

Inhibitory effect of edible films and coatings containing selected amino acids on the formation of
2-amino-1-methyl-6-phenylimidazo[4-5-b]pyridine (PhIP) in pan-fried chicken breasts

by

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B.S., Shahid Beheshti University of Medical Sciences, 2014
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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

2022

Abstract

This study was conducted to investigate the inhibitory effects of edible films and coatings containing amino acids (AAs) on the formation of 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP) in chicken breasts and to evaluate the physicochemical properties of the chicken. Heated whey protein isolate (HWPI) solution (100g) was made by heating 5 g whey protein isolate (WPI) solution at 90 °C for 30 min (in a water bath). After cooling down the solution, it was mixed with 2.5 g glycerol (GLY), and tryptophan (Trp) or lysine (Lys) were added at 0.25%, 0.5%, and 0.75% (w/w of the coating solution) concentrations, subsequently. Unheated whey protein isolate (UHWPI)-based casting solution was prepared with the same method but without heating of WPI solution. Chicken breasts were cut were covered with the prepared edible films and pan-fried at 195 °C for 7.5 min on each side using an electrical frying pan with adjustable heat. The average PhIP level decreased from 78.47 ppb to 6.69-8.31 ppb for chicken covered with Lys containing HWPI edible films, and to 16.09-46.80 ppb for chicken covered with Trp containing ones. For chicken covered with UHWPI edible films the PhIP decreased to 7.75-40.32 ppb for Lys containing ones and to 28.4-56.04 ppb for Trp containing ones. Moreover, chicken breasts covered with HWPI edible films had lower cooking loss and improved tenderness compared to the chicken breasts with no edible film.

Furthermore, the effect of potato starch (PS) coating containing AAs on the formation of PhIP in chicken breasts was studied, and physicochemical properties of chicken were evaluated. The PS coating solution (100 g) was made by heating 5 g of PS in water solution at 80 °C for 30 min (in a water bath). After cooling down, GLY (2 g/100 g w/w of PS), and Trp or Lys were added to the solution at 0.25%, 0.5%, and 0.75% (w/w of the coating solution). Chicken breasts cuts were dipped in the PS coating solution for 15 min before frying. Chicken coated with PS

with no AA and chicken with no coating were used for comparison. After frying the chicken at 195 °C for 7.5 min on each side, PhIP level, color, cooking loss percentage, tenderness, and texture profile, viscosity of the PS coating solution and coating pickup percentage by chicken were measured. Triangle test was also conducted for consumer discrimination. The average PhIP concentration was decreased from 92.62 ppb for the control chicken breast without coating to 6.30 ppb (0.25% Lys), 6.76 ppb (0.5% Lys), and 11.98 ppb (0.75% Lys), respectively accounting for a 89%-92% reduction in PhIP level compared to the chicken breast without coating. However, dipping in Trp containing PS coating had significantly lower ($p < 0.05$) PhIP reduction effect (34-67%). Moreover, there was not a significant difference ($p > 0.05$) in cooking loss percentage, tenderness, texture profile parameters, color parameters of PS coated chicken compared to the chicken breasts with no coating, after frying. Triangle test results showed that consumers cannot find a significant difference between the PS coated and uncoated chicken breasts at ($p < 0.001$).

PS-0.25% Lys coating solution was selected to evaluate the effect of dipping time of chicken breast on the formation of PhIP and its possible inhibitory mechanism. Lys (0.25% w/w of solution) was added to the gelatinized 5% (w/w) PS solution. The chicken breasts with the same dimensions were dipped in the coating solution for different times (15 min, 30 min, 1 h, 3 h, and 6 h) and after draining the excess coating chicken were kept under hood for 20 min to dry the coating on them. Chickens were subsequently pan-fried at 195 °C for 7.5 min (on each side). The PhIP level, tenderness, texture profile, color and cooking loss were evaluated for chicken breasts after pan-frying. Phenylacetaldehyde (PheAce), and volatile compounds were also determined using GC-MS for a selected samples. Chicken with no coating was used for comparison. Results showed that dipping time does not significantly decrease ($p > 0.05$) the PhIP

level suggesting that 15 min dipping time is enough for PhIP reduction compared to the control chicken without coating. Moreover, phenylacetaldehyde (PheAce) was shown to increase in chicken breast coated with PS-0.25% Lys, suggesting that there should be another pathway to prevent PhIP by PS-0.25% Lys. Volatile compounds analysis showed an increase in several aroma compounds such as pyrazines in the coated chicken. Moreover, no significant differences ($p>0.05$) was shown between cooking loss percentage, color parameters, texture profile and tenderness of chicken with PS-0.25% coating and chicken without coating. Overall, the results of this study suggested that application of edible films and coatings containing Lys and Trp to chicken breasts could effectively mitigate the PhIP formation during cooking.

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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 toxicology. I am greatly thankful to Dr. Jayendra Amamcharla 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. Kelly Getty for being in my committee and her support for conducting experiments and Dr. Xrong Wu for serving on my committee and providing his valuable input during this research.

I would like to thank my lab mates, and other 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, and my brother, 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 dedicate my dissertation to all brave Iranian women who are fighting for their basic rights.

Chapter 1 - Review of Literature

Introduction-Heterocyclic amines

Heterocyclic amines (HCAs) (Table 1), which are mutagenic or carcinogenic compounds consisted of hydrogen, carbon and nitrogen atoms, are formed during cooking of meat at high temperatures and have been the subject of many studies on human cancer during recent decades (Oz, Kızıl, Zaman, & Turhan, 2016; Sugimura, 2000). Many researches have proved the association between high intake of HCAs and humans cancer (J. S. Felton, Knize, Salmon, Malfatti, & Kulp, 2002). The International Agency for Research on Cancer considers one the HCAs as a probable human carcinogen (class 2B), and some of them as potential human carcinogens (class 2A) (Humans, Cancer, & Organization, 1993). Therefore minimal exposure to these compounds is recommended by this agency. Based on the information from The U.S National Toxicology Program (NTP), many HCAs such as 2-amino-1-methyl-6-phenyl-imidazo [4,5-b] pyridine (PhIP), 2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQ), and 2-Amino-3-methylimidazo[4,5-*f*]quinoline (IQ) are potential human carcinogens (Bucher & Portier, 2004). Norharman and harman are other types of HCAs that are considered to have no genotoxicity or mutagenicity; however, they can increase the toxicity of other HCAs (Beer, et al., 2019). More than 25 HCAs are found in different foods such as meat and fish with molecular weight of 160-250 and pKa values of 3.5 to 8.6 (Alaejos & Afonso, 2011; Gibis, 2016; Herraiz*, 2004; M. Knize, Cunningham, Griffin Jr, Jones, & Felton, 1994). The HCAs can be categorized in two groups, polar and non-polar ones. Generally, the polar ones can be divided into three subgroups such as imidazopyridine-type (such as DMIP, TMIP, and PhIP), imidazo-quinoxalines-type (IQx) (such as MeIQx, IQx), and imidazo-quinolines-type (IQ) (such as MeIQ, and IQ) (K. W. Cheng, Chen, & Wang, 2006).

Polar ones can be formed at temperatures between 100-300 °C and are called thermic HCAs. Among them PhIP is the most abundant and common one found in foods, specifically meat products (Iwasaki, et al., 2010; Warzecha, et al., 2004). Studies have shown different evidences of diseases caused by PhIP such as tumors in animal models and cancers (N. Ito, et al., 1991; Shirai, et al., 1997; Sinha, et al., 2000; Sinha, et al., 2005). The precursors of the formation of these compounds are sugar, creatine/creatinine, free amino acids, and reducing sugars such as glucose.

PhIP formation mechanism

HCAs are reported to mainly be produced as byproducts in cooked foods, especially muscle food such as fish, pork, poultry, and beef heated under normal domestic cooking conditions such as baking, grilling, pan-frying, roasting, and broiling (Sinha, et al., 2000; Sinha, Knize, et al., 1998; Sinha, et al., 1995).

There are many studies on the formation of polar HCAs in heated model systems that contains common components of muscle tissues, such as dipeptides, sugars, creatinine, and free amino acids. Maillard reaction which is a non-enzymatic heat-induced browning reaction and results in a distinct color and flavor, is the pathway in which most of the HCAs are formed in cooked meats (Martins, Jongen, & Van Boekel, 2000; Murkovic, 2004; Pais & Knize, 2000; Sinha, Knize, et al., 1998). The formation mechanism of HCAs is not still clear; however, there are some assumptions about it (M Jägerstad, Skog, Arvidsson, & Solyakov, 1998; Weisburger, 2002; Yaylayan, 2003). Generally, heating causes dehydration in free amino acids (amino group) and reducing sugars (carbonyl group) in meat which results in the formation of vinyl-pyrazines or vinyl-pyridines after Strecker degradation. Aldol condensation reactions with creatin(in)e result

in the formation of amino-imidazo which is part of PhIP, IQ-, and IQx-type HCAs (Murkovic, 2004; Pearson, Chen, Gray, & Aust, 1992). The reactions that occur are as follows in details: after the formation of Schiff's base through the reaction of the amino group of amino acids with the carbonyl group of reducing sugars, the Amadori rearrangement results in the formation of glycol-aldehyde alkylimine (enol type), which can be further condensed with carbonyl group to form dialkylpyrazine radicals or oxidized and form glyoxal monoalkylimine which can then be condensed with glyoxal to produce pyridine radicals. Moreover, cyclization with Strecker aldehydes and creatinine (i.e., CH_3CHO or HCHO), results in the formation of IQ- type and IQx- HCAs form final products in the Maillard reaction.

Table 1. Abbreviations, molecular weights, chemical names and polarity of HCAs.

