-phenylalanine (98%), L-leucine (98%), L-isoleucine (98%), L-proline (99%), L-methionine (99.5%), L-threonine (98%), L-valine (99.5%), L-aspartic acid (98%) (chemical structure and abbreviated name of these amino acids are provided in Appendix A, Figure A-1), D-glucose (99.5%), creatinine, diethylene

glycol, triethylamine, diethyl ether (GC grade), and internal standard n-decanal (≥ 98%, GC grade) were purchased from the Sigma-Aldrich (St. Louis, MO, USA). Toluene (GC grade), the internal standard, acetonitrile (HPLC grade), n-hexane (GC grade), acetic acid (HPLC grade), methanol (HPLC grade), and syringe filters (nylon, 0.2 µm) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Deionized water was prepared using a Sybron/Barnstead PCS filtration unit (Barnstead/Thermolyne, Inc, Dubuque, IA, USA).

Model systems preparation

Model systems used to study PhIP formation are consist of a reducing sugar (typically glucose), creatinine, and phenylalanine to mimic precursors present in the meat (Skog and Jägerstad, 1991). Many studies have assessed how the precursor amounts affect PhIP formation in a model system, and it was found that when glucose was added in half molar quantity of the other 2 precursors, the highest amount of PhIP was formed (Skog and Jägerstad, 1991). The naturally occurring sugar in meat is approximately half molar amount to the AAs and creatinine (Gibis, 2016), therefore, the molar ratio used in this study was 0.5:1:1 to maximize the PhIP formation in the control group.

Two different model systems were prepared, with 60 model systems combinations in each. In the first part of experiments, the effect of each AAs on formation of PhIP was tested in a PhIP-producing model system consisted of 0.02 mmol D-glucose (Glc), 0.04 mmol creatinine (Cre), and 0.04 mmol phenylalanine (Phe) mixed with 6 levels of 0.4 M AAs solutions: 0 (Control), 13.75, 27.5, 55, 110, and 220 µL, to get a final molar ratio of Glc:Cre:Phe:AA at 0.5:1:1:0, 0.5:1:1:0.125, 0.5:1:1:0.25, 0.5:1:1:0.5, 0.5:1:1:1, and 0.5:1:1:2. The second part of experiments examined the reactivity of each AAs to the total PheAce in PheAce-containing model system, with 0.04 mmol PheAce was used as precursor reacted with 0 (Control), 13.75,

27.5, 55, 110, and 220 µL of 0.4 M AAs to get a final molar ratio of PheAce:AA at 1:0, 1:0.125, 1:0.25, 1:0.5, 1:1, and 1:2. The total assay volume was brought to 770 µL using diethylene glycol (DEG):water (90:10, v/v) and all assays were prepared in triplicates. All model systems were run in a 1 mL conical reaction vial placed inside a cylindrical brass vessel with 2 screw caps on the top/bottom side, and 4 holes (1 cm x 1cm) on the cylindrical body to increase the heat transfer efficiency to the vial. The reaction mixtures were vortexed vigorously prior to the heat treatment to ensure all solids were well suspended into the liquid medium and vessels were hand tightened placed into an oven (HP 5890; Agilent Technologies Inc., Santa Clara, CA, USA). Oven temperature and internal reaction temperature were monitored using a USB-TC thermocouple and Tracer DAQPro software (Measurement Computing™, Waltham, MA, USA). After heating at 180 °C for 1 h, the vessels were removed and immediately cooled on ice for 5 min prior to further analysis.

Identification and quantification of HCAs

The reaction solution was diluted 1:20 in methanol, syringe filtered (Nylon, 0.2 µm) and submitted to HPLC analysis. The HCAs analysis was carried out on an HP 1050 Series HPLC (Agilent Technologies, Santa Clara, CA, USA) coupled with an HP 1050 series diode array UV-visible detector and an HP 1046A fluorescence detector. The HCAs separation was achieved using a "core-shell" reversed-phase Kintex EVO C18 column (150 x 4.6 mm, 5 µm, 100 Å) from Phenomenex Inc. (Torrance, CA, USA). The column temperature was set at 40 °C. A linear HPLC gradient at a flow rate of 1 mL/min was used according to Puangsombat, Gadgil, Houser, Hunt, and Smith (2012) with a slight modification. Mobile phase A was 0.01 M triethylamine in water (pH 3.6), and mobile phase B was acetonitrile. The initial mobile phase percentage started with 10 % mobile phase B, held constant for 4 min, and then linearly increased to 25 % over a

course of 30 min. The percentage was then decreased to 10 % in 5 min followed by a post-run of 5 min to equilibrate the column. The fluorescence detector settings for PhIP detection were set at 229 nm (excitation) and 437 nm (emission), while the UV detection wavelength for IQx, IQ, MeIQx, and 4,8-DiMeIQx was at 258 nm (Jackson & Hargraves, 1995). Data were analyzed using ChemStation software Rev A.10.02. The identification of HCAs was achieved by comparing retention times to external standards, and HCAs content was quantified using the calibration curve prepared using HCAs standards. The coefficient of determination (R^2) for HCAs standard curve was 0.998 (PhIP), 0.9999 (IQx), 0.9997 (IQ), 0.9999 (MeIQx), and 0.9999 (4,8-DiMeIQx). The limit of detection (LOD) and limit of quantification (LOQ) of PhIP was 0.1 ng/g and 0.3 ng/g.

Liquid/liquid extraction

PheAce was extracted and analyzed according to Cheng et al. (2008) with some modifications. Briefly, 200 µL of the reaction mixture were transferred into a salinized vial and mixed with 10 µL of internal standard solution (5 mg of n-decanal in 1 mL of 2:1 mixture of diethyl ether in hexane). The mixture was then subsequently extracted with 500 µL of diethyl ether: hexane (2:1, v/v) by vigorously shaking for 1 min (2X), and centrifuged at 300 g for 2 min. A 100 µL of the supernatant (top layer) from the two-phase solution was diluted with 900 µL of diethyl ether: hexane (2:1, v/v) to obtain 1:10 dilution and 80 µL of the diluted solution was finally transferred into a GC vial subjected to GC-MS analysis for the residual PheAce level. A model reaction of Glc:Cre:Phe:AA (0.5:1:1:1) was selected to evaluate the volatile compounds formation. The reaction mixture (200 µL) spiked with 10 µL of internal standard toluene (5 µL/5 mL diethyl ether: hexane (2:1, v/v)) was directly extracted by using 500 µL of diethyl ether:

hexane (2:1, v/v) twice and subsequently centrifuged at 300 g for 2 min, with an 80 µL of the supernatant was collected and subjected to GC-MS analysis.

GC-MS analysis

All samples were analyzed using an HP 5890 GC series II Plus gas chromatograph (Agilent Technology Inc., Santa Clara, CA, U.S.A) connected to an HP 5972 mass spectral detector operating in an electron impact (EI) ion source. Compounds separations were performed on an HP-5 MS column (60 m × 0.25 mm I.D. × 0.25 µm 5%-phenyl)-methylpolysiloxane film thickness) (Agilent Technology Inc., Santa Clara, CA, U.S.A) with a constant flow rate at 1 mL/min (high-purity helium, carrier gas). The analysis parameters for PheAce were as follows: 1 µL of sample was injected in a splitless mode with the inlet temperature set at 300 ℃. The oven temperature program was set at 50 ℃ for 2 min, ramped to 180 ℃ at a rate of 10 ℃/min, and then increased to 280 ℃ at a rate of 25 ℃/min, and held for 5 min. The MS detector and transfer line temperature were 180 ℃ and 300 ℃. Selective ion monitoring (SIM) mode was used to quantify PheAce and n-decanal (IS) based on peak area using the molecular ion(s) and three product ion(s) (full scan mass spectrum are presented in Appendix A, Figure A-5). The limit of detection (LOD) and limit of quantification (LOQ) of PheAce was 0.007 µg/g and 0.02 µg/g. The analysis parameters for volatile compounds were as follow: 1 µL of sample was injected in a splitless mode; inlet temperature was set at 250 ℃; the oven temperature program was 5 min at 40 ℃, increased to 110 ℃ at 3℃/min, then ramped to 150 ℃ at 4 ℃/min, and then raised to 220 ℃ at 10 ℃/min, and held for 5 min. The MS full scan operating condition was as follow: MS detector and transfer line temperature were 180 ℃ and 300 ℃. The volatile compounds were identified by comparing the mass spectra to the NIST Mass Spectral Library (2008), with a minimum library match score of > 90%.

Browning

The browning intensity of the reaction was monitored at a wavelength of 420 nm (Karim and Smith 2015) using a Genesys 10vis spectrophotometer (Thermo Electron Corporation, Waltham, MA, USA).

STATISTICAL ANALYSIS

The experiment was performed using randomized complete block design (RCBD) with days serving as blocks. The statistical significance was compared between the control and treatment groups by one-way analysis of variance (ANOVA) using SAS version 9.3 (2013, SAS Inst. Inc., Cary, NC, USA). The multiple pairwise comparisons among the treatment levels were made with Tukey-Kramer adjustments to determine the difference, with the level of significance (*P*-value) defined as $P < 0.05$.

RESULTS AND DISCUSSION

Effect of AAs on the formation of PhIP and PheAce in PhIP-producing (Glc:Cre:Phe:AA) reaction mixtures

The PhIP quantification in the control and treatment groups are summarized in Table 7. The results showed that adding different AAs into PhIP-producing model affected PhIP formation after heat treatment at 180 °C for 1 h. When the molar ratio of AA to Glc:Cre:Phe (0.5:1:1) was 2 (the highest level tested), Trp, Lys, Pro, and Leu exhibited the most pronounced inhibitory effects. The PhIP content was reduced from 2788.1 ± 150.6 ng/g (control) to below of detection (LOD), 3270.8 ± 71.5 ng/g (control) to below of detection (LOD), 3154.9 ± 91.1 ng/g (control) to 195.8 ± 11.4 ng/g, and 2446.0 ± 39.4 ng/g (control) to 319.4 ± 11.8 ng/g, which represent 100%, 100%, 90%, and 87% of PhIP inhibition ($P < 0.05$), respectively. These results were in

good agreement with previously reported results of Jones and Weisburger (1988b), where Pro and Trp were found to have inhibitory effects on the mutagen formation in Maillard model system. Furthermore, Met, Val, and Ile also showed significant reductions ($P < 0.05$) by 79%, 70%, and 55% in the PhIP concentrations at 0.5:1:1:2 molar ratio, but were less effective than Trp, Lys, Pro, and Leu. Compared to the control, when the molar ratios were ≥ 0.5:1:1:1 (Thr) and ≥ 0.5:1:1:0.5 (Phe), PhIP content at the highest level was significantly reduced ($P < 0.05$) by 41% and 30%, but with no significant difference ($P > 0.05$) observed at lower molar ratios. It was observed in this experiment that poor solubility of Thr at a molar ratio of 0.5:1:1:1 and 0.5:1:1:2 (110 µL and 220 µL of added Thr) after heat treatment may be used to explain why there was a significant increase ($P < 0.05$) in PhIP reduction at higher levels. Interestingly, with the presence of Asp, PhIP formation seemed to be slightly promoted by 9% from 2591.0 ± 68.3 ng/g (control) to 2836.4 ± 121.0 ng/g at 0.5:1:1:2 ($P < 0.05$). Overall, the inhibitory efficiency of AAs on PhIP formation at the highest concentration was ranked as: Trp = Lys > Pro > Leu > Met > Val > Ile > Thr > Phe > Asp.

Unlike our results, Zamora and others (2013) found adding Trp, Pro, Met, Thr, Asp, Cys, and Ser into aqueous model system containing Cre and Phe significantly increased the amount of PhIP produced upon heating, whereas the other AAs including Lys, Leu, Ile, and Val did not show any significant effects. Different types of precursors or reaction mediums used in a model system may account for the observed conflicting results. It is notable that our model system was prepared in DEG:Water rather than sodium phosphate (pH 8) (Zamora, Alcón, & Hidalgo, 2013), and was composed of Glc, Cre, and Phe, in which Glc was considered to be an essential precursor in this study. In addition, no other HCAs (IQx, IQ, MeIQx, and 4,8-DiMeIQx) were formed in the PhIP-producing model system with adding AAs while PhIP was predominantly

reduced, showing that the tested AAs may not lead to new or more mutagens formation and AAs can, therefore, be anticipated to possess inhibitory effects against a wide spectrum of HCAs in the Maillard reaction.

Table 7. Effect of AA concentrations on PhIP formation in Glc:Cre:Phe:AA model heated at 180 °C for 1 h.

AA added	Molar ratio of AAs relative to Glc:Cre:Phe (0.5:1:1)					
	PhIP Conc. (ng/g)[1]					
	0	0.125	0.25	0.5	1	2
Trp	2788.1 ± 150.6[a]	1909.1 ± 144.3[b]	1362.1 ± 42.8[c]	735.4 ± 90.8[d]	ND	ND
Lys	3270.8 ± 71.5[a]	1837.2 ± 64.5[b]	1042.2 ± 51.5[c]	903.4 ± 22.5[c]	259.9 ± 9.3[d]	ND
Pro	3154.9 ± 91.1[a]	2237.7 ± 105.6[b]	1855.2 ± 126.7[b]	1284.5 ± 79.0[c]	441.4 ± 17.1[d]	195.8 ± 11.4[d]
Leu	2446.0 ± 39.4[a]	2220.0 ± 117.6[ab]	2154.6 ± 110.1[b]	1536.8 ± 88.9[c]	481.2 ± 25.7[d]	319.4 ± 11.8[d]
Met	3368.1 ± 160.4[a]	3049.8 ± 159.9[a]	2484.6 ± 191.2[b]	1757.8 ± 36.8[c]	1023.4 ± 103.8[d]	691.0 ± 24.5[d]
Val	3207.3 ± 38.2[a]	2688.0 ± 55.8[b]	2139.4 ± 55.5[c]	1969.8 ± 148.9[c]	1481.4 ± 55.5[d]	959.6 ± 78.0[e]
Ile	2489.7 ± 113.5[a]	2328.6 ± 48.5[ab]	2174.9 ± 105.7[b]	1858.7 ± 73.5[c]	1400.1 ± 73.5[d]	1122.2 ± 51.4[e]
Thr	2737.8 ± 144.5[a]	2666.1 ± 102.7[a]	2627.0 ± 86.2[a]	2645.7 ± 57.4[a]	1891.0 ± 125.4[b]	1607.9 ± 78.4[b]
Phe	2614.8 ± 158.3[a]	2507.9 ± 123.2[a]	2298.7 ± 35.8[ab]	2066.4 ± 82.4[bc]	2005.7 ± 94.9[bc]	1823.5 ± 96.7[c]
Asp	2591.0 ± 68.3[a]	2552.6 ± 176.2[a]	2444.6 ± 40.6[ab]	2629.4 ± 67.5[ab]	2812.6 ± 111.4[ab]	2836.4 ± 121.0[b]

[1] Values are means ± standard deviation (n=3).

[a-e] Means with different uppercase letter within the same row are significantly different ($P < 0.05$).

ND = below limit of detection (LOD).

In order to understand the effect of each AAs on the relationship between the PhIP and its intermediate PheAce formation, the concentration of PheAce from PhIP-producing model with and without added AAs after thermal treatment (180 ℃ for 1 h) was evaluated. As shown in Table 8, compared to the control, the added AAs significantly decreased the PheAce concentration ($P < 0.05$) at a molar ratio of 0.5:1:1:0.125, with the exception of Met and Asp. As the level of the assayed AAs increased, the percentage reductions on PheAce formation were also gradually increased. In particular, when Pro, Trp, Val, Lys, and Thr levels were increased to the highest molar ratio (0.5:1:1:2), the amount of PheAce was significantly decreased ($P < 0.05$) by 95%, 92%, 88%, 80%, and 79%, respectively, from control ranged from 10.0 ± 0.4 to 13.2 ± 1.0 μg/g to below of quantification (LOQ), 1.0 ± 0.2 μg/g, 1.2 ± 0.0 μg/g, 2.3 ± 0.1 μg/g, and 2.1 ± 0.2 μg/g. Leu, Met, Ile, and Phe were also capable of decreasing PheAce formation, but had a relatively lower reduction efficiency, with 77%, 77%, 65%, and 42% reduction ($P < 0.05$) was observed at 0.5:1:1:2. Furthermore, Asp did not have any effects on PheAce formation as no significant difference was observed between control and treatment groups among all levels ($P > 0.05$). It was worth pointing out that reduction trend of PheAce from the model system with presence of AA inhibitors (Table 8) has a good agreement with the PhIP's reduction trend from the same model system (Table 7), indicating a competitive effect of the added AAs on intermediate PheAce formation; therefore, PhIP inhibition mechanism may be related to the decreasing in PheAce formation in the PhIP formation pathway.

Table 8. Effect of AA concentrations on PheAce formation in Glc:Cre:Phe:AA model heated at 180 °C for 1 h.

AA added	Molar ratio of AAs relative to Glc:Cre:Phe (0.5:1:1)					
	PheAce Concentration (μg/g)[1]					
	0	0.125	0.25	0.5	1	2
Trp	13.2 ± 1.0[a]	10.6 ± 0.6[b]	6.4 ± 0.4[c]	4.3 ± 0.2[d]	1.7 ± 0.3[e]	1.0 ± 0.2[e]
Lys	10.9 ± 0.9[a]	5.8 ± 0.5[b]	3.9 ± 0.3[c]	2.7 ± 0.1[cd]	2.1 ± 0.3[d]	2.3 ± 0.1[d]
Pro	13.0 ± 0.4[a]	6.2 ± 0.2[b]	2.9 ± 0.2[c]	1.6 ± 0.2[d]	0.9 ± 0.1[e]	ND
Leu	11.2 ± 0.3[a]	9.9 ± 0.2[b]	9.0 ± 0.5[b]	6.7 ± 0.1[c]	3.4 ± 0.3[d]	2.6 ± 0.1[e]
Met	10.4 ± 0.7[a]	9.3 ± 0.7[a]	6.9 ± 0.3[b]	4.4 ± 0.0[c]	3.0 ± 0.0[d]	2.9 ± 0.2[d]
Val	10.0 ± 0.4[a]	7.9 ± 0.4[b]	6.4 ± 0.1[c]	4.2 ± 0.1[d]	1.9 ± 0.2[e]	1.2 ± 0.0[e]
Ile	10.6 ± 0.3[a]	8.9 ± 0.1[b]	7.7 ± 0.3[c]	4.4 ± 0.1[d]	2.9 ± 0.2[e]	3.7 ± 0.1[e]
Thr	10.4 ± 0.1[a]	8.6 ± 0.4[b]	7.7 ± 0.2[b]	5.9 ± 0.4[c]	2.7 ± 0.2[d]	2.1 ± 0.2[d]
Phe	11.7 ± 0.6[a]	11.0 ± 0.9[b]	10.8 ± 0.5[c]	9.4 ± 0.3[d]	7.1 ± 0.6[e]	6.8 ± 0.8[e]
Asp	12.4 ± 0.8[a]	12.8 ± 0.0[a]	12.6 ± 0.5[a]	12.6 ± 0.9[a]	13.1 ± 0.4[a]	13.2 ± 0.5[a]

[1]Values are means ± standard deviation (n=3).

[a-e] Means with different uppercase letter within the same row are significantly different ($P < 0.05$).

ND = below limit of qualification (LOQ).

In general, during the Maillard reaction, AAs are thermally degraded throughout Strecker degradation into different by-products, in which Strecker aldehydes and α-keto acids showed the most active reactivity due to their reactive carbonyl group (Yaylayan, 2003). Zamora et al. (2013) investigated the effect of different α-keto acids generated from AAs on the PhIP formation in aqueous model reaction (Phe: Cre = 1:1), and results revealed that most α-keto acids produced by their promoter AAs including Gly, Met, Phe, Ile, and Leu were reactive and prone to induce the Strecker degradation of Phe to form PhAce, and contributed significantly to PhIP

formation. However, it was not in accordance with results reported in our study. None of tested AAs showed a positive enhancement on PhIP formation, except for Asp. As the α-keto acids and carbonyl compounds generated from added AAs were not measured in our model systems, the relation between results reported here and the findings in previous papers (Zamora, Alcón & Hidalgo, 2013) is not clear, but the availability of Glc or reaction medium (DEG:water or methanol or buffer) may have a significant effect on both quantity and type of produced Maillard intermediates and/or degradation compounds of AAs, and hence, of PhIP formation. In addition, Hidalgo et al. (2013) simplified the model reaction with only Phe as a precursor to investigate the effects of AAs on PheAce formation. They reported that only addition of Ser and Cys could significantly impact the PheAce conversion from Phe in aqueous system heat-treated at 200 °C /1 h. Their results can be further inferred that the thermal decomposition compounds from most AAs were unable to either induce or suppress the conversion of Phe into PheAce, particularly for AAs with β-carbon substituted with methyl group and hydroxyl carbonyl groups, such as Thr and Asp. Linked to our study, it is reasonable to believe that the observed changes in the amount of PhIP along with PheAce formation with the presence of AAs should be considered as a consequence of other reactions involved, e.g., PheAce-AA or PhIP-AA adducts formation. According to studies done by Kataoka et al. (2010 and 2012), free PhIP can form protein adducts in cooked foods (fish, beef, chicken, pork, and egg) with cooking temperatures (100-300 °C) and times (2-10 min). Their later results also revealed that PhIP can bind with various AAs by addition of the nucleophilic group of AA to the C=C double bond of PhIP to form PhIP-AA adducts in either methanol/water solution or dry system, in which Trp, Pro, Leu, Ile, Met, Phe, Gly, and Ala showed the most reactive ability. Therefore, from the first part of our experiments, it can be hypothesized that PhIP-AA and/or PheAce-AA adducts formation could both directly

contribute to the reduction of PhIP when AAs were introduced into model systems, but scavenging of available PheAce after it was formed from Phe may be primarily responsible for PhIP reduction.

Browning

Browning of the model system was also investigated. From the results (Table 9), browning was observed in all combination treatments and most AAs had a significant relationship ($P < 0.05$) with browning development. Trp at the highest molar ratio was able to significantly ($P < 0.05$) induce the browning from 14.5 ± 0.1 (control) to 26.4 ± 1.3 after heating at 180 °C for 1 h. Other AAs including Lys, Pro, Leu, Val, Ile, and Thr showed a decrease in browning formation as increasing AA concentrations with a significant difference ($P < 0.05$) observed between the control and the highest concentration. Met and Asp showed constant browning formation throughout the treatment levels. However, the addition of Phe did not produce significant changes in browning formed in model systems ($P > 0.05$).

Though the Maillard browning formation was well studied in model systems, little research has evaluated the browning change when the different free AAs were introduced into the Maillard reaction containing precursors of Glc, Cre, and Phe. Many of previous research studies emphasized on the browning intensity changes with various sugar types/molar ratios or buffering reagents (Laroque, Inisan, Berger, Vouland, Dufossé, & Guérard, 2008; Bell, 1997). During the thermal treatment of AAs, different types of intermediate products are generated via glycation processes between the free amino group of AAs and sugar fragmentation in the Maillard reaction. And it is possible that some of those intermediate products might undergo a rapid conversion to the final brown compounds. For instance, Lys, Tyr (Ashoor & Zent, 1984), Trp (Fry & Stegink, 1982), and Ser, Thr (Piloty & Baltes, 1979), were reported to induce

browning formation in model systems. On the other hand, generation of some other intermediates may be less reactive to give browning with AAs, as found by Leu, Ile, Met, and Phe (Ashoor & Zent, 1984), Pro (Fry & Stegink, 1982), and Asp (Piloty & Baltes, 1979). It was also indicated that the acidic, hydroxyl, and SH-containing AAs induced less Maillard browning reaction (Friedman & Molnar-Perl, 1990; Ashoor & Zent, 1984). Linked to our study, the more intense browning found in Trp compared to control was likely due to the higher formation of intermediate compounds, or because of its complex structure (indole ring, two nitrogen), or its high reactivity favored the browning formation (Ashoor & Zent, 1984); while Ile, Leu, Val, Met, Phe, Thr, and Asp as the less reactive AAs displayed decreasing in browning. In addition, the reason for the least degree of browning observed in Lys, which was supposed to give a higher intensity of browning due to α- and Ɛ- amino groups was unclear; it might be because the change in the matrix pH or the fact that Lys was strongly thermal degraded during heating compared to Trp (Ajandouz & Puigserve, 1999). Labuza and Massaro (1990) reported that Trp generated more browning than Lys. Although the development of browning was not apparently changed by the addition of Met, Phe, and Asp in systems, most of other AAs seemed to significantly prevent or retard the formation of browning ($P < 0.05$). These results also suggested that the ratio of reducing sugar (Glc) to the added AAs or AA varieties significantly determine the rate of browning development in Maillard reaction.

Table 9. Browning results in Glc:Cre:Phe:AA model heated at 180 °C for 1 h.

AA added	Molar ratio of AAs relative to Glc:Cre:Phe (0.5:1:1)					
	Browning (420 nm)[1]					
	0	0.125	0.25	0.5	1	2
Trp	14.5 ± 0.1^{a}	17.6 ± 0.5^{a}	19.7 ± 0.8^{b}	22.4 ± 0.5^{c}	25.3 ± 1.0^{c}	26.4 ± 1.3^{d}
Lys	15.0 ± 0.2^{a}	12.0 ± 0.2^{b}	10.1 ± 0.4^{c}	8.7 ± 0.3^{d}	7.4 ± 0.3^{e}	6.6 ± 0.2^{e}
Pro	14.6 ± 0.5^{a}	14.1 ± 0.2^{ab}	13.5 ± 0.4^{abc}	12.8 ± 0.5^{d}	12.6 ± 0.2^{d}	12.0 ± 0.4^{d}
Leu	14.6 ± 0.6^{a}	13.8 ± 0.8^{ab}	14.0 ± 0.7^{ab}	11.6 ± 0.8^{b}	9.1 ± 0.7^{c}	7.8 ± 0.6^{c}
Met	12.7 ± 0.2^{a}	12.2 ± 0.1^{ab}	12.3 ± 0.3^{ab}	12.0 ± 0.1^{ab}	12.0 ± 0.2^{b}	12.3 ± 0.0^{b}
Val	12.3 ± 0.3^{a}	11.4 ± 0.5^{ab}	10.6 ± 0.6^{bc}	10.0 ± 0.3^{bc}	9.4 ± 0.3^{c}	7.7 ± 0.6^{d}
Ile	14.7 ± 0.1^{a}	14.2 ± 0.3^{ab}	13.5 ± 0.1^{b}	11.7 ± 0.3^{c}	9.8 ± 0.3^{d}	8.4 ± 0.3^{e}
Thr	15.1 ± 0.6^{a}	14.4 ± 0.2^{b}	14.0 ± 0.2^{c}	14.0 ± 0.1^{d}	13.0 ± 0.1^{e}	13.3 ± 0.1^{e}
Phe	13.4 ± 0.1^{a}	13.5 ± 0.3^{a}	12.7 ± 0.6^{a}	13.0 ± 0.6^{a}	12.4 ± 0.6^{a}	12.5 ± 0.2^{a}
Asp	14.7 ± 0.0^{a}	14.0 ± 0.2^{ab}	14.3 ± 0.4^{ab}	13.9 ± 0.4^{bc}	13.2 ± 0.2^{c}	13.4 ± 0.2^{c}

[1]Values are means ± standard deviation (n=3).

$^{a\text{-}e}$ Means with different uppercase letter within the same row are significantly different ($P < 0.05$).

Effects of AAs on PheAce formation in PheAce-containing (PheAce:AA) reaction mixtures

The scavenging activity of AAs toward PheAce was evaluated by monitoring the concentration of PheAce in PheAce-containing model with the presence of the same AAs previously used. Results are presented in Table 10. All AAs were capable of significantly reducing PheAce concentration by 93% to 98%, except of Asp, at the highest molar ratio (PheAce:AA = 1:2), with positive correlation observed between total PheAce content and AA concentrations ($P < 0.05$). At 1:0.25 molar ratio, the concentration of PheAce was greatly reduced from 1376.5 ± 71.9 μg/g in the control to 404.0 ± 35.3 μg/g, 1375.9 ± 154.6 μg/g in the control to 82.2 ± 4.1 μg/g, 1094.7 ± 70.9 μg/g in the control to 328.8 ± 28.3 μg/g, and 1138.9 ± 61.0 μg/g in the control to 639.5.0 ± 27.3 μg/g after adding Trp, Lys, Val, and Pro,

corresponding with 94%, 71%, 70%, and 41% reduction, respectively. This can be explained by the high reactivity of AAs toward the carbonyl group of PheAce. To the best of our knowledge, no study has been done to detect and quantitate the amount of PheAce in DEG:water system with the added AAs. The observed higher affinity of Trp was probably due to its indole-ring structure, which was in line with the previously reported articles. As described in Jones and Weisburger (1988c), Trp and the selected indole and indole-3-carboxaldehyde displayed similar inhibitory effects on mutagen formation and the various effectiveness was depending on the specific indoles. Furthermore, because of the chemical reactivity as it follows the electrophilicity of aldehyde with amines known as Schiff base formation, the substituent at the side chain of AAs such as two amino groups in Lys and pyrrolidine ring in Pro may show the analogous reactivity to PheAce to a different extent. Upon heating, the active carbonyl group of PheAce formed a covalent bond with indole-ring nitrogen and/or free amino of AAs to generate PheAce-AA and/or PheAce-AA-PheAce complexes, which caused the PheAce unavailable for aldol condensation with Cre, and hence, reduced the PhIP produced.

In addition, Ile, Thr, Leu, Phe, Asp, and Met had relatively less impacts on the PheAce (Table 10). It appeared that the percent change in PheAce content was increased from the control to 1:0.25 molar ratio with only 36%, 35%, 28%, 27%, 20%, and 15% reduction observed, and then sharply increased to 96%, 97%, 93%, 93%, 66%, and 98% at 1:2 molar ratio. This phenomenon could be explained by the low reactivity of AAs to carbonyl groups or it might be due to poor solubility of Thr, Phe, and Asp at higher molar ratios (1:1 and 1:2). Although the order of effectiveness was slightly different for some AAs observed in the PhIP-producing model, Trp and Lys were the most effective AAs and showed the highest reactivity to PheAce.

Table 10. PheAce values formed in PheAce:AA model heated at 180 °C for 1 h.

AA added	Molar ratio of AAs relative to PheAce					
	PheAce Concentration (μg/g)[1]					
	0	0.125	0.25	0.5	1	2
Trp	1376.5 ± 71.9[a]	759.0 ± 28.9[b]	404.0 ± 35.3[c]	83.9 ± 1.2[d]	36.6 ± 0.9[d]	36.3 ± 5.4[d]
Lys	1375.9 ± 154.6[a]	397.2 ± 17.7[b]	82.2 ± 4.1[c]	57.7 ± 3.7[c]	45.8 ± 3.3[c]	48.9 ± 2.4[c]
Pro	1138.9 ± 61.0[a]	723.0 ± 35.7[b]	639.5 ± 27.3[b]	252.6 ± 7.7[c]	35.3 ± 3.7[d]	24.0 ± 1.1[d]
Leu	1419.7 ± 34.0[a]	997.0 ± 53.6[b]	1024.3 ± 73.3[b]	436.2 ± 31.7[c]	63.7 ± 4.5[d]	92.7 ± 1.8[d]
Met	1098.2 ± 25.1[a]	1036.1 ± 38.9[ab]	933.9 ± 46.6[b]	746.5 ± 39.7[c]	33.0 ± 1.2[d]	47.7 ± 2.1[d]
Val	1094.7 ± 70.9[a]	770.8 ± 27.3[b]	328.2 ± 28.3[c]	117.9 ± 15.8[d]	27.0 ± 3.9[d]	27.7 ± 3.0[d]
Ile	1054.2 ± 101.9[a]	776.9 ± 45.5[b]	675.7 ± 40.8[b]	249.6 ± 9.9[c]	24.7 ± 2.0[d]	44.0 ± 3.1[d]
Thr	1486.9 ± 59.6[a]	1237.8 ± 51.9[b]	961.0 ± 50.3[c]	874.9 ± 51.2[d]	510.3 ± 29.0[e]	42.4 ± 2.8[e]
Phe	1245.8 ± 181.2[a]	937.0 ± 52.8[b]	914.2 ± 17.2[c]	264.3 ± 19.1[d]	85.9 ± 2.1[e]	81.3 ± 5.7[e]
Asp	1174.8 ± 50.5[a]	1138.0 ± 35.8[b]	971.8 ± 48.3[c]	813.0 ± 29.7[d]	552.0 ± 41.3[e]	408.8 ± 20.6[e]

[1]Values are means ± standard deviation (n=3).

[a-e] Means with different uppercase letter within the same row are significantly different ($P < 0.05$).

The relationship between PheAce-scavenging ability of AAs and PhIP inhibition

To further confirm our hypothesis and elucidate the relationship between the effects of AAs on PhIP formation and their PheAce-scavenging ability in the model systems, the amount of PhIP reduced in the presence of AAs in PhIP-producing model system (Glc:Cre:Phe:AA) was plotted against the amount PheAce reduced by the same AAs in PheAce model system (PheAce:AA). A strong correlation was observed with Trp (R^2=0.9181), Lys (R^2=0.863), Pro (R^2=0.9476), Leu (R^2=0.8743), Met (R^2=0.8915), Val (R^2=0.8225), Thr (R^2=0.8311), and Phe (R^2=0.8416), and a moderate correlation was observed with Ile (R^2=0.6427) indicating that the inhibitory ability of Trp, Pro, Lys, Leu, Met, Val, Ile, Thr, and Phe was highly correlated with their reactive scavenging ability to PheAce. The AA that exhibited the least relationship between reduction of PhIP and reactivity to PheAce was Asp (R^2=0.4263). Thus, the observed positive and linear correlation supported that the scavenging reactivity between PheAce and AAs may explain, in part, the noted strong inhibitory activity of AAs on PhIP. A postulated pathway for the inhibitory activity of Lys on PhIP formation is illustrated in Figure 8. Because of the aldol condensation between Cre and PheAce as a critical step for PhIP formation was halted by forming of the PheAce-Lys adducts in Maillard reaction, the content of the end product PhIP was reduced in a dose-dependent fashion.

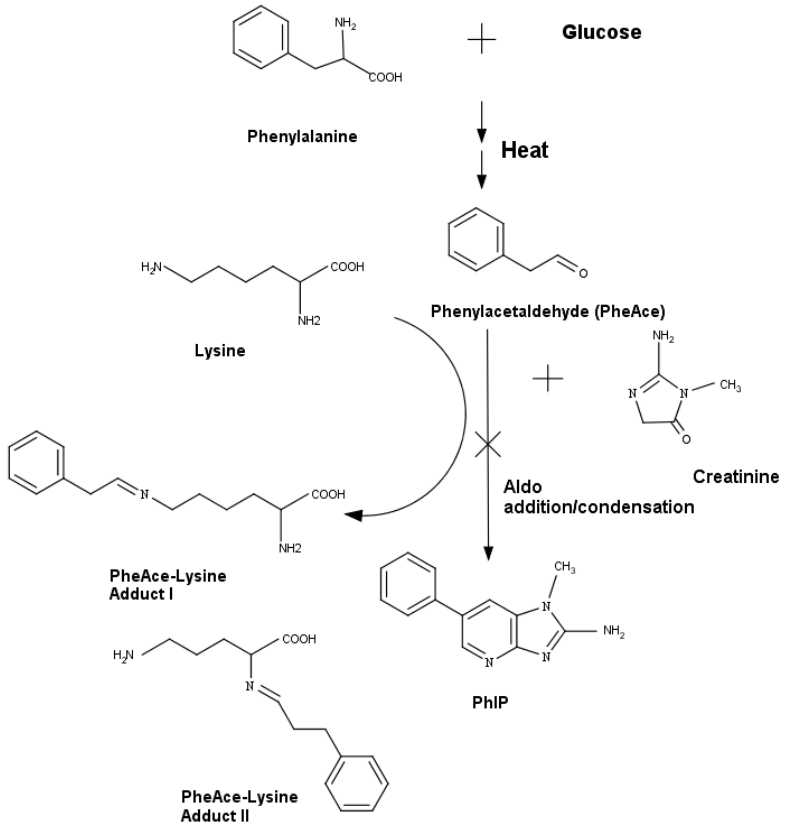


Figure 8. Postulated pathway for inhibitory activity of Lys on PhIP formation.

Volatile compounds analysis

There is far less information available on the formation of pyrazines derivatives from the Maillard reaction composed of 2 different AAs, and considering the competition may happen between Phe and added AA for the sugar fragments, the final part of this study was to investigate this reaction. The PhIP-producing model containing Glc:Cre:Phe with a fixed molar ratio (0.5:1:1) was selected as a reference, and 10 different AAs were individually added into the model system at 0.5:1:1:1 to compete with Phe for the reaction of Glc in the volatile compounds generation. Results are reported in Figure 9. Pyrazines are a group of heterocyclic nitrogen-containing compounds and are believed to contribute significantly to the unique roasted, nutty,

and smoky aroma in cooked foods (Maga & Sizer, 1973). Analysis of pyrazines formation with different AAs present enables to evaluate the involvement of each AAs, and it is useful to predict change in the aroma profile when an AA is added into foods. Many previous studies indicated there is more than one pathway of pyrazines formation during the Maillard reaction, but the most accepted mechanism is involved in a condensation reaction between a reducing sugar and AAs. The self-condensation and/or condensation with other α-aminoketones compounds (such as 1-aminopropanal) generated from the Strecker degradation of AAs, and α-dicarbonyl compounds of sugar could yield different varieties of alkylpyrazines (Adams, Polizzi, Boekel, & Kimpe, 2008; Mottram, 2007). Therefore, different alkylpyrazines distribution generated from the added AAs to sugar fragments would be highly affected by the chemical structure of the AAs reactant. From Figure 9, it can be derived that compared to the reaction system containing Phe only, Trp and Lys are the greatest inhibitors among the tested AAs, yielding the significant reduction ($p < 0.05$) in the amount of pyrazine, ethyl- and methyl-pyrazine ranged from 75 to 100%, 53 to 100%, and 53 to 57% observed; while both Thr and Asp are the smallest contributors to ethyl- and methyl-pyrazine formation, showing no significant impacts ($P > 0.05$).

In addition, 4 different alkylpyrazines were identified from the model system. The 2,5(6)-diemthylpyrazine, 2,3-diemthylpyrazine, and 3-ethyl-2,5-diemthylpyrazine were important nutty, roasted, and earthy aromas generated in Maillard reaction with odor thresholds range of 1140 – 2430, 590-1170, and 2.4-4.8 ng/L in air (Wagner, Czerny, Bielohradsky, & Grosch, 1999). For 2,5(6)-diemthylpyrazine, it was found that addition of Lys significantly promoted ($P < 0.05$) its formation by 573% enhancement upon reacting with Glc:Cre:Phe, followed with Thr (169%), Ile (111%), Val (87%), Trp (79%), Leu (69%), and Asp (63%). But, there was no significance difference observed in Pro, Met, and Phe ($P > 0.05$). The formation of 2,3-diemthylpyrazine was

significantly induced with Ile, Pro, Val, and Lys by 33%, 29%, 16%, and 14% increment; however, Trp and Phe showed negative effects with 20% decrease observed ($P < 0.05$). Figure 9 also shows that Trp, Lys, Met, Thr, or Asp produces 3-ethyl-2,5-diemthylpyrazine, and Leu specifically produces 3-butyl-2,5-dimethylpyrazine.

The variety of AAs and their relative reactivity to Glc highly determine the pyrazines derivatives formed, and this may be why a great distribution of alkypyrazines was observed when each AAs was added. It appears that Lys, Thr, Val, or Ile can produce a synergistic effect with Phe and Glc on alkylpyrazines formation. Lys is generally considered as a highly reactive species in Maillard reaction due to the presence of nucleophilic Ɛ-amino group on the side chain (Ashoor & Zent, 1984). And it is also known that the sugar degradation and dehydration are catalyzed by amino groups (Mottram, 2007). With α- and Ɛ-amino groups, Lys can easily initiate sugar fragmentation and participate in the Strecker degradation including decarboxylation and/or deamination with generation of a group of new compounds (e.g., Strecker aldehyde and α-aminoketones), which contributed greatly to the alkylpyrazines formation (Mottram, 2007). Adams et al. (2008) observed similar results, showing the highest yield in the alkylpyrazines content was produced when Lys was added into the model system. On the other hand, the high alkylpyrazines production of Thr, Val, and Ile was probably due to the flexible side chain or nonpolar property leading to a faster deamination rate, although Piloty and Baltes (1979) reported that the hydroxyl and basic AAs were more reactive to α-dicarbonyl compounds than nonpolar and acidic AAs. Surprisingly, when the added Phe was in equimolar ratio to other precursors, it performed as an inhibitor to the alkylpyrazines formation. In other words, the benzene group of the side chain in Phe may make it harder to transfer the α-amino group to α-diketones during the Strecker degradation. This result can further confirm that it is important to

maintain the optimal ratio among different precursors in Maillard reaction, and an extra amount of precursor (in the case of Phe) negatively impact on volatile compounds formation. Moreover, some higher molecular weight alkylpyrazines were found to be specifically generated in certain types of AA. Overall, the most contribution to the total yields of pyrazines formation from the model reactions at a molar ratio of 0.5:1:1:1 was ranked as follows: Leu > Thr > Lys > Asp > Met > Ile > Pro > Trp > Phe, showing that the variety and quantity of the pyrazines derivatives formation is highly depending on the reactivity and type of added AAs.

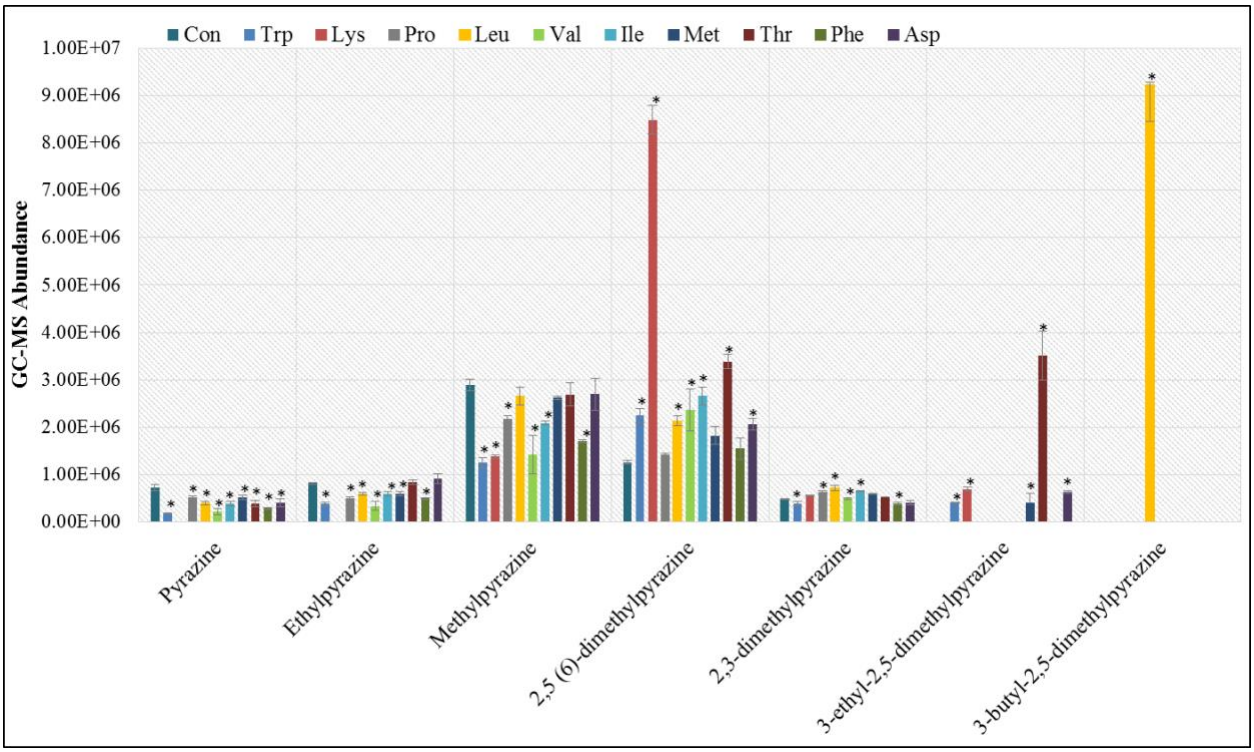


Figure 9. GC-MS chromatogram of volatile compounds detected in reaction mixture containing Glc:Cre:Phe:AA at 0.5:1:1:1 molar ratio heated at 180 °C for 1 h. Bars with an asterisk have a significant difference compared to control ($P < 0.05$).

CONCLUSION

This study provides a basic understanding of mutagenic PhIP inhibition by added AAs during the Maillard reaction. The results showed that adding AAs into a model system can significantly inhibit the PhIP formation and the inhibitory effects of each AAs were highly depended on their chemical structure as well as concentration. The establishment of 2 Maillard model systems has offered strong evidence that the PhIP inhibition with presence of AAs was highly correlated to the scavenging of PheAce intermediate. The possible PheAce-AA adducts formation are responsible for the reduction in PhIP formed. Trp and Lys showed the strongest inhibition of both PhIP and PheAce. Future studies will be aiming at isolation and future characterization of those adducts. This paper demonstrated that, most essential and non-essential AAs besides their nutritional properties and aroma/browning enhanced functionality could also be used as an effective strategy to prevent PhIP formation and help in maintaining the high quality of foods and compensate for the loss of nutritive values during processing.

REFERENCES

Adams, A., Polizzi, V., Boekel, v., T, & Kimpe, d., N (2008). Formation of Pyrazines and a Novel Pyrrole in Maillard Model Systems of 1,3-Dihydroxyacetone and 2-Oxopropanal. *Journal of Agricultural and Food Chemistry*, *56*(6), 2147-2153.

Ajandouz, E.H., & Puigserver, A. (1999). Nonenzymatic Browning Reaction of Essential Amino Acids: Effect of pH on Caramelization and Maillard Reaction Kinetics. *Journal of Agricultural and Food Chemistry*, *47*(5), 1786-1793.

Alaejos, M. S., & Afonso, A. M. (2011). Factors That Affect the Content of Heterocyclic Aromatic Amines in Foods. *Comprehensive Reviews in Food Science and Food Safety*, *10*(2), 52-108.

Ashoor, S.H., Dietrich, R.A., Chu, F.S., & Pariza, M.W. (1980). Proline enhances mutagen formation in ground beef during frying. *Life Science*, *26*, 1801-1805.

Ashoor, S.H., & Zent, J.B. 1984. Maillard browning of common amino acids and sugars. *Journal of Food Science*, *49*, 1206-1207.

Bell, L.N. (1997). Maillard reaction as influenced by buffer type and concentration. *Food Chemistry*, *59*(1), 143-147.

Belitz, H.D., Grosch, W., & Schieberle, P. (2004). Food Additives. In O.R. Fennema (Eds), *Food Chemistry* (pp. 433-435). New York: Springer.

Cheng, K.W., Wong, C.C., Cho, C.K., Chun, I.K., Sze, K.H., Lo, C., et al. (2008). Trapping of Phenylacetaldehyde as a Key Mechanism Responsible for Naringenin's Inhibitory Activity in Mutagenic 2-Amino-1-methyl-6-phenylimidazo [4,5-b]Pyridine Formation. *Chemical Research in Toxicology*, *21*(10), 2026-2034.

Damašiusa, J., Venskutonisa, P.R., Ferracaneb, R., & Foglianob, V. (2011). Assessment of the influence of some spice extracts on the formation of heterocyclic amines in meat. *Food Chemistry, 126*(1), 149-156.

DeBruin, L.S., Martos, P.A., Josephy, P.D. (2001). Detection of PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine) in the milk of healthy women. *Chemical research in toxicology, 1*4(11), 1523-1528.

Felton, J.S, Knize, M.G., Shen, N.H., Andresen, B.D., & Bjeldanes, L.F. (1986). Identification of the mutagens in cooked beef. *Environmental Health perspectives*. *67*, 17-24.

Friedman, M., & Molnar-Perl, I. (1990). Inhibition of browning by sulfur amino acids. 1. Heated amino acid-glucose systems. *Journal of Agricultural and Food Chemistry, 38*(8), 1642-1647.

Fry, L.K., Stegink, L.D. (1982). Formation of Maillard reaction products in parenteral alimentation solutions. *Journal of Nutrtion, 112*(8), 1631-1637.

Gerhardsson de Verdier, M., Hagman, U., Peters, R.K., Steineck, G., & Overvik, E. (1991). Meat, cooking methods and colorectal cancer: a case-referent study in Stockholm. *International Journal of Cancer, 49*(4), 520-525.

Hidalgo, F.J., Alcón, E., & Zamora, R. (2013). Cysteine- and serine-thermal degradation products promote the formation of Strecker aldehydes in amino acid reaction mixtures. *Food Research International, 54*(2), 1394-1399.

IARC (1993). Some naturally occurring substances: food items and constituents, heterocyclic aromatic amines and mycotoxins. *IARC Monographs on the Evaluation of Carcinogenic Risk to Humans*. *56*, 165-195.

Jackson, L.S., & Hargraves, W.A. (1995). Effects of time and temperature on the formation of MeIQx and DiMeIQx in a model system containing threonine, glucose, and creatine. *Journal of Agricultural and Food Chemistry*, *43*(6), 1678–1684.

Jones, R.C., & Weisburger, J. (1988a). L-Tryptophan inhibits formation of mutagens during cooking of meat and in laboratory models. *Mutation Research*, *206*(3), 343-349.

Jones, R.C., & Weisburger, J. (1988b). Inhibition of aminoimidazo-quinoxaline-type and aminoimidazol-4-one type mutagen formation in liquid-reflux models by the amino acids L-proline and/or L-tryptophan. *Environmental and Molecular Mutagenesis*, *11*, 509-514.

Jones, R.C., & Weisburger, J. (1988c). Inhibition of aminoimidazo-quinoxaline-type and aminoimidazol-4-one-type mutagen formation in liquid reflux models by L-tryptophan and other selected indoles. *Japanese Journal of Cancer Research*, *79*, 222-230.

Karim, F., & Smith, J.S. (2015). Detection and Quantification of 4(5)-Methylimidazole in Cooked Meat. *Journal of Food Science*, *80*(2), T465-471.

Kataoka, H., Miyake, M., Nishioka, S., Matsumoto, T., Saito, K., & Mitani, K. (2010). Formation of protein adducts of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine in cooked foods. *Molecular Nutrition & Food Research*, *5*(7), 1039-1048.

Kataoka, H., Miyake, M., Saito, K., & Mitani, K. (2012). Formation of heterocyclic amine–amino acid adducts by heating in a model system. *Food Chemistry*, *130*(3), 725-729.

Labuza, T.P., & Massaro, S.A. (1990). Browning and amino acids losses in model total parenteral nutrition systems with specific reference to cysteine. *Journal of Food Science*, *55*(3), 821-826.

Laroque, D., Inisan, C., Berger, C., Vouland, E., Dufossé, L., & Guérard, F. (2008). Kinetic study on the Maillard reaction. Consideration of sugar reactivity. *Food Chemistry*, 111(4), 1032-1042.

Maga, J.A., & Sizer, C.E. (1973). Pyrazines in foods. A review. *Journal of Agricultural and Food Chemistry*, *21*, 22-30.

Mottram, D.S. (2007). The Maillard Reaction: Source of Flavour in Thermally Processed Foods. In Anonymous *Flavours and Fragrances* (pp. 269-283). Berlin, Heidelberg: Springer Berlin Heidelberg.

Murkovic, M. (2004). Formation of heterocyclic aromatic amines in model systems. *Journal of Chromatography B*, *802*(1), 3-10.

National Toxicology Program (NTP), U.S. Dept. of Health and Human Services, Public Health Service. (2011). Heterocyclic amines (selected). *Report on Carcinogens*, 12th ed., 220-224.

Piloty, M., & Baltes, W. (1979). Investigations on the reaction of amino acids with α-dicarbonyl compounds. I. Reactivity of amino acids in the reaction with α-dicarbonyl compounds. *Zeitschrift Fur Lebensmittel-Untersuchung Und -Forschung*. *168*, 368-373.

Puangsombat, K., Gadgil, P., Houser, T.A., Hunt, M.C., & Smith, J.S. (2012). Occurrence of heterocyclic amines in cooked meat products. *Meat Science*, *90*(3), 739-746.

Smith, J. S., Ameri, F., & Gadgil, P. (2008). Effect of Marinades on the Formation of Heterocyclic Amines in Grilled Beef Steaks. *Journal of Food Science*, *73*(6), T105.

Sugimura, T., Wakabayashi, K., Nakagama, H., & Nagao, M. (2004). Heterocyclic amines: Mutagens/carcinogens produced during cooking of meat and fish. *Cancer Science*, *95*(4), 290-299.

Tsen, S.Y., Ameri, F., & Smith, J.S. (2006). Effect of rosemary extracts on the reduction of heterocyclic amines in beef patties. *Journal of Food Science, 71*(8), C469-C473.

Vitaglione, P., & Fogliano, V. (2004). Use of antioxidants to minimize the human health risk associated to mutagenic/carcinogenic heterocyclic amines in food. *Journal of Chromatography B Analytical Technologies in the Biomedical and Life Science, 802*(1), 189-199.

Wagner, R., Czerny, M., Bielohradsky, J., & Grosch, W. (1999). Structure-odour-activity relationships of alkylpyrazines. *Z Lebensm Unters Forsch A. 208*, 308-316.

Wakabayashi, K., Ushiyama, H., Takahash, I.M., Nukaya, H., Kim, S.B., Hirose, M., et al. (1993). Exposure to heterocyclic amines. *Environmental Health Perspectives, 99*, 129-134.

Yaylayan, V. A. (2003). Recent Advances in the Chemistry of Strecker Degradation and Amadori Rearrangement: Implications to Aroma and Color Formation. *Food Science and Technology Research*, *9*(1), 1-6.

Zamora, R., Alcón, E., & Hidalgo, F.J. (2013). Effect of amino acids on the formation of 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP) in creatinine/phenylalanine and creatinine/phenylalanine/4-oxo-2-nonenal reaction mixtures. *Food Chemistry*, *141*(4), 4240-4245.

Zeng, M., He, Z., Zheng, Z., Qin, F., Tao, G., Zhang, S., et al. (2014). Effect of six Chinese spices on heterocyclic amine profiles in roast beef patties by ultra-performance liquid chromatography-tandem mass spectrometry and principal component analysis. *Journal of Agricultural and Food Science*, *62*(40), 9908-9915.

Zöchling, S., Murkovic, M., & Pfannhauser, W. (2002). Effects of industrially produced flavours with pro- and antioxidative properties on the formation of the heterocyclic amine PhIP in a model system. *Journal of Biochemical and Biophysical Methods*, *53*(1), 37-44.

CHAPTER 3 - DETERMINATION OF HETEROCYCLIC AMINES IN MEAT MATRICES USING ENHANCED MATRIX REMOVAL - LIPID EXTRACTION AND LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY[1]

ABSTRACT

A simple, fast, and efficient method, "Enhanced Matrix Removal of Lipids" (EMR-Lipid), was proposed, optimized, and validated for identifying five polar heterocyclic amines (HCAs) in meat samples that ranged from high-protein (beef and chicken) to high-fat (pork bacon) matrices. The protocol involves an initial solid-liquid phase extraction followed by a rapid dispersive solid-phase extraction using EMR-Lipid sorbents and salting-out partitioning. Acetonitrile containing formic acid at two levels (1% and 2%) efficiently extracted HCAs from different meat matrices. Liquid chromatography-tandem mass spectrometry with selective reaction monitoring mode was developed for qualitative and quantitative analysis. The highest MS/MS responses and better peak separation of analytes were achieved by adjusting mobile phases to pH 3.0 with instrumental detection limits between 0.01and 0.05 ng/mL. Good linearity of standard curves was obtained in both pure solvents and post-spiked meat extracts between 0.5 and 50.0 ng/mL. The validation results showed good precision and sensitivity for detecting HCAs in spiked meat samples. Satisfactory recoveries of four HCAs were achieved: 65 to 111% in beef, 71 to 106% in bacon, and 42 to 77% in chicken. Matrix effects were also assessed and showed less than -20% of ion suppression in bacon extract, while a medium to high signal suppression was observed in beef (-37 to -55%) and chicken (-28% to -52%). This optimized EMR-Lipid method provides acceptable results and advantages for determining trace level HCAs in complex meat matrices.

1- Reproduced with permission from: Linghu, Z., Karim, F., & Smith, J. S. 2019. Determination of heterocyclic amines in meat matrices using enhanced matrix removal - lipid extraction and liquid chromatography-tandem mass spectrometry. J. Food Sci. 84 (x): xxx-xxx. (in press).

INTRODUCTION

Heterocyclic amines (HCAs) are in a large group of organic compounds that have been extensively studied since their mutagenic properties were discovered in smoke condensates of broiled fish and beef steak (Ames, Mccann, & Yamasaki, 1975). These compounds are known to form in cooked protein-rich foods, such as meat and seafood, during frying, baking, roasting, grilling, and broiling (Herraiz, 2004; Knize, Cunningham, Griffin, Jones, & Felton, 1994; Murray, Lynch, Knize, & Gooderham, 1993). To date, more than 25 HCAs have been isolated and characterized in different cooked foods (Cheng, Chen, & Wang, 2006). HCAs can be classified as polar or nonpolar HCAs based on their different chemical properties (Alaejos & Afonso, 2011). Formation of polar HCAs (also known as thermic HCAs) has received much more attention than non-polar HCAs in research because they can be formed by the Maillard reaction of free amino acids, creatine/creatinine, and reducing sugars at temperatures from 100 to 300 °C (Cheng et al., 2006). In addition to the formation mechanism, toxicity studies have showed that many polar HCAs induce tumors in a wide range of organs and tissues in mice and rats (Sugimura, Wakabayashi, Nakagama, & Nagao, 2004). Some epidemiological studies have also revealed that high exposure to these HCAs through cooked meats may lead to an increased risk of colorectal, breast, and pancreatic cancers in humans (Cross et al., 2005; Felton et al., 2007). Several polar HCAs, including 2-Amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), 2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) and 2-Amino-1-methyl-6-phenyl-imidazo[4,5-b]pyridine (PhIP) have been classified as possible human carcinogens (Group 2B) with 2-Amino-3-methylimidazo [4,5-f]quinoline (IQ) as a probable human carcinogen (Group 2A) by the International Agency for Research on Cancer (IARC, 1993).

Given the high complexity of meat matrices and the diverse chemical properties of HCAs at the ppb level (Gibis, 2016), detecting and quantifying trace-level polar HCAs present a tough analytical challenge. The traditional extraction and purification method for HCA analysis uses a protocol developed by Gross and Grüter (1992), which involves liquid-liquid extraction (LLE) followed by tandem solid-phase extraction (SPE) to achieve sample matrix clean up and HCA extraction from complex sample matrices. As a standard method, the major disadvantages of the Gross method are that the procedures are complicated and extremely time-consuming; it requires intensive clean up steps and uses large amounts of dichloromethane and ethyl acetate, both toxic chemicals. Although modifications of the Gross method have reduced the extraction and clean up time (Messner & Murkovic, 2004; Turesky & Le Marchand, 2011), the recovery rates of polar HCAs within meat matrices remained at 25 to 80% because most of the analytes were lost during the diatomaceous earth extraction (Pais & Knize, 2000; Puangsombat, Gadgil, Houser, Hunt, & Smith, 2012). We must develop a simple, fast, and efficient analytical method that, with high sensitivity and reproducibility, can identify polar HCAs within meat products.

In recent years, one advanced extraction method–the Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) method–has been widely used to analyze multi-class pesticides and veterinary drugs in produce because it provides high matrix clean up with high analyte recovery (Castillo, González, & Miralles, 2011; Lehotay, Mastovská, & Lightfield, 2005; Park et al., 2012; Rejczak & Tuzimski, 2015). QuEChERS has been accepted as an AOAC Official Method (Lehotay et al., 2005) and European Standard Method (2007). The QuEChERS protocol was initially developed by Anastassiades, Lehotay, Štajnbaher, and Schenck (2003), and sample preparation used a salting-out extraction followed by dispersive solid-phase extraction (dSPE). By taking advantage of porous dSPE sorbents, such as primary secondary amines (PSA),

octadecylsilane (C18), zirconia-containing sorbents, and/or graphitized carbon black (GCB) (Lehotay, 2011), QuEChERS provides high capacity and selectivity as it removes lipids, polar pigments, sugars, and other substances that interfere with the matrix, leaving the target analytes in solution for subsequent analysis (Castillo et al., 2011; Cunha et al., 2007; He et al., 2017; Lehotay et al., 2010). The final clean up extracts can be directly injected into analytical instruments like liquid chromatography-tandem mass spectrometry (LC-MS/MS) and gas chromatography-mass spectrometry (GC-MS) for identification and quantification of analytes (Lehotay et al., 2010). Its simplicity, flexibility, the speed of analysis, as well as efficiency in removing unwanted food matrices with little consumption of solvents make QuEChERS methodologies promising and reliable extraction methods for analyzing HCAs in meat products.

Hsiao, Chen, and Kao (2017) used QuEChERS methodologies in HCA analysis, optimizing a QuEChERS method for extracting HCAs from fried pork fiber using PSA, C18, and $MgSO_4$ as matrix clean up sorbents. Good analyte recoveries ranged from 58.9 to 117.4%, and extraction precision was less than 30%. However, significant ion suppression of HCAs was found in LC-MS/MS detection, suggesting that the sorbents had insufficient capacity to remove co-extractive lipophilic substances (mostly lipids) from high-fat meat matrices. More recently, a novel Enhanced Matrix Removal (EMR)-Lipid dSPE sorbent was introduced into Agilent Bond Elut QuEChERS protocol to remove matrices in fatty samples. The EMR-Lipid dSPE sorbents and polish salts for the trace-level analytes have been used to detect veterinary drugs, polycyclic aromatic hydrocarbons, and pesticides in complex food samples using this method (Zhao & Lucas, 2015a, b; Lucas & Zhao, 2015).

To our knowledge, our research is the first to apply EMR-Lipid to prepare samples for determining of low concentrations of polar HCAs in meat samples. The objective of this study

was to evaluate how well the EMR-Lipid method worked in analysis of five HCAs in three different types of meat (i.e., beef, chicken, and bacon): IQ, IQx, MeIQx, 4,8-DiMeIQx, and PhIP. The target HCA analytes were chosen because of their high levels in the cooked meats and their carcinogenicity/mutagenicity, thus posing threats to consumers (Puangsombat et al., 2012). Table 11 summarizes their chemical properties. In addition, our research allowed us to validate this optimized method, determining its linearity, precision, matrix effects, recovery, instrument detection limits (IDLs), limits of detection (LODs), and limits of quantification (LOQs). The HCAs were quantified using the selective reaction monitoring (SRM) acquisition mode with LC-MS/MS.