Abbreviations and Chemical Names			Molecular Weights (g/mol)	Polarity
Imidazoquinoxalines	MeIQx	2-Amino-3-methylimidazo[4,5- <i>f</i>]quinoxaline	213.3	Polar
	IQx	2-Amino-3,8-dimethylimidazo[4,5- <i>f</i>]quinoxaline	199.3	Polar
	4,7,8-TriMeIQx	2-Amino-3,4,7,8-tetramethylimidazoline[4,5- <i>f</i>]quinoxaline	241.3	Polar
	7,8-DiMeIQx	2-Amino-3,7,8-trimethylimidazo[4,5- <i>f</i>]quinoxaline	227.3	Polar
	4,8-DiMeIQx	2-Amino-3,4,8-trimethylimidazo[4,5- <i>f</i>]quinoxaline	227.3	Polar
Imidazopyridines	PhIP	2-Amino-1-methyl-6-phenylimidazo[4,5- <i>b</i>]pyridine	224.3	Polar
	TMIP	Trimethylimidazopyridine	176.2	Polar
	DMIP	Dimethylimidazopyridine	162.2	Polar
Imidazoquinolines	IQ	2-Amino-3-methylimidazo[4,5- <i>f</i>]quinoline	192.2	Polar

	MeIQ	2-Amino-3,4-dimethylimidazo[4,5- <i>f</i>]quinoline	212.3	Polar
β-amino-carbolines	Harman	9H-pyrido[4,3- <i>b</i>]indole	168.2	Nonpolar
	Norharman	1-methyl-9H-pyrido[4,3- <i>b</i>]indole	182.2	Nonpolar
α-amino-carbolines	MeAαC	2-Amino-3-methyl-9H-pyrido[2,3- <i>b</i>]indol	197.2	Nonpolar
	AαC	2-Amino-9H-pyrido[2,3- <i>b</i>]indol	183.2	Nonpolar
δ-amino-carbolines	Glu-P-2	2-Amino-dipyrido[1,2- <i>a</i> :3'2'- <i>d</i>]imidazole	184.3	Nonpolar
	Glu-P-1	2-Amino-6-methyldipyrido[1,2- <i>a</i> :3'2'- <i>d</i>]imidazole	198.3	Nonpolar
γ-amino-carbolines	Trp-P-2	3-Amino-1-methyl-5H-pyrido[4,3- <i>b</i>]indole	197.2	Nonpolar
	Trp-P-1	3-Amino-1,4-dimethyl-5H-pyrido[4,3- <i>b</i>]indole	211.3	Nonpolar

Due to the faster formation of dialkylpyrazine radicals compared to that of pyridine radicals, IQx-type HCAs form in larger quantities in meat and fish than IQ-type HCAs. Researchers (Margaretha Jägerstad, Skog, Grivas, & Olsson, 1991) indicated that this reaction mechanism of HCAs is highly dependable on meat type, precursor types and concentrations, cooking time and temperatures. There are several studies about the presence of an alternative route for the HCA formation a direct condensation reaction between creatinine and Strecker aldehydes of amino acids occurs (Murkovic, 2004). The Strecker degradation is known as a part of the oxidative decarboxylation of amino acids in the Maillard reaction, in which an α -amino acid is being converted into a product with aldehyde-containing side chain (Yaylayan, 2003). Phenylacetaldehyde (PheAce) formation takes place through thermal degradation of its precursor amino acid in the model system. Subsequently it undergoes an aldol-condensation with creatinine and then dehydration. Finally, PhIP forms when Schiff's base combines with an amino group of creatinine or phenylalanine (Figure 1). Thus, PheAce is an indispensable precursor for PhIP. It was shown that PhIP can still form when phenylalanine is replaced by PheAce in the model system containing creatinine (Vitaglione & Fogliano, 2004).

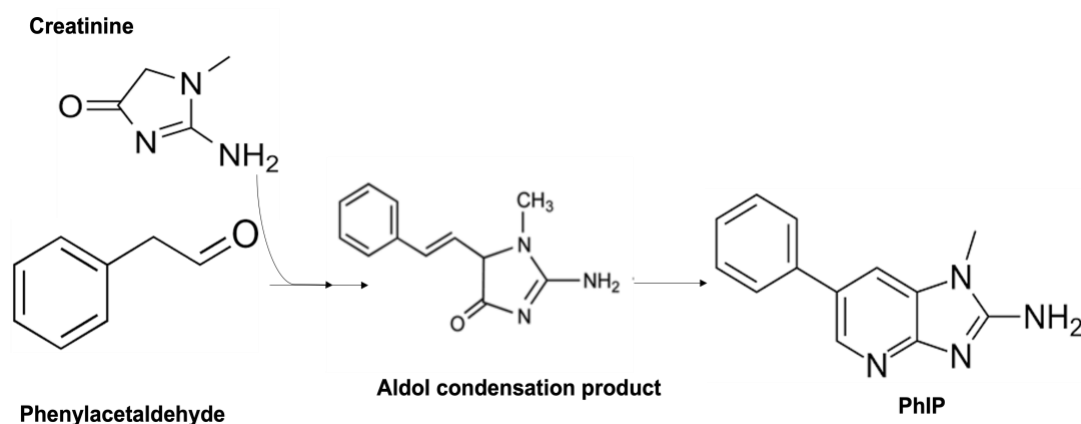


Figure 1. PhIP formation pathway (Yu & Yu, 2016)

Precursors of PhIP formation

Due to the complex matrix of meat, model systems are being used to study concurrent chemical reactions that occur when meat is cooked at high temperatures (Zöchling & Murkovic, 2002). There are several model systems which provide a better understanding of the formation of the amines. These model systems are helpful to determine the foods and cooking conditions in which heterocyclic amines are formed and to develop strategies to prevent or reduce their formation (Johansson & Jägerstad, 1996; Pais, Salmon, Knize, & Felton, 1999). The major precursors of heterocyclic amines' formation in the model system are usually sugars, mixture of amino acids and creatinine/creatine, in a similar molar ratio to those found in meat products. However, the concentration of these compounds are usually higher in the model systems (~20 times) to accelerate HCA formation and determination (Arvidsson, Van Boekel, Skog, & Jägerstad, 1997; Pais, et al., 1999). The usual heating temperatures for the model systems range from 100 to 300 °C, whereas times range from 0.5 to 120 min. IQ-type, PhIP, and IQx-type HCAs are the most studied (Arvidsson, et al., 1997). Various model systems were developed in sealed tubes (Hayatsu, Arimoto, & Wakabayashi, 1991; Shioya, Wakabayashi, Sato, Nagao, & Sugimura, 1987; Wakabayashi, et al., 1997) and kinetics (KI Skog, Johansson, & Jägerstad, 1998). For PhIP formation the temperature of 37 °C was reported; However, in another study with the same condition, no PhIP was formed after keeping the model system at room temperature for two weeks. Chicken breast has 3.54 mg creatinine and 0.47 mg glucose per gram of chicken, and contains different amount of amino acids. When these compounds are mixed and heated for 30 minutes at 225 °C, some heterocyclic amines are formed. The cooked chicken model was shown to produce the largest amount of PhIP (Zöchling & Murkovic, 2002)

Phenylalanine, creatinine and glucose were shown to be probable precursors of PhIP (Shioya, et al., 1987). In a model system using dry heating of ^{13}C -labelled creatinine and phenylalanine it has been shown that phenylalanine and creatinine are precursors of PhIP (Hayatsu, et al., 1991). It was found that PhIP can be produced without the presence of glucose and by heating creatine together with tyrosine, isoleucine, and leucine (KI Skog, et al., 1998). However, its formation is affected by glucose depending on its concentration (either increasing or preventing) (Kerstin Skog & Jägerstad, 1990) and also during dry heating (Hayatsu, et al., 1991). It was reported that tetrose (erythrose) is the most active carbohydrate when it is heated ($37\text{ }^{\circ}\text{C}$ and $60\text{ }^{\circ}\text{C}$) along with phenylalanine and creatinine dissolved in water. The other carbohydrates such as ribose, arabinose, galactose and glucose are not as active. PhIP also was found in heated mixtures of creatinine, phenylalanine and aldehydes (Manabe, Kurihara, Wada, Tohyama, & Aramaki, 1992), as well as in mixtures of creatinine, nucleic acids, and phenylalanine (Manabe, Suzuki, Wada, & Ueki, 1993). The 4'-hydroxy derivative of PhIP was found in an analogous reaction using tyrosine instead of phenylalanine (Manabe, et al., 1993). In a model system consisted of phenylalanine and creatinine kept at room temperature for two weeks no PhIP was formed. Thus, degradation of phenylalanine to PheAce is the possible pathway of PhIP formation. In the mentioned model system the energy was not enough for the degradation which was in accordance with the other studies (Manabe, et al., 1992). A slight increase in the temperature of the reaction to $37\text{ }^{\circ}\text{C}$ and $60\text{ }^{\circ}\text{C}$ did not increase the formation of PhIP. These temperatures were low for PhIP formation even after addition of carbohydrates (e.g. glucose) to this model system. The PhIP formation in the simple model system consisted of phenylalanine and creatinine starts with the formation of PheAce which is the Strecker aldehyde. The second step is an aldol condensation of the aldehyde

with creatinine subsequent dehydration. The origin of the nitrogen forming the pyridine moiety of PhIP can be either the amino group of creatinine that can react with the oxo group of the intermediate and the amino group of phenylalanine or even free ammonia (Zöchling & Murkovic, 2002). Felton's group who investigated the PhIP formation by heating phenylalanine and creatine reported that the phenyl ring from phenylalanine is incorporated intact. They also indicated that amino nitrogen from phenylalanine and the 3-carbon atom are incorporated into PhIP. Using labelled creatine, they found that the methyl-carbon, 1-nitrogen, and the amino-nitrogen from creatine are each incorporated into PhIP (Taylor, Fultz, Morris, Knize, & Felton, 1988). From these experiments it can be expected that the carbon atoms of phenylalanine can form a part of the pyridine moiety and that creatine can form the imidazol part. In another study PhIP was formed in a reaction consisted of the aldol addition product of creatinine and PheAce which was heated in diethylene glycol at 200 °C for 60 min. This confirms that in the course of the formation of PhIP, the aldol addition product acts as an intermediate. The nitrogen of the pyridine moiety of PhIP must come from the creatinine part of the aldol addition product. The origin of the nitrogen in the pyridine ring was assumed to be from the nitrogen in creatinine, probably its amino group. This was also confirmed by the PhIP formation through a reaction with PheAce and creatinine where there was no additional nitrogen. It was indicated that in the model reaction consisted of dl-phenylalanine-15N, no 15N was incorporated in 10% of the formed PhIP which shows that phenylalanine nitrogen was not incorporated into PhIP. Moreover, 13% of the formed PhIP incorporated two 15N, and 77% of the formed PhIP had only one atom of 15N. This shows the possibility of the exchange of the amino group of creatinine by the amino group of phenylalanine. These results indicate that the origin of majority of the

pyridine nitrogen is phenylalanine. In the case of the absence of this nitrogen, the nitrogen of creatinine can be inserted in PhIP.

As it was mentioned free amino acids are one of the precursors of the formation of HCA in the Maillard reaction. Researchers (H. Li, et al., 2009) stated that free amino acids were involved in the formation of HCAs in cooked pork floss. It was found that there was a positive correlation between HCA formation and certain amino acids' reduction such as threonine, serine, glutamic acid, isoleucine, tyrosine, and phenylalanine. Other researchers (Övervik, Kleman, Berg, & Gustafsson, 1989) reported that addition of threonine, alanine, serine, leucine, phenylalanine, and tyrosine to raw pork increased the formation of PhIP, MeIQ_x, and TMP during cooking. Moreover, threonine 1% (w/w) increased the total mutagenicity by 43 times. The effect of free amino acids was investigated on the formation of HCAs. Glycine, phenylalanine, serine, threonine, proline, alanine, tyrosine, and leucine were all reported as precursors of different HCAs. Recently, the effect of 20 amino acids on PhIP formation was investigated in model systems consisted of phenylalanine and creatinine as precursors. Addition of cysteine, tyrosine, methionine, serine, threonine, tryptophan, proline, asparagine, and aspartic acid significantly increased the formation of PhIP upon heating (Zamora, Alcón, & Hidalgo, 2013). It was shown that active carbonyl compounds such as α -keto acids, being produced as a result of thermal degradation of amino acids, are the potential inducers of the formation of HCA by converting phenylalanine into PheAce and formation of PhIP. These findings indicate that PhIP could be generated over an alternative reaction pathway in the presence of different amino acids. Furthermore, the effect of amino acid concentration on HCA production was even more than the effect of glucose and creatinine in model systems (Bordas, Moyano, Puignou, & Galceran, 2004). For instance, (K Skog, Solyakov, & Jägerstad, 2000) found that increase in tryptophan

content by five times have a significant effect on the formation of IQx and β -carbolines in a meat juice model system. Moreover, a mixture of amino acids such as alanine, glycine, and phenylalanine showed an increase in the PhIP and MeIQx levels in both dry- and wet- based model systems (Bordas, et al., 2004). However, a recent study in the model system and beef patties showed the inhibitory effect of free amino acids on the HCAs formation. They reported the inhibitory effect of nine different amino acids in the Maillard model system, showing a concentration-dependent inhibitory effect of amino acids on formation of PhIP. Results of these studies showed that adding selected amino acids to the surface of ground beef patties can significantly decrease PhIP formation after cooking at 230 °C/15 min; among these amino acids, Trp and Lys were more effective compared to Leu and Pro in suppressing PhIP formation (Albashabsheh, Smith, Linghu, & Karim, 2020; Linghu, Karim, & Smith, 2017; Linghu, et al., 2020).