Table 11. Abbreviations, chemical names, molecular weights, and properties of polar HCAs (Gibis, 2016).

Abbreviations and Chemical Names			**Molecular Weights (g/mol) and Properties** [1]
Quinolines	IQ	2-Amino-3-methylimidazo[4,5-f]quinoline	198.2, Polar, p*Ka* 5.86
Quinoxalines	IQx	2-Amino-3-methylimidazo[4,5-*f*]quinoxaline	199.3, Polar, p*Ka* 1.96
	MeIQx	2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline	213.3, Polar, p*Ka* 2.32
	4,8-DiMeIQx	2-Amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline	227.3, Polar, p*Ka* 2.56
Pyridines	PhIP	2-Amino-1-methyl-6-phenyl-imidazo[4,5-b]pyridine	224.3, Polar, p*Ka* 7.72

[1] At 25 °C.

MATERIALS AND METHODS

Chemicals and reagents

HCA standards 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), 2-amino-3-methylimidazo[4,5-f]quinoxaline (IQx), 2-amino-3-methylimidazo[4,5-f]quinolone (IQ), 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline (4,8-DiMeIQx), and 2-amino-3,4,7,8-tetramethyl-imidazo [4,5-f]quinoxaline (4,7,8-TriMeIQx) as the internal standard (IS) were purchased from Toronto Research Chemicals, Inc. (Ontario, Canada). Optima® LC-MS grade acetonitrile methanol, acetic acid, formic acid, ammonium acetate, and syringe filters (nylon, 0.2 µm) were purchased from Fisher Scientific (Fair Lawn, N.J., U.S.A.). Deionized water was prepared using a Sybron/Barnstead PCS filtration unit (Barnstead/Thermolyne, Inc, Dubuque, Iowa, U.S.A.). Bond Elut Enhanced Matrix Removal (EMR) - Lipid was purchased from Agilent Technologies (Santa Clara, U.S.A.) included Lipid dSPE (p/n 5982-1010) and Lipid-Polish (p/n 5982-010).

Chemical analyses

The crude protein measurement of each raw meat sample was determined with LECO FP-2000 protein analyzer (Leco Corp, St Joseph, Miss., U.S.A.) according to AOAC Int Method 992.15 (AOAC International, 2002). Fat and moisture content were determined according to AOAC Int method 2008.06 (AOAC International, 2002) using rapid a CEM Smart Trac system (CEM Corporation, Matthews, N.C., U.S.A.).

Meat samples preparation and cooking procedure

Fresh ground beef (7% fat), chicken (breast without skin), and pork bacon were purchased from grocery stores in Manhattan, Kans., U.S.A. These meat products were selected because of their different protein and fat compositions. Ground beef (80 g) was first formed into

beef patties with a Petri dish (4.52 x 2 cm) and then fried in a thermostat-controlled, Teflon-coated, square frying pan at 230 °C (446 °F) for 7.5 min on each side. Chicken breast (310-315 g) were sliced into 105-125 g, 3-cm thick portions, and then placed in the frying pan at 230 °C (446 °F) for 7.5 min per side. Bacon slices (16-20 g) were fried at 172 °C (341 °F) for 3 min per side. The frying pan surface temperature was monitored with a flat surface thermometer probe. All meats were cooked one sample at a time, and 2 replications were performed on the same day. The crusts on the beef and chicken (approximately 2 mm) were cut off from cooled samples, while the bacon sample was treated as a whole. All cooked samples were frozen in liquid nitrogen, ground in a commercial Waring blender (Fisher, Pittsburgh, PA, U.S.A.), and stored at -18 °C until the analysis on the following day.

EMR-Lipid extraction procedure

In this study, the EMR-Lipid extraction procedure was carried out according to the manufacture protocol (Zhao & Luca, 2015b) with some modifications. The extraction solvents acetonitrile (MeCN) at concentrations of 0, 1, and 2% and methanol (MeOH) with formic acid (FA) at the same concentrations were tested. Briefly, 3.0 g of finely ground meat crusts (beef, chicken, and bacon) were extracted in a 50 mL polypropylene tube with 15 mL of extraction solvent for an extraction ratio of 1:5 (w/v). The tube was then shaken vigorously on a mechanical shaker for 30 min and centrifuged for 10 min at 4 °C and 10,000 rpm. Afterwards, 12 mL of the supernatant was collected and cleaned up using EMR-Lipid dSPE sorbents (1 g) pre-activated with 5 mL of ammonium acetate buffer (5 mM). The tube was immediately hand shaken vigorously for 2 min to disperse the sample and then, after centrifugation for 10 min at 4 °C and 10,000 rpm, a polishing step (known as salting-out extraction/partitioning) was carried out by transferring 10 mL of cleaned up supernatant into a 50-mL polypropylene tube containing 2 g

EMR-Lipid Polish salts (NaCl: MgSO4, 1:4, w/w). After the tube was vigorously hand shaken for 2 min, it was centrifuged again for 10 min at 4 ℃ and 10,000 rpm, and the final extract (1 mL of top layer) was collected and dried under nitrogen. Finally, the extract was reconstituted in an autosampler vial with 100 µL of MeOH and 10 µL of IS at 50 ng/mL, filtered through a 0.2 µm nylon filter, and analyzed using the LC-MS/MS system per the following description.

LC-MS/MS analysis

A Finnigan Surveyor Plus HPLC system (Thermo Finnigan, San Jose, Calif., U.S.A.) equipped with the Finnigan TSQ Quantum UltraTM EMR triple quadrupole mass spectrometer (Thermo Finnigan, San Jose, Calif., U.S.A.) was used for this study. For chromatographic separation of HCAs, we used a Supelco Ascentis® Express C18 column (15 cm × 2.1 mm, 2.7 µm, 80 Å) (Sigma-Aldrich, Bellefonte, PA, U.S.A.). The column temperature was set at 30 ºC, and a 10 µL sample was injected into the HPLC. Chromatographic separation and ionization efficiencies for HCAs were studied using mobile phases containing (A) 10 mM ammonium acetate buffer with different pHs of 6.0, 5.0, 4.0, 3.5, and 3.0 adjusted with acetic acid, and (B) MeCN. A linear gradient was used following Shin, Strasburg, and Ustunol (2003) with a slight modification as follows: 0-6 min, 90% A and 10% B; 6-12 min, 75% A and 25% B; 12-17 min, 70% A and 30% B; and 17-24 min, 90% A and 10% B. The column was then re-equilibrated with 90% A and 10% B for 1 min before the next injection. The flow rate was 0.25 mL/min, and the total run time was 25 min. The MS/MS system was operated with an electron spray ionization (ESI) source in positive mode; the optimized ion source parameters were as follows: spray voltage, 4.0 kV; N_2 sheath gas flow, 50 arbitrary units; auxiliary gas flow rate, 15 arbitrary units; and capillary temperature, 275 ºC.

Compounds were optimized by directly injecting each HCA standard solution into ESI source using a syringe pump at a flow rate of 10 µL/min. The SRM transition (precursor ion and product ion) of each HCA was optimized under different collision energies to achieve the highest intensity; Table 12 shows the optimized SRM parameters (full scan product ion mass spectrum of analytes are presented in Appendix A, Figure A- 9-10). Xcalibur version 2.0.7 software was used for instrument control and data acquisition.

Table 12. Optimized SRM parameters for HCAs with LC-MS/MS.

Analytes	Precursor Ion (m/z) $[M+H]^+$	Product Ion (m/z)	Collision Energy (eV)	Polarity
IQx	200	185	20	Positive
IQ	199	184	22	Positive
MeIQx	214	199	20	Positive
4,8-DiMeIQx	228	213	20	Positive
PhIP	225	210	25	Positive
4,7,8-TriMeIQx (IS)	242	227	25	Positive

Method validation

Linearity and calibration curve. The HCA standard mixed solutions containing IQ, IQx, MeIQx, 4,8-DiMeIQx, and PhIP were prepared at five concentration levels (0.5, 1.0, 5.0, 10.0, and 50.0 ng/mL) in methanol, and each standard was mixed with IS at a fixed concentration of 50 ng/mL. After analysis with LC-MS/MS using SRM mode, each standard curve was prepared by plotting the concentration against its peak area. The coefficient of determination (R^2) and slope of calibration curves were determined using the linear regression equation.

Matrix effects. To evaluate the impact of the matrix components on mass detection of the analytes in ESI (+), meat extracts after EMR-Lipid clean up were post-spiked with HCA

standards at concentrations of 0.5, 1.0, 5.0, 10.0, and 50.0 ng/mL. After LC-MS/MS analysis, matrix-matched calibration curves for each meat sample were obtained, and linearity was assessed using the linear regression equation. Matrix effect (%) was calculated using the following equation (Hsiao et al., 2017):

Matrix effects (%) = [(slope of matrix-matched calibration curve / slope of standard solvent calibration curve) -1] × 100%

IDLs, LODs, and LOQs. To evaluate the sensitivity of the current LC-MS/MS method in SRM scan mode, IDLs were determined using the linear regression analysis (Evard, Kruve, & Leito, 2016). In this analysis, IDL values were calculated as $3.3 \times S_y/b$, where b is the slope and S_y is the standard deviation of the y-intercept of calibration curves prepared for the HCA standards at five concentrations (0.025 to 0.5 ng/mL, n = 3). In different meat extracts, the method LODs and LOQs were also determined using the linear regression analysis. Blank meat extracts with no HCAs were prepared and spiked with all five HCA standards at concentrations of 0.05, 0.1, 0.25, 0.5, and 1.0 ng/mL (n = 3). After LC-MS/MS-SRM analysis, meat matrix-matched calibration curves were made and the LOD and LOQ levels were calculated as $3.3 \times S_y/b$ and $10 \times S_y/b$, respectively, where b is the slope and S_y is the standard deviation of the y-intercepts of linear regression lines.

Precision. The intra- and inter-day precision of the method was validated by analyzing spiked beef samples at 1.0 ng/g of HCA standard mixtures and calculating the relative standard deviation (%RSD) for the analysis of meat extract within the same day (n = 9) and then for three consecutive days (n = 3).

Recovery. The recovery was determined by spiking each meat sample (beef, chicken, and bacon) with a low (10.0 ng/g) and high (50.0 ng/g) concentration of HCA standard mixtures (n =

3 at each level) before extracting using the optimized EMR-Lipid method. Recovery (%) of each HCA was calculated based on the equation:

Recovery (%) = (HCA concentration in the spiked sample – HCA concentration in the original sample) / amount of HCA added ×100%

STATISTICAL ANALYSIS

All experiments were performed in triplicate, and the data were analyzed by one-way analysis of variance (ANOVA) using SAS version 9.3 (2013, SAS Inst. Inc., Cary, NC, U.S.A.). The mean value comparisons were done using Tukey-Kramer adjustments to determine any differences with the level of significance (*P*-value) defined as $P < 0.05$.

RESULTS AND DISCUSSION

Composition analysis

Table 13 summarizes the chemical composition of raw meat products. Total fat and protein differed significantly among the three tested meat samples ($P < 0.05$). To be specific, the chicken had the most total protein (22.88%) followed by beef (20.04%) and bacon (9.51%). The total fat content was highest for bacon (42.60%), 5 to 16 times the fat in beef (9.59%) and chicken (2.70%). The moisture levels of raw meat samples ranged from 40.07% to 73.81% ($P < 0.05$). These results indicated that meat matrices could vary greatly depending on their different compositions. These meat products may serve as excellent representatives of high-protein and high-fat sample matrices for evaluating the EMR-Lipid protocol.

Table 13. Chemical compositions of three raw meat samples. Values are means ± standard deviation.

Meat type	Moisture (%)	Fat (%)	Protein (%)
Beef	69.65 ± 0.14[a]	9.59 ± 0.09[a]	20.04 ± 0.21[a]
Chicken	73.81 ± 0.00[b]	2.70 ± 0.01[b]	22.88 ± 0.02[a]
Bacon	40.07 ± 0.09[c]	42.60 ± 0.09[c]	9.51 ± 1.54[b]

[a-c] Means with a different upper case letter within the same column are significantly different ($P < 0.05$).

Optimization of extraction solvents in EMR-Lipid

Using the EMR-Lipid method has not been described in HCA analysis. Because published protocols and/or application notes provided by manufacturers differ from each other due to the different chemical properties of target analytes and sample matrices (Dias, Cutillas, Lozano, Pizzutti, & Fernández-Alba, 2016; Zhao & Lucas, 2015 a, b; Lucas & Zhao, 2015), using the EMR-Lipid method for polar HCA analysis was quite a challenge in meat matrices. After preliminary studies, 3 g of well-homogenized meat sample was used as a test sample and the extraction solvent volume was optimized to 15 mL to achieve the optimal sample—an organic ratio of 1:5 (w/v). Maximizing the sample surface areas greatly improved extraction efficiency while reducing sample material needed. Moreover, different extraction parameters that could affect extraction efficiency and sample matrix clean up, such as type of extraction solvent and acid modifier, were also optimized for the three different meat matrices. Typically, MeCN is the most commonly used extraction solvent in the EMR-Lipid protocol. Because it has a wide polarity range, a broad group of polar compounds can be easily extracted from each matrix,

while lipids, proteins, and other lipophilic co-extractives are not trapped because they have limited solubility (Anastassiades et al., 2003; Rejczak & Tuzimski, 2015). Upon adding polish salts, MeCN allows separation from the water phase more easily than other solvents (i.e., acetone and ethyl acetate) during the salting-out step. More defined phase separation occurs in this step as more target analytes are forced into the organic phase from water, thus leading to high analyte recovery and minimizing interference from other compounds. Thus, the matrix effect with MeCN is relatively low (Rejczak & Tuzimski, 2015). In addition, MeOH is often used to extract polar compounds in conventional solid-liquid extraction and shows great selectivity for polar HCA extraction and purification in tandem with the SPE method (Gross & Grüter, 1992). Therefore, MeCN and MeOH were both selected as extraction solvents and tested in this study. Yan et al. (2014) mentioned that organic extraction solvent with pH < 3.0 shows great solubility of polar HCAs. In QuEChERS methodologies, using an acidified extraction solvent is often an advantage because it can enhance the efficiency of protein precipitation (Rejczak & Tuzimski, 2015; Zhao, Lucas, Long, Richter, & Stevens, 2018). Taking this into account, FA with its good LC-MS/MS compatibility, was chosen as an acid modifier (Zhao & Lucas, 2015b).

The effects of extraction solvents with FA at different percentages (0, 1, and 2%, v/v) on HCA recovery were examined by comparing the total peak area obtained from LC-MS/MS. Figure 10 illustrates the total HCA extraction expressed as peak area units from the three tested meat matrices using MeCN with FA. The major HCAs extracted from three tested meat samples were MeIQx, 4,8-DiMeIQx, and PhIP, with no IQ and IQx detected; which has been confirmed by many previous studies (Pais & Knize, 2000; Puangsombat et al., 2012). Statistical analysis showed that adding FA to MeCN-based extraction solvent had no significant effect on total HCA extraction from meat matrices ($P > 0.05$). This is probably because MeCN with 0% FA has a pH

of 4.45, less than many HCAs' p*Ka* values ranging from1.96 to 7.72, and therefore, can ionize most HCAs within the sample matrix. Also, the small variation in pH from 0% FA (pH 4.45), 1% FA (pH 1.98), to 2% FA (pH 0.65) in MeCN does not substantially improve the ionization efficiency of HCAs in MeCN solvent. However, HCA extraction was still improved by adding acid. As Figure 10 shows, total peak areas (n = 3) from MeIQx, 4,8-DiMeIQx, and PhIP extracted from beef were slightly higher when 1% FA was used in MeCN compared with 0% and 2% FA. Using 2% FA in MeCN was more effective in extracting the total HCAs from chicken and bacon than 0% and 1% FA. Chicken contained the most protein (22.88%) and bacon had the most fat (42.6%). Hsiao et al. (2017) reported similar results using MeCN with 1% FA in a QuEChERS protocol, with significantly higher HCA recovery in roast duck meat compared to solvents such as acetone and ethyl acetate. ***In the case of MeOH extraction***, please note that adding polish salts ($NaCl/MgSO_4$) did not form a water-MeOH partition in the final extracts probably because MeOH is too miscible with water to be easily separated, suggesting that methanol might not be compatible with the EMR-Lipid protocol. The final MeOH extract was also very viscous after methanol was removed under nitrogen, appearing to form a gel. For these reasons, we did not analyze the extracts for acidic methanol in this study.

Overall, the results demonstrated that the EMR-Lipid protocol using MeCN as the extraction solvent could successfully extract the most representative polar HCAs (MeIQx, 4,8-DiMeIQx, and PhIP) from pan-fried beef, chicken, and bacon samples. In the subsequent validation studies, 1% FA was selected as the solvent modifier in MeCN for beef extraction, while chicken and bacon samples used the higher level of 2% FA in MeCN for extraction.

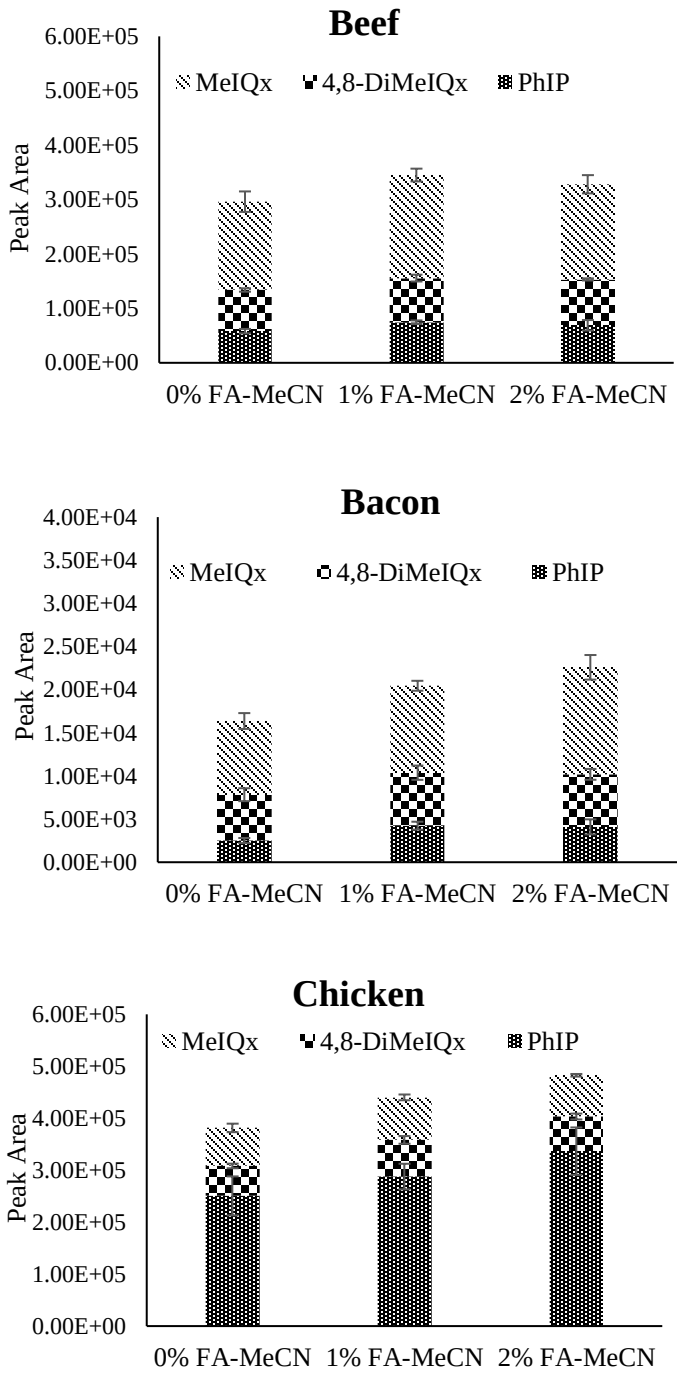


Figure 10. Total HCA extraction (peak areas) from three different meat matrices using MeCN with FA at different percentages (0, 1, and 2%, v/v) during the EMR-Lipid extraction. Errors bars represent the SD. Values with a same upper case are not significantly different ($P > 0.05$).

Optimization of LC-MS/MS Conditions

The LC separation of HCAs in extract has usually been carried out using C18 column (Linghu, Karim, & Smith, 2017; Ni, McNaughton, LeMaster, Sinha, & Turesky, 2008; Yan et al., 2014), and mobile phases consisting of a mixture of MeCN and water with ammonium acetate buffer are also commonly used in reports on the LC-MS/MS methods because of its satisfactory separation and high resolution of HCAs (Barceló-Barrachina et al., 2006; Ni et al., 2008; Shin et al., 2003). In general, mass detection using ESI (+) requires analytes to be ionized in the mobile phases to obtain the highest sensitivity in MS detector. To enhance the ionization process, the acidic mobile phase is used with pH less than the p*Ka* values of compounds, although low pH is not ideal for chromatographic separation on C18 column because retention times of the analytes can be affected and considerably shortened (Barceló-Barrachina et al., 2006).

During method optimization, given the properties of HCAs with a wide pKa range from 1.96 to 7.72, we chose five different mobile phase pH levels (6.0, 5.0, 4.0, 3.5, and 3.0) to evaluate the separation, retention, and MS signal intensity of HCAs. To properly detect and quantify HCAs, the SRM scan mode was used because of its selectivity and sensitivity (Sanz Alaejos, Ayala, González, & Afonso, 2008). Considering that an extremely high concentration of ammonium salt would affect MS performance, ammonium acetate buffer at a low concentration of 10 mM was used. The mobile phases flow rate was optimized at 0.25 µL/min and gradient elution program was adopted from the previous paper by Shin et al. (2003). Figure 11 shows the LC/ESI (+)-MS/MS chromatograms of all five HCAs obtained at different mobile phase pHs (6.0, 5.0, 4.0, 3.5, and 3.0) and Table 14 summarizes the MS/MS response (peak areas) of each HCAs. When decreasing the mobile phase pH from 6.0 to 5.0, the peak area of each HCA

increased significantly by 11 to 31% ($P < 0.05$). Polar HCAs have 2-amino-imidazole moiety with nitrogen atoms (N-) would be mostly deprotonated at a pH higher than their p*Ka* values; thus, at high pH, non-ionized HCAs gave low responses as noted at pH 6.0. However, decreasing the mobile phase pH from 5.0 to 4.0 and from 4.0 to 3.5 did not significantly affect MS/MS responses for HCAs ($P > 0.05$); while the peak intensity of IQ was increased significantly at pH 3.5 ($P < 0.05$). The highest signal responses of all five HCAs were observed when mobile phase pH was dropped to 3.0, where IQx, MeIQx, and 4,8-DiMeIQx showed a significant signal enhancement compared to pH 3.5 ($P < 0.05$). Presumably, in the positive ion detection, lowering the mobile phase pH by 2 units below the p*Ka* values of HCAs can cause 99.5% of the analytes to be ionized ($^{+}$NH-), which may explain why IQx, IQ, MeIQx, and 4,8-DiMeIQx with p*Ka* ranged from 1.96 to 5.86 had the highest MS/MS intensity at pH 3.0; conversely, more than 99.5% of HCAs will be non-ionized (N-) in mobile phases where the pH is 2 units higher than their p*Ka* values, resulting in low MS/MS intensity. In addition, variations in mobile phase pH did not change the elution order of HCAs on the C18 column, but the retention time was increased with the increasing of pH from 3.0 to 6.0 (Table 14, Figure 11). The separation of less-polar PhIP was better than that with polar IQx, IQ, MeIQx, and 4,8-DiMeIQx under any acidic conditions; compared with high pH, the peak separation of IQ and MeIQx was improved at a low pH level between 3.5 and 3.0 (Figure 11). Hsiao et al. (2017) also evaluated the separation of HCAs using different mobile phase pHs and found that ammonium acetate (10 mM) mobile phase at pH 2.9 resulted in better peak separation of HCAs on C18 column. Linked to the MS intensity results, the mobile phase at pH 3.0 was ultimately selected for simultaneous detection of five polar HCAs in the final method.

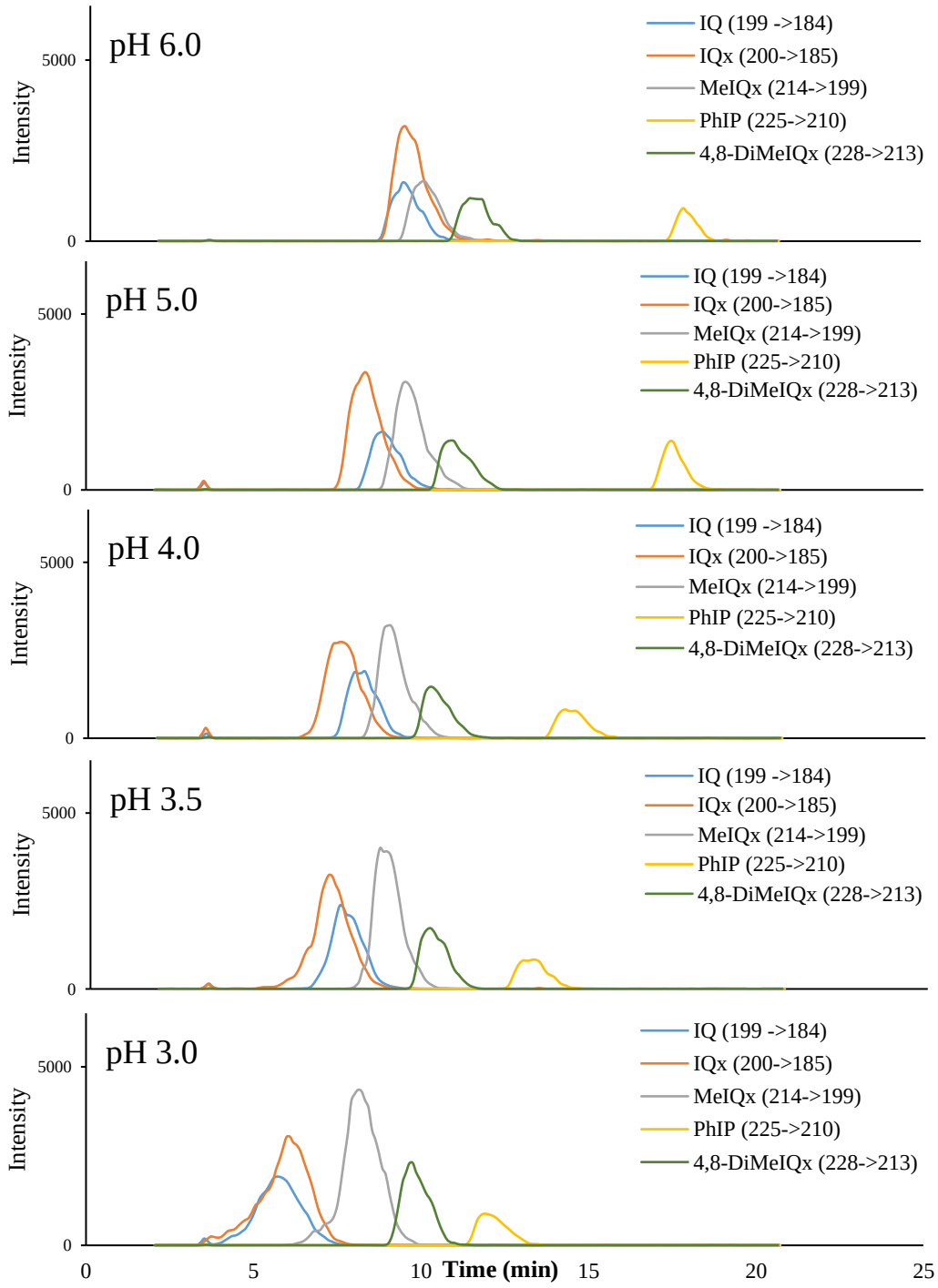


Figure 11. LC (ESI+)-MS/MS chromatogram (SRM mode) of a HCA mixed standard (5 ng/mL) separated at different mobile phase pHs (6.0, 5.0, 4.0, 3.5, and 3.0). The SRM transitions (precursor → product ion) for detection and quantification are indicated.

Table 14. Effects of mobile phase pHs on retention time and signal response (peak area units × 10^5) of HCA standard solution (5 ng/mL) by LC-MS/MS in SRM mode.

Analyte	pH 6.0		pH 5.0		pH 4.0		pH 3.5		pH 3.0	
	RT (min)	Peak Area[1]	RT (min)	Peak Area[1]	RT (min)	Peak Area[1]	RT(min)	Peak Area[1]	RT(min)	Peak Area[1]
IQx	9.20	1.85±0.09[a]	8.35	2.15±0.06[b]	7.64	2.20±0.09[b]	7.18	2.38±0.09[b]	6.12	2.74±0.12[c]
IQ	9.77	0.91±0.01[a]	8.93	1.07±0.03[b]	8.16	1.18±0.06[b]	7.42	1.54±0.06[c]	5.94	1.70±0.05[c]
MeIQx	10.56	1.12±0.06[a]	9.72	1.86±0.06[b]	8.93	2.05±0.09[bc]	8.65	2.38±0.23[c]	8.24	3.19±0.43[d]
4,8-DiMeIQx	11.83	0.77±0.01[a]	10.86	0.86±0.03[b]	10.37	0.87±0.03[bc]	10.18	0.90±0.02[c]	9.59	1.20±0.07[d]
PhIP	17.86	0.44±0.02[a]	17.44	0.61±0.04[b]	14.20	0.58±0.04[b]	13.23	0.55±0.02[b]	12.04	0.52±0.04[b]

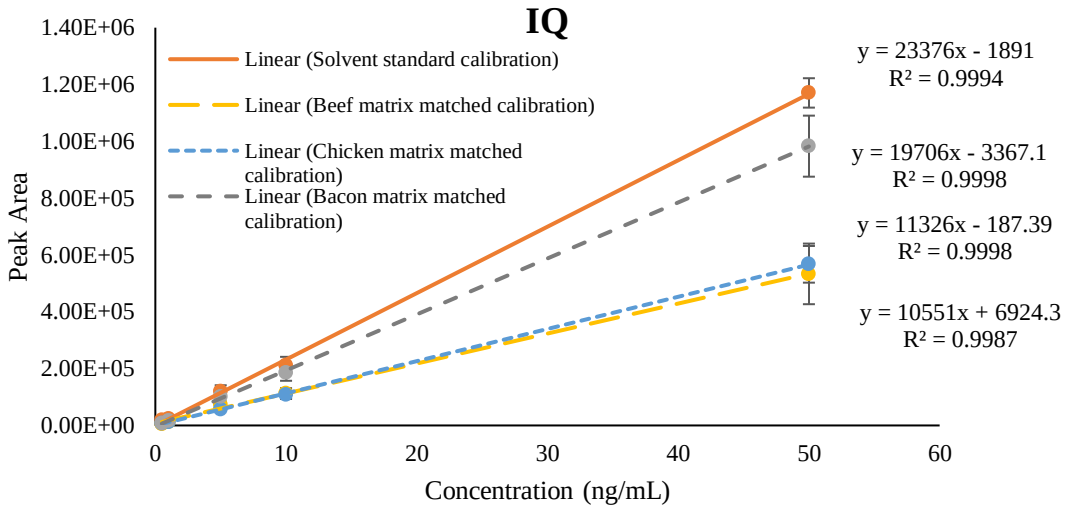
[1] Values are means ± standard deviation (n = 3).

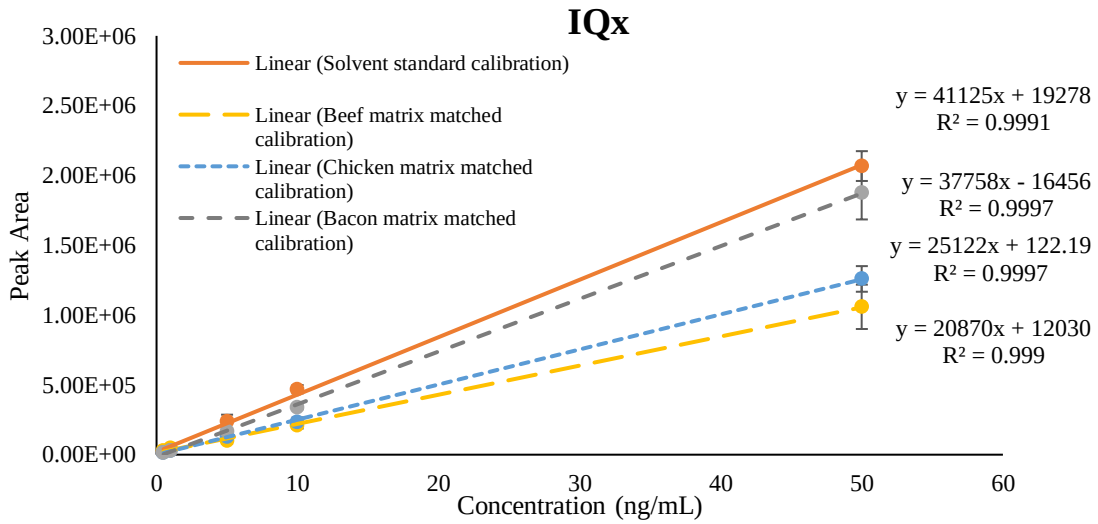
[a-d] Means with a different upper case letter within the same row are significantly different ($P < 0.05$).

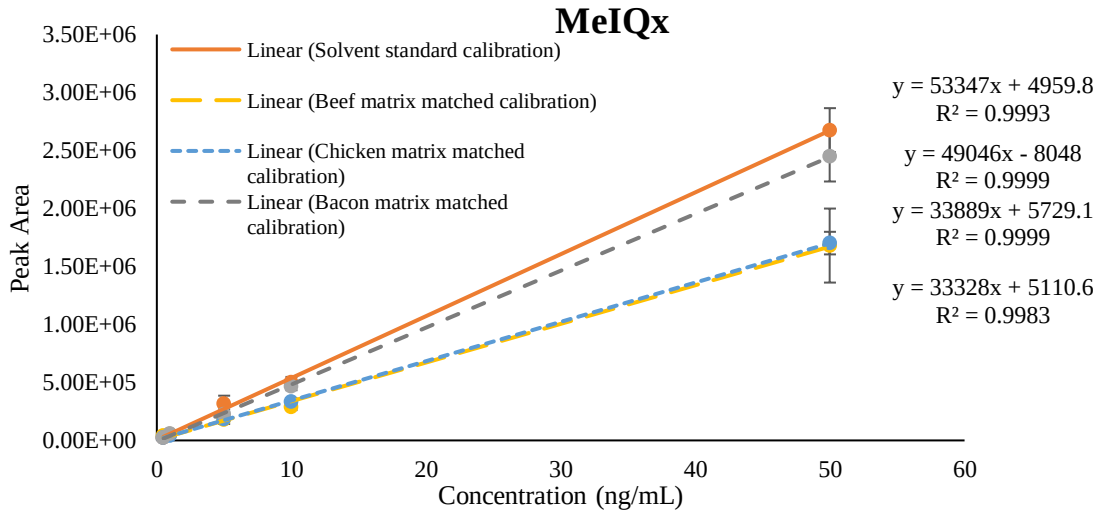
Method validation

The performance of optimized EMR-Lipid extraction coupled with LC-ESI-MS/MS IDLs/LODs/LOQs, precision, matrix effects, and HCA recovery in three meat matrices.

Linearity, Calibration curves, and IDLs/LODs/LOQs. Figure 12 illustrates the linear regression equations and R^2 obtained in the analysis of HCAs in pure standards, as well as beef, chicken, and bacon extracts. Good linearity of calibration curves in the solvent ($R^2 \geq 0.999$) were beef, chicken, and bacon showed good linearity for each HCA in this range, giving R^2 between 0.9941 and 0.999. The IDLs for HCAs in solvent ranged from 0.02 to 0.05 ng/mL (Table 15). The method LOQs and LOQs results are shown in Table 15. The obtained LODs ranged from 0.07 to 0.18 ng/mL in the beef extracts with LOQs ranged from 0.21 to 0.56 ng/mL; in the chicken extracts, HCAs have LODs that ranged from 0.05 to 0.16 ng/mL and LOQs ranged from 0.16 to 0.47 ng/mL. The differences in analyte sensitivity were due to the high complexity of the meat matrices that reduced ionization efficiency and caused more background noise in the chromatogram. R^2


IQ
1.40E+06
1.20E+06
1.00E+06
8.00E+05
6.00E+05
4.00E+05
2.00E+05
0.00E+00
Peak Area
0
10
20
30
40
50
60
Concentration (ng/mL)
Linear (Solvent standard calibration)
Linear (Beef matrix matched calibration)
Linear (Chicken matrix matched calibration)
Linear (Bacon matrix matched calibration)
y = 23376x - 1891
R² = 0.9994
y = 19706x - 3367.1
R² = 0.9998
y = 11326x - 187.39
R² = 0.9998
y = 10551x + 6924.3
R² = 0.9987


IQx
3.00E+06
2.50E+06
2.00E+06
1.50E+06
1.00E+06
5.00E+05
0.00E+00
Peak Area
0
10
20
30
40
50
60
Concentration (ng/mL)
Linear (Solvent standard calibration)
Linear (Beef matrix matched calibration)
Linear (Chicken matrix matched calibration)
Linear (Bacon matrix matched calibration)
y = 41125x + 19278
R² = 0.9991
y = 37758x - 16456
R² = 0.9997
y = 25122x + 122.19
R² = 0.9997
y = 20870x + 12030
R² = 0.999


MeIQx
3.50E+06
3.00E+06
2.50E+06
2.00E+06
1.50E+06
1.00E+06
5.00E+05
0.00E+00
Peak Area
0
10
20
30
40
50
60
Concentration (ng/mL)
Linear (Solvent standard calibration)
Linear (Beef matrix matched calibration)
Linear (Chicken matrix matched calibration)
Linear (Bacon matrix matched calibration)
y = 53347x + 4959.8
R² = 0.9993
y = 49046x - 8048
R² = 0.9999
y = 33889x + 5729.1
R² = 0.9999
y = 33328x + 5110.6
R² = 0.9983

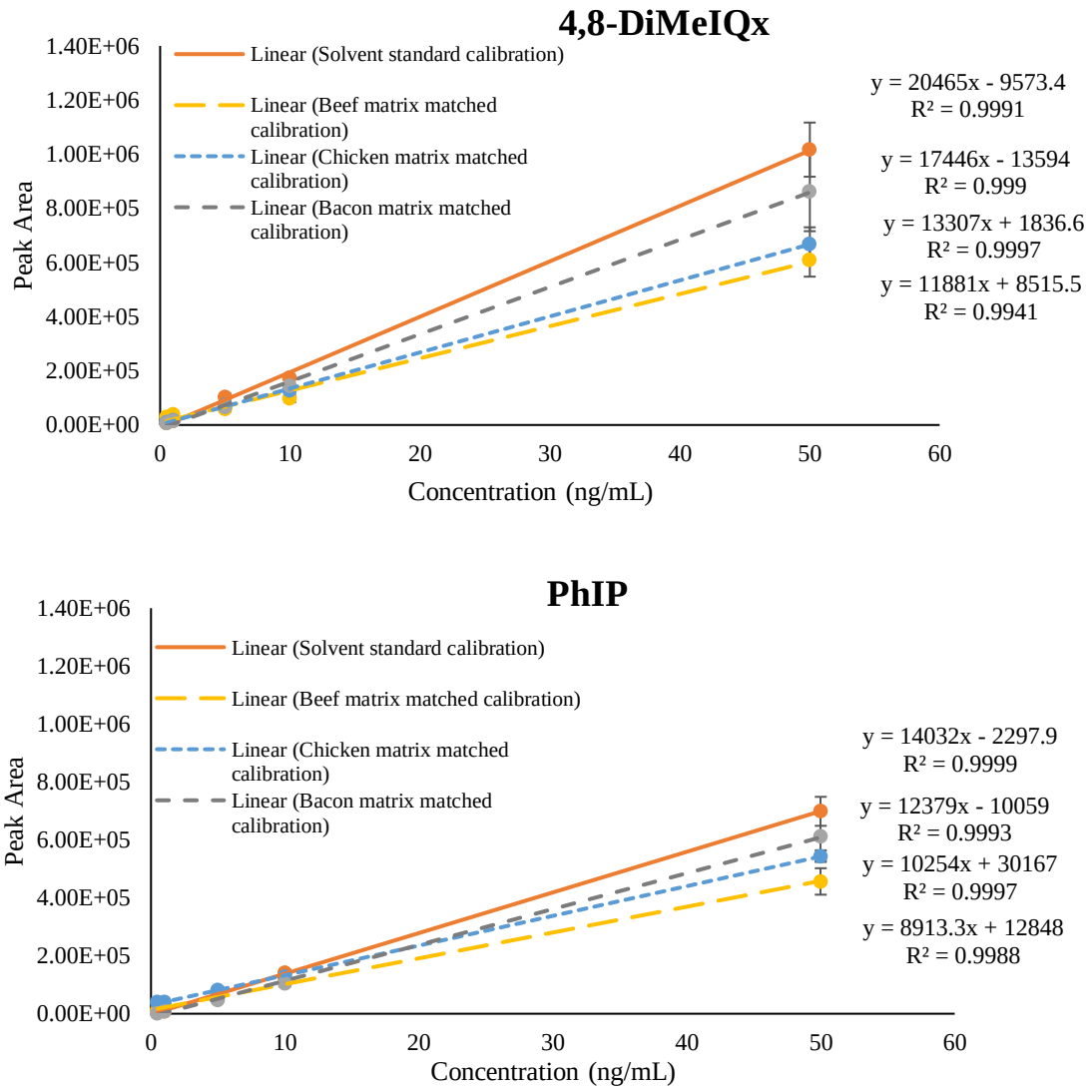


Figure 12. Calibration curves for IQ, IQx, MeIQx, 4,8-DiMeIQx, and PhIP in standard solutions and spiked meat samples at concentrations ranging from 0.5 to 50 ng/mL.

Table 15. IDLs, LODs, and LOQs in analyzing HCAs using LC-MS/MS-SRM.

Analyte	Standards	Beef extract		Chicken extract	
	IDLs (ng/mL)	LODs (ng/mL)	LOQs (ng/mL)	LODs (ng/mL)	LOQs (ng/mL)
IQx	0.05	0.17	0.53	0.11	0.35
IQ	0.02	0.18	0.56	0.16	0.47
MeIQx	0.04	0.14	0.43	0.09	0.26
4,8-DiMeIQx	0.05	0.15	0.45	0.13	0.41
PhIP	0.03	0.07	0.21	0.05	0.16

Precision. The overall method was repeatable and valid, shown by the analysis of a spiked beef sample at 1.0 ng/g of HCA standard mixture on the same day and on three consecutive days. The intraday precision gave excellent results with a %RSD less than 10%; inter-day precision also showed satisfactory, repeatable results (%RSD < 14%).

Matrix Effect Assessment. Matrix effects often occur in LC-MS/MS due to the co-extracted matrix compounds that interfere with the ionization process of analytes in the MS detector, thereby negatively affecting the reproducibility, accuracy, and sensitivity of the quantitative analysis (Van Eeckhaut, Lanckmans, Sarre, Smolders, & Michotte, 2009). Compounds that interfere with the matrix, especially those with high polarity, mass, and basicity, neutralize and deprotonate the analyte ions and thus, cause signal suppression, making it difficult to quantify the analytes in real samples (Van Eeckhaut et al., 2009). To estimate the extent of matrix effects after using the EMR-Lipid clean up, we post-spiked the HCA standard solutions with each of the meat extracts. The quantitative matrix effect (%ME) was determined by comparing the slopes of the calibration curve of the HCA pure standards and meat matrix spiked at the same concentration range of 0.5 - 50.0 ng/mL. Signal suppression would occur if the %ME value is negative; if it is positive, it would indicate signal enhancement (Economou, Botitsi,

Antoniou, & Tsipi, 2009). Furthermore, the matrix effect can be classified into 3 categories based on the calculated %ME values: low matrix effect (between -20% and +20%), medium matrix effect (between -50% and -20%; between +20% and +50%), and strong matrix effect (less than -50% or more than +50%) (Economou et al., 2009).

As Table 16 shows, all polar HCAs had ion suppression in the three tested meat matrices. The %ME of HCAs in bacon matrices ranged from -15 to -8%, which indicated a low matrix effect might occur in LC-MS/MS analysis. In more complex meat matrices, HCAs showed medium to strong matrix effects in chicken (-39 to -28% ME) and beef (-48 to -37% ME) matrices while IQ showed the highest ion suppression with %ME > -50%. These results showed that the degree of ion suppression may vary from analyte to analyte, and depend highly on the sample matrix composition. However, the reduced matrix ion suppression of HCAs in bacon matrices confirmed that the EMR-Lipid sorbents can effectively remove co-extractive lipids. The matrix profiles of chicken and beef are complicated by lipids and meat proteins, so the capacity of sorbents to remove proteins is limited. A recent study by Hsiao et al. (2017) reported that using the traditional PSA, C18, and $MgSO_4$ as dSPE sorbents could only reduce matrix effects of IQx, MeIQx, 4,8-DiMeIQx, and PhIP to a range between -37.98 and -8.69% in pork fiber. Similar high ion suppression of IQ with -44.18%ME was also observed in their study. Overall, the optimized EMR-Lipid extraction method demonstrates its efficiency in removing high co-extractives in high-fat meat samples. We continued to use matrix-matched calibration curves to quantify HCAs in the final method to reduce quantification error.

Table 16. Matrix effect (%) for HCAs using the optimized EMR-Lipid method.

Analyte	Range (ng/mL)	Beef Extracts	Chicken Extracts	Bacon Extracts
IQx	0.5 – 50.0	-48%	-39%	-8%
IQ	0.5 – 50.0	-55%	-52%	-16%
MeIQx	0.5 – 50.0	-37%	-36%	-8%
4,8-DiMeIQx	0.5 – 50.0	-42%	-35%	-15%
PhIP	0.5 – 50.0	-37%	-28%	-13%

Recovery. To evaluate analyte recovery in the optimized EMR-Lipid method, all three meat matrices were spiked with low (10.0 ng/g) and high (50.0 ng/g) concentrations of HCA standards and extracted following the procedure described previously. The concentrations of HCAs were quantified using matrix-matched calibration curves as shown in Figure 12, Figure 13 shows the results of % recoveries. The EMR-Lipid method showed different recovery rates of HCAs in different meat matrices. In the bacon and beef matrices, all polar HCAs other than IQ (< 20%), showed good results (65 to 111%) at both spiking levels, with 4,8-DiMeIQx and PhIP showing the highest recovery rates > 80%. However, chicken matrices showed relatively low HCA recovery ranging from 42 to 77%, although IQ was below 15%, at both spiking levels. Chicken is well known for its matrix complexity, especially protein, compared to bacon and beef; presumably, proteins within the chicken matrices might have a strong and different binding affinities to polar HCAs, preventing them from partitioning in the MeCN phase during extraction and leading to low analyte recovery. Nevertheless, we found that polar IQ had the lowest recovery rate (9 to 19%) in all three meat matrices, indicating that low recovery is independent of meat type. Unlike traditional dSPE sorbents (i.e., PSA, C18, and GCB) used in QuEChERS,

the EMR-Lipid sorbents show high selectivity for lipid-like compounds in the matrix based on a combination of size exclusion and hydrophobic interaction. The structure of the sorbents is unclear because it is a trade secret, but IQ containing a 2-amino-3-methyl-imidazole and a quinoline ring (a benzene fused with a pyridine) might also be trapped by sorbents during extraction, which might explain why IQ had such a low extraction recovery regardless of meat matrix. On the other hand, these results also indicated the challenge in optimizing a method used to analyze multiple HCAs in different matrices and achieving the desired results. Apart from IQ, the overall recovery of HCAs conform well to the AOAC standards, which define an acceptable recovery range as 70 to 125% for analyte concentrations at the 10 ng/g level and 75 to 120% for analyte content at 1 ng/g level in the sample (AOAC, 2002). The analyte recovery using the optimized EMR-Lipid method in our research is similar to values reported in the literature for the traditional Gross or modified methods (Gibis, 2016; Lee et al., 2015; Oz, 2011; Puangsombat et al., 2012; Smith, Ameri, & Gadgil, 2008; Yan et al., 2014).

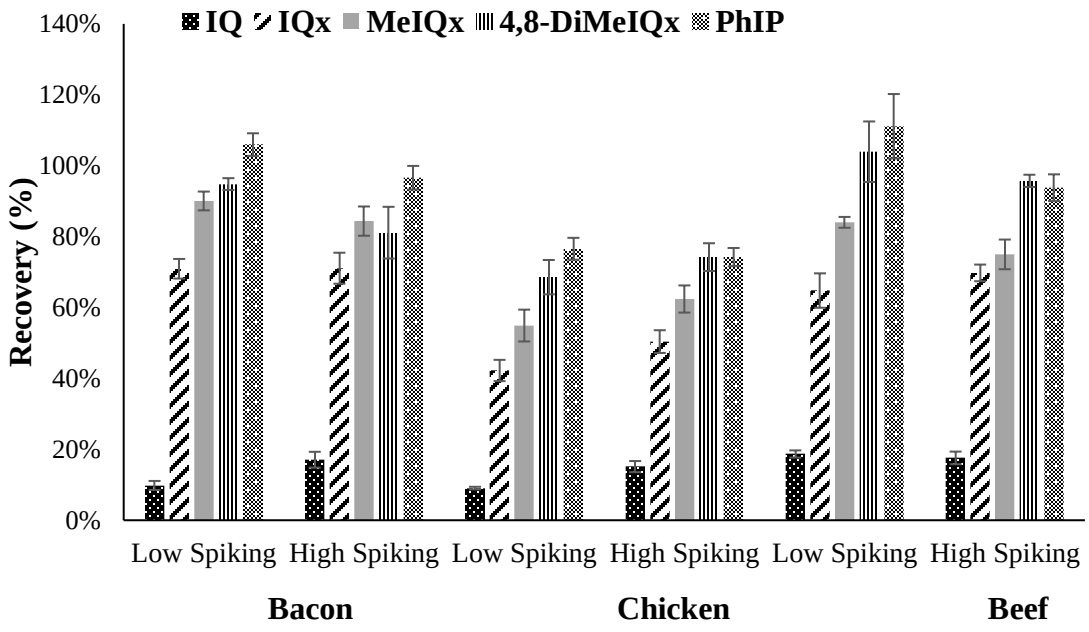


Figure 13. Average recovery (%) of HCAs in spiked beef, chicken, and bacon matrices using the EMR-Lipid method. Errors bars represent the SD.

CONCLUSION

This study is the first research paper on determining levels of polar HCAs in cooked meat products using a novel Agilent Bond Elut EMR-Lipid method. A simple solid-liquid extraction using acidified MeCN followed by EMR-Lipid matrix clean up and salting-out extraction using polish salts ($NaCl/MgSO_4$) was developed and validated for HCA analysis in three different meat samples (beef, chicken, and bacon). Identifying HCAs was carried out using LC-ESI-MS/MS operating in SRM scan mode. This optimized method (EMR-Lipid-LC-MS/MS) showed good precision (< 14% RSD) and sensitivity for detecting HCAs in the spiked meats. The validation results demonstrated the high capacity and selectivity of EMR-Lipid sorbents for meat matrix clean up with a low matrix effect (< -20% ME) in bacon and medium to high matrix effects in

beef (-37 to -55%) and chicken (-28% to -52%). The method provided acceptable recovery of four HCAs (IQx, MeIQx, 4,8-DiMeIQx, and PhIP) in spiked beef and bacon ranging from 65 to 111%, as well as 42 to 77% recovery in chicken. However, IQ showed poor recovery in all three meat matrices. Overall, this work demonstrates that EMR-Lipid coupled with LC-MS/MS may be a promising option for polar HCA extraction and detection in fatty meat products.

REFERENCES

Alaejos, M. S., & Afonso, A. M. (2011). Factors That Affect the Content of Heterocyclic Aromatic Amines in Foods. *Comprehensive Reviews in Food Science and Food Safety*, *10*(2), 52–108.

Anastassiades, M., Lehotay, S. J., Štajnbaher, D., & Schenck, F. J. (2003). Fast and Easy Multiresidue Method Employing Acetonitrile Extraction/Partitioning and "Dispersive Solid-Phase Extraction" for the Determination of Pesticide Residues in Produce, 20.

AOAC International. (2012). George W. Latimer Jr. editor. *Official methods of analysis*. 19th ed. Rockville, MD.

Barceló-Barrachina, E., Moyano, E., Galceran, M. T., Lliberia, J. L., Bagó, B., & Cortes, M. A. (2006). Ultra-performance liquid chromatography–tandem mass spectrometry for the analysis of heterocyclic amines in food. *Journal of Chromatography A*, 1125(2), 195–203.

Castillo, M., González, C., & Miralles, A. (2011). An evaluation method for determination of non-polar pesticide residues in animal fat samples by using dispersive solid-phase extraction clean-up and GC-MS. *Analytical and Bioanalytical Chemistry*, *400*(5), 1315–1328.

Cheng, K.-W., Chen, F., & Wang, M. (2006). Heterocyclic amines: Chemistry and health. *Molecular Nutrition & Food Research*, *50*(12), 1150–1170.

Cross, A. J., Peters, U., Kirsh, V. A., Andriole, G. L., Reding, D., Hayes, R. B., & Sinha, R. (2005). A prospective study of meat and meat mutagens and prostate cancer risk. *Cancer Research*, *65*(24), 11779–11784.

Cunha, S. C., Lehotay, S. J., Mastovska, K., Fernandes, J. O., Beatriz, M., & Oliveira, P. P. (2007). Evaluation of the QuEChERS sample preparation approach for the analysis of pesticide residues in olives. *Journal of Separation Science*, *30*(4), 620–632.

Dias, J. V., Cutillas, V., Lozano, A., Pizzutti, I. R., & Fernández-Alba, A. R. (2016). Determination of pesticides in edible oils by liquid chromatography-tandem mass spectrometry employing new generation materials for dispersive solid phase extraction clean-up. *Journal of Chromatography A*, 1462, 8–18.

Economou, A., Botitsi, H., Antoniou, S., & Tsipi, D. (2009). Determination of multi-class pesticides in wines by solid-phase extraction and liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A*, *1216*(31), 5856–5867.

Evard, H., Kruve, A., & Leito, I. (2016). Tutorial on estimating the limit of detection using LC-MS analysis, part I: Theoretical review. *Analytica Chimica Act*a, *942*, 23–39.

Felton, J. S., Knize, M. G., Wu, R. W., Colvin, M. E., Hatch, F. T., & Malfatti, M. A. (2007). Mutagenic potency of food-derived heterocyclic amines. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, *616*(1), 90–94.

Gibis M. 2016. Heterocyclic aromatic amines in cooked meat products: causes, formation, occurrence, and risk assessment. *Compr Rev Food Sci Food Safety, 15*, 265–302.

Gross, G. A., & Grüter, A. (1992). Quantitation of mutagenic/carcinogenic heterocyclic aromatic amines in food products. *Journal of Chromatography*, 592(1–2), 27.

He, Z., Wang, Y., Wang, L., Peng, Y., Wang, W., & Liu, X. (2017). Determination of 255 pesticides in edible vegetable oils using QuEChERS method and gas chromatography tandem mass spectrometry. *Analytical and Bioanalytical Chemistry*, *409*(4), 1017–1030.

Herraiz, T. (2004). Relative exposure to beta-carbolines norharman and harman from foods and tobacco smoke. *Food Additives and Contaminants*, *21*(11), 1041–1050.

Hsiao, H.-Y., Chen, B.-H., & Kao, T.-H. (2017). Analysis of Heterocyclic Amines in Meat by the Quick, Easy, Cheap, Effective, Rugged, and Safe Method Coupled with LC-DAD-MS-MS. *Journal of Agricultural and Food Chemistry*, *65*(42), 9360–9368.

[IARC] Intl. Agency for Research on Cancer. 1993. Monographs on the Evaluation of Carcinogenic Risk to Humans. Vol. 56. Lyon, France: IARC.

Knize, M. G., Cunningham, P. L., Griffin, E. A., Jones, A. L., & Felton, J. S. (1994). Characterization of mutagenic activity in cooked-grain-food products. *Food and Chemical Toxicology: An International Journal Published for the British Industrial Biological Research Association*, *32*(1), 15–21.

Lehotay, S. J., Mastovská, K., & Lightfield, A. R. (2005). Use of buffering and other means to improve results of problematic pesticides in a fast and easy method for residue analysis of fruits and vegetables. *Journal of AOAC International*, *88*(2), 615–629.

Lehotay, S. J., Son, K. A., Kwon, H., Koesukwiwat, U., Fu, W., Mastovska, K., … Leepipatpiboon, N. (2010). Comparison of QuEChERS sample preparation methods for the analysis of pesticide residues in fruits and vegetables. *Journal of Chromatography A*, *1217*(16), 2548–2560.

Linghu, Z., Karim, F., & Smith, J. S. (2017). Amino Acids Inhibitory Effects and Mechanism on 2-Amino-1-Methyl-6-Phenylimidazo [4,5-b]Pyridine (PhIP) Formation in the Maillard Reaction Systems. *Journal of Food Science*, *82*(12), 3037–3045.

Lucas, D., & Zhao, L. (2015). PAH Analysis in Salmon with Enhanced Matrix Removal. *Agilent Technologies Application Note*, publication number 5991-6088EN.

Messner, C., & Murkovic, M. (2004). Evaluation of a new model system for studying the formation of heterocyclic amines. *Journal of Chromatography B*, *802*(1), 19–26.

Murray, S., Lynch, A. M., Knize, M. G., & Gooderham, M. J. (1993). Quantification of the carcinogens 2-amino-3,8-dimethyl- and 2-amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine in food using a combined assay based on gas chromatography-negative ion mass spectrometry. *Journal of Chromatography*, *616*(2), 211–219.

Ni, W., McNaughton, L., LeMaster, D. M., Sinha, R., & Turesky, R. J. (2008). Quantitation of 13 Heterocyclic Aromatic Amines in Cooked Beef, Pork, and Chicken by Liquid Chromatography−Electrospray Ionization/Tandem Mass Spectrometry. *Journal of Agricultural and Food Chemistry*, *56*(1), 68–78.

Pais, P., & Knize, M. G. (2000). Chromatographic and related techniques for the determination of aromatic heterocyclic amines in foods. *Journal of Chromatography. B, Biomedical Sciences and Applications*, *747*(1–2), 139–169.

Park, K. H., Choi, J.-H., Abd El-Aty, A. M., Cho, S.-K., Park, J.-H., Kwon, K. S., … Shim, J.-H. (2012). Development of QuEChERS-based extraction and liquid chromatography–tandem mass spectrometry method for quantifying flumethasone residues in beef muscle. *Meat Science*, *92*(4), 749–753.

Parrilla Vázquez, P., Hakme, E., Uclés, S., Cutillas, V., Martínez Galera, M., Mughari, A. R., & Fernández-Alba, A. R. (2016). Large multiresidue analysis of pesticides in edible vegetable oils by using efficient solid-phase extraction sorbents based on quick, easy, cheap, effective, rugged and safe methodology followed by gas chromatography–tandem mass spectrometry. *Journal of Chromatography A*, *1463*, 20–31.

Puangsombat, K., Gadgil, P., Houser, T. A., Hunt, M. C., & Smith, J. S. (2012). Occurrence of heterocyclic amines in cooked meat products. *Meat Science*, *90*(3), 739–746.

Rejczak, T., & Tuzimski, T. (2015). A review of recent developments and trends in the QuEChERS sample preparation approach. *Open Chemistry*, *13*(1).

Sanz Alaejos, M., Ayala, J. H., González, V., & Afonso, A. M. (2008). Analytical methods applied to the determination of heterocyclic aromatic amines in foods. *Journal of Chromatography B*, 862(1–2), 15–42.

Shin, H.-S., Park, H., & Park, D. (2003). Influence of Different Oligosaccharides and Inulin on Heterocyclic Aromatic Amine Formation and Overall Mutagenicity in Fried Ground Beef Patties. *Journal of Agricultural and Food Chemistry*, *51*(23), 6726–6730.

Smith, J. S., Ameri, F., & Gadgil, P. (2008). Effect of Marinades on the Formation of Heterocyclic Amines in Grilled Beef Steaks. *Journal of Food Science*, *73*(6), T100–T105.

Sugimura, T., Wakabayashi, K., Nakagama, H., & Nagao, M. (2004). Heterocyclic amines: Mutagens/carcinogens produced during cooking of meat and fish. *Cancer Science*, *95*(4), 290–299.

Turesky, R. J., & Le Marchand, L. (2011). Metabolism and biomarkers of heterocyclic aromatic amines in molecular epidemiology studies: lessons learned from aromatic amines. *Chemical Research in Toxicology*, *24*(8), 1169–1214.

Van Eeckhaut, A., Lanckmans, K., Sarre, S., Smolders, I., & Michotte, Y. (2009). Validation of bioanalytical LC-MS/MS assays: evaluation of matrix effects. *Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences*, *877*(23), 2198–2207.

Yan, Y., Zeng, M.-M., Zheng, Z.-P., He, Z.-Y., Tao, G.-J., Zhang, S., … Chen, J. (2014). A novel one-step extraction method for simultaneously determining eleven polar heterocyclic aromatic amines in meat products by UHPLC-MS/MS. *Analytical Methods*, *6*(16), 6437–6444.

Zhao, L., & Lucas, D. (2015a). Multiresidue Analysis of Pesticides in Avocado with Agilent Bond Elut EMR—Lipid by LC/MS/MS. *Agilent Technologies Application Note*, publication number 5991 6098EN.

Zhao, L., & Lucas, D. (2015b). Multiresidue Analysis of Veterinary Drugs in Bovine Livers by LC/MS/MS. *Agilent Technologies Application Note*, publication number 5991-6096EN.

Zhao, L., Lucas, D., Long, D., Richter, B., & Stevens, J. (2018). Multi-class multi-residue analysis of veterinary drugs in meat using enhanced matrix removal lipid cleanup and liquid chromatography-tandem mass spectrometry. *Journal of Chromatography. A, 1549*, 14–24.