Cooking method, temperature and time effect on formation of heterocyclic amines

Cooking condition (temperature and time) are important factors affecting the HCA formation in all kinds of meat types. In general, the formation of HCA increases by increasing the cooking temperature to more than 150 °C (Jackson & Hargraves, 1995). It was reported that, (Bordas, et al., 2004) at cooking temperatures below 150 °C, less 4,8-DiMeIQx, PhIP, and MeIQx were being formed in a model system even at cooking time of an hour, however their concentration increased noticeably by increasing the temperature to 200 °C even for 30 min. Thus heating parameters also affect the type of heterocyclic amines being formed. Researchers (Arvidsson, et al., 1997) reported that at temperatures less than 200 °C, HCAs formation is rapid,

then they reach a plateau after 5-10 min of heating, but at a cooking temperature of 225 °C, IQx and PhIP production was not identified, whereas 7,8-DiMeIQx and MeIQx began to decrease instantly after peaking, showing that polar HCAs can degrade at higher temperatures. This was more significant for IQx-type and PhIP HCAs once the temperature reaches to higher than 200 °C. It was also shown that the formation PhIP was more temperature-sensitive and the least stable one at high temperatures, compared to other HCAs (Ahn & Grün, 2005a).

In bouillon and boiled chicken meat systems, only harman and norharman (0.5 ng/g) were identified. However, in deep-fried chicken breast, MeIQx, 4,8-DiMeIQx and PhIP were also detected (Solyakov & Skog, 2002). The reason for the formation of higher level of PhIP and MeIQx in most cooked foods, is the highest rate constant (k) of its formation at all tested temperatures, whereas IQ and MeIQ showed the lowest k values. Moreover, in pan-fried chicken breast, PhIP was shown to be the most abundant HCA going from non-detectable to almost 40 ng/g based on cooking time and temperature. The highest level of PhIP was formed at pan-fried chicken breast cooked at 190 °C for 34 min (40 ng/g). However, decreasing the cooking time to 31 min and 18 min, only 19 ng/g and 10.5 ng/g of PhIP was formed, respectively. Pan-frying of the same sample at 220 °C for 12 min resulted in 30 ng/g of PhIP formation. Decreasing the temperature to below 190 °C has considerably decreased the PhIP level to less than 0.1 ng/g even for a 20 min cooking time. Harman, norharman, 4,8-DiMeIQx, and MeIQx were also detected in most of the samples; however, the amounts of 8-DiMeIQx and MeIQx were below 1 ng/g and below 2 ng/g, respectively. The amounts of norharman and harman were also less than 10 ng/g. Moreover, in pan residues of corresponding chicken breasts, no MeIQx was detected, but low amounts of PhIP (trace–0.2 ng/g) was detected (Table 2).

Commercially barbecued chicken meat had traces of MeIQx, harman, and norharman. The chicken skin also had low amounts of 4,8-DiMeIQx, MeIQx, and PhIP (up to 2.3 ng/g), and harman and norharman (up to around 13 ng/g). Boiling of chicken showed non-detectable amounts of 4,8-DiMeIQx, MeIQx, and PhIP and non-detectable to 0.5 ng/g of harman and norharman. (Solyakov & Skog, 2002). Direct-heating methods such as barbecuing, grilling, pan-frying, and oven-broiling produced the most HCAs in fish and meat products at temperatures higher than 150 °C for a short time (4-20 min) (Chen & Chiu, 1998). In another study (Sinha, Knize, et al., 1998) it was reported that grilling or barbecuing to induce more HCAs formation in meat compared to other cooking methods due to the more efficient transfer of heat from the grill/metal grates to the meat surface (Sinha, Knize, et al., 1998). Researchers in another study (Puangsombat, Gadgil, Houser, Hunt, & Smith, 2012) also reported higher levels HCAs in pan-frying beef products as compared to broiling and baking. Moreover significantly higher mutagenic activity was shown in hamburgers and pan-fried chicken fillets prepared by a convection oven, whereas less mutagenicity was generated in boiling and steaming cooking methods, which can be due to the lower surface temperature of meat in these methods (Kerstin Skog, Eneroth, & Svanberg, 2003).

Table 2. PhIP amount in chicken breasts pan-fried in different times and temperatures (Solyakov & Skog, 2002).

Chicken	Method	Temperature (°C)	Time (min)	PhIP (ng/g)
I, breast	Pan-frying	170	16	0.7±0.2
I, breast	Pan-frying	190	18	10.5±3.0
I, breast	Pan-frying	220	12	29.7±1.4
I, breast	Pan-frying	190	34	38.2±2.0
II, breast	Pan-frying	190	31	12.2±0.4
III, breast	Pan-frying	190	31	19.3±0.1
IV, breast	Pan-frying	170	20	nd
IV, breast	Pan-frying	220	20	1.8±0.1

Dietary HCA toxicity

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). Two classes of aromatic amines (amino-carbolines, and amino-imidazo-azaarenes (AIA)) are related to cooked-food mutagens. The mutagenicity of a group of 23 amino-carbolines were evaluated with mutagenic potency of over 200,000-fold. Studies showed that the mutagenic potency depended on the position of amino group and presence and position of a pyridine-type nitrogen in one of the rings, and the methyl substitutions on ring carbon atoms (Hatch, Knize, Felton, & Morgan, 1991). The AIAs are classified as the most predominant mutagenic compounds in well-done meat, thus they are the most relevant mutagens for human exposures from foods. AIA class of mutagens are derived from creatine which is a constituent of muscle, and contributes the amino group on the second carbon of the imidazole ring which is common in the structure of these

AHAs. Other than nonharman and harman, all HCAs acted as strong mutagens after metabolically activation with the S-9 microsomes mix in the Ames test, thus inducing frameshift and base pair substitution mutations in *Salmonella Typhimurium* strains TA98 and TA100. The mutagenic potency level of each HCA varied in the test with IQ and MeIQ showing the highest mutagenicity, followed by MeIQx, Trp-P-1, Trp-P-2, Glu-P-1, Glu-P-2, and PhIP. Three of the most frequently studied AHAs, MeIQx, PhIP, and IQ can be found in well-done meat. The principal attributes related to higher mutagenic potency of these isomeric amines are: (1) a little dipole moment, (2) ring fusion and the N3-methyl group combination (3) a lower pi electron system energy (4) a more stable nitrenium ion, and (5) a little energy gap between the highest and lowest unoccupied molecular orbitals of the amine (J. S. Felton, et al., 2007). Table 3 shows the mutagenicity of different HCAs in the Ames/*Salmonella* TA98 and TA100 strains test.

Table 3. Mutagenicity of HCAs in the Ames/*Salmonella* TA100 and TA98 strains test (Sugimura, Wakabayashi, Nakagama, & Nagao, 2004).

HCAs	Revertants/ μ g	
	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

Mutagenicity of PhIP isomers

The N-methyl group of PhIP's position is an important factor to be considered in the mutagenicity of PhIP isomers. The Ames/*Salmonella* assay showed that PhIP's mutagenic potency (with methyl group at the 1-position) and its 3-Me-PhIP isomer differs by ~100-fold. Slight differences in two isomer's structure was considered as important characteristics for compound's interaction with the enzyme active sites phase II enzymatic conjugation or oxidation. Moreover, it was shown that the slower oxidation step (conversion of the PhIP isomers from the 3-Me-PhIP isomer to their N-OH intermediate) could be the reason for a part of (5-fold) the 100-fold difference in their potency for the mutagenicity and the remaining difference (20-fold) could be clearly observed by mutagenic potency of NOH derivatives in the Ames/*Salmonella* test. The differences in reactive intermediates stability, and the way they fit into the sulfotransferase and N-acetyltransferase active sites may affect further activation. Adduct's stability, and its removal, and specificity for base targets in the histidinol dehydrogenase gene, may all affect the mutagenic potency (J. S. Felton, et al., 2007).

Generally, mouse liver microsomes metabolizes the PhIP to two main metabolites, one of which is a direct-acting mutagen (N-OH-PhIP) to Chinese hamster ovary cells and *Salmonella* cells. Another metabolite is hydroxylated at the position 4 of the phenyl ring and showed to be a detoxification product (Turteltaub & Dingley, 1998). Therefore, it is critical to identify factors that cause the formation of these metabolites because the ratio will alter the level of reactive intermediates that are available for mutation and DNA binding (adduct formation).

PhIP requires metabolic activation (by CYP1A2) to induce mutagenicity which includes important alterations in the cell's biochemistry that result in a number of genes being differentially expressed. The early consequences of activated PhIP exposure would involve DNA

damage (H. Zhu, Boobis, & Gooderham, 2000). Cells that were able to survive these primary consequences were shown to keep a damaged gene set which subsequently could either lead to their death by apoptosis or survival with a mutated genome. It was also indicated that at very low doses (10^{-9} to 10^{-11} M), PhIP could induce estrogenic activity which could stimulate a mitogenic response (Lauber, Ali, & Gooderham, 2004). Due to the fact that human consumption of cooked meat can result in PhIP accumulation in body fluids such as milk (DeBruin, Martos, & Josephy, 2001), there is a particular possibility that low-dose PhIP is mitogenic and estrogenic in humans. In vitro studies showed that acetylation is not needed for the formation of active electrophiles of PhIP. Thus, mutagenicity of N-hydroxy-PhIP is dependent on bacterial sulfotransferase activity but not on acetylation. PhIP was significantly highly mutagenic compared to IQ and MeIQx in *Salmonella* strains (Buonarati & Felton, 1990).

Carcinogenicity of PhIP

Long term animal trials have been shown that all of the mutagenic HCAs (MeIQ, IQ Trp-P-1, Trp-P-2, Glu-P-1, PhIP, Glu-P-2, A α C, and MeA α C) other than DiMeIQx were carcinogenic to rats and mice, inducing formation of mammary and colon carcinomas (Schut & Snyderwine, 1999). Aromatic amine's carcinogenic risk in humans was first discovered by a physician during a research on the occurrence of urinary bladder cancer. Laboratory researches showed that mice and rats who administered specific azo dyes arylamines or derivatives developed cancer, specifically in the liver (Weisburger, 2002).

Biochemical activation to more reactive N-hydroxy compounds was shown to occur, which mostly occurs in the liver, through prostaglandin synthetases and the cytochrome P450 enzyme systems. Guinea pigs were shown to be resistant to carcinogenesis due to the low titer of

the required activating enzymes. In target tissues, a second essential reaction such as acylation or sulfate ester formation was required. The produced reactive compounds indicate attributes of genotoxicity in suitable test systems. Research about this classification has been expanded after discovery of the formation of mutagens (HCAs) at the surface of cooked meat or fish by (Sugimura, et al., 2004). HCAs has shown to have similar activation reactions to other arylamines. Meat eaters were shown to have the potential of a higher risk of breast and colon cancer and HCAs were shown to induce cancer in these organs of rats in the pancreas, and the prostate. Moreover, some studies indicated their effect on the vascular system. Findings showed that only creatine containing foods, mostly meat and fish, would produce HCAs. PhIP was shown to be a mammary gland carcinogen in rats (Nagao, et al., 2002; Snyderwine, Venugopal, & Yu, 2002). A major series of bioassays were conducted by Ito and coworkers (J. S. Felton & Gentile, 1997; Hirose, Nishikawa, Shibutani, Imai, & Shirai, 2002; N. Ito, et al., 1997). In rats, certain HCAs induced cancer in pancreas, colon, prostate, and mammary gland, target sites in humans that are often linked to dietary factors. Research on rats showed that the HCAs could be the genotoxic carcinogens related to human cancer in these target organs. It was stated that (de Verdier, Hagman, Peters, Steineck, & Övervik, 1991) regular consumption of well-done fried meat could result in rectum, colon, and pancreas cancer occurrence, imitating the animal findings. Findings by other researchers (Sinha & Rothman, 1997) further showed the involvement of the consumption well-done cooked meat and specifically MeIQx and PhIP exposure in human breast and colon cancers. In Sweden, and at IARC similar findings were made and reviewed (Augustsson, Skog, Jägerstad, Dickman, & Steineck, 1999; Kerstin Skog, Steineck, Augustsson, & Jägerstad, 1995). There are lots of problems in interpreting epidemiologic results for colorectal cancer as a function of meat intake. Some reports did not

include the effect of cooking time. It was shown in many studies that only well-done and very well done meat constitutes enough amounts of HCAs (Grivas, Nyhammar, Olsson, & Jägerstad, 1985; M Jägerstad, et al., 1983; Sinha, Rothman, et al., 1998; K Skog, 1993; Kerstin Skog, et al., 1995). Moreover, colorectal cancer is a complex disease including multiple genetic and etiologic factors. Nutritional habits, including type and amount of fats and HCA intake mainly affect the occurrence of distal colon cancer and to some extent of rectal cancer. However, nutrition has little influence on proximal colon cancer. Biochemical approaches also suggested that heart and aorta are possible targets (Övervik, Ochiai, Hirose, Sugimura, & Nagao, 1991; Thorgeirsson, Farb, Virmani, & Adamson, 1994; Wakabayashi, 1990). Life-long vegetarians have shown lower incidence of heart disease and various types of cancer, such as colon or breast cancers (Dwyer, 1999; Fraser, 1999). The presence of HCAs have been shown in cooked foods by a number of investigators, which included the risk of several important types of cancer in consumers (J. S. Felton, et al., 1992; Gold, Slone, Manley, & Ames, 1994; Övervik & Gustafsson, 1990; Stavric, 1994). The general consensus from these studies is that well-done meats intake is related to an increased risk of certain types of cancers.

PhIP, has been indicated to particularly induce colon, breast and prostate tumors in rats (N. Ito, et al., 1991; Shirai, et al., 1997), which account for the three most common diet-associated cancer sites in Western society. In this context, the following points need to be highlighted: (i) PhIP particularly stimulates tumours of the prostate, breast, and colon; (ii) the major sites of diet related cancer in humans are colon, breast, and prostate; (iii) each of these tissues are highly affected by hormonal estrogenic chemicals; (iv) the main endogenous estrogens are known to consider as cancer burden in these tissues.

Estrogens play an essential role in growth regulation, functioning and differentiation of many reproductive tissues including the uterus, vagina, breast, and ovary (Stone, 1994). The ability of estrogens to induce cell proliferation is well-known and the main physiological estrogen, 17 β -estradiol (E2) is known to be one of the major risk factors of the breast cancer development, and has also been affected the etiology of prostate and colorectal cancer (English, Stewart, & Hewison, 2001; Stone, 1994). Both PhIP and estrogen's tissue particular carcinogenicity and the ability of PhIP to imitate the selective estrogen activities and the strong PhIP genotoxicity are captivating factors considering its potential to cause cancer. In provoking PhIP's effects, it may be affecting the required processes of cell signaling, particularly the transduction pathways that are involved in proliferation. It should be stated that in animal trials the tested HCA dosages are much higher than the their quantity in foods that are consumed by humans.

N-Hydroxylation of PhIP is catalyzed by phase I hepatic cytochrome P450, largely by CYP1A2, subsequently followed by phase II esterification by N-sulfotransferase and N-acetyltransferase. Arylnitrenium ions produced from N-sulfonyloxy-PhIP or N-acetoxy-PhIP react with nucleophilic sites in DNA, primarily at guanine bases, resulting in the formation of dG-C8-PhIP adducts (Figure 2).

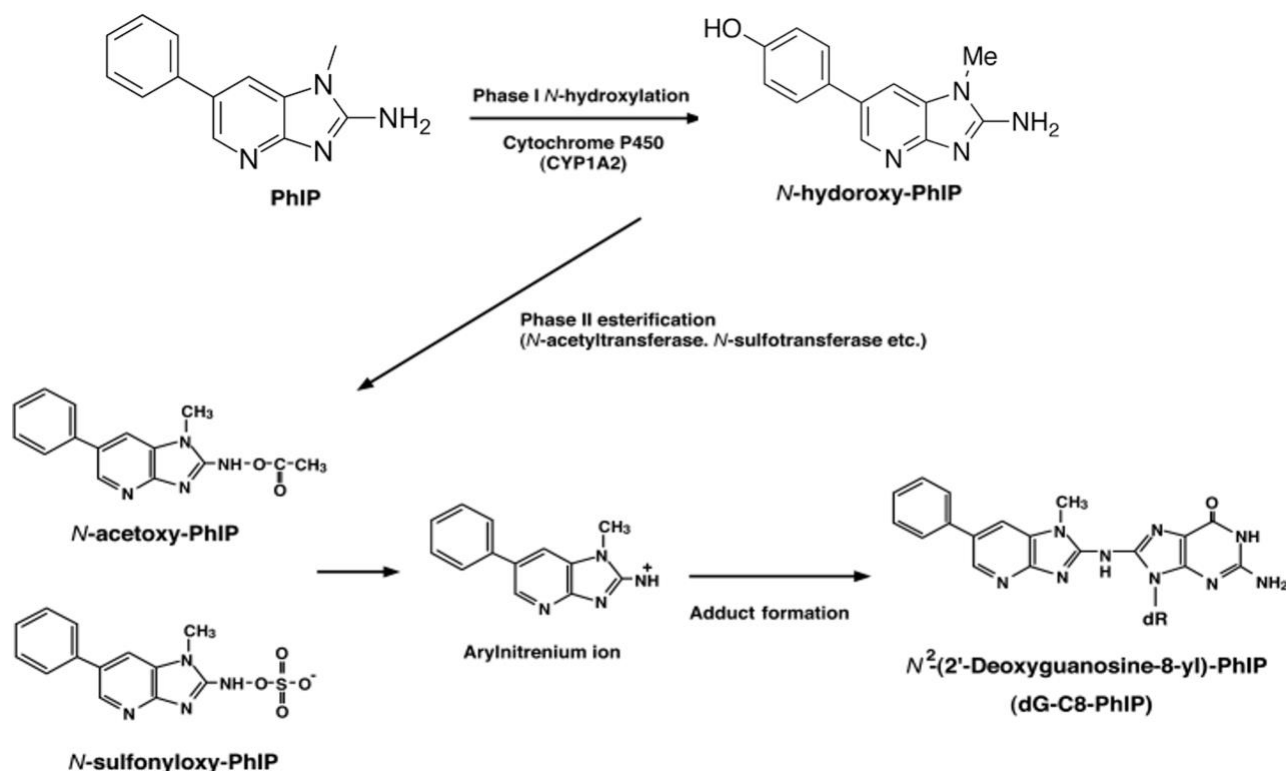


Figure 2. Chemical pathway and structure of PhIP's metabolic activation resulting in DNA adduct formation (Rosenberg, Giardina, & Tanaka, 2009).

Target organs of PhIP

A long-term experimental protocol was used to investigate the *in vivo* carcinogenic potential of PhIP against rodents. PhIP preferentially targets the colon and prostate in male rats, mammary glands in female rats, and lymphoid tissues in both male and female rats (N. Ito, et al., 1991). It should be noted that human colon, mammary glands, and prostate cancers are common in Western countries, and are now increasing in Japan in which dietary habits are more Westernized. Lauber et al. recently reported that steroid hormone receptors, such as progesterone receptor (PR), and estrogen receptor α and β (ER α and ER β) are being upregulated in tumors induced by PhIP. The PhIP has estrogenic action, inducing cell proliferation in an ER-dependent

pathway, which activates the mitogen-activated protein kinase (MAPK) signaling pathway through mRNA induction of *c-myc* and *PR* genes (Lauber, et al., 2004). This could be a contributing genetic issue. Compared to rats, the organ spectrum of PhIP in mice is more predominant and is restricted to lymphoid tissues (Sugimura, et al., 2004).

Colon carcinogenesis in rodents by PhIP

As it was mentioned before, the colon is one of the most targeted organs for cancer induced by PhIP in rats especially in male rats. In contrary, mice rarely develop colon cancers after standard long-term feeding protocols by PhIP. It was stated that short-term feeding protocol with PhIP with a diet high in fats resulted in the development of small-intestinal cancers in C57BL/6 N mice, but not colon cancers. Feeding rats with 25–400 ppm PhIP for a short time was shown to cause aberrant crypt foci (ACF), and candidate neoplastic lesions in the colon. A subset of ACF features dysplastic compounds in cryptic cells, and dysplastic ACF usually show the accumulation of β -catenin in either the nucleus or cytoplasm. Some of these can result in mutations in the β -catenin codon gene which are responsible for the stability of the β -catenin protein. Specifically, β -catenin mutations originating from lesions shows nuclear accumulation of β -catenin and more dysplastic characteristics. These lesions were categorized as high-grade dysplastic ACF. Mutation spectra determined in dysplastic ACF, particularly in high-grade dysplastic ones, were indicated to be similar to those that were stimulated by PhIP in colon cancers. Moreover, mucin identified by alcian blue-periodic acid Schiff (AB-PAS) staining method was practically entirely depleted in high-grade dysplastic ACFs. Long-term standard animal experiments indicated that F344 male rats could regularly develop colon carcinomas, with the incidence of 50% during 52 weeks of continuously feeding animals with a diet

consisted of 400 ppm PhIP (N. Ito, et al., 1991). Feeding with 100 ppm PhIP took approximately two years to get a ~50% cancer occurrence in the colon, but almost no colon carcinomas were detected with two years of feeding with 25 ppm of PhIP (Hasegawa, et al., 1993). These findings indicated that an obvious dose-dependency is present for colon cancer induced by PhIP, which usually occurs in the middle or distal part of the colon, and mostly characterize the polypoid growth. Tumors are histologically determined and are appropriately differentiated by tubular adenocarcinomas, and the lesions rarely invaded into submucosal layers or induce metastasize to other organs. In colon cancers induced by PhIP, the Paneth cells have been frequently monitored. It was found that a subgroup of genes their expression is characteristic of Paneth cells, such as the matrilysin and intestinal-type defensins, were overexpressed in colon cancers. The molecular mechanisms of Paneth cell differentiation in colon cancer tissues have not been fully clarified yet, and activation of the Wnt/Apc/ β -catenin signaling pathway could be a contributing event. Van Es *et al.* has recently shown the Wnt signaling activation to stimulate maturation of Paneth cells in intestinal crypts (van Es, et al., 2005). The Paneth cell appearance could reveal the aberrant differentiation of colonic stem cells in cancer tissues.