CHAPTER 4 - AMINO ACIDS EFFECTS ON HETEROCYCLIC AMINES FORMATION AND PHYSIOCHEMICAL PROPERTIES IN PAN-FRIED BEEF PATTIES[1]

ABSTRACT

The effects of surface application of amino acids on the formation of heterocyclic amines (HCAs) and meat quality properties were evaluated in pan-fried beef patties (230 °C/15 min). Tryptophan, lysine, leucine, and proline at three concentrations, 0.05%, 0.20%, and 0.50% (w/w), were tested. The meat crusts were analyzed for HCA content using liquid chromatography - tandem mass spectrometry. Results showed that surface application of all tested amino acids significantly reduced total HCA content ($P < 0.05$), and the interaction of amino acid type and concentration significantly affected ($P < 0.05$) both individual and total HCA formation. Tryptophan at 0.50% reduced total HCAs the most (0.92 ng/g, 93% inhibition), followed by 0.50% lysine (1.94 ng/g, 84% inhibition), while leucine (3.95 ng/g, 64%) and proline (4.71 ng/g, 56%) were less effective at 0.50%. In addition, applying amino acids to meat surface significantly influenced ($P < 0.05$) pH and surface color change of beef crusts; particularly, lysine at 0.20% and 0.50% increased pH and a* (redness) but reduced b* (yellowness), while tryptophan and leucine at 0.50% increased L* (whiteness). No significant effect was observed on cooking loss. Adding amino acids at 0.50% affected ($P < 0.05$) formation of aldehydes and pyrazines (as the key flavor compounds of fried beef). Overall, the results of this study suggested that adding amino acids to ground beef patties could effectively mitigate mutagenic HCA formation during cooking.

1- Presented in part at IFT Annual Meeting 2017. Toxicology and Safety Evaluation Division.

INTRODUCTION

Heterocyclic amines (HCAs) are a group of highly mutagenic and carcinogenic compounds that typically form on cooked, muscle-rich foods like beef, pork, poultry, and fish under normal cooking temperatures (Cheng, Chen, & Wang, 2006). High cooking temperature (> 150 °C), prolonged heating time, and thermal treatment, especially pan-frying, grilling, and broiling to well- and very well-done levels, are of the most concern because more HCAs are produced under these circumstances (Puangsombat, Gadgil, Houser, Hunt, & Smith, 2012). Many epidemiological studies have shown that consuming many well-done and fried meat products, red meat in particular, is associated with increased risks of human cancers including stomach, colon, breast, prostate, and pancreatic cancers (Cross et al., 2005; Felton et al., 2007). Among the known HCAs (now more than 25 of them), 2-amino-1-methyl-6-phenyl-imidazo[4,5-b]pyridine (PhIP), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), and 2-amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline (4,8-DiMeIQx) are most abundantly formed in cooked meat, followed by 2-amino-3-methyl-imidazo [4,5-f]quinoline (IQ) and 2-amino-3-methylimidazo [4,5-f]quinoxaline (IQx) (Gibis, 2016; Knize et al., 1994). These compounds have shown strong mutagenic effects (i.e., frameshift and base-pair substitution mutations) in the Ames/*Salmonella* test and are carcinogenic to a wide spectrum of organs/tissues, mainly the liver, mammary glands, skin, and lungs in long-term animal feeding studies (Sugimura, Wakabayashi, Nakagama, & Nagao, 2004). Therefore, the International Agency for Research on Cancer (IARC, 1993) classified PhIP and MeIQx as *possible human carcinogen*s (Group 2B) and IQ as a *probable human carcinogen* (Group 2A).

Methods to mitigate HCA production in cooked meat typically involve reducing cooking temperature and time, modifying cooking methods, and using food additives with antioxidant

properties like natural plant-based additives (i.e., herbs, spices) (Cheng et al., 2007; Damašius, Venskutonis, Ferracane, & Fogliano, 2011; Jinap, Iqbal, & Selvam, 2015), plant-extracts (Cheng et al., 2007; Damašius et al., 2011), as well as pure antioxidants (Alaejos & Afonso, 2011). Although there have good results, an increasing number of studies have revealed that adding natural herbs/spices and/or pure antioxidants can also enhance HCA formation, possibly because of their pro-oxidant activity at higher levels (Damašius et al., 2011; Zochling, 2002).

Major thermic HCAs form through the Maillard reaction of free amino acids, reducing sugars, and creati(ni)ne in meats during high-temperature treatment (Murkovic, 2004; Pais & Knize, 2000; Sugimura, Wakabayashi, Nakagama, & Nagao, 2004). Modulating HCA precursor profiles, such as types and concentrations of precursors before heating, might help minimize HCA formation. Several studies have shown that adding reducing sugars (i.e., glucose, fructose, and lactose), carbohydrates, and honey can effectively reduce HCA content in fried beef patties (150-200 °C) (Skog, Johansson, & Jägerstad, 1998) and pork sausage (Abdulkarim & Smith, 1998). However, minimal research has been done on modulating free amino acid profile. Ashoor et al. (1980) mixed proline with ground beef patties and found that mutagen formation rose after frying. On the other hand, Jones and Weisburger (1988a) blended tryptophan into a commercial beef sauce and found that mutagenic IQ- and IQx-type HCA formation was significantly inhibited in fried beef patties after the beef sauce was spread on top of the patties. In another study, Jones and Weisburger (1988b) also showed that mixing proline with tryptophan was more effective in reducing IQx-type HCA formation than using proline by itself in a model system. In a previous study, nine amino acids (tryptophan, proline, lysine, leucine, isoleucine, methionine, threonine, phenylalanine, and valine) inhibited PhIP formation after heating glucose, creatinine, and phenylalanine for 1 h at 180 °C (Linghu, Karim, & Smith, 2017). A more pronounced effect

was observed with tryptophan, lysine, leucine, and proline, which led to more than 87% reduction while aspartic acid actually increased PhIP formation. The mechanism for amino acid's inhibitory activity might be to form amino acid-phenylacetaldehyde adducts and to scavenge phenylacetaldehyde, which is the key precursor (reactive carbonyl species) to PhIP formation during Maillard reaction.

To the best of our knowledge, the literature does not cover how adding those free amino acids affect the formation of PhIP and other mutagenic HCAs like MeIQx and 4,8-DiMeIQx in cooked meat products. Therefore, in this study, we investigated the potential effects of the amino acids tryptophan, lysine, leucine, and proline on mutagenic HCA formation in a real meat system. Surface application of the amino acids were set at 0.05, 0.20, and 0.50% (w/w) following previously reported concentrations in beef patties (Jones &Weisburger, 1988a). In addition, how these amino acids influenced certain quality characteristics of cooked meat was also evaluated; cooking loss, surface color, pH, and the volatile profile were included. The results were expected to reveal effects of adding amino acid on HCA formation and the development of cooked meat color and flavors.

MATERIALS AND METHODS

Chemicals and reagents

HCA standards 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), 2-amino-3-methylimidazo[4,5-f]quinoxaline (IQx), 2-amino-3-methylimidazo[4,5-f]quinolone (IQ), 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo[4,5-f]quinoxaline (4,8-DiMeIQx), and 2-amino-3,4,7,8-tetramethyl-imidazo [4,5-f]quinoxaline (4,7,8-TriMeIQx) as the internal standard (IS) were purchased from Toronto Research

Chemicals, Inc. (Ontario, Canada). L-tryptophan (98%), L-lysine (99%), L-proline (99%), and L-leucine (98%) were purchased from the Sigma-Aldrich (St. Louis, Mo., U.S.A.). Optima® LC-MS grade acetonitrile, methanol, acetic acid, formic acid, ammonium acetate, and syringe filters (nylon, 0.2 µm) were purchased from Fisher Scientific (Fair Lawn, N.J., U.S.A.). Deionized water was prepared using a Sybron/Barnstead PCS filtration unit (Barnstead/Thermolyne, Inc, Dubuque, Iowa, U.S.A.). Carboxen/polydimethylsiloxane (CAR/PDMS) fiber was purchased from Supelco Inc. (Bellefonte, Pa., U.S.A). Bond Elut Enhanced Matrix Removal (EMR) - Lipid was purchased from Agilent Technologies (Santa Clara, U.S.A.) included Lipid dSPE (p/n 5982-1010) and Lipid-Polish (p/n 5982-010).

Preparation and frying of beef patties

Fresh ground beef (7% fat) was purchased from local stores in Manhattan, Kans., U.S.A. The proximate analysis showed the ground beef contained 9.55% fat content, 19.83% protein content, and 69.77% moisture content (data not shown). The ground beef was shaped into patties using a petri dish (4.52 cm diameter and 2 cm thickness); and each patty weighed 80 ± 0.1 g. Four different amino acids, tryptophan (Trp), lysine (Lys), leucine (Leu), and proline (Pro), were prepared at three concentrations each: 0.05%, 0.20%, and 0.50% (w/w), which represents 20 mg, 80 mg, and 200 mg per side of patty. Each amino acid was spread evenly on the surface of the beef patty using a spatula and then sat at room temperature for 10 min before cooking. Beef patties with no added amino acids were prepared as controls. All beef patties were pan-fried at 230 ± 3 °C for 7.5 min on each side in a thermostat-controlled, Teflon-coated, square frying pan as described in Abdulkarim and Smith (1998). No other food additives (like salts or spices) or oil was added to meat samples during cooking. The surface temperature of the frying pan was monitored using an infrared thermometer. After frying, meat samples were allowed to cool for 10

min at room temperature, and then meat surface color and percentage of cooking loss (measured as the weight difference before and after frying) were measured. Approximately 2 mm of meat crust was then cut off from beef samples, immediately frozen in liquid nitrogen, and ground well in a commercial Waring blender (Fisher, Pittsburgh, Pa., U.S.A.). A total of 15 g meat crust was collected from each meat sample and stored at -18 °C until analysis the following day. All experiments were repeated three times.

Surface color measurement

The surface color change of beef patties was measured using a HunterLab MiniScan XE colorimeter (Hunter Associates Laboratory Inc., Reston, Va., U.S.A.), with full spectral data obtained as L* (lightness-darkness), a* (redness-greenness), and b* (yellowness-blueness). The colorimeter was calibrated with white and black standards provided with the instrument. The color measurement was carried out after cooked beef patties were cooled for 10 min at room temperature. Five measurements were taken on each surface of beef patty, and an average value for L*, a*, and b* was calculated from the mean of ten readings per sample.

pH determination

The pH values of fried meat crusts were determined according to Fan et al. (2019). A 1 g sample of meat was homogenized with 9 mL of distilled water for 1 min at medium speed using an Omni TH homogenizer (Omni International, Kennesaw, Ga., U.S.A.). The pH was then measured for each sample at room temperature using a portable pH meter (Accumet AP115; Accumet Probe Model 13-620-AP61; Fisher, Pittsburgh, Pa., U.S.A.).

HCA extraction using EMR-Lipid method

HCAs were extracted from the meat crust using QuEChERS "Enhanced Matrix Removal of Lipids" (EMR-Lipids) extraction method as described in Chapter 3. Briefly, meat crust (3.0 g)

was mixed in a 50 mL polypropylene (PP) tube with 15 mL of 1% formic acid-acetonitrile and shaken on a mechanical shaker for 30 min at room temperature. After centrifugation (11,300 x g at 4 °C) for 10 min, 12 mL of supernatant was collected and cleaned up using EMR-Lipid dSPE sorbents (1 g) that was pre-activated with 5 mL of ammonium acetate buffer (5 mM). The tube was then vigorously hand shaken for 2 min followed by centrifugation for 10 min at 11,300 x g and 4 °C. A salting-out extraction was then carried out by transferring 10 mL of supernatant into a 50 mL PP tube and mixing with 2 g EMR-Lipid Polish salts ($NaCl:MgSO_4$, 1:4, w/w) to further clean up the extract. After another hand shaking for 2 min, followed by centrifugation (11,300 x g at 4 °C) for 10 min, the final extract (1 mL of the top layer) was collected and evaporated to dryness under a constant flow of nitrogen. Finally, the extract was reconstituted with 100 µL of methanol and 10 µL of IS at 50 ng/mL, filtered through a 0.2 µm nylon filter and analyzed using a LC-MS/MS system.

Liquid Chromatography- tandem mass spectrometry (LC-MS/MS) analysis

A Finnigan Surveyor Plus HPLC system (Thermo Finnigan, San Jose, Calif., U.S.A.) equipped with a Finnigan TSQ Quantum Ultra EMR triple quadruple mass spectrometer (Thermo Finnigan, San Jose, Calif., U.S.A.) was used for HCA analysis in this study. A 10 µL of the sample was injected into the HPLC, the chromatographic separation of HCAs was achieved using a Supelco Ascentis® Express C18 column (15 cm × 2.1 mm, 2.7 µm, 80 Å) (Sigma-Aldrich, Bellefonte, Pa., U.S.A.). The column temperature was set at 30 ºC for the whole run. Separation was achieved with binary mobile phases with a flow rate of 0.25 mL/min. Mobile phase (A) was 10 mM ammonium acetate adjusted to pH 3.0 with acetic acid, and mobile phase (B) used acetonitrile. The linear gradient was as follows: 0-6 min, 90% A and 10% B; 6-12 min, 75% A and 25% B; 12-17 min, 70% A and 30% B; 17-24 min, 90% A and 10% B. The column

was then re-equilibrated with 90% A and 10% B for 1 min before the next injection. The total run time was 25 min. The MS/MS system was operated with an electron spray ionization (ESI) source in positive mode; the optimized ion source parameters were as follows: spray voltage, 4.0 kV; N_2 sheath gas flow, 50 arbitrary units; auxiliary gas flow rate, 15 arbitrary units; capillary temperature, 275 ºC. The SRM transitions for HCAs along with collision energies were as follows: 185 → 200 (20 eV) for IQx; 210 → 225 (25 eV) for IQ; 199 → 214 (20 eV) for MeIQx; 213 → 228 (20 eV) for 4,8-DiMeIQx; 210 → 225 (25 eV) for PhIP; and 227 → 242 (25 eV) for 4,7,8-TriMeIQx. Xcalibur version 2.0.7 software was used for instrument control and data acquisition. The quantification of HCAs was performed using beef matrix-matched calibration curves containing HCA standards at five calibrant concentrations of 0.5, 1.0, 5.0, 10.0, and 50.0 ng/mL. The coefficient of determination (R^2) for matrix-matched standard curves was between 0.9941 and 0.999. The limit of detection (LOD) and limit of quantification (LOQ) of HCAs ranged from 0.07 to 0.48 ng/mL and 0.21 to 0.56 ng/mL.

Volatile compound extraction

Volatile compounds in the fried beef crust were extracted using headspace-solid phase microextraction (HP-SPME) and analyzed using gas chromatography-mass spectrometry (GC-MS). Preliminary studies were carried out to select HS-SPME extraction conditions that generated meat volatiles, in particular, pyrazine derivatives. An SPME fiber coated with 75 µm CAR/PDMS was used as recommended by Gianelli, Salazar, Mojica, and Friz (2012). Sampling temperatures, times, salt, and water addition were all investigated (results not shown). The final HP-SPME method was as follows: 1.5 g of meat crust was weighed into a 10 mL headspace vial and sealed with a PTFE/red rubber septum. The samples were then heated in an aluminum heating block at 70 °C for 20 min to pre-equilibrate the headspace. The SPME fiber was exposed

in the upper space of vial for 40 min to adsorb volatiles in the headspace. After that, the fiber was withdrawn into a manual holder and injected manually into the GC injector for desorption and subsequent GC-MS analysis.

GC-MS analysis

An HP 5890 GC series II Plus gas chromatograph (Agilent Technology Inc.) was used coupled with an HP 5972 mass spectral detector operating in an electron impact (EI) ion source (Agilent Technology Inc.). The analysis of meat volatile compounds was carried out using an HP-5 MS fused silica capillary column (60 m × 0.25 mm i.d. x 0.25 μm film thickness; Agilent Technologies, Santa Clara, CA, U.S.A). The carrier gas was helium using a constant flow rate of 1 mL/min (high-purity helium, carrier gas). The SPME fiber was desorbed in a splitless mode at inlet temperature of 280 °C for 5 min. The oven temperature program was as follows: 40 °C for 2 min, increased to 80 °C at a rate of 3 °C/min, and increased again to 150 °C at a rate of 5 °C/min, and finally to 250 °C at a rate of 10 °C/min and held for 1 min. The MS detector and GC-MS transfer line temperatures were 150 °C and 250 °C. The MS data processing was detected with 3 min solvent delay and recorded by monitoring the total ion currents in the 45-550 m/z mass range at a rate of 1 scan/s. Volatile compounds were identified by comparing the mass spectra with NIST Mass Spectral Library (2008) using the ChemStation software (G1701BA, version B.01.00), with a minimum library match score of > 90%.

STATISTICAL ANALYSIS

Experiments were performed in a two-way factorial arrangement with completely randomized design (CRD), having 3 replications to each treatment combination. Two-way analysis of variance (ANOVA) was used to determine the significant effect of amino acids (Trp, Lys, Leu, and Pro), concentrations (0, 0.05, 0.20, and 0.50%), and their interactions on HCA

formation, cooking loss, pH, and surface color (L*, a*, and b*) change using SAS version 9.3 (SAS Inst. Inc., Cary, N.C., U.S.A., 2013); significant differences in volatile compound formation among the treatments were compared using one-way analysis of variance (ANOVA). The level of significance (*P*-value) was defined as $P < 0.05$. When significant differences were observed among treatments, Tukey's multiple comparison test was applied to the data.

RESULTS AND DISCUSSION

Effect of amino acids on HCA formation in pan-fried beef patties

LC-MS/MS analysis was used to detect the HCA contents. Table 17 summarizes how much of each HCA (expressed as ng/g trimmed meat crust) in pan-fried beef patties when different types and concentrations of amino acids were added. In this study, we conducted analyses for all five thermic HCAs, IQ, IQx, PhIP, MeIQx, and 4,8-DiMeIQx. As Table 17 shows, all cooked beef patties including control and amino acid treated beef patties contained PhIP, MeIQx, and 4,8-DiMeIQx, but IQ and IQx were not detected in any meat samples. Similar results were also reported by Abdulkarim and Smith (1998), Sabally, Sleno, Jauffrit, Iskandar, and Kubow (2016), and Zeng et al. (2018), who also found no IQ and IQx forming in beef patties (5% -15% fat) or beef steaks after frying or grilling at 190 to 250 ℃ for 10 to 20 min. Puangsombat et al. (2012) also showed that beef patties broiled and/or fried to medium-rare and well-done generated no IQ and IQx. However, IQ and/or IQx have been detected, in a range from 0.02 to 3.65 ng/g, in fried beef when cooked at high temperatures, 250 to 275 ℃ for 5 to 15 min (Felton et al., 1986; Oz & Kaya, 2011), suggesting that IQ and IQx tend to form in beef samples at high cooking temperatures, which could explain its absence in the present work.

In this study, PhIP was the most abundant HCA forming in fried beef patties, with concentrations ranging from 3.76 to 4.89 ng/g for Trp, Lys, Leu, and Pro batches (Table 17). Abdulkarim and Smith (1998) reported similar results with PhIP forming in the crust of beef patties with 5% fat ranging from 3.13 to 3.30 ng/g after frying at 230 to 240 °C for 7 to 15 min. When amino acids were applied to meat surface at the lowest concentration (0.05%), PhIP content in controls were reduced significantly by 61%, 42%, 51%, and 25%, for Trp, Lys, Leu, and Pro ($P < 0.05$). Significantly more reduction of PhIP was observed when surface concentrations of amino acids increased from 0.05% to 0.20% and from 0.20% to 0.50% ($P < 0.05$). The highest reduction of PhIP was observed when 0.50% of Trp and/or Lys were added, leading to a 99% and 97% inhibition, which was significantly higher ($P < 0.05$) than 0.50% Leu (75% inhibition) and 0.50% Pro (54% inhibition). As in our findings, Linghu et al. (2017) reported a similar reduction in PhIP after adding nine different amino acids in the Maillard model system, indicating a concentration-dependent inhibitory effect of amino acids on PhIP formation. Our results clearly demonstrated that adding selected amino acids to the surface of ground beef patties can significantly reduce PhIP formation after cooking at 230 °C/15 min; among these amino acids, Trp and Lys were more effective than Leu and Pro in suppressing PhIP formation. The inhibition mechanism was probably involved the reaction of these amino acids with the precursor phenylacetaldehyde requisite for PhIP formation, thus rendering the phenylacetaldehyde unavailable for reaction with creatinine, leading to less PhIP being produced (Linghu et al., 2017). In addition, Trp, Leu, and Pro have been reported to form an adduct with PhIP in meat products (i.e., beef, fish, chicken, and pork) during cooking (Kataoka et al., 2009). Kataoka, Miyake, Saito, and Mitani (2012) also demonstrated in a model system that the PhIP-amino acid adduct formation helped remove PhIP once temperatures reached > 200 °C with

heating time > 5 min. Therefore, PhIP- and phenylacetaldehyde-amino acid adduct formation could both directly contribute to the reduction of PhIP in cooked meat.

IQx-type HCAs like MeIQx and 4,8-DiMeIQx were also present at high levels in fried beef patties, ranging from 3.35 to 3.97 ng/g and 3.38 to 3.90 ng/g for Trp, Lys, Leu, and Pro treated batches (Table 17). These concentrations were comparable to those obtained in previous studies (Persson, Sjöholm, Nyman, & Skog, 2004; Puangsombat, Jirapakkul, & Smith, 2011) that reported 0.3 to 7.00 ng/g of MeIQx in the crust of beef patties when fried at 200-230 °C for 6-15 min. No 4,8-DiMeIQx was, however, detected in these studies. We could, thus, evaluate the effect of amino acids on both MeIQx and 4,8-DiMeIQx formation in this study. Compared to controls, applying all amino acids to beef patty surfaces produced an inhibitory effect on formation of MeIQx and 4,8-DiMeIQx after frying ($P < 0.05$). Statistical analysis showed that the effect at 0.50% was more pronounced than that at 0.20% and 0.05% levels ($P < 0.05$). Please note that Trp was better than Lys, Leu, and Pro in inhibiting MeIQx and 4,8-DiMeIQx at all three concentrations. At the lowest concentration (0.05%), Trp treated patties showed decreases up to 63% in MeIQx and 4,8-DiMeIQx compared to controls ($P < 0.05$); Lys, Leu, and Pro treatments showed less than 50% decrease with no significant differences among treatments ($P > 0.05$). Increasing Trp concentration from 0.05% to 0.50% showed the most reduction in MeIQx (up to 90%) and 4,8-DiMeIQx (up to 91%), while Lys, Leu, and Pro treatments had less reduction ($P < 0.05$), showing percentages of 70-81%, 55-60%, and 49-64%. These results indicated that adding amino acids not only inhibited PhIP formation but also IQx-type HCAs, including MeIQx and 4,8-DiMeIQx, in fried meat. Jones and Weisburger (1988a) reported similar results after surface application of essential amino acid Trp (0.25%, w/w) to ground beef patties before frying, significantly inhibiting (up to 100%) both IQ- and IQx-type HCA

formation and mutagenicity. Furthermore, Trp and Pro both inhibited MeIQx and 4,8-DiMeIQx formation in model systems (Jones & Weisburger, 1988b, c); that research also indicated a synergistic HCA inhibitory effect between Pro and Trp. However, the mechanism involved in this observed inhibitory activity on IQx-type HCAs is still not clear. Jones and Weisburger (1988c) hypothesized that Trp might block the reaction of precursor aldehydes like methylglyoxal with creatinine via its indole-ring nitrogen, thus making precursors unavailable in MeIQx/4,8-DiMeIQx formation pathway. According to a previous study by Kataoka et al. (2012), a reduction of MeIQx content in cooked meat might occur because a condensation reaction forms MeIQx and amino acid adducts. For type of amino acid, Pro showed the strongest binding affinity to MeIQx in model systems once the molar ratio of amino acid was 10 times higher than MeIQx.

Figure 14 shows the total content of HCAs based on the averaged PhIP, MeIQx, and 4,8-DiMeIQx levels. It also shows that the total amounts of HCAs in beef patties decreased significantly as amino acid concentrations increased ($P < 0.05$), suggesting that the effect of amino acids on reducing HCA formation was concentration dependent. Trp, Lys, and Pro at 0.50% and 0.20% levels inhibited significantly more formations of total HCAs than concentrations of 0.05% ($P < 0.05$), whereas Leu at 0.50% inhibited more HCA formation ($P < 0.05$) than at 0.20% and 0.05%. Furthermore, the statistical analysis showed the interaction between amino acid type and concentration significantly affected ($P < 0.05$) total HCA reduction. Beef patties containing 0.50% Trp had the lowest total HCA content (0.92 ng/g), for a maximum 93% inhibition rate followed by beef patties containing 0.50% Lys (84% inhibition). Leu and Pro at the 0.50% level were less effective at reducing total HCA content in the patties, showing 64% and 56% inhibition. Because Trp and Lys at 0.50% are the most effective

inhibitors of mutagenic HCA formation in cooked meat, followed by Leu and Pro, a specific structure-activity relationship might also exist.

Table 17. Effect of different concentrations of amino acids on PhIP, MeIQx, and 4,8-DiMeIQx formation (expressed as ng/g trimmed meat crust) in pan-fried beef patties.

Amino Acid	Concentration	PhIP		MeIQx		4,8-DiMeIQx	
		ng/g ± SD[1]	% Reduction	ng/g ± SD[1]	% Reduction	ng/g ± SD[1]	% Reduction
Control	0.00%	4.89 ± 1.23 [a]	--	3.49 ± 0.12 [ab]	--	3.90 ± 0.40 [a]	--
Trp	0.05%	1.92 ± 0.46 [fg]	61%	1.47 ± 0.04 [efg]	63%	1.46 ± 0.17 [f]	63%
	0.20%	1.32 ± 0.54 [hg]	73%	1.11 ± 0.15 [g]	76%	0.92 ± 0.09 [gh]	76%
	0.50%	ND [j]	99%	0.52 ± 0.06 [h]	90%	0.38 ± 0.02 [i]	91%
Control	0.00%	4.49 ± 0.58 [ab]	--	3.97 ± 0.39 [a]	--	3.38 ± 0.13 [b]	--
Lys	0.05%	2.61 ± 0.28 [de]	42%	2.07 ± 0.42 [cd]	48%	2.34 ± 0.07 [cd]	31%
	0.20%	0.44 ± 0.08 [ij]	90%	1.89 ± 0.78 [de]	52%	1.17 ± 0.17 [e]	65%
	0.50%	0.12 ± 0.03 [j]	97%	1.19 ± 0.24 [gf]	70%	0.63 ± 0.15 [ih]	81%
Control	0.00%	3.87 ± 0.16 [bc]	--	3.64 ± 0.68 [ab]	--	3.41 ± 0.30 [b]	--
Leu	0.05%	1.88 ± 0.13 [fg]	51%	2.11 ± 0.20 [cd]	42%	1.96 ± 0.35 [e]	43%
	0.20%	1.27 ± 0.15 [hi]	67%	2.04 ± 0.17 [de]	44%	1.92 ± 0.16 [e]	44%
	0.50%	0.93 ± 0.12 [hi]	75%	1.64 ± 0.04 [defg]	55%	1.38 ± 0.23 [f]	60%
Control	0.00%	3.76 ± 0.26 [c]	--	3.35 ± 0.23 [b]	--	3.49 ± 0.15 [b]	--
Pro	0.05%	2.82 ± 0.11 [d]	25%	2.55 ± 0.21 [c]	24%	2.65 ± 0.34 [c]	24%
	0.20%	2.07 ± 0.15 [ef]	45%	2.53 ± 0.23 [c]	25%	2.06 ± 0.15 [de]	41%
	0.50%	1.74 ± 0.10 [fg]	54%	1.71 ± 0.09 [def]	49%	1.27 ± 0.14 [fg]	64%

[1]Values are represented as mean ± standard deviation (n = 3).
[a-j] Means with a different upper case letter in a column are significantly different ($P < 0.05$). ND = below limit of detection (LOD).

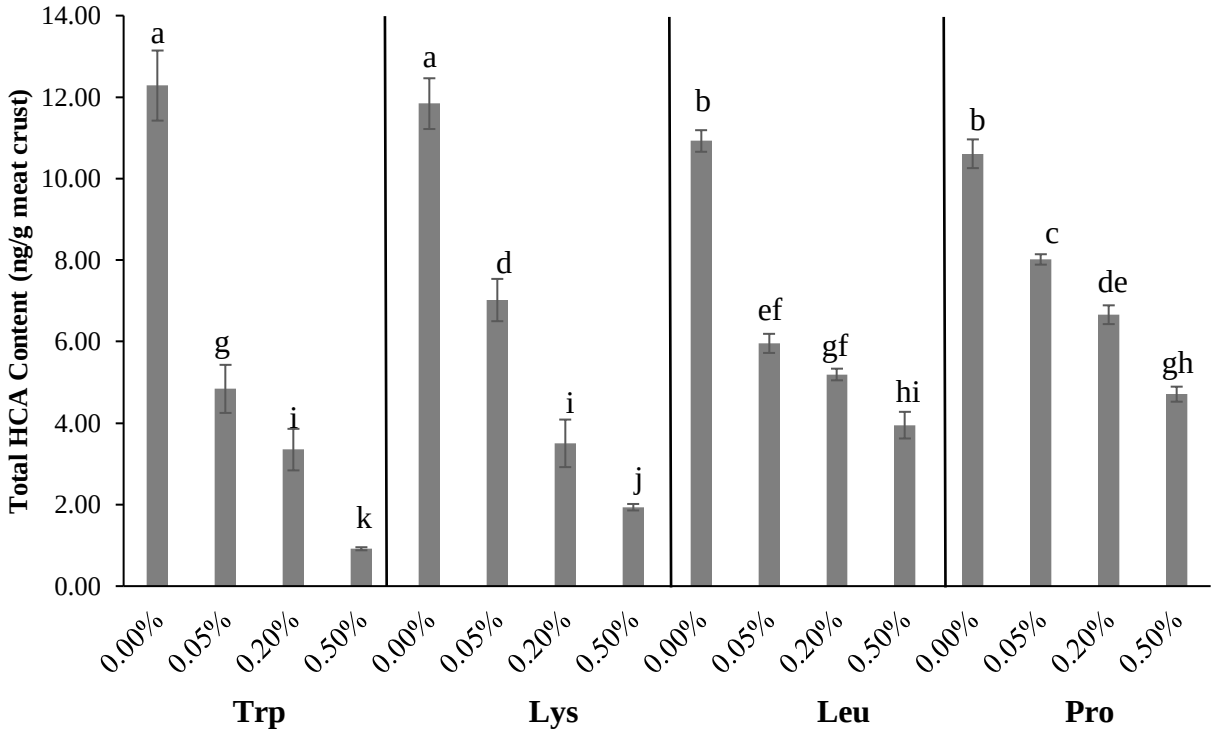


Figure 14. Total amount of HCAs (PhIP, MeIQx, and 4,8-DiMeIQx) formation in pan-fried beef patty crust treated with different concentrations of amino acids (0.00%, 0.05%, 0.20%, and 0.50%). [a-k] Bars with a different upper case letter differ significantly ($P < 0.05$).

Effect of amino acids on cooking loss

Cooking loss is an important parameter of meat characteristics during cooking. Table 18 summarizes our results for cooking loss. The averaged cooking loss in fried control patties ranged from 39.00% to 40.67%. Similar results were also reported by Persson et al. (2004). Cooking losses in fried beef patties treated with 0.05%, 0.20%, and 0.50% of amino acids did not differ significantly from the controls, although a small variation (2 - 10%) was observed ($P > 0.05$). Adding Lys, Leu, and Pro at the highest concentration (0.50%) to beef patties seems to reduce cooking loss the most ($P > 0.05$). In general, cooking causes reduction in weight of meat products, mainly because of water evaporation and/or loss of fat, protein, collagen, salt

polyphosphates and other flavor compounds during heating (Gerber, Scheeder, & Wenk, 2009). In this sense, our results indicate that applying amino acids to the surface of beef patties did not affect the water-holding capacity of meat during cooking. On the other hand, many previous studies have indicated that high cooking loss is related to the increased HCA formation in cooked meat because more water-soluble precursors migrate from the center to the high-temperature surface of the meat and thus induce more HCA formation (Knize et al., 1994). Our study results confirmed that while amino acids did inhibit HCA formation in fried beef patties, this was not a result of reduced cooking loss during frying.