Breast cancer

Estrogens play an essential role in growth regulation, differentiation and functioning of many reproductive tissues such as uterus, breast, ovary and vagina (Stone, 1994). The ability of estrogens to induce cell proliferation is well known and the principal physiological estrogen, 17 β -estradiol (E2) is considered to be an important risk factor in breast cancer development, and has also been implicated in the etiology of colorectal and prostate cancer (English, et al., 2001; Noble, 1977; Stone, 1994). The tissue specific carcinogenicity of both estrogen and PhIP, the

ability of PhIP to imitate selective estrogen activities and the powerful genotoxicity of PhIP are factors to be considered as potential cause of cancer. In provoking its effects, PhIP may be affecting the fundamental processes of cell signaling, particularly the transduction pathways involved in proliferation. Detection of PhIP metabolism by MCL-5 cells or any other evidences showing PhIP's toxicity was not detectable at low concentrations of PhIP (10^{-10} to 10^{-6} M) (N. Gooderham, Creton, Lauber, & Zhu, 2007). It is well-known that both MCF10A and MCF-7 cells, such as primary epithelial breast cells, could express very low levels of CYP1B1 and CYP1A1 (Sun, et al., 2004), both of which can metabolize PhIP. Therefore, for imitating the *in vivo* situation of breast epithelial cells that are exposed to circulating PhIP, experiments related to low doses of PhIP were accomplished with two target cells (MCF-7 or MCF10A) without the presence of co-culture with MCL-5 cells. It was shown that low concentrations (nanomolar) of PhIP were able to induce cell multiplying and invasion in ER+ human breast cancer cells. These effects were shown to be due to the estrogenic properties of PhIP, and anti-estrogen administration could inhibit these effects, and PhIP was also indicated to stimulate the transcription of an estrogen receptor α reporter gene (N. J. Gooderham, Zhu, Lauber, Boyce, & Creton, 2002; Lauber, et al., 2004). Considering the fact that estrogens are a major risk factor in breast cancer development, and have also been induced the colorectal etiology and prostate cancer (Bonkhoff, Fixemer, Hunsicker, & Remberger, 2001; Leo, Messa, Cavallini, & Linsalata, 2001), the PhIP's ability to imitate the selective estrogen activity even in low doses indicates that the estrogenicity of PhIP might be an important contributing factor to the site specific carcinogenicity of the compound. Findings (Lauber, et al., 2004) showed that exposure to low doses of PhIP results in significantly different cellular responses compared to the DNA damage and cell cycle induced by high doses.

Pancreatic cancer

According to American Cancer Society (2005), pancreatic cancer is known as the fourth leading cause of cancer death in both women and men in the USA. Diagnosis of this cancer usually takes place in the late phases of the natural history of the tumour, and the mortality rate of pancreatic cancer is almost the same as its incidence rate; around 86% of patients usually die during the first year of diagnosis, and by five years after diagnosis almost all of the patients have died (Warshaw & Castillo, 1992). A semi-quantitative analysis showed that a significantly higher level of PhIP-DNA adducts was identified in the cancer patients compared to the control patients. Moreover, a significant association between the PhIP-DNA adducts levels in pancreatic tissues and the risk of pancreatic cancer was observed. More investigations are needed to see if PhIP is responsible for human pancreatic cancer or not. Because of the difficulties in extracting pancreatic tissue samples to do research, tissue samples from different sources were used. Epidemiological studies have shown that cooking methods of meats may affect the risk of development of pancreatic cancer. HCAs have been indicated to stimulate pancreatic tumor in tested animals. However, it is still unknown whether HCA exposure contributes to the development of pancreatic cancer among individuals with a diet high in animal meat. Early experiments of data from 144 controls and 147 cases have indicated that the average daily intake (g/day) of red and white meat was close between cases and controls, but people in cases consumed more well-done meat compared to controls and the median level of DiMeIQx, MeIQx, and PhIP, among controls was 1.7, 32.5, and 99.8 ng/day, respectively. It was estimated that the race-, age-, sex-, and smoking-adjusted odd ratios of pancreatic cancer were 2.2 (1.0-4.9) for PhIP, 1.9 (1.1-3.4) for MeIQx, 2.2 (1.2-3.8) for DiMeIQx, and 2.6 (1.2-5.9) for total HCAs (all P values were < 0.05) (D. Li, Xie, Wolff, & Abbruzzese, 2004).

Evidence of human exposure to PhIP

The PhIP amounts in cooked meat and fish varies from 0.29 to 182 ng/g (Sugimura, et al., 2004), and the daily intake of it is reported between 0 to 865 ng/day, with 72 ng/day the mean value (Augustsson, Skog, Jägerstad, & Steineck, 1997). Until now, a substantial number of reports have been done on the presence of low levels of PhIP and PhIP-DNA adducts in human tissues such as hair, urine, and feces. Measuring the level of PhIP-DNA adducts in tissues is another way of the quantification of the human exposure to PhIP. The dG-C8-PhIP compound is a major DNA adduct of PhIP (Ghoshal, Preisegger, Takayama, Thorgeirsson, & Snyderwine, 1994), and P-postlabeling (Takayama, Yamashita, Wakabayashi, Sugimura, & Nagao, 1989), HPLC detection (D. Lin, et al., 1992) and an immunohistochemical procedure using an anti-PhIP-adducted DNA polyclonal antibody (Shirai, et al., 1998), have been widely used for identification of PhIP-adducted DNA. Gas chromatography (GC)/MS (Friesen, et al., 1994) and accelerator MS (AMS) (Dingley, et al., 1999) analyses have also been applied to detect a small amount of PhIP-DNA adducts, at sensitivities of 10^{-8} and 10^{-12} , respectively. PhIP-adducts detected in samples obtained from human colon using the P-postlabeling method are about three adducts per 10^8 nucleotides (Friesen, et al., 1994). Considering cooking methods, pan-frying was shown to produce the most HCAs, followed by grilling/BBQ, and broiling, which were shown to be higher compared to the oven-roasting or baking because HCAs tend to form more often at higher temperatures. Furthermore, higher levels of HCAs in cooked meat compared to cooked fish, and chicken was shown to produce higher levels of PhIP (up to 480 ng/g) and MeIQx during high temperature cooking compared to pork and beef. Moreover, cooked lamb, turkey, and fish muscle also contain a very low or untraceable amounts of HCAs (Alaejos & Afonso, 2011; M. Knize, Cunningham, et al., 1994). Moreover, the amount of HCAs that is formed in

cooked meats can be different and can range from more than 10 to 100-fold. PhIP was shown to be the most abundant in almost all cooked foods, with levels ranging from not detected (nd) to as high as 480 ng/g (M. Knize, Cunningham, et al., 1994) followed by MeIQx and 4,8-DiMeIQx ranging from nd to 18.3 ng/g and nd to 29 ng/g, respectively. Other HCAs, usually are present at a relatively low concentrations ranging from nd to 10.5 ng/g (M. Knize, Cunningham, et al., 1994). In some studies, the non-mutagenic β -carbolines including norharman and harman were also found in cooked meat products (up to 30 ng/g) (Gross & Grüter, 1992; Smith, Ameri, & Gadgil, 2008).

Another sources of HCAs are pan residues and meat drippings after frying of different meat products. High amounts of HCAs was reported in the drippings from bacon fat and the scrapings of the pan after grilling meat and fish (Gross & Grüter, 1992). The PhIP, MeIQx, and DiMeIQx levels were significantly higher (10 to 100 times) than what was found in the corresponding meat products. It was reported (Wakabayashi, et al., 1993) that the average total amount of HCAs in the U.S. diet as 9 ng/kg/day, with the PhIP having the average daily intake of 100-13,800 ng/person and MeIQx having daily intake of 200-2,600 μ g/person. PhIP was shown to account for about two-thirds of the total HCAs' intake from dietary sources (Bogen & Keating, 2001). Poultry with 38 g/day and beef with 40.4 g/day are the most commonly consumed meats in the U.S., followed by pork with 20.3 g/day) and seafood with 11.8 g/day. Human's exposure to dietary HCA was not considerably affected by intrinsic differences in the meat types (Pouzou, Costard, & Zagmutt, 2018). However, cooking method highly affect the exposure levels to HCAs. Pan-frying is the most common cooking method being used in US which contributes to 367.7 ng/day of PhIP and MeIQx in the diet, followed by baking which contributes to 72.4 ng/day, barbecuing contributing to 60.9 ng/day, broiling accounting for 33.5 ng/day, and roasting

which contributes to 12.8 ng/day (M. Knize, Dolbeare, Carroll, Moore II, & Felton, 1994) (Table 4 and 5). Consumers who want to reduce their daily exposure to HCAs need to consider their methods of preparation and cooking meat, not the type of meat itself (Pouzou, et al., 2018; Kerstin Skog, et al., 2003).

Table 4. Estimated amounts of PhIP formed in meat cooked with different methods (Alaejos & Afonso, 2011; M. Knize, Dolbeare, et al., 1994; Smith, et al., 2008).

Muscle Type	Cooking Method	PhIP (ng/g)
Beef patty	Pan-fried	nd-33.1
	Grilled/BBQ	nd-16.27
	Broiled	nd-18.40
Beef steak	Pan-fried	nd-33.8
	Grilled	nd-6.99
Hamburger	Pan-fried	nd-4.9
	Charbroiled	1.8-290
Whole chicken	Fried	8.7-48.55
	Barbecued	78.52-304.71
	Oven-broiled	5.6-71.96
Chicken breast without skin	Fried	1.70-72.00
	Grilled/BBQ	27-480
	Oven-broiled	6-150
Pork patty	Pan-fried	nd-13.40
	Roasted	0.5-2.3
Bacon	Pan-Fried	nd-36.4
	Oven-broiled	nd-15.91
Sausage	Fried	nd-5.83
	Grilled/BBQ	nd-2.35
Pork chop	Fried	nd-3.27
	Fried	nd-17
Fish	Grilled/BBQ	nd-50.3
	Baked	4.31-5.67

Table 5. Total exposure per day (ng/day) to PhIP (M. Knize, Dolbeare, et al., 1994)

Cooking Methods	PhIP (ng/day)
Fried	317
Baked	49.2
Barbecued	54.6
Broiled	28.8
Roasted	9.6
Smoked	8.0
Deep Fried	2.9
Boiled	1.8
Microwaved	0.8
Dried	0.9
Sum	473.6

Bioavailability of dietary heterocyclic amines in humans

HCAAs are usually present in cooked meats at a low concentration, so human exposure is chronic with HCAAs consumed daily over a lifetime. Therefore, it is critical to understand the way that HCAAs are absorbed in human subjects, and how they can be eliminated in human food. In a study (Lynch, et al., 1992), in which 10 healthy male volunteers consumed fried beef patties containing known quantities of PhIP, MeIQx, and DiMeIQx over 14 months on four separate circumstances, it was found within the first 8 h of consumption that most of the ingested HCAAs were identified, and within 24 h of the test meal, just 1.1% of ingested PhIP and 2.1% of ingested MeIQx were recovered intact in the urine. However, DiMeIQx was barely detectable in its native form. Moreover, (Krul, et al., 2000) invented a gastrointestinal tract chemical model that imitated human digestion and discovered that 50% of ingested HCAAs, including PhIP, MeIQ, MeIQx, and IQ, were removed within 2 h from an *in vitro* human GI model, and 95% of ingested HCAAs were removed after 6 h, and 94% were recovered in dialysates, ileal, and jejunal. Recently, the model system by (H. S. Kim & Hur, 2018) showed that almost 52% of HCA intake from pork patties were reduced after digestion in the stomach and 90% were reduced after digestion in large intestine, suggesting that the ingested HCAAs were degraded in the GI tract or/and by enterobacteria such as *Lactobacillus sakei*; however, digestive enzymes were also metabolized HCAAs to form active mutagenic metabolites in small intestine, stomach, and large intestine.

Method of quantifying heterocyclic amines

QuEChERS (quick, easy, cheap, effective, rugged, and safe) method

Researchers (Anastassiades, Lehotay, Štajnbaher, & Schenck, 2003) first developed QuEChERS as a sample preparation method for multi-class pesticide analysis in milk, fruits, avocado, soybeans, flaxseeds, olive oil, peanuts, vegetables, and eggs (Castillo, González, & Miralles, 2011; Lehotay, et al., 2010; K. H. Park, et al., 2012; Pizzutti, de Kok, Hiemstra, Wickert, & Prestes, 2009; Rejczak & Tuzimski, 2015). Based on the types of salt, this method can be normally classified into three groups: original unbuffered (NaCl, MgSO₄), acetate-buffering (NaAcetate, MgSO₄) (AOAC Official Method 2007.01) and citrate-buffering (NaCl, MgSO₄, disodium citrate sesquihydrate, NaCitrate) nominated as European Standard EN 15662 (2007) (Lehotay, et al., 2010). Generally, the QuEChERS protocol has two steps: first step is salting-out extraction/partitioning by addition of anhydrous salts and a dispersive solid-phase extraction (dSPE) (Anastassiades et al., 2003). Salts are usually used in the early extraction step to separate water from organic extraction solvents (such as acetonitrile, acetone, or methanol), which leads to a high recovery of polar analytes. Consequently, a mixture of porous sorbents such as C18, major secondary amines, zirconia-containing sorbents such as Z-Sep+, Z-Sep, and/or graphitized carbon black (GCB) are used in the dSPE clean-up step to remove organic acids, lipids, sugars, and polar pigments (which cause maximum interference with detection) from matrix. Many research papers have evaluated and compared the performance of different QuEChERS methods to investigate their precision, extraction efficiency, LODs, and the ability to remove the co-extracted interference in different kinds of foods (Cunha, et al., 2007; He, et al., 2017; Lehotay, et al., 2010; Pouech, et al., 2012). For instance, (Castillo, et al., 2011) reported that the extraction recoveries of pesticides from ovine, swine, and poultry with the

QuEChERS method (PSA/C18 as dSPE sorbents, MgSO₄ as salt) was between 70%-120%. Meat extraction through the QuEChERS method resulted in a lower fat content and improved cleanliness of the chromatogram and reduced matrix co-extractives and background noise. The QuEChERS methodology showed to be a highly efficient sample preparation method, and because of its efficiency, flexibility, and low solvent consumption it has seen a wide applications in the food industry. Modified QuEChERS methods have been successfully applied for analyzing acrylamide (Mastovska & Lehotay, 2006), veterinary drugs (K. H. Park, et al., 2012), mycotoxin (Pizzutti, et al., 2009), alkaloids (Krska, Stubbings, Macarthur, & Crews, 2008), polycyclic aromatic hydrocarbons (João Ramalhosa, Paíga, Morais, Delerue-Matos, & Prior Pinto Oliveira, 2009) and hormones (Pouech, et al., 2012). The QuEChERS-based methods was also applied to HCA extraction in meat. This method's application was studied for extracting HCAs in fried pork fiber using a modified QuEChERS technique (C18, PSA, and MgSO₄ as sorbents). The extraction recoveries of HCAs ranged from 59-117%, which was comparable to the recovery results from traditional Gross method using tandem SPE (Hsiao, Chen, & Kao, 2017). Nevertheless, matrix effect was relatively high in the QuEChERS treated meats due to the interference of co-eluting substances, specifically, PhIP, IQx-, and IQ-type HCAs, showing that the existing commercially available dSPE sorbents which lack selectivity are not sufficient to remove high amounts of co-extracted lipids from foods with high-fat levels (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 suitable method of extraction for maximum removal of lipid in samples and high extraction efficiency of target substance even in low amounts (Vázquez, et al., 2016) In this method nano-particles are used as dSPE sorbents that after activation in buffer solution or water, they develop a combination of size exclusion and hydrophobic interaction with lipid-like functional groups such as compounds with bulky branches or ring structures (such as pesticides, PAH, veterinary drugs, and vitamin D). Thus, EMR-Lipid could be the most appropriate analytical method to isolate and separate most aliphatic groups containing analytes, specifically with foods with complex matrices (Lucas & Zhao, 2015). EMR-Lipid is an easy method to use and using EMR-Lipid dSPE for extraction followed by addition of EMR polish salts (such as NaCl/MgSO₄) has been successfully applied to analyze low-level analytes in food products such as vegetable oils with high lipid content (Vázquez, et al., 2016) and avocado (L. Zhao & Lucas, 2015) by GC-MS/MS or LC-MS/MS. Moreover, it was stated (Han, Matarrita, Sapozhnikova, & Lehotay, 2016) that the efficiency of co-extractive removal in the EMR-Lipid method was 83-89% in meats and avocado and 79% in kale.

Reducing formation of dietary heterocyclic amines

Generally, there are two different approaches to mitigate dietary HCAs in foods: 1. modifying cooking methods 2. addition of HCA inhibitors.

Modifying cooking methods and marination

Cooking method can be modified by microwaving (3 to 9 times less) or marinating the meat before frying to prevent the formation of HCAs such as IQ, DiMeIQx, and PhIP (J. S. Felton, et al., 1986). Comparable results for grilled chicken products were reported (Jinap, Iqbal, & Selvam, 2015) which were pre-treated in a microwave oven. The mechanism of HCAs reduction with this pretreatment may be due to leaching out of HCA precursors such as sugars, creatine, free amino acids, water, and fat during the microwave process. Moreover, marinating meat, which is done as a pre-cooking treatment to intensify flavor and improve tenderness/juiciness of meat products, might greatly decrease HCAs in chicken and beef. Marinating chicken with different types of sugars showed to affect the HCAs levels differently. Results showed that all types of HCA (other than IQx) in samples marinated with table sugar were significantly higher ($p < 0.006$) compared to those marinated with brown sugar; however, marination with honey resulted in lower HCA concentrations with considerable reduction in PhIP, MeIQ, IQx, DiMeIQx, IQ, and norharman concentrations (Hasnol, Jinap, & Sanny, 2014). Red wine marination was also shown to decrease the formation of PhIP, up to 88%, although the formation of harman was increased (Busquets, Puignou, Galceran, & Skog, 2006). Marinating chicken with a mixture of sugar, vinegar, olive oil, mustard, garlic, salt, and lemon juice, resulted in 92%-99% decrease in PhIP of chicken (Salmon, Knize, & Felton, 1997). It was also shown that (Nerurkar, Marchand, & Cooney, 1999) marinating beef steaks with turmeric-garlic containing sauce or teriyaki could reduce the formation of MeIQx and PhIP by 70% and 45%. It was also reported that (Gu, et al., 2001) grilling chicken at 100 °C which was marinated with a commercial seasoning consisted of sesame oil, soy sauce, garlic, onion powder, and sugar could significantly reduce the mutagenicity of all HCAs except for the well-done chicken. In another

study HCA levels varied according to cooking methods, PhIP levels varied in well-done and non-marinated meats which was specifically high for churrasco (31.8 ng/g in the outer part of the meat), with lower levels for grilled (16.3 ng/g), and pan-fried beef (0.58 ng/g). Chicken showed to contain higher levels of HCA compared to other non-marinated samples (Iwasaki, et al., 2010).

Addition of synthetic and natural compounds

Antioxidants which are free-radical scavengers, were shown to be preventive on the lipid peroxidation process (Balasaheb Nimse & Pal, 2015). Synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tert-butylhydroquinone (TBHQ), propyl gallate (PG), and tert-butylhydroquinone (TBHQ), and 1-O-hexyl-2,3,5,-trimethylhydroquinone (HTHQ) were shown to have HCA inhibitory activity in model systems as well as real meat products (Vitaglione & Fogliano, 2004). The formation of PhIP was reduced by 62.9-99.2% in a model system consisted of 1,000 ppm of BHA (Moon & Shin, 2013). However, addition of BHT was shown to increase the mutagenic activity of HCAs depending on its concentration. MeIQx formation was increased by 100 ppm in TBHQ treated meat (Johansson & Jägerstad, 1996). In real meat systems, it was reported that (Ahn & Grün, 2005b) a mixture of 0.02% (w/w) of BHT and BHA could significantly inhibit the formation of MeIQx by 57% and PhIP by 22% in pan-fried beef patties while having no significant effect on IQ, MeIQ, and DiMeIQx formation. Recently, there is more interest toward natural compounds to be used to prevent HCAs formation (Saito, Sakagami, & Fujisawa, 2003). Western spices/Asian/herb, variety of fruits, vegetables, and their extracts have been widely investigated on inhibiting the formation of HCA. For instance, (Oz, 2011; Puangsombat, et al., 2012; Zeng, et al., 2014)

reported that addition level of 1% (w/w) of finger root, turmeric, galangal, black pepper, and prickly ash peel to raw beef patties could reduce the formation of HCAs by 29.2% to 100% in pan-fried samples. In addition, Cheng et al. (2007) indicated that active compounds such as phloridzin, proanthocyanidins, and chlorogenic acids were responsible for the inhibitory properties of grape seed extracts. In model systems, it was shown that treatment with different levels of *Adinandra nitida* leaf extract (0, 15, 30, 45, 60 mg) resulted in the most effective inhibition of investigated HCAs with reduction levels of 47.88%, 49.73% and 29.63% treated with 45 mg, 60 mg and 60 mg of PhIP, norharman and harman, respectively (T. Zhao, Xi, Zhang, Ma, & Wang, 2021). Addition of 2 g of onion to beef patties, was shown to prevent the formation of 4,8-DiMeIQx, PhIP Glu-P-1, MeIQ, and Norharman, by 100%, 79%, 96%, 88%, and 74%, respectively (Dong, Lee, & Shin, 2011). Pomegranate seed extract was also shown to have high inhibitory effect on the formation of HCAs with 68% for PhIP, 45% for IQ, 18% for harman, 57% for MeIQx, and 24% for norharman (Keşkekoğlu & Üren, 2014).