Table 18. Effects of different concentrations of amino acids on cooking loss and pH in pan-fried beef patties.

Amino Acid	Concentration	%Cooking Loss[1]	pH[2]
Control	0.00%	40.67 ± 1.25 [a]	6.04 ± 0.08 [cde]
Trp	0.05%	38.33 ± 2.36 [ab]	5.94 ± 0.05 [cdf]
	0.20%	38.67 ± 1.70 [ab]	6.00 ± 0.08 [cde]
	0.50%	37.67 ± 1.70 [ab]	6.09 ± 0.11 [cd]
Control	0.00%	40.00 ± 0.82 [ab]	6.01 ± 0.07 [cde]
Lys	0.05%	38.67 ± 1.70 [ab]	6.13 ± 0.11 [bc]
	0.20%	38.67 ± 2.05 [ab]	6.41 ± 0.15 [b]
	0.50%	36.00 ± 2.94 [b]	6.94 ± 0.11 [a]
Control	0.00%	39.00 ± 0.82 [ab]	6.00 ± 0.09 [cde]
Leu	0.05%	37.00 ± 1.63 [ab]	5.77 ± 0.22 [ef]
	0.20%	38.33 ± 3.87 [ab]	5.86 ± 0.12 [cdef]
	0.50%	36.33 ± 2.87 [ab]	5.96 ± 0.10 [cdef]
Control	0.00%	40.33 ± 1.25 [ab]	5.99 ± 0.11 [cdef]
Pro	0.05%	39.00 ± 2.16 [ab]	5.71 ± 0.32 [f]
	0.20%	38.00 ± 2.16 [ab]	5.81 ± 0.20 [def]
	0.50%	36.67 ± 2.87 [ab]	5.99 ± 0.01 [cdef]

[1,2] Values are represented as mean ± standard deviation (n = 3).

[a-f] Means with a different upper case letter in a column are significantly different ($P < 0.05$).

Effect of amino acids on pH after frying

Table 18 provides the changes in the pH value of beef crust treated with different concentrations of amino acids. While the initial pH of fresh ground beef (7% fat) was 5.84, comparable to previously reported values in raw beef (Oz & Zikirov, 2015; Tengilimoglu-Metin & Kizil, 2017), pan-frying at 230 °C for 15 min led to expected increases in pH values of patties (5.99 - 6.04). In general, pH values of meat will increase during cooking, probably due to the

cleavage of bonds involving imidazole, sulfhydryl, and hydroxyl groups (Girard, 1992). After frying, these pH values did not differ significantly ($P > 0.05$) between controls and patties treated with amino acids, other than Lys at all three concentrations. For beef patties treated with Lys, 0.20% and 0.50% concentrations led to a significant increase in pH to 6.41 and 6.94 compared to controls ($P < 0.05$). Unlike the other amino acids, Lys contains a side chain of Ɛ- amino group (p*Ka* 10.5) and is classified as a basic amino acid at neutral pH, so the increased pH in Lys-treated beef crusts could be due to the presence of Lys residues or the result of high concentrations of amines generated by thermal degradation of Lys (α- and Ɛ- amino groups).

Surface color change after frying

Color is also an important influence on consumer acceptance of cooked meat products. Figure 15 shows images of pan-fried control beef patties and patties containing different amounts (0.05%, 0.20%, and 0.50%) of amino acids. Beef patties containing 0.20% and 0.50% Lys have a more intense red color at the meat surface, while patties containing 0.50% Trp and 0.50% Leu have more whiteness than the control patties. To confirm the visual observation of color change, instrumental color parameters (L*, a*, and b* values) of all fried beef patties were measured; Table 19 summarizes the results. Surface application of Lys and Pro at all tested concentrations had no significant effect on L* values (brightness) of beef patties after cooking ($P > 0.05$), but L* values increased significantly ($P < 0.05$) in beef patties containing 0.50% Trp and 0.50% Leu, indicating that observations of a more white appearance was justified (see Figure 15).

The a* values, indicating redness, increased significantly from 8.30 (control) to 9.57 and 11.91 in Lys treated beef patties at 0.20% and 0.50% levels ($P < 0.05$). Surface application of Lys do enhance the redness of beef patties after cooking. Moreover, other amino acids had a

slight but non-significant increase in a* values (redness) at the higher concentrations ($P > 0.05$) (see Table 19). The a* value is often associated with myoglobin, oxymyoglobin, and metmyoglobin pigments of meat, and a high a* value (red color) is inversely related to the percentage of denatured myoglobin in cooked meat (Roldán, Antequera, Martín, Mayoral, & Ruiz, 2013). Furthermore, Trout (1989) noted that a high pH (> 6.0) in raw meats, including beef, pork, and turkey, would reduce the percentage of denatured myoglobin formed by cooking, resulting in a red cooked meat color. In our study, the higher a* values in patties treated with Lys was probably due to their higher pH values (6.41 to 6.94) in cooked meat, thus producing a high-intensity red meat appearance. Also, Lys might induce the formation of nitrosomyoglobin by producing nitric oxide (NO) (Lewis, 2004), which may also induce red color formation in meat products during cooking.

Adding Lys also affected the b* values (yellowness) in fried beef patties, significantly decreasing b* values from 10.14 (control) to 8.26 and 8.08 at 0.20% and 0.50% levels ($P < 0.05$); no significant difference was observed between control and Trp, Leu, and Pro treated patties ($P > 0.05$). This indicates that Lys, in addition to enhancing red color formation, also adversely affects yellow color formation at the meat surface. Linghu et al. (2017) also reported that Lys seems to be more reactive than Trp, Leu, and Pro in retarding the Maillard browning reaction in model systems (180 ℃ for 1h); a significant decrease in browning (Abs 420) occurred when Lys content was 4 times less than other precursors (i.e., glucose and creatinine). Therefore, our results clearly indicated that applying most amino acids, including Trp, Leu, and Pro at levels of 0.05% to 0.20%, did not alter surface color in fried beef patties. However, Lys at high concentrations may increase red color (a*) and decrease yellow color (b*) during frying.

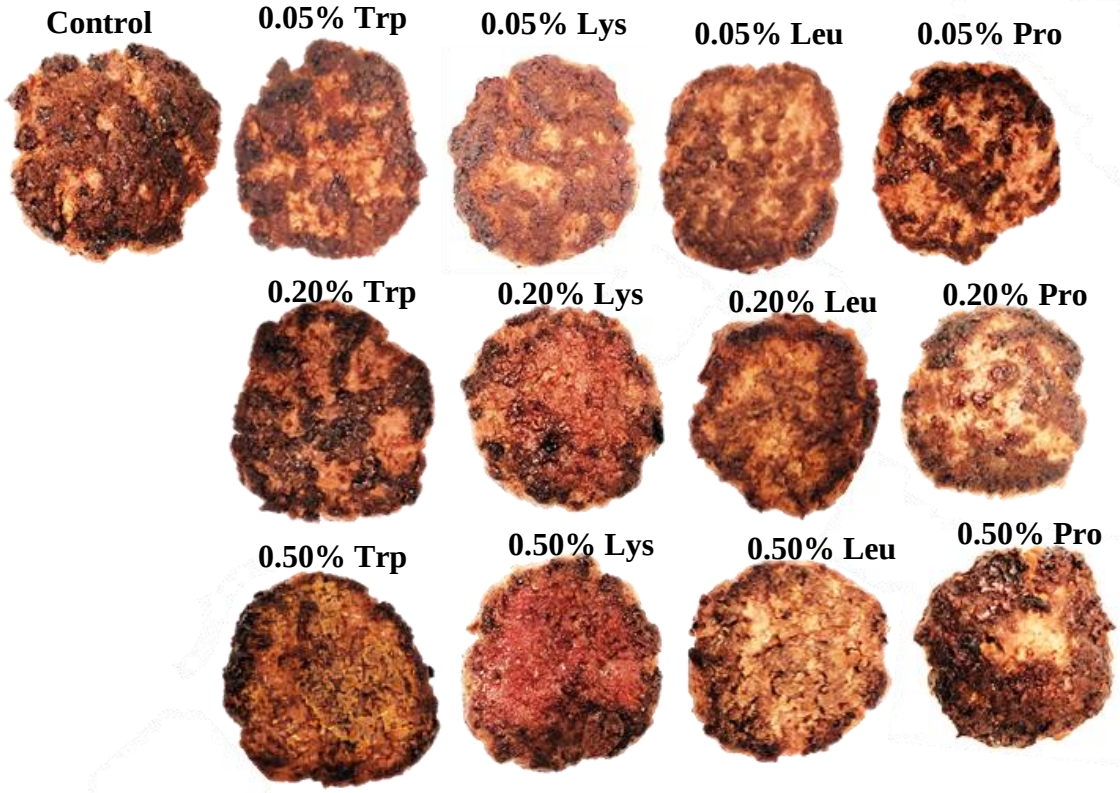


Figure 15. Images of the control beef patties (with no amino acids) and treated patties containing different amounts of amino acids (Trp, Lys, Leu, and Pro) after pan-frying at 230 °C for 15 min.

Table 19. Surface color measurement (L*, a*, and b*) results in pan-fried beef patty crusts containing different concentrations of amino acids.

Amino Acid	Concentration	L* [1]	a* [2]	b* [3]
Control	0.00%	26.11 ± 1.46 [b]	8.24 ± 0.28 [de]	10.42 ± 0.74 [bcd]
Trp	0.05%	26.31 ± 1.46 [b]	8.14 ± 0.25 [de]	9.85 ± 0.23 [dc]
	0.20%	26.34 ± 0.67 [b]	8.05 ± 0.45 [de]	9.31 ± 0.60 [de]
	0.50%	29.55 ± 1.13 [a]	8.61 ± 0.49 [cde]	11.57 ± 1.17 [ab]
Control	0.00%	25.82 ± 1.51 [b]	8.30 ± 0.36 [cde]	10.14 ± 0.81[cd]
Lys	0.05%	26.90 ± 0.89 [b]	8.73 ± 0.49 [cd]	9.10 ± 0.89 [de]
	0.20%	25.54 ± 0.25 [b]	9.57 ± 0.15 [b]	8.26 ± 0.30 [e]
	0.50%	25.33 ± 0.64 [b]	11.91 ± 0.40 [a]	8.08 ± 0.49 [e]
Control	0.00%	27.14 ± 1.21 [b]	8.15 ± 0.10 [de]	10.99 ± 0.45 [abc]
Leu	0.05%	27.01 ± 0.39 [b]	8.47 ± 0.28 [cde]	8.21 ± 0.43 [e]
	0.20%	27.32 ± 1.30 [b]	8.59 ± 0.42 [cde]	10.07 ± 0.30 [cd]
	0.50%	28.87 ± 1.10 [a]	9.02 ± 0.17 [bc]	11.97 ± 0.67 [a]
Control	0.00%	26.42 ± 1.56 [b]	8.30 ± 0.36 [cde]	10.88 ± 0.82 [abc]
Pro	0.05%	26.59 ± 0.82 [b]	8.43 ± 0.38 [cde]	9.16 ± 0.79 [de]
	0.20%	27.32 ± 1.03 [b]	7.99 ± 0.34 [e]	10.20 ± 0.80 [bcd]
	0.50%	27.30 ± 1.09 [b]	8.44 ± 0.15 [cde]	9.80 ± 0.95 [dc]

[1,2,3] Values are represented as mean ± standard deviation (n = 3).

[a-e] Means with a different upper case letter in a column are significantly different ($P < 0.05$).

Volatile profile change after frying

During cooking, various volatile compounds are released from meat products, primarily through the Maillard reaction of amino acids, lipid oxidation, and degradation, as well as the interaction between lipid-oxidation products with Maillard intermediates (Mottram, 1998). As Figures 16-19 illustrate, the differences in the volatile compound profile of fried beef crust (230 °C/15 min) treated with amino acids (Trp, Lys, Leu, and Pro) were characterized with GC-MS

data (express as peak area) (their GC-MS chromatogram are presented in Appendix A, Figure A-13-14). Using the SPME-GC-MS method, 16 volatile compounds, including 7 aldehydes (benzaldehyde, nonanal, hexanal, heptanal, decanal, phenylacetaldehyde and 2-methylbutanal), 5 pyrazines (methylpyrazine, 2,5(6)-dimethylpyrazine, 3-ethyl-2,5-dimethylpyrzines, 2-ethyl-6-methylpyrazine, and 2-ethyl-5-methylpyrazine), 2 alcohols (1-octen-3-ol and 1-octanol), 1 sulfur compound (methanethiol), and 1 furan (2-pentyl furan) were identified in the control; among the volatile compounds, the most abundant were aldehydes and alkylpyrazines, while the alcohols, sulfur compound, and furan were relatively low (Figures 16-19). The overall volatile profile of fried beef patties was also comparable with previously reported research of cooked beef products (Moon, Cliff, & Li-Chan, 2006; Wang et al., 2018).

Pyrazines are the Maillard reaction products that contribute savory, nutty, roasted, and burnt odors in the volatiles of cooked beef (Mottram, 1988). Many studies have demonstrated that pyrazines form through the α-dicabonyls and Strecker degradation of amino acids in the Maillard reaction (Mottram, 1998). As Figures 16-19 show, applying amino acids to the patty surfaces significantly influenced the amounts and compositions of pyrazines in fried beef patties. Lys at 0.50% level significantly enhanced the formation of methylpyrazine (nutty and roast odor; Van, Hwang, Jeong, & Touseef, 2012), 2,5(6)-dimethylpyrazine (popcorn and roasted odor; Van et al., 2012), 3-ethyl-2,5-dimethylpyrazine (nutty and roast odor; Van et al., 2012), and 2-ethyl-5-methylpyrazine (fruity and sweet odor; Van et al., 2012) compared to controls ($P < 0.05$). However, Pro at 0.50% significantly inhibited 2,5(6)-dimethylpyrazine and 3-ethyl-2,5-dimethylpyrzine formation by 46% and 63% ($P < 0.05$); while Trp and Leu appeared to contribute the least to pyrazines, showing no significant differences to the controls ($P > 0.05$). These findings suggested that adding high concentrations of Lys can promote formation of nutty

and roasted odors in fried meat, while Pro at high levels decreased the intensity of those the aromas. Please note that adding Leu to beef patties at 0.20% and 0.50% levels produced two new pyrazines: 3-butyl-2,5-dimethylpyrazine and 2-isoamyl-6-methylpyrazine. Adding Lys to beef patties also produced a new pyrazine: trimethylpyrazine (nutty and roasted odor; Moon et al., 2006). Linghu et al. (2017) reported similar findings, confirming that adding Leu and Lys to a model system (glucose: creatinine: phenylalanine) can alter the Maillard reaction by forming new pyrazine compounds. Much research has revealed that pyrazines like 3-ethyl-2,5-dimethylpyrzine, 2-ethyl-6-methylpyrazine, trimethylpyrazine, 2-ethyl-3,5-dimethylpyrazine, 2,3-diethyl-5-methylpyrazine, and 2,5-diethyl-3,6-dimethylpyrazine are important odor-active compounds in fried, grilled, and baked beef (Amrani-Hemaimi, Cerny, & Fay, 1995; Leod & Ames, 1986; Moon et al., 2006), but no pyrazines have been identified in boiled or raw beef (Moon et al., 2006). Different cooking conditions (temperature and time), meat composition, and extraction conditions of volatile compounds may affect pyrazine formation in cooked beef. However, 3-butyl-2,5-dimethylpyrazine and 2-isoamyl-6-methylpyrazine have not been previously identified in volatile compounds of cooked beef although they were found in simulated beef flavor (Moon et al., 2006).

Aldehydes are also important odor-active compounds, providing grassy, fresh, and fruity notes (Van et al., 2012), with great impact on the whole aroma profile of cooked beef. As Figures 16-19 show, lipid-oxidation derived aldehydes, including benzaldehyde, nonanal, hexanal, heptanal, and decanal, showed no significant differences among the treatments ($P > 0.05$), which indicated that adding amino acids to beef patties before cooking did not affect the production of volatile compounds originating from lipid oxidation. On the other hand, phenylacetaldehyde (green and floral odor), which is a Strecker degradation product of

phenylalanine, was reduced significantly when all tested amino acids were added, with controls for comparison ($P < 0.05$); 2-methylbutanal showed no significant differences ($P > 0.05$). Interestingly, adding Leu to beef patties released a new aldehyde named 3-methylbutanal (see Figure 18). Gianelli et al. (2012) reported that Leu is the precursor of 3-methylbutanal produced via Strecker degradation in cooked beef patties. This indicates that the added amino acids were involved in the Maillard reaction during cooking and influenced formation of different Strecker aldehydes. Moreover, adding most amino acids did not affect alcohols, sulfur compound (as methanethiol), and furan (as 2-pentyl furan) in the treatments ($P > 0.05$); Trp, however, at 0.50% significantly increased 1-octen-3-ol (mushroom odor; Van et al., 2012) and 2-pentyl furan (metallic, green, earthy odor; Van et al., 2012) formation ($P < 0.05$). These compounds mainly originate from lipid oxidation or Maillard reactions (Mottram, 1988). Furthermore, GC-MS analysis of beef crust revealed that two new volatile compounds, 3-methylindole and indole, formed in patties treated with high concentrations (0.20% and 0.50%) of Trp (see Figure 16). 3-methylindole (also known as skatol) and indole have been previously reported as microbial degradation products of Trp in the rumen and are described as pungent, fecal, and animal-like rancid odors (Kamili et al., 2010). In our study, thermal degradation of Trp might contribute to their formation, which has been confirmed in a previous study (Linghu et al., 2017). As for their unpleasant fecal-like odor, which is perceptible at extremely low odor thresholds (0.8-40 ng/L in air) (Kamili et al., 2010), Trp treated beef patties might not be acceptable to consumers. Our results indicate that applying amino acids to meat surfaces greatly affects the volatile profile of fried beef patties and more effectively influence modulating the Maillard-type volatile compounds than volatiles generated via lipid oxidation.

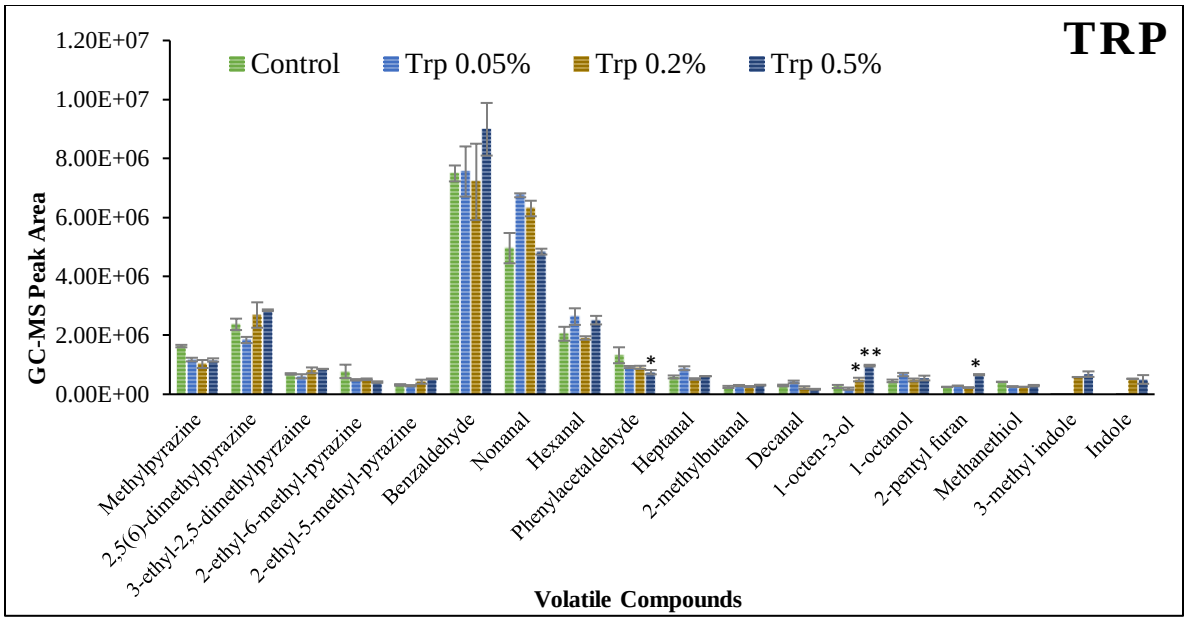


Figure 16. GC-MS analysis data of volatile compounds generated from pan-fried beef crust treated with 0.05%, 0.20%, and 0.50% of Trp and control. Bars with an asterisk have a significant difference compared to control ($P < 0.05$).

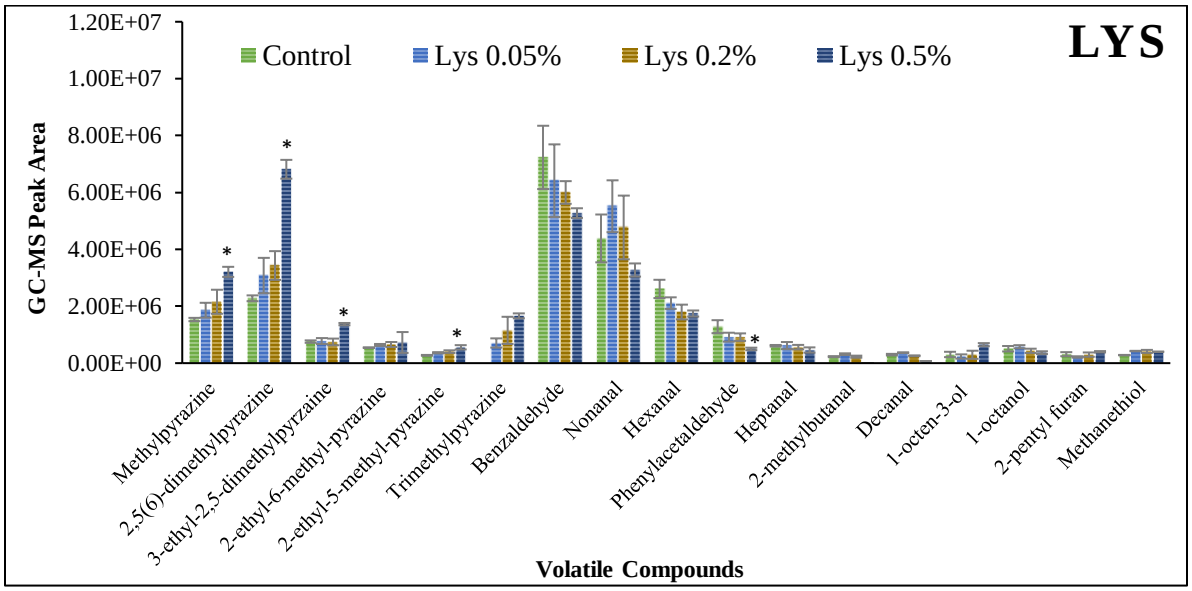


Figure 17. GC-MS analysis data of volatile compounds generated from pan-fried beef crust treated with 0.05%, 0.20%, and 0.50% of Lys and control. Bars with an asterisk have a significant difference compared to control ($P < 0.05$).

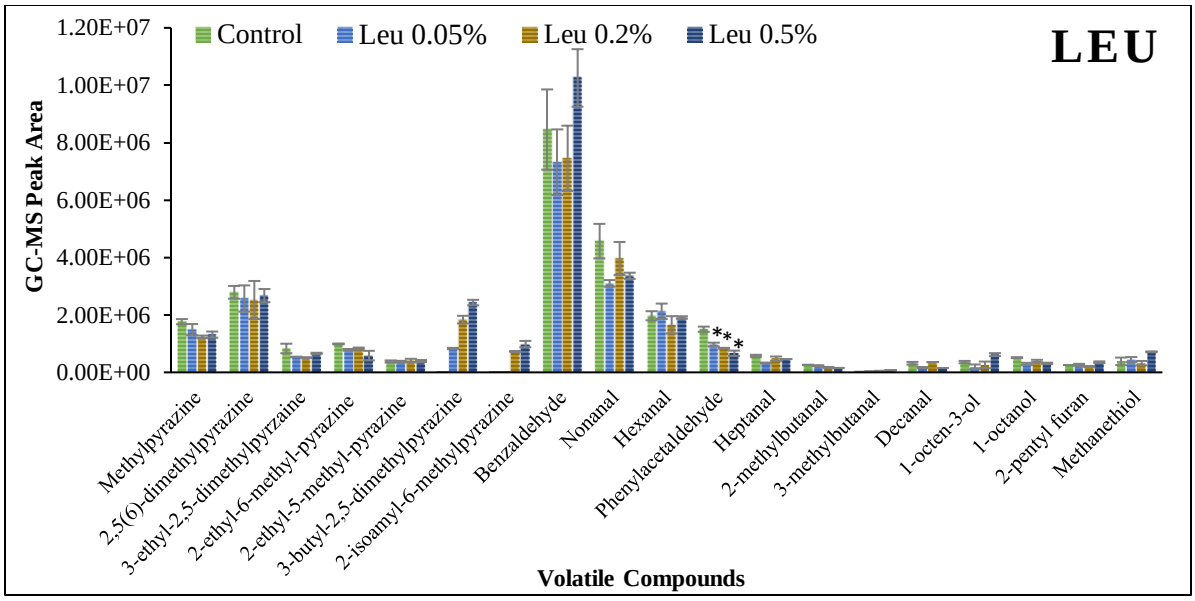


Figure 18. GC-MS analysis data of volatile compounds generated from pan-fried beef crust treated with 0.05%, 0.20%, and 0.50% of Leu and control. Bars with an asterisk have a significant difference compared to control ($P < 0.05$).

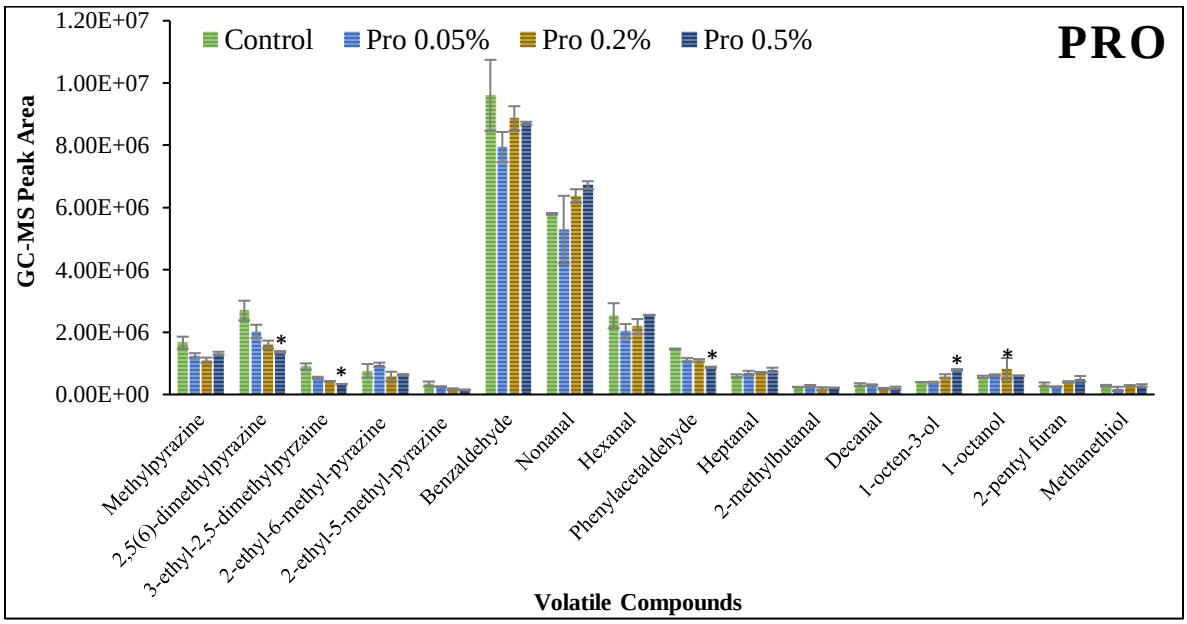


Figure 19. GC-MS analysis data of volatile compounds generated from pan-fried beef crust treated with 0.05%, 0.20%, and 0.50% of Pro and control. Bars with an asterisk have a significant difference compared to control ($P < 0.05$).

CONCLUSION

This study demonstrated that surface application of amino acids (Trp, Lys, Leu, and Pro) at three concentrations of 0.05%, 0.20%, and 0.50% can significantly inhibit total HCA formation in beef patties when frying the patties at 230 °C for 15 min. Three major HCAs including PhIP (3.76-4.89 ng/g), MeIQx (3.35-3.97 ng/g), and 4,8-DiMeIQx (3.38-3.90 ng/g) were detected and quantified from fried beef crusts using LC-MS/MS; IQ and IQx, however, were not detectable. Statistical analysis showed that the interaction of amino acid type and concentration significantly affected the reduction of HCAs ($P < 0.05$). Trp at 0.50% reduced PhIP (up to 99%), MeIQx (up to 90%), and 4,8-DiMeIQx (up to 91%), the highest reduction for all four amino acids, followed by 0.50% Lys (70%-97% inhibition) and 0.50% Leu (55%-75% inhibition); while 0.50% Pro (49%-64% inhibition) was less effective. On the other hand, cooking loss did not differ significantly from controls ($P > 0.05$). Other meat quality properties, such as pH, surface color, and volatile compounds, were significantly affected (induced/suppressed) by adding Trp, Lys, and Lue ($P < 0.05$). Lys especially appeared to increase redness in the meat as well as more aromatic volatile compounds in meat crust. Pro showed no effect.

REFERENCES

Abdulkarim, B. G., & Smith, J. S. (1998). Heterocyclic Amines in Fresh and Processed Meat Products. *Journal of Agricultural and Food Chemistry*, *46*(11), 4680–4687.

Alaejos, M. S., & Afonso, A. M. (2011). Factors That Affect the Content of Heterocyclic Aromatic Amines in Foods. *Comprehensive Reviews in Food Science and Food Safety*, *10*(2), 52–108.

Amrani-Hemaimi, M., Cerny, C., & Fay, L. B. (1995). Mechanisms of Formation of Alkylpyrazines in the Maillard Reaction. *Journal of Agricultural and Food Chemistry*, *43*(11), 2818–2822.

Ashoor, S.H., Dietrich, R.A., Chu, F.S., & Pariza, M.W. (1980). Proline enhances mutagen formation in ground beef during frying. *Life Science*, *26*, 1801-1805.

Cheng, K.W., Chen, F., & Wang, M. (2006). Heterocyclic amines: Chemistry and health. *Molecular Nutrition & Food Research*, *50*(12), 1150–1170.

Cheng, K.W., Wu, Q., Zheng, Z. P., Peng, X., Simon, J. E., Chen, F., & Wang, M. (2007). Inhibitory Effect of Fruit Extracts on the Formation of Heterocyclic Amines. *Journal of Agricultural and Food Chemistry*, *55*(25), 10359–10365.

Cross, A. J., Peters, U., Kirsh, V. A., Andriole, G. L., Reding, D., Hayes, R. B., & Sinha, R. (2005). A prospective study of meat and meat mutagens and prostate cancer risk. *Cancer Research*, *65*(24), 11779–11784.