Other natural compounds such as vitamins, mono-, oligo-, and poly-saccharides may also inhibit the HCA formation. Three water soluble vitamins (niacin, pyridoxamine, and ascorbic acid) were shown to significantly inhibit the formation of PhIP and MeIQx by more than 40% in both beef patties and model systems (Wong, Cheng, & Wang, 2012). It was reported that (Shin, Park, & Park, 2003) addition of fructo-, galacto-, isomalto- oligosaccharide, and inulin at 1.5% (w/w) levels in fried beef patties could decrease the total HCA formation by up to 54%. Direct addition of 1% (w/w) chitosan also could reduce the total HCA content in pan-fried beef chops up to 100% (Oz & Cakmak, 2016). Addition of free amino acids which are HCAs precursors also were shown to reduce the production of HCAs. Researchers (R. Jones & J. Weisburger, 1988) reported that blending of tryptophan into a commercial beef sauce significantly inhibited the

formation of mutagenic IQx- and IQ- in fried beef patties. In another study, a mixture of tryptophan and proline was used in model system to increase the inhibitory activity of IQx-type HCAs (R. C. Jones & J. H. Weisburger, 1988). Though, the mechanism of inhibitory effect of free amino acids on formation of HCA remains unclear. In another study on the effect of free amino acids on HCAs formation in beef patties, it was shown that tryptophan at 0.50% had the highest inhibitory effect (93% inhibition), followed by 0.50% lysine (84% inhibition), 0.50% leucine (64% inhibition) and 0.50% proline (56% inhibition) (Linghu, et al., 2020).

Mechanism of antioxidant to prevent HCAs

The mechanism by which antioxidants inhibit the formation of HCA is related to free-radical scavenging through free radical pathways (such as Maillard free-radical intermediates and pyrazine/pyridine radicals) during the HCA formation. Many studies have suggested a non-antioxidant preventive mechanism due to the insufficiency of the effect of the antioxidant capacity by itself (K.-W. Cheng, et al., 2007; Oguri, Suda, Totsuka, Sugimura, & Wakabayashi, 1998). Antioxidants could react with the reactive carbonyl species that were generated during Strecker degradation, their carbonyl scavenging/trapping functions, therefore they can change the formation pathways of HCAs (Arvidsson, et al., 1997; Kikugawa, 1999; Pearson, et al., 1992). Naringenin was shown to prevent the formation of PhIP by trapping its key intermediate, PheAce and converting it into different non-reactive adducts. Two adducts from naringenin and PheAce were identified and characterization by one- and two-dimensional NMR, showing that C-6 and C-8 are two active sites for naringenin in adduct formation. It was reported that scavenging or trapping carbonyl compounds was an important mechanism by which flavonoids can inhibit the formation of PhIP. Seven dietary flavonoids such as luteolin, apigenin, genistein, EGCG,

quercetin kaempferol, and phlorizin have demonstrated the ability to trap PheAce in both meat and model systems (Pearson, et al., 1992). Other than aldehyde adducts, epicatechin (EC) formed covalent bonds with hydroxycarbonyl and dicarbonyl sugar fragments such as glycolaldehyde, glyoxal, glyceraldehyde, methylglyoxal in the Maillard reaction; the structures of EC-methylglyoxal conjugates were identified using NMR (Totlani & Peterson, 2006). In another study, EGCG and catechin were shown to trap the reactive imine intermediates such as glycoaldehyde imine and glyoxal imine through their A-rings, therefore affecting pyrazinium radicals' formation (Bin, Peterson, & Elias, 2012).

Food-based carriers for natural and synthetic compounds- edible films and coatings

The encapsulation of active compounds has shown to be relatively recent, but its fast and significant advances have allowed its use in the most diverse branches of the industry, mainly food, cosmetic, and pharmaceutical sectors. In encapsulation the substance or an active agent is entrapped into another one, which is considered as the wall material. Encapsulation is usually conducted with the purpose of delivering the active compounds, by allocating them in an appropriate carrier providing controlled release of them by different time intervals independent of the bioactive nature or mass molecular of them, modifying effectiveness and safety since it has benefits of protecting the bioactive compound. Food safety and quality are the main concerns in the food industry. Preventing microbiological and chemical deterioration, as well as the exposure of active biomolecules such as vitamins, colorants, essential oils to extreme humidity and temperature conditions, which leads them to degradation (Ponce, Buera, & Elizalde, 2010). The interest for developing novel methods of prolong the food shelf life has increased recently.

Edible films are solid sheets that can be applied on the surface of several foods and can be a good alternative to modify food quality by acting as selective barriers to moisture transfer, carbon dioxide permeability, oxygen uptake, lipid oxidation, and losses of flavors and volatile aromas (Di Pierro, Sorrentino, Mariniello, Giosafatto, & Porta, 2011; Lacroix & Vu, 2014). These films are thin layers of edible compounds such as proteins, polysaccharides and lipids. Studies showed that incorporation of natural active compounds in edible film may expand their functionality by providing a protection for the product from microbial spoilage and therefore extending their shelf-life (Alboofetileh, Rezaei, Hosseini, & Abdollahi, 2014; Emiroğlu, Yemiş, Coşkun, & Candoğan, 2010; T. McHugh & Senesi, 2000; Peretto, et al., 2014). An edible coating is stated as a thin layer of edible material that is applied to the surface of a food, providing a barrier against migration of lipids, oxygen moisture, aromas, carbon dioxide, and other solutes (Biquet & Labuza, 1988; Cuq, Gontard, & Guilbert, 1995; Kester & Fennema, 1986).

Moreover, there are more innovative applications of edible coatings such as encapsulating the bioactive compounds to improve food products' quality, providing controlled release (Pothakamury & Barbosa-Cánovas, 1995). This application of edible films is being used to extend shelf-life and to decrease the risk of pathogen growth on food surfaces, and providing functional product with health benefits to the consumer.

Bioactive compounds are considered as non-nutritional constituents that usually are present in small quantities in foods. Generally, these compounds are found in millions of species of animals, marine, plants, and organisms, which can be extracted by different methods of extraction (Kris-Etherton, et al., 2002). Bioactive compounds can be used to produce novel functional foods, extend its shelf life, increase nutritional quality and their consumer acceptance (Ayala-Zavala, et al., 2011; Muranyi & Fraunhofer, 2013). Bioactive compounds incorporation

into foods provides several advantages for food preservation contributing to functional foods' development. However, there are some disadvantages related to bioactive compounds such as promoting off-flavors and loss of functionality in early stages (Ayala-Zavala, et al., 2011; Silva-Weiss, Ihl, Sobral, Gómez-Guillén, & Bifani, 2013).

Encapsulation through edible coatings is a promising method to overcome the disadvantages related to the application of bioactive compounds as food additives. There are different methods of encapsulation for a bioactive compound, depending on the purpose and nature of it. For instance, the bioactive compound can be incorporated: a) on the outer surface of the edible film, b) at the interface of the edible film and the food, c) among the edible coatings' layers, and d) among different sections of the edible film. These methods are more effective compared to a direct application on the food surface, due to the delay they cause in the migration of the agents from the surface, which helps them to maintain a high concentration of bioactive compounds wherever is needed. The structural material of edible coatings varies on proteins (such as animal- and plant- based proteins), lipids (such as waxes and glycerol esters), polysaccharides (such as chitosan, starch cellulose derivates, and alginate) which may be used alone or in combination, based on the properties of the compound that are going to be encapsulated and the purpose of encapsulation. The functionality of these materials may be different due to different properties that each compound offers to the composite matrix. The barrier and mechanical properties of these edible films depends on the types of compounds, their compatibility, and on cohesion forces, including ionic bonds, H-bonding, and covalent bonds between polymer molecules that are forming the coating (Janjarasskul & Krochta, 2010).

To improve the mechanical properties of edible films, plasticizers and emulsifiers can be used. Plasticizers are usually hydrophilic agents with small molecular weight which are applied

to improve coatings properties. These compounds position themselves in edible film's polymeric network and they compete to form chain-to-chain H-bonding along the polymer chains. Glycerol (Gly), sorbitol, surfactants, phospholipids, polyethylene glycols, and fatty acids are common plasticizers that are being used in edible films (J. M. Krochta, Baldwin, Nisperos-Carriedo, & Park, 1995). Cellulose, pectin, starch derivatives, gums, chitosan, and seaweed extracts are examples of polysaccharides being used to form edible films (Baldwin, Hagenmaier, & Bai, 2011; Bourtoom, 2008; Espitia, Du, de Jesús Avena-Bustillos, Soares, & McHugh, 2014). However, these compounds are very hydrophilic that result in poor gas barrier and water vapor properties in moist conditions. Most of the polysaccharides have good barrier properties at RH <25% conditions (Baldwin, et al., 2011). Due to a large number of hydrophilic moieties and hydroxyl groups present in the polysaccharides' structure, H-bonds have an essential role in the formation of edible film and its characteristics. During the co-acervation process, interactions among long-chain polymer segments are being disrupted, and new intermolecular H-bonding and hydrophilic-bonding are formed upon evaporation of the solvent to produce a coating matrix (Janjarasskul & Krochta, 2010).

Proteins, on the other hand, are more desirable for the formation of edible coatings, as they possess Excellent mechanical and optical properties and they have good barrier properties against the transportation of CO₂ and O₂ (Janjarasskul & Krochta, 2010). However, the mechanical and barrier properties are compromised by moisture due to their intrinsic hydrophilic nature. Proteins can participate in different chemical reactions through covalent and non-covalent interactions (J. Krochta & Hernández-Izquierdo, 2008). Moreover, hydrophobic interactions can occur among non-polar groups of amino acids chains (Guerrero, Retegi, Gabilondo, & De la Caba, 2010). Interlinkages between proteins that participate in the formation of a film can

improve film properties. Lipids are another compounds being used in the formation of edible films. Lipids are consisted of natural wax, acetylated monoglycerides, and surfactants. Paraffin wax and beeswax are the most effective lipid compounds being used (Debeaufort & Voilley, 2009). The main function of a lipid coating is to block transportation of moisture due to their relative low polarity. In contrary, the hydrophobic characteristic of lipid result in the formation of thicker and more brittle films; thus, they need to be combined with other proteins or cellulose derivates. Lipid-based films are usually sustained on a matrix with polymer structure, such as polysaccharide, to provide better mechanical strength.