Damašius, J., Venskutonis, P. R., Ferracane, R., & Fogliano, V. (2011). Assessment of the influence of some spice extracts on the formation of heterocyclic amines in meat. *Food Chemistry*, *126*(1), 149–156.

Fan, X.J., Liu, S.Z., Li, H.H., He, J., Feng, J.-T., Zhang, X., & Yan, H. (2019). Effects of Portulaca oleracea L. extract on lipid oxidation and color of pork meat during refrigerated storage. *Meat Science*, *147*, 82–90.

Felton, J. S., Knize, M. G., Shen, N. H., Andresen, B. D., Bjeldanes, L. F., & Hatch, F. T. (1986). Identification of the Mutagens in Cooked Beef. *Environmental Health Perspectives*, *67*, 17.

Felton, J. S., Knize, M. G., Wu, R. W., Colvin, M. E., Hatch, F. T., & Malfatti, M. A. (2007). Mutagenic potency of food-derived heterocyclic amines. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, *616*(1), 90–94.

Gerber, N., Scheeder, M. R. L., & Wenk, C. (2009). The influence of cooking and fat trimming on the actual nutrient intake from meat. *Meat Science*, *81*(1), 148–154.

Gianelli, M. P., Salazar, V., Mojica, L., & Friz, M. (2012). Volatile compounds present in traditional meat products (charqui and longaniza sausage) in Chile. *Brazilian Archives of Biology and Technology*, *55*(4), 603–612.

Gibis, M. (2016). Heterocyclic Aromatic Amines in Cooked Meat Products: Causes, Formation, Occurrence, and Risk Assessment: Heterocyclic amines in cooked meat products…. *Comprehensive Reviews in Food Science and Food Safety*, *15*(2), 269–302.

Girard, J.P. (1992). Cooking. Technology of meat and meat products France, 32–83.

[IARC] Intl. Agency for Research on Cancer. 1993. Monographs on the Evaluation of Carcinogenic Risk to Humans. Vol. 56. Lyon, France: IARC.

Jinap, S., Iqbal, S. Z., & Selvam, R. M. P. (2015). Effect of selected local spices marinades on the reduction of heterocyclic amines in grilled beef (satay). *LWT - Food Science and Technology*, *63*(2), 919–926.

Jones, R.C., & Weisburger, J. (1988a). L-Tryptophan inhibits formation of mutagens during cooking of meat and in laboratory models. *Mutation Research*, *206*(3), 343-349.

Jones, R.C., & Weisburger, J. (1988b). Inhibition of aminoimidazo-quinoxaline-type and aminoimidazol-4-one type mutagen formation in liquid-reflux models by the amino acids L-proline and/or L-tryptophan. *Environmental and Molecular Mutagenesis*, *11*, 509-514.

Jones, R.C., & Weisburger, J. (1988c). Inhibition of aminoimidazo-quinoxaline-type and aminoimidazol-4-one-type mutagen formation in liquid reflux models by L-tryptophan and other selected indoles. *Japanese Journal of Cancer Research*, *79*, 222-230.

Kamili, A., Wat, E., Chung, R. W., Tandy, S., Weir, J. M., Meikle, P. J., & Cohn, J. S. (2010). Hepatic accumulation of intestinal cholesterol is decreased and fecal cholesterol excretion is increased in mice fed a high-fat diet supplemented with milk phospholipids. *Nutrition & Metabolism*, *7*, 90.

Kataoka, H., Miyake, M., Nishioka, S., Matsumoto, T., Saito, K., & Mitani, K. (2009). Formation of protein adducts of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine in cooked foods. *Molecular Nutrition & Food Research*, *54*(7), 1039–1048.

Kataoka, H., Miyake, M., Saito, K., & Mitani, K. (2012). Formation of heterocyclic amine–amino acid adducts by heating in a model system. *Food Chemistry*, *130*(3), 725–729.

Knize, M. G., Cunningham, P. L., Griffin, E. A., Jones, A. L., & Felton, J. S. (1994). Characterization of mutagenic activity in cooked-grain-food products. *Food and Chemical Toxicology: An International Journal Published for the British Industrial Biological Research Association*, *32*(1), 15–21.

Leod, G. M., & Ames, J. M. (1986). Capillary Gas Chromatography-Mass Spectrometric Analysis of Cooked Ground Beef Aroma. *Journal of Food Science*, *51*(6), 1427–1434.

Lewis, R.J. Sr. (2004). *Sax's Dangerous Properties of Industrial Materials* (11th ed). Hoboken, NJ: Wiley-Interscience, Wiley & Sons, Inc.

Linghu, Z., Karim, F., & Smith, J. S. (2017). Amino Acids Inhibitory Effects and Mechanism on 2-Amino-1-Methyl-6-Phenylimidazo [4,5-b]Pyridine (PhIP) Formation in the Maillard Reaction Model Systems. *Journal of Food Science*, *82*(12), 3037–3045.

Moon, S.Y., Cliff, M. A., & Li-Chan, E. C. Y. (2006). Odour-active components of simulated beef flavour analysed by solid phase microextraction and gas chromatography–mass spectrometry and –olfactometry. *Food Research International*, *39*(3), 294–308.

Mottram, D. S. (1998). Flavour formation in meat and meat products: a review. *Food Chemistry*, *62*(4), 415–424.

Oz, F., & Kaya, M. (2011). HETEROCYCLIC AROMATIC AMINES IN MEAT: HCAS AND MEAT. *Journal of Food Processing and Preservation*, *35*(6), 739–753.

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

Persson, E., Sjöholm, I., Nyman, M., & Skog, K. (2004). Addition of various carbohydrates to beef burgers affects the formation of heterocyclic amines during frying. *Journal of Agricultural and Food Chemistry*, *52*(25), 7561–7566.

Puangsombat, K., Gadgil, P., Houser, T. A., Hunt, M. C., & Smith, J. S. (2012). Occurrence of heterocyclic amines in cooked meat products. *Meat Science*, *90*(3), 739–746.

Puangsombat, K., Jirapakkul, W., & Smith, J. S. (2011). Inhibitory activity of Asian spices on heterocyclic amines formation in cooked beef patties. *Journal of Food Science*, *76*(8), T174-180.

Roldán, M., Antequera, T., Martín, A., Mayoral, A. I., & Ruiz, J. (2013). Effect of different temperature–time combinations on physicochemical, microbiological, textural and structural features of sous-vide cooked lamb loins. *Meat Science*, *93*(3), 572–578.

Sabally, K., Sleno, L., Jauffrit, J.-A., Iskandar, M. M., & Kubow, S. (2016). Inhibitory effects of apple peel polyphenol extract on the formation of heterocyclic amines in pan fried beef patties. *Meat Science*, *117*, 57–62.

Skog, K. I., Johansson, M. A., & Jägerstad, M. I. (1998). Carcinogenic heterocyclic amines in model systems and cooked foods: a review on formation, occurrence and intake. *Food and Chemical Toxicology: An International Journal Published for the British Industrial Biological Research Association*, *36*(9–10), 879–896.

Sugimura, T., Wakabayashi, K., Nakagama, H., & Nagao, M. (2004). Heterocyclic amines: Mutagens/carcinogens produced during cooking of meat and fish. *Cancer Science*, *95*(4), 290–299.

Tengilimoglu-Metin, M. M., & Kizil, M. (2017). Reducing effect of artichoke extract on heterocyclic aromatic amine formation in beef and chicken breast meat. *Meat Science*, *134*, 68–75.

Trout, G. R. (1989). Variation in Myoglobin Denaturation and Color of Cooked Beef, Pork, and Turkey Meat as Influenced by pH, Sodium Chloride, Sodium Tripolyphosphate, and Cooking Temperature. *Journal of Food Science*, *54*(3), 536–540.

Van, H., Hwang, I., Jeong, D., & Touseef, A. (2012). Principle of Meat Aroma Flavors and Future Prospect. In I. Akyar (Ed.), *Latest Research into Quality Control*. InTech.

Wang, X., Zhu, L., Han, Y., Xu, L., Jin, J., Cai, Y., & Wang, H. (2018). Analysis of volatile compounds between raw and cooked beef by HS-SPME–GC–MS. *Journal of Food Processing and Preservation*, *42*(2), e13503.

Zeng, M., Wang, J., Zhang, M., Chen, J., He, Z., Qin, F., … Chen, J. (2018). Inhibitory effects of Sichuan pepper (Zanthoxylum bungeanum) and sanshoamide extract on heterocyclic amine formation in grilled ground beef patties. *Food Chemistry*, *239*, 111–118.

Zochling, S. (2002). Formation of the heterocyclic aromatic amine PhIP: identification of precursors and intermediates. *Food Chemistry*, *79*(1), 125–134.

CHAPTER 5 - SUMMARY AND FUTURE WORK

SUMMARY

This project has provided a fundamental understanding of the influence of AAs on HCA formation in cooked meat products. HCAs are a class of highly mutagenic and potentially carcinogenic compounds that typically form in cooked protein-rich foods, like beef, pork and poultry, through the Maillard reaction. Among the known HCAs, PhIP is formed most abundantly in cooked meat, followed by MeIQx, and 4,8-DiMeIQx. We hypothesized that modifying precursor profiles of HCAs, such as concentrations and types of AAs, in the Maillard reaction, might be a proactive approach to minimizing dietary HCA intakes.

Through the use of different Maillard model systems, Trp, Lys, Leu, and Pro were the most reactive AAs, showing the strongest inhibitory effect on PhIP formation, followed by Met, Val, Ile, Thr, Phe, and Asp. Moreover, AAs were also found to exhibit the similar suppression with an intermediate Maillard product, PheAce, which is the key precursor (reactive carbonyl species) to PhIP formation. This suggests that AAs may inhibit PhIP formation through scavenging the available PheAce by forming different PheAce-AA adducts. Therefore, this work highlights that scavenging PheAce was a key mechanism of amino acids to inhibit PhIP formation in the Maillard reaction. This study demonstrates that most essential and some non-essential AAs could be used as an effective strategy to prevent PhIP formation. In a different study, EMR-Lipid extraction method coupled with LC-MS/MS was proposed, developed, and validated for HCA analysis in different meat matrices including beef, chicken, and pork bacon. Results from this study demonstrate that optimized EMR-Lipid method coupled with LC-MS/MS provides a simple, fast, and efficient technique for the extraction and quantitation of trace-level HCAs in both high-protein and high-fat meat products. Lastly, study of the surface

application of AAs at different concentrations on HCA formation in pan-fried beef patties provides insights into AA type selection and applied usage to reduce the major HCAs including PhIP, MeIQx, and 4,8-DiMeIQx in cooked meat. Results showed that Trp at 0.50% reduced total HCAs the most followed by 0.50% Lys, while 0.50% Leu and Pro were less effective. Applying amino acids to meat surface also plays a key role in influencing meat quality properties, such as pH, surface color, and generation of several volatile compounds (pyrazines and aldehydes).

FUTURE WORK

The project has majorly focused on the effect of 8 essential and 2 non-essential AAs on mutagenic PhIP formation in model systems; however, other non-essential AAs, like L-Arginine containing Ɛ-amino group on the side chain, may also have strong inhibitory activity against PhIP formation via scavenging its intermediate PheAce. Thus, future work can focus on the screening of non-essential AAs and investigating their PheAce scavenging ability in model systems. In addition, the identification of AA-PheAce adducts from the Maillard model systems may provide a chemical basis for understanding the structure activity relationship between AAs and carbonyl compounds.

Furthermore, this project primarily investigated the inhibitory effects of individual AAs including Trp, Lys, Leu, and Pro on total mutagenic HCA formation in a real meat system; pan-fried beef patty was the only meat sample tested in the work of Chapter 4. However, other meat products like chicken breast and pork bacon prepared under pan-frying, grilling, and broiling have also been well reported to generate high amounts of total HCAs. Thus, future work can also focus on the synergistic effect of AAs on HCA reduction in different meat products to address: 1) whether tested AAs could also inhibit HCA formation in other cooked meat products; 2)

reduction of HCAs could be enhanced by applying binary AAs, like Pro+Lys, at lower concentrations; 3) how the surface application of AAs influences overall taste (e.g., bitter attribute) and meaty flavor profile of different meat products.

APPENDIX A

LIST OF FIGURES

LIST OF TABLES

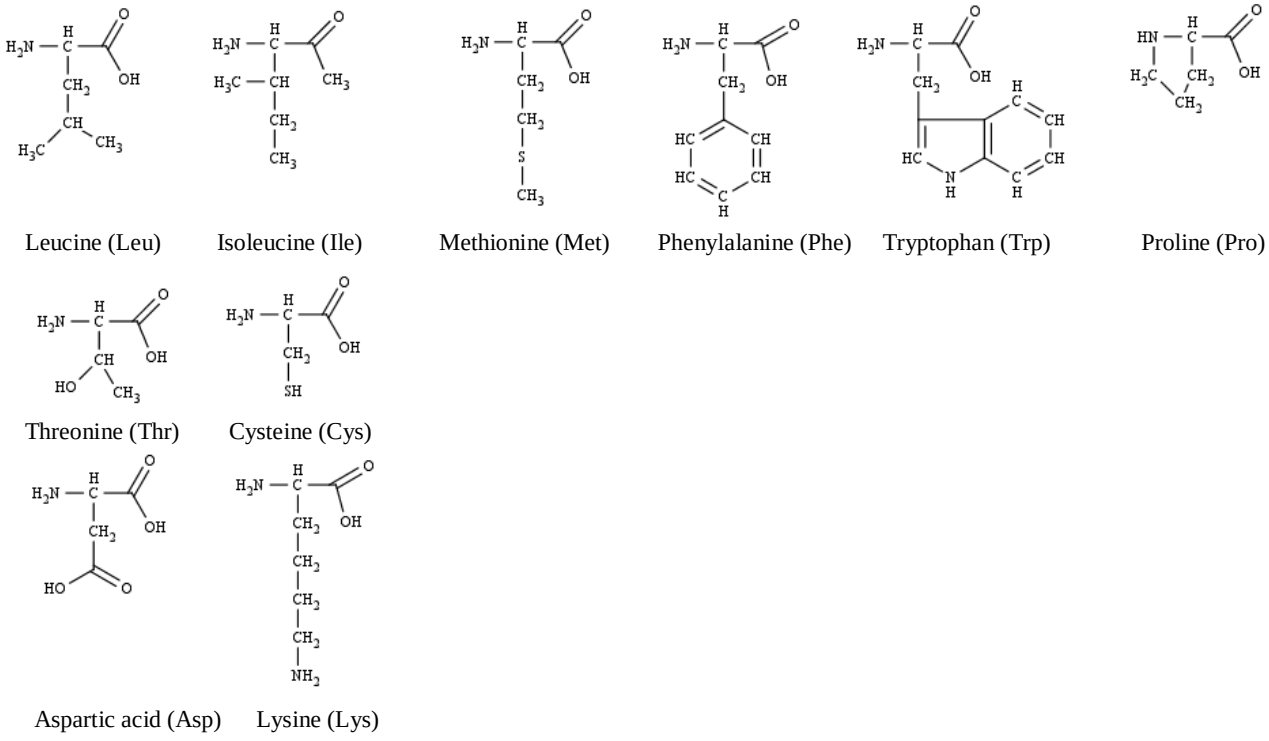


Figure A- 1. Chemical structure and abbreviated name of amino acids.

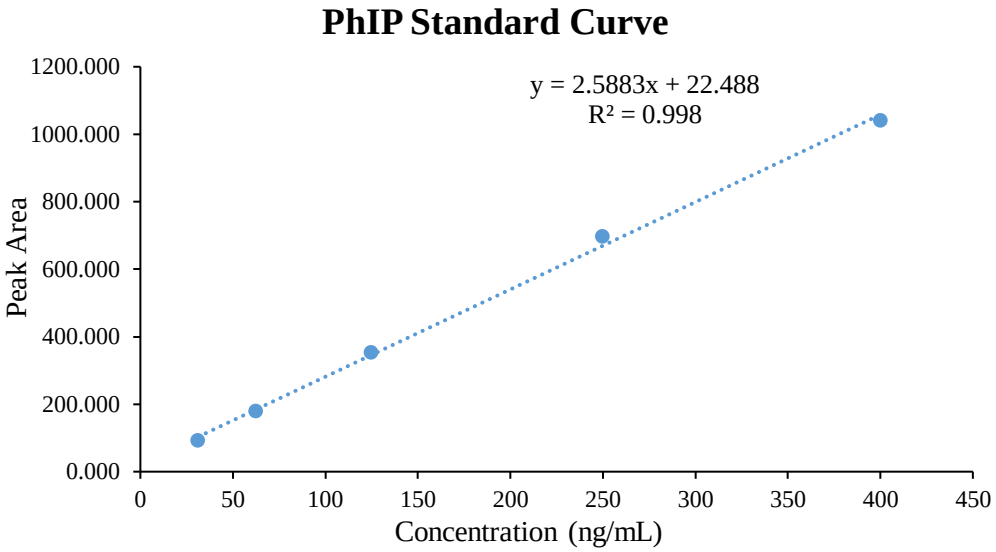


Figure A- 2. Standard curve of PhIP, concentration range from 31.25 - 400 ng/mL measured using HPLC fluorescent detector (Ex=229 nm, Em=437 nm).

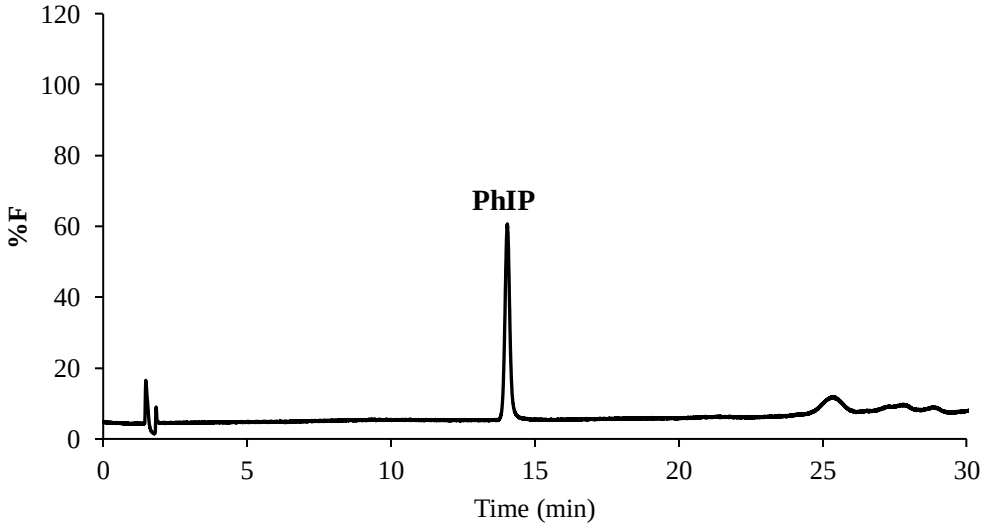


Figure A- 3. HPLC chromatogram of PhIP pure standard separated with a Kintex EVO C18 column (150 x 4.6 mm, 5 µm). The fluorescent detector was set up at 229 nm (excitation) and 437 nm (excitation). The flow rate was 1 mL/min and injection volume was 10 µL.

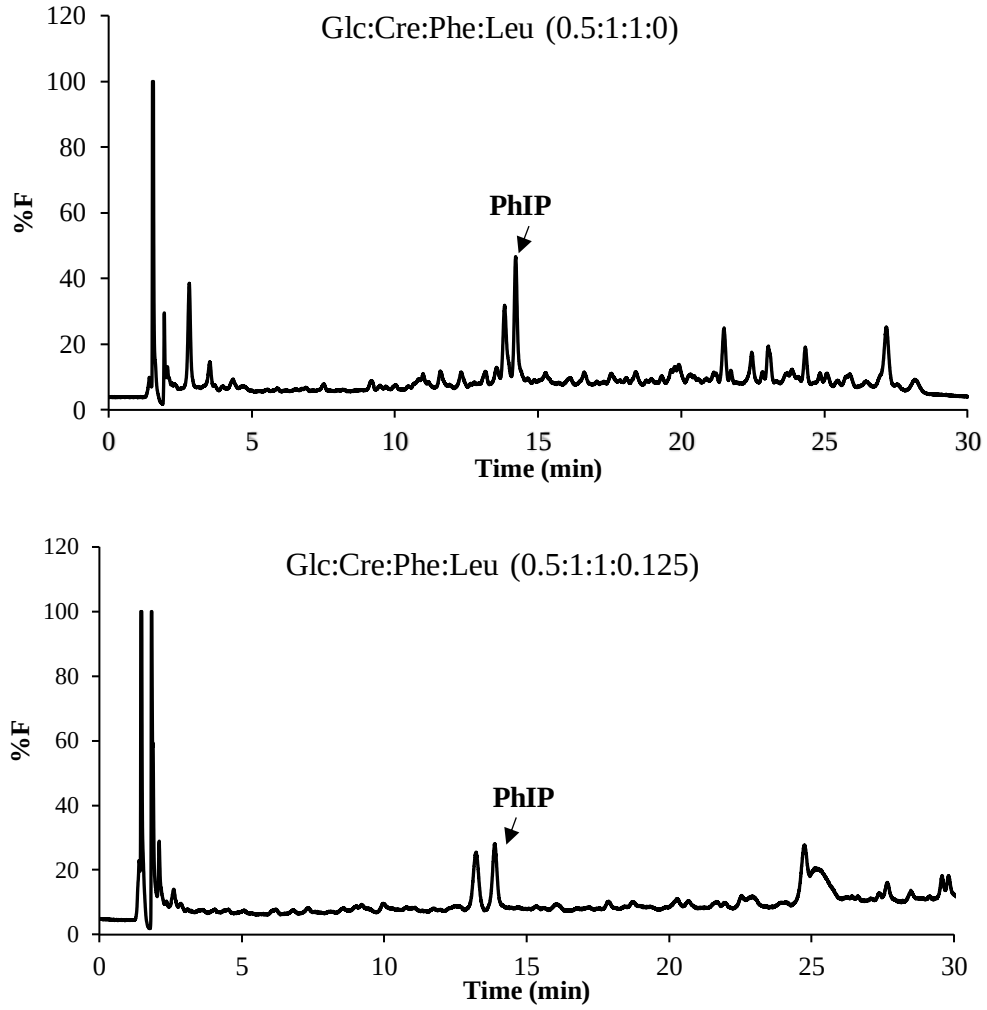


Figure A- 4. HPLC chromatogram of PhIP extracted from Glc:Cre:Phe:Leu model systems (180 °C/1 h) at a molar ratio of 0.5:1:1:0 (Control) and 0.5:1:1:0.125, separated with a Kintex EVO C18 column (150 x 4.6 mm, 5 μm). The fluorescent detector was set up at 229 nm (excitation) and 437 nm (excitation). The flow rate was 1 mL/min and injection volume was 10 µL.

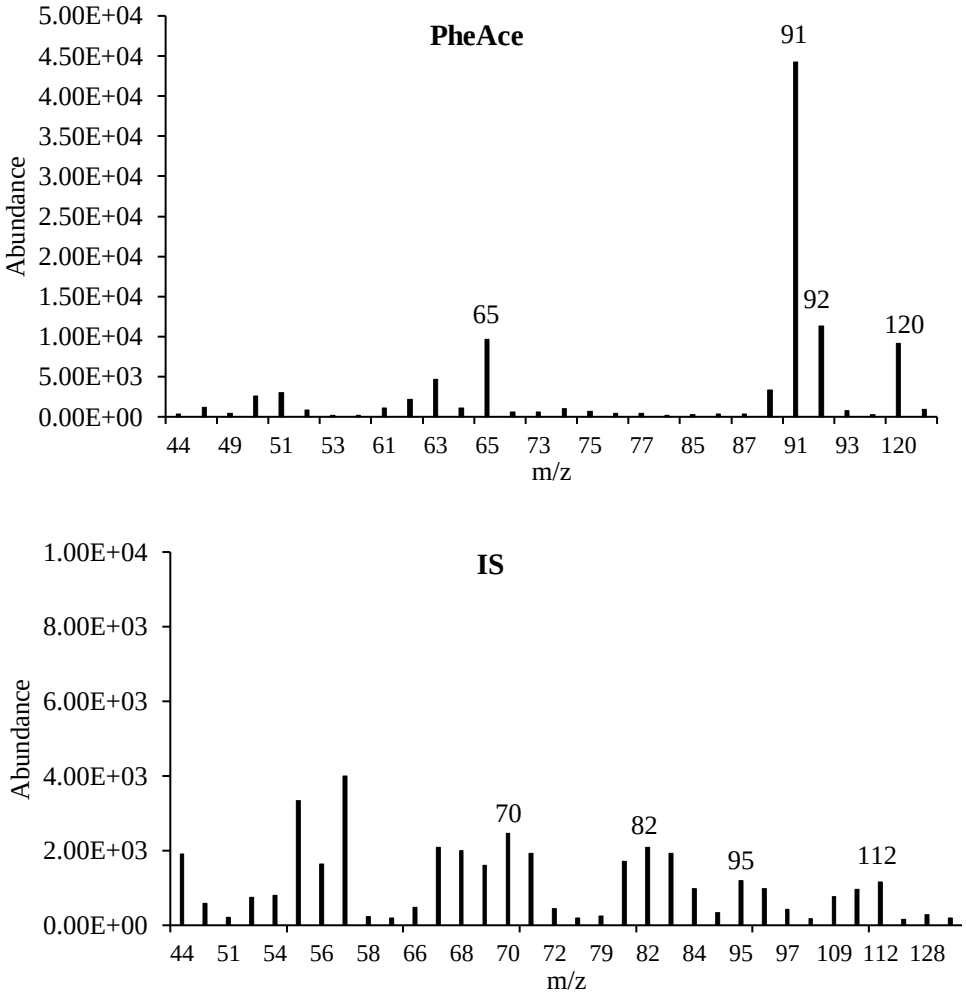


Figure A- 5. Mass spectrum of PheAce and n-decanal (IS) obtained in full scan mode of GC-MS.

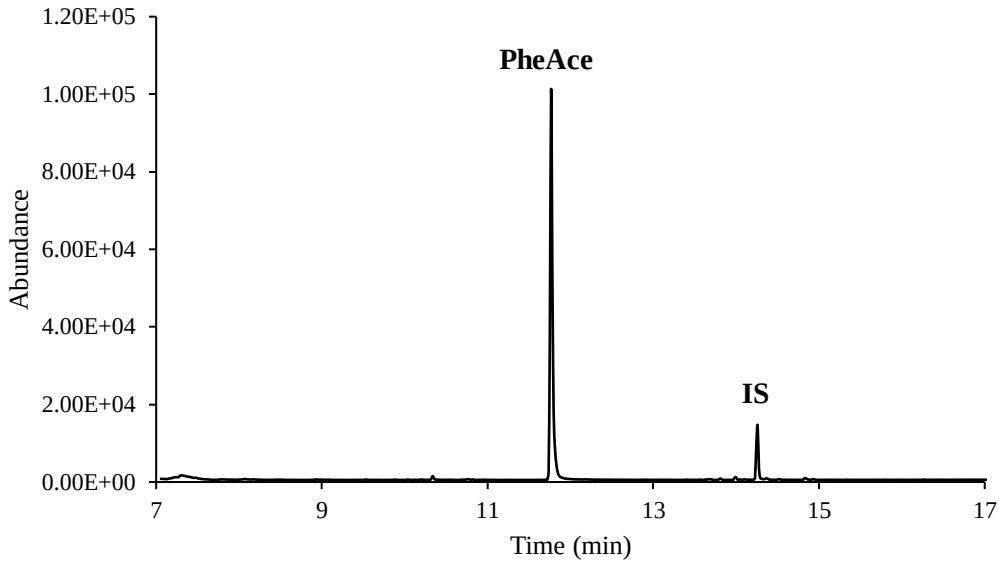


Figure A- 6. GC-MS-SIM chromatogram of 5 ppm PheAce standard for ions *m/z* 120, 91, 92, 65 and 5 ppm IS for ions *m/z* 112, 95, 82,70 separated on an HP-5MS column (60 m x 0.25mm I.D. x 0.25 µm) with a flow rate at 1 mL/min. The oven temperature program: 50 °C for 2 min, ramped to 180 °C at a rate of 10 °C/min, and increased to 280 °C at a rate of 25 °C/min, and held for 5 min.

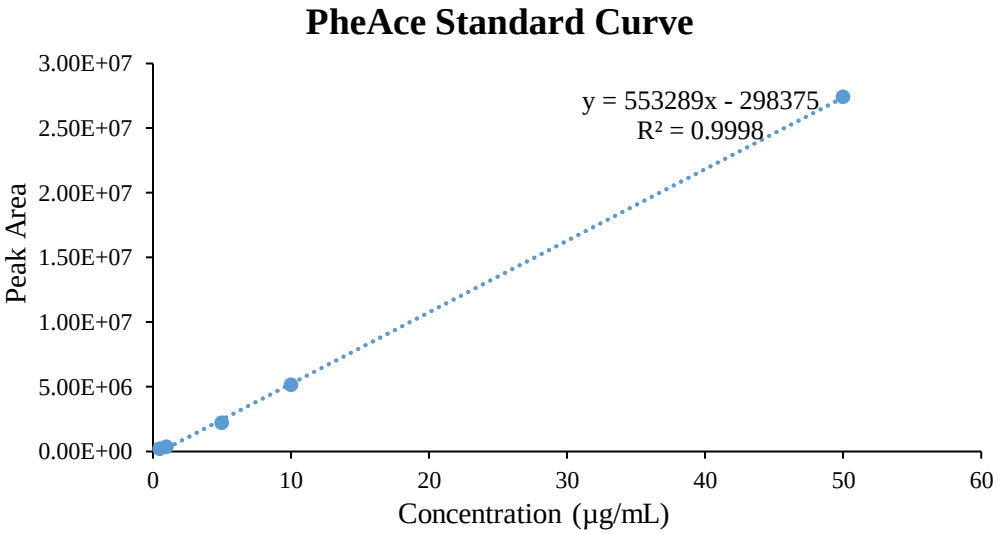


Figure A- 7. Standard curve of PheAce, concentration range from 0.5 – 50 µg/mL by GC-MS.

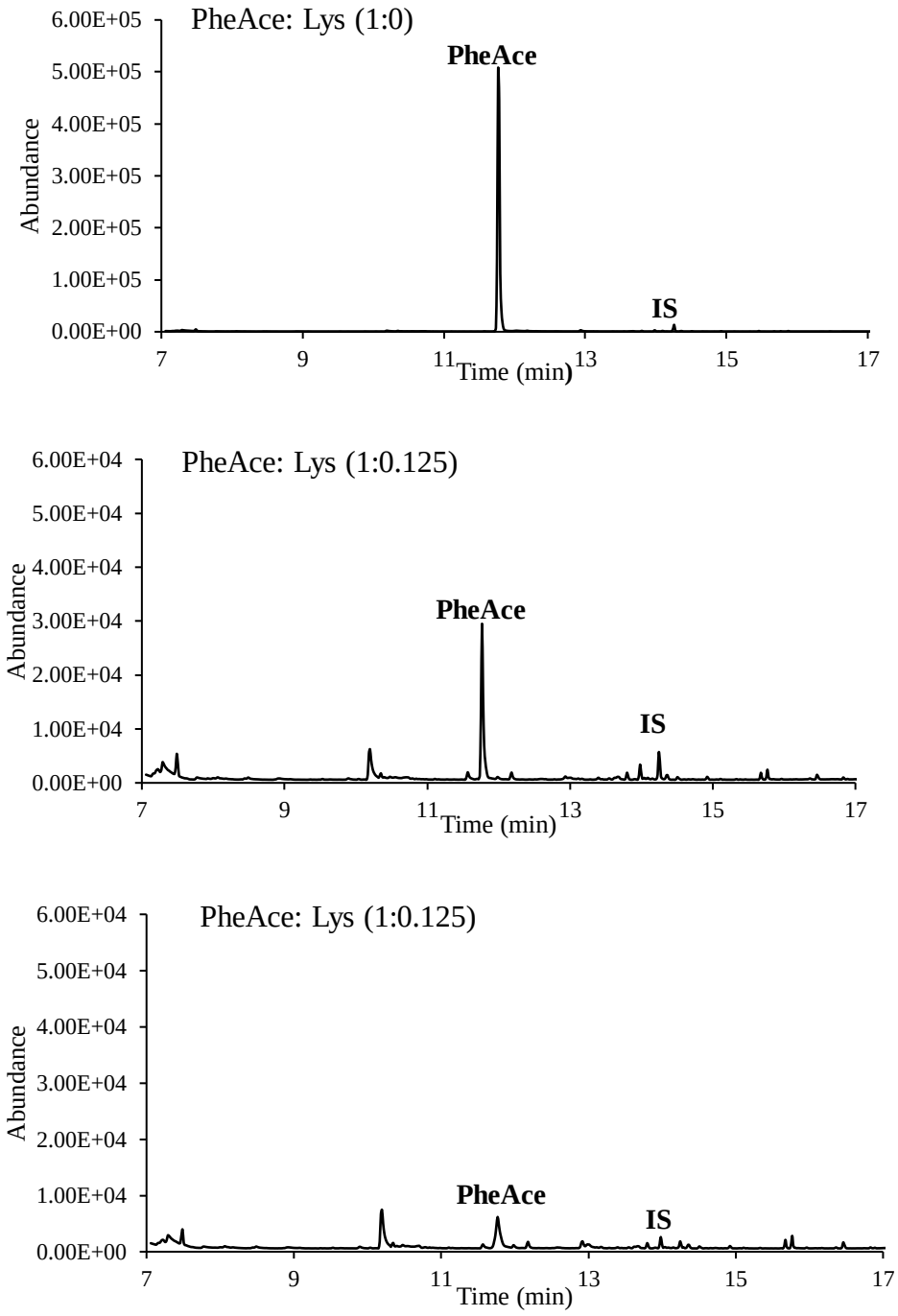


Figure A- 8. GC-MS chromatogram (SIM mode) of PheAce extracted from PheAce:Lys model systems (180 ℃/1 h) at a molar ratio of 1:0 (Control), 1:0.125, and 1:2, separated on an HP-5MS column. The oven temperature program: 50 ℃ for 2 min, ramped to 180 ℃ at a rate of 10 ℃/min, and increased to 280 ℃ at a rate of 25 ℃/min, and held for 5 min.

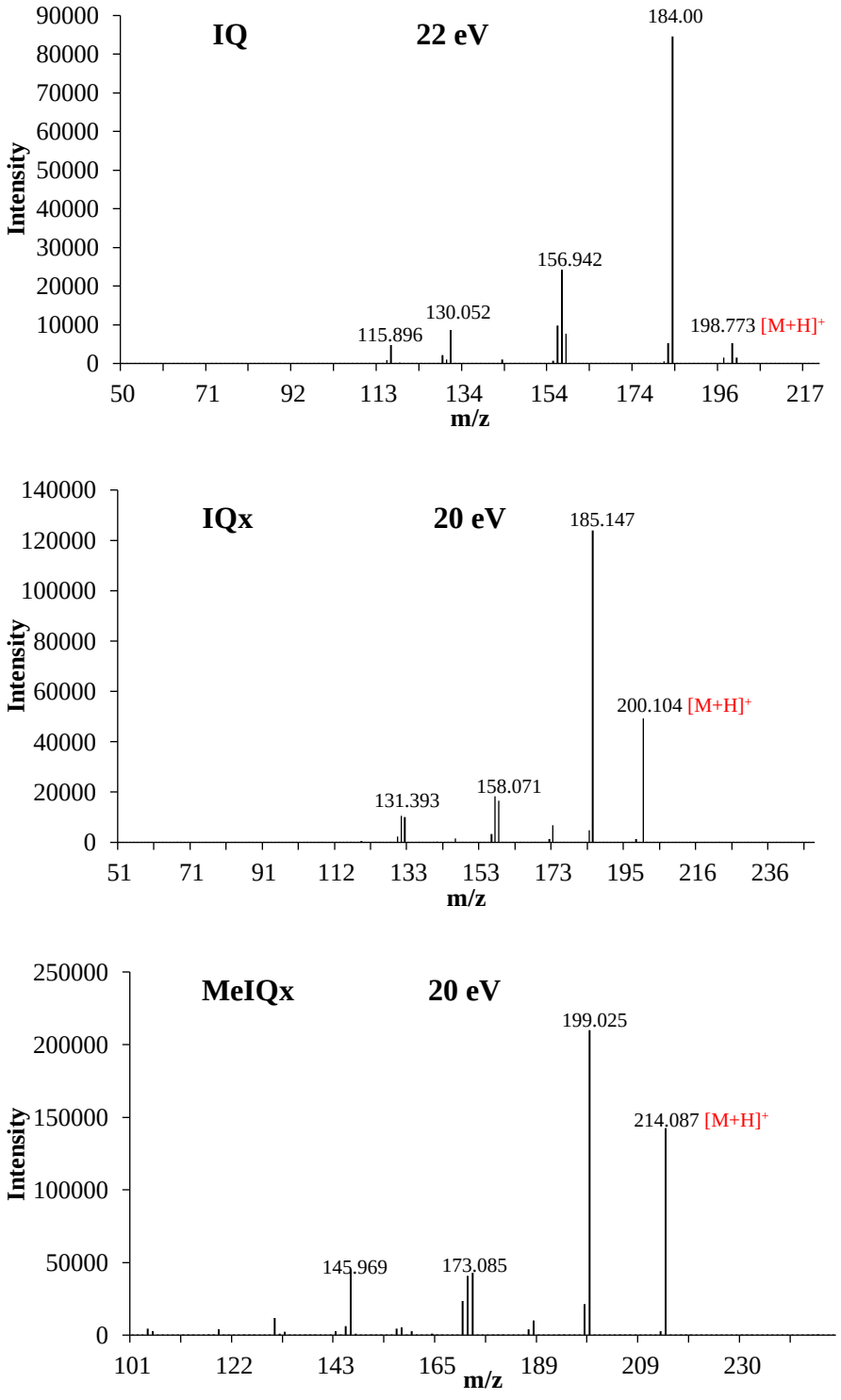


Figure A- 9. LC/ESI (+)-MS full scan product ion mass spectrum of IQ, IQx, and MeIQx standards obtained in TSQ Quantum Ultra TM EMR triple quadrupole.

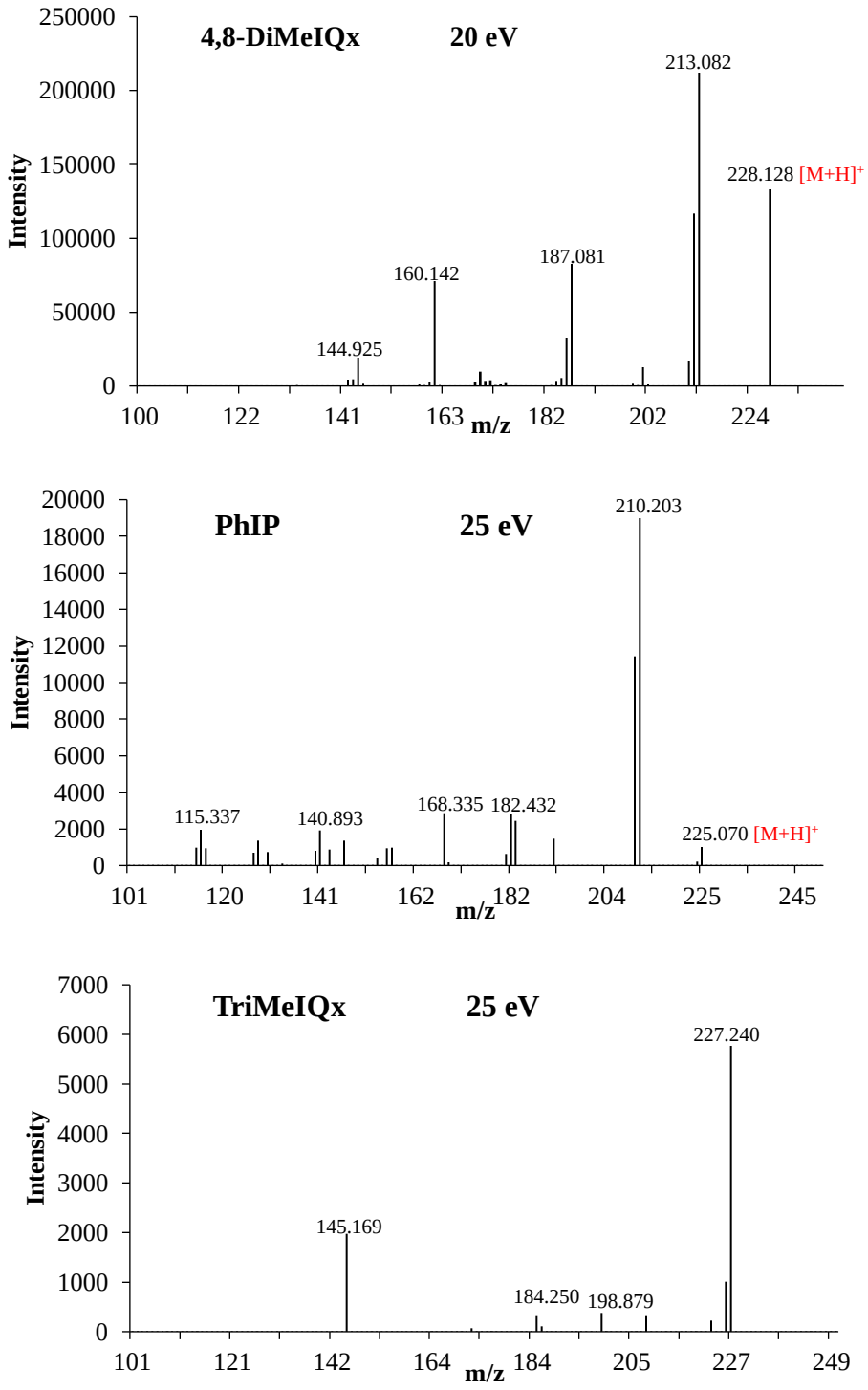


Figure A- 10. LC/ESI (+)-MS full scan product ion mass spectrum of 4,8-DiMeIQx, PhIP, and TriMeIQx standards obtained in TSQ Quantum Ultra TM EMR triple quadrupole.

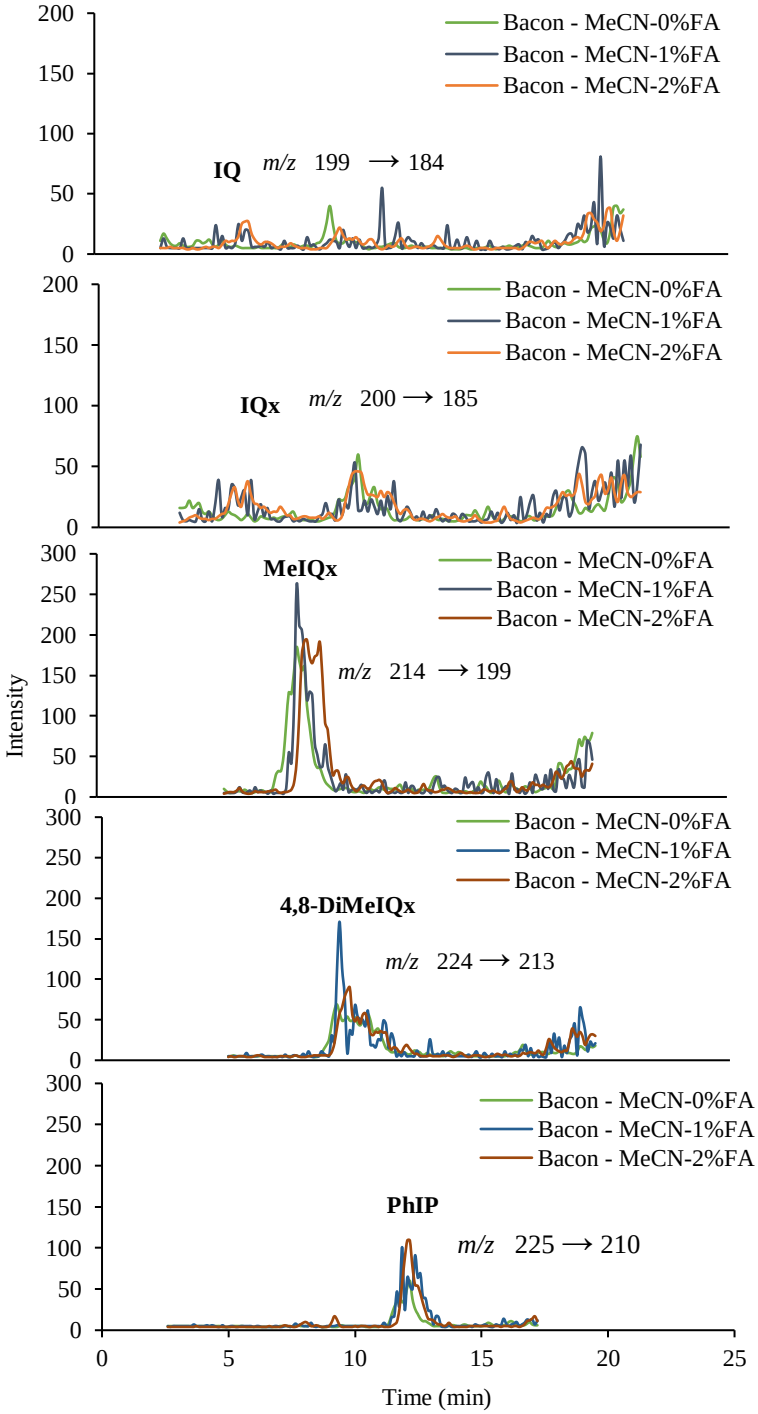


Figure A- 11. LC/ESI (+)-MS/MS chromatogram (SRM mode) of IQ, IQx, MeIQx, 4,8-DiMeIQx, and PhIP in pan-fried bacon samples extracted using EMR-Lipid method with MeCN at different FA concentrations (0, 1, and 2%, v/v). SRM transitions (precursor → product ion) for detection and quantification are indicated in each window.

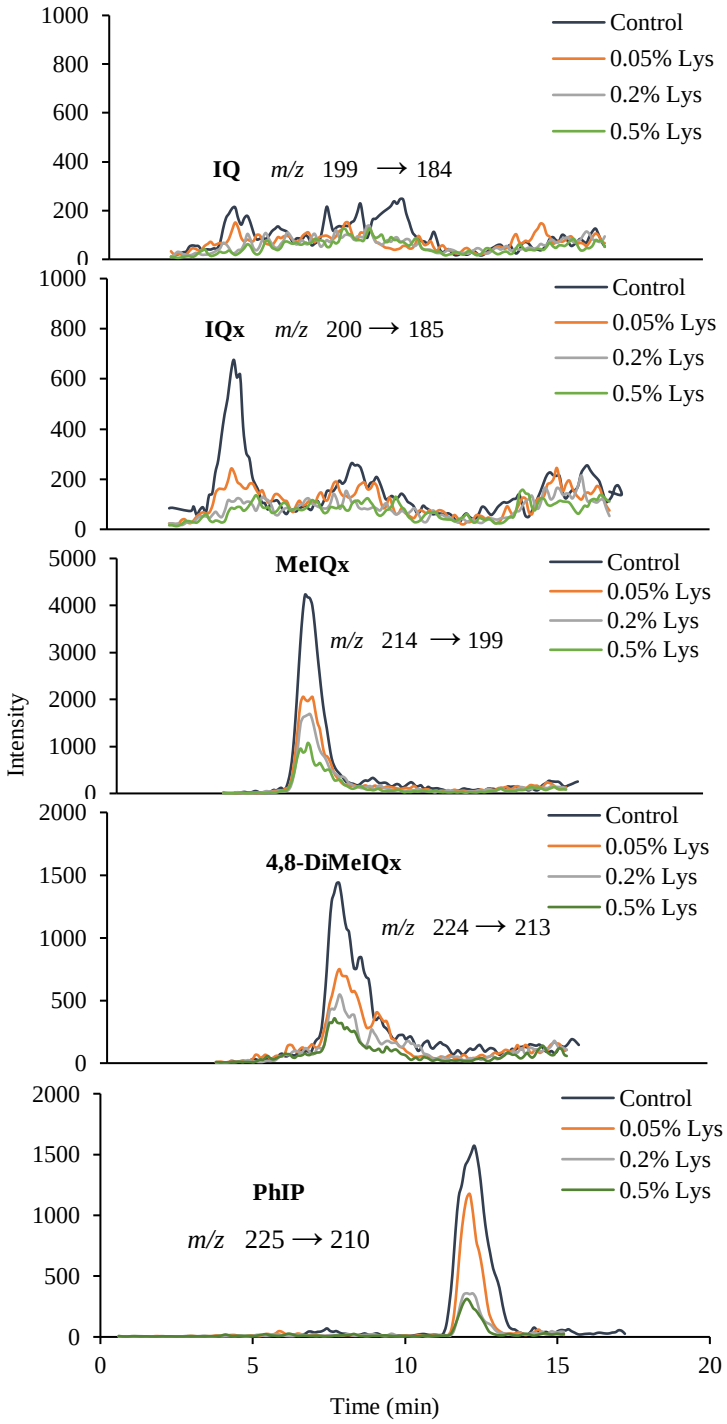


Figure A- 12. LC/ESI (+)-MS/MS chromatogram (SRM mode) of IQ, IQx, MeIQx, 4,8-DiMeIQx, and PhIP in pan-fried beef crust treated with 0% (control), 0.05%, 0.20%, and 0.50% Lys. SRM transitions (precursor → product ion) for detection and quantification are indicated in each window.

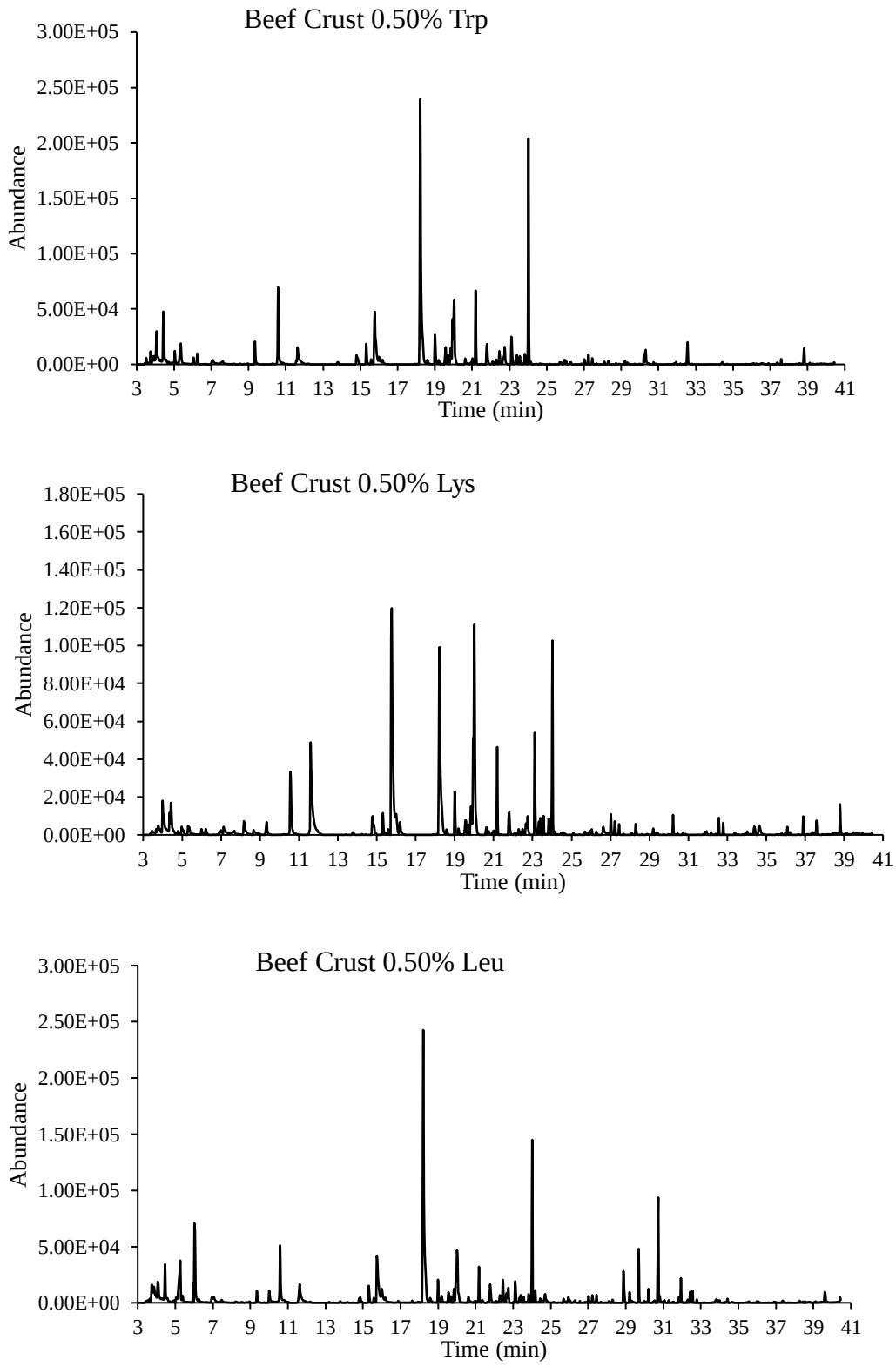


Figure A- 13. HS-SPME GC-MS total ion chromatogram obtained from pan-fried beef crust treated with Trp, Lys, and Leu at 0.50% (w/w).

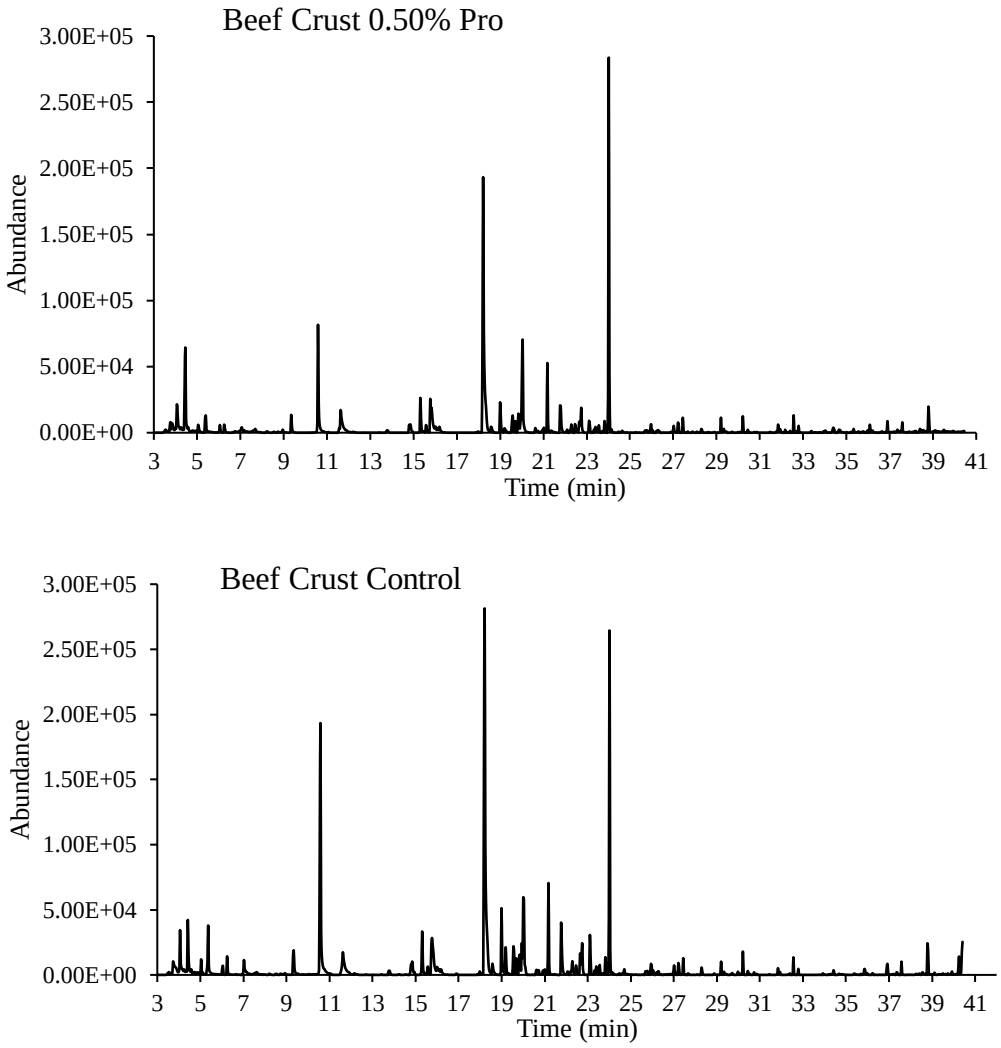


Figure A- 14. HS-SPME GC-MS total ion chromatogram obtained from pan-fried beef crust treated with Pro at 0.50% (w/w) and control.

Table A- 1. SAS code example used in Chapter 2.

I. Model # 1 (Glc:Cre:Phe, 0.5:1:1): one-way ANOVA

```
data Proline;
input Rep$ Molar_ratio$ PHIP PheAce Browning;
datalines;
1     1:0   3281.65     12.63 13.920
2     1:0   3071.57     12.66 14.600
3     1:0   3111.58     13.57 15.230
1     1:0.125     2635.83     6.46  13.820
2     1:0.125     2566.05     6.04  14.210
3     1:0.125     2090.70     6.04  14.330
1     1:0.25      2150.14     2.63  13.002
2     1:0.25      2247.26     3.05  13.610
3     1:0.25      1579.56     3.07  13.930
1     1:0.5 1198.39     3.00  12.100
2     1:0.5 1505.16     3.00  13.330
3     1:0.5 802.32      3.00  13.020
1     1:1   417.19      3.00  12.320
2     1:1   452.12      3.00  12.790
3     1:1   454.80      3.00  12.680
1     1:2   210.54      3.00  12.110
2     1:2   182.72      3.00  11.440
3     1:2   194.26      3.00  12.300
;
run;

proc print data=Proline;
run;

proc glimmix data=Proline;
class Rep Molar_ratio;
model PheAce= Molar_ratio ;
random Rep;
lsmeans Molar_ratio/ ADJUST=TUKEY pdiff adjust=Tukey plot=meanplot(join cl);
run;

proc mixed covtest data=Proline;
class Rep Molar_ratio PHIP;
model PHIP= Molar_ratio ;
random Rep;
lsmeans Molar_ratio/ ADJUST=TUKEY pdiff;
run;

proc mixed covtest data=Proline;
class Rep Molar_ratio Browning;
model Browning= Molar_ratio ;
random Rep;
lsmeans Molar_ratio/ ADJUST=TUKEY pdiff;
run;

proc gplot data=Proline;
plot PheAce*Molar_ratio ;
plot Browning*Molar_ratio ;
plot PheAce*PHIP;
```

```
run;

*proc glimmix data=Proline;
*class Rep Molar_ratio Browning;
*model Browning= Molar_ratio ;
*random Rep;
*lsmeans Molar_ratio/ pdiff adjust=tukey;
*run;


*proc reg data=Proline  plots(only)=(residuals residualbypredicted qqplot);
 *model PheAce= Molar_ratio;
 *model Browning= Molar_ratio;
 *run;
```

II. Model # 2: one-way ANOVA

```
data Proline2;
input Rep$ Molar_ratio$ PheAce;
datalines;
1     1:0   1138.64
2     1:0   1006.75
3     1:0   1133.70
1     1:0.125     772.38
2     1:0.125     707.55
3     1:0.125     689.01
1     1:0.25      602.83
2     1:0.25      647.43
3     1:0.25      668.29
1     1:0.5 244.79
2     1:0.5 249.86
3     1:0.5 263.03
1     1:1   32.05
2     1:1   33.40
3     1:1   40.50
1     1:2   23.38
2     1:2   23.08
3     1:2   25.63
;
run;

proc print data=Proline2;
run;


proc mixed covtest data=Proline2;
class Rep Molar_ratio PheAce;
model PheAce= Molar_ratio ;
random Rep;
lsmeans Molar_ratio/ADJUST=TUKEY pdiff;
run;

proc gplot data=Proline2;
plot PheAce*Molar_ratio ;
run;
```

Table A- 2. SAS code example used in Chapter 4.

I. Two-way ANOVA: Rep, Level, Amino acid type, Level*AminoAcid

```
data ziyi;
input Rep$  Level $ AminoAcid $      L*;
datalines;
1.00  0.00% Trp   24.08
2.00  0.00% Trp   26.77
3.00  0.00% Trp   27.48
1.00  0.00% Lys   23.71
2.00  0.00% Lys   26.63
3.00  0.00% Lys   27.12
1.00  0.00% Leu   26.06
2.00  0.00% Leu   26.53
3.00  0.00% Leu   28.83
1.00  0.00% Pro   24.47
2.00  0.00% Pro   26.51
3.00  0.00% Pro   28.28
1.00  0.05% Trp   25.49
2.00  0.05% Trp   27.12
3.00  0.05% Trp   26.41
1.00  0.05% Lys   25.89
2.00  0.05% Lys   26.74
3.00  0.05% Lys   28.06
1.00  0.05% Leu   27.29
2.00  0.05% Leu   26.46
3.00  0.05% Leu   27.28
1.00  0.05% Pro   26.14
2.00  0.05% Pro   27.74
3.00  0.05% Pro   25.90
1.00  0.20% Trp   27.00
2.00  0.20% Trp   25.02
3.00  0.20% Trp   26.99
1.00  0.20% Lys   25.77
2.00  0.20% Lys   25.19
3.00  0.20% Lys   25.67
1.00  0.20% Leu   27.44
2.00  0.20% Leu   25.67
3.00  0.20% Leu   28.86
1.00  0.20% Pro   25.86
2.00  0.20% Pro   28.10
3.00  0.20% Pro   28.00
1.00  0.50% Trp   30.23
2.00  0.50% Trp   30.45
3.00  0.50% Trp   27.96
1.00  0.50% Lys   26.13
2.00  0.50% Lys   24.56
3.00  0.50% Lys   25.29
1.00  0.50% Leu   29.25
2.00  0.50% Leu   28.95
3.00  0.50% Leu   31.42
1.00  0.50% Pro   26.59
2.00  0.50% Pro   26.46
3.00  0.50% Pro   28.84
```

```
;
proc print;
run;
*Rep$ Level $ AminoAcid $      L*;


proc mixed data=ziyi;
class Level AminoAcid Rep;
model L*=Level|AminoAcid;
*random Rep Rep*Level Rep*AminoAcid Level*AminoAcid;
Lsmeans Level|AminoAcid/pdiff;
run;


proc glimmix data=ziyi;
class level aminoacid rep;
model L*=Level|AminoAcid;
lsmeans level|aminoacid/pdiff lines;
covtest GLM;
run;
```