Edible films based on proteins-whey protein isolate

Animal and plant proteins, such as gelatin, collagen, wheat gluten, milk proteins, corn zein, peanut protein, and soy protein have been used for edible films (Gennadios, Hanna, & Kurth, 1997). Films based on protein can adhere well to hydrophilic surfaces which provide good barriers for carbon dioxide and oxygen; however, they do not have resistance for water diffusion compared to polysaccharide films (Cutter, 2006). Many studies have been conducted to solve the problem associated with the poor water-vapor barrier properties of edible films based on protein (Kokoszka, Debeaufort, Lenart, & Voilley, 2010; L. Liu, Kerry, & Kerry, 2006; Min, Janjarasskul, & Krochta, 2009; Monedero, Fabra, Talens, & Chiralt, 2009). These studies indicated that by addition of a lipid component to protein- and polysaccharide-based films, the barrier and physical properties were increased. In their native states, proteins normally exist as either fibrous or water insoluble proteins which serve as an important structural materials of animal tissues, or globular or soluble proteins (in water or aqueous solutions of acids, bases or salts) which widely function in living systems (Scopes, 1982). Fibrous proteins are fully

extended and are closely associated with each other in parallel structures to form fibers (usually through hydrogen bonding). The globular proteins fold into complicated spherical structures which are held together by a combination of covalent (disulfide), hydrogen, ionic, and hydrophobic bonds (Scopes, 1982).

The physical and chemical properties of these proteins is dependent on the relative amounts of the amino acid residues and their location on the protein polymer chain. Protein films are generally formed from dispersions or solutions of the protein as the solvent evaporates. The solvent is usually limited to water, ethanol or their mixtures (Kester & Fennema, 1986). Generally, to form the more extended structures required for the formation of film by proteins, they should be denatured by acid, base, heat, and/or solvent. After extension, ionic, hydrophobic, hydrogen, and covalent bonding can be formed. The degree of chain extension determines the chain-to-chain interaction that produces cohesive films. Uniform distribution of hydrophobic, polar, and/or thiol groups along the polymer chain enhance the probability of the respective interactions. Increased polymer chain-to chain interactions result in stronger films with less flexibility and less permeability to vapors, liquids, and gases (Kester & Fennema, 1986). Polymers with groups that can associate through ionic and hydrogen bonding result in films with Excellent oxygen barriers but susceptible to moisture (Yam, 2010). Thus, protein films are considered as films with good barrier to oxygen at low relative humidities. Various types of protein have been used as edible films. These include casein, soy protein, gelatin, corn zein, whey protein, mung, and wheat gluten.

There is higher interest about using whey protein isolate (WPI) films due to their transparent properties with high barrier to lipids, oxygen, and flavors (Maté & Krochta, 1996; T. H. McHugh & Krochta, 1994; Miller & Krochta, 1997). There is a need to add plasticizers to

WPI films to be functional; however, it could migrate during storage and subsequently it can affect the film properties. The most usual plasticizers being used are mono-, di-, and oligosaccharides, and polyols. Changes can occur in the moisture content and mechanical properties of WPI-based films plasticized with Gly during their storage at 50% RH (Anker, Stading, & Hermansson, 2001), which resulted in stiffer and less flexible edible films due to the migration of Gly to the film surface. It was shown that the properties of WPI-based films with sorbitol remained stable. However, other study showed that mechanical properties and the appearance of films plasticized with Gly did not change by six months storage time. However, edible films that were plasticized with sorbitol became stiffer and less flexible with time which could be attributed to sorbitol crystallization (Osés, Fernández-Pan, Mendoza, & Maté, 2009).

Plasticizers function by weakening the intermolecular forces between adjacent polymer chains, which results in an increased film flexibility and extensibility decreased mechanical resistance, elasticity, and barrier properties. The Gly, as a plasticizer, has been incorporated into many hydrocolloid-based edible films. This compound is a polar, water-soluble, protein miscible, and non-volatile plasticizer with high boiling point. These properties make glycerol an appropriate plasticizer to be used with a compatible water-soluble polymers (Banker, 1966). Depending on the structural cohesion, WPI-based edible films has wide range of functional properties. Native WPI (NWPI) films were the subject of less research studies in comparison to the heat-denatured ones. Early investigations have shown that protein denaturation is an essential step for making WPI-based edible films (Banker, 1966). Thermal denaturation is being used to unfold the globular structure of whey proteins for producing a cohesive structural film matrix by exposure of the internal hydrophobic and sulfhydryl groups and promoting free sulfhydryl oxidation, hydrophobic bonding, and disulfide bond interchange. The heated whey protein isolate

(HWPI) films were stated to be transparent, glossy, flexible, bland, and water-insoluble with decent barrier and mechanical properties at low relative humidity (RH) (Banker, 1966). A method was developed for quantification the protection ability of WPI-based edible films applied on food with a high polyunsaturated fatty acids (PUFA) content from lipid oxidation. It was indicated that the plasticizer decreased the protective effect of the edible film, by increasing their moisture content, which decreases their protection ability (Osés, Fernández-Pan, Ziani, & Maté, 2008). Researchers could successfully produce an edible, entirely water-soluble, bland, glossy, clear, and odorless NWPI films, although the range of WPI to GLY ratio was narrower in these edible films, compared to heat-denatured, and water-insoluble counterparts. The difference in the water solubility of the these two edible films makes them suitable for different food applications. Complete solubility of the NWPI-based edible film pouches showed their potential commercial application for dry food ingredients (Janjarasskul, Tananuwong, Phupoksakul, & Thaiphanit, 2020).

Edible films and coatings based starch-potato starch

Polysaccharides such as starches, cellulose derivatives, gums, and chitosan have been used to prepare coatings and edible films which can be used as packaging material for food preservation (Aider, 2010; Elsabee & Abdou, 2013; Vargas & González-Martínez, 2010). The packaging materials based on polysaccharides must be accessible and functional with specific properties and low cost.

The polysaccharides-based biodegradable edible films are known to provide good barrier to gas transference such as O₂ and CO₂ (Bertuzzi, Vidaurre, Armada, & Gottifredi, 2007); However, due to their hydrophilic nature they have poor water vapour barrier properties.

These edible films are highly hygroscopic and can be applied on the surface of food as a relatively thick films to absorb water which provides a temporary protection to inhibit moisture loss from the food. Thus, until dehydration of the coating, the product does not lose significant moisture (Bourtoom, 2008, Embuscado and Huber, 2009).

The types of polysaccharide and the wide range of additives that can be incorporated during the preparation of biodegradable films may allow enhancing the shelf-life of ready-to-eat foods. Starch possess the ability of forming edible films with appropriate mechanical resistance, and it can be applied as a coating to food products providing a good barrier to moisture loss or protecting them from unfavorable oxidation. Moreover, starch edible films were shown to be tasteless, odorless, and transparent, with very decent oxygen barrier properties, which makes them extremely suitable for food preservation applications (Acosta, et al., 2016; Fei, et al., 2013).

There are different sources being used to make starch-based edible films such as cassava, corn, wheat, waxy corn, potato, and sweet potato starch (Dai, Zhang, & Cheng, 2019). There are also other non-conventional sources of starches such as pumpkin fruits, quinoa, and lentil seeds that were shown to be good alternative for producing edible films with good tensile strength and elongation at breaks varying between 8.98 and 13.85 MPa, and 3.35–4.44%, respectively (Galindez, Daza, Homez-Jara, Eim, & Váquiro, 2019; Pająk, Przetaczek-Rożnowska, & Juszcak, 2019). Moreover, in order to improve the packaging properties, a mixture of starch with other plant sources such as eggplant flour, pomegranate peel and grape juice can be used (Ali, et al., 2019; Nouraddini, Esmaili, & Mohtarami, 2018; Yıldırım-Yalçın, Şeker, & Sadıkoğlu, 2019). Starch-based films have Excellent O₂ and CO₂ barrier properties (Kester & Fennema, 1986). Due to these properties, films based on starch are very appropriate to preserve

fresh vegetable and fruit by decreasing their exposure to environmental O₂ and increasing the internal CO₂ content. Botanical source, and the concentration of starch as well as type of plasticizer had substantial effect on the physical properties of starch-based films. Moreover, mechanical treatment (stirring speeds and time) during film preparation can be used to modify starch films (Santacruz, Rivadeneira, & Castro, 2015).

Amylose is a branched carbohydrate consisted of $\alpha(1-4)$ bonds with 10^5 to 10^6 molecular weight of anhydroglucose units. The number of macromolecular configurations that are based on $\alpha(1-6)$ linkage is directly proportional to the amylose's molecular weight (Phan, Debeaufort, Luu, & Voilley, 2005). On the other hand, amylopectin is a multiple-branched polymer with a higher molecular weight of 10^7 to 10^9 anhydroglucose units. The size of starch granules is in the range of 2–150 μm , the amylose content is from 0% to 5%, but normally it is 20–25% (w/w) (Phan, et al., 2005). Waxy starch has low or even no amylose; however, high-amylose starch consisted of more than 50% of the linear polymer. Functional, physical, and chemical properties of coatings and edible films depend on the ratio of amylose to amylopectin (Parker & Ring, 2001; Xie, Halley, & Avérous, 2012). Better gas barrier and mechanical properties was reported for high amylose starch such as pea (Palviainen, et al., 2001). Researchers (Joshi, et al., 2013) showed that in comparison to potato and corn starches, lentil starch (30% amylose) has a higher tendency to form gel at a relatively low concentration. Regardless of the botanical origin, starch shows different disadvantages such as strong water sensitivity (hydrophilic properties), which limits its application. Potato starch (PS)-based films provide a greater barrier for water vapour and oxygen; however, the films had weaker mechanical properties compared to films based on corn and wheat starch.

Higher amylose content of starch species resulted in better mechanical resistance, lower wettability properties, that is highly dependent on the water content as a result of the hydrophilic nature of starch films, so they could be applied on products with higher water activity, such as fruits, vegetables, and cheese (Basiak, Lenart, & Debeaufort, 2017). There are different applications of edible films for food products. It was stated (Garcia, Martino, & Zaritzky, 1998) that the shelf life of strawberry can be increased from 14 to 21 days by applying a starch coating. Moreover, films based on starch have the potential to protect foods with high lipid content that are susceptible to oxidation (Wu, Weller, Hamouz, Cuppett, & Schnepf, 2001).

In another study walnuts were coated with films based on modified starch and the sensory evaluation indicated that the oxidative rancidity was retarded during storage (Jokay, Nelson, & Powell, 1967). Among all types of starch, PS has amylose:amilopectine content ratio of 21:79 which is lower compared to that of other starches. Therefore, PS has lower tendency to retrogradation (O. R. Fennema, 1996). Moreover, PS granules can quickly get disintegrated, providing a homogeneous matrix in a very short time. Another study showed that PS were able to form films without the need to add plasticizers. However, addition of plasticizer such as glycerol could improve the mechanical properties of the films based on PS which was compatible with PS (Rodríguez, Osés, Ziani, & Maté, 2006).

Edible film application on meat

One of the main concerns of the meat, seafood, and poultry industry is raw products contamination with gram-negative foodborne pathogens such as *E. coli* O157:H7, *Salmonella*, and *Campylobacter*. A possible solution to control these foodborne pathogens could be edible films incorporated with antimicrobial agents (Shefet, Sheldon, & Klaenhammer, 1995;

Stevens, Sheldon, Klapes, & Klaenhammer, 1991). Coatings and edible films may be applied to poultry and meat products by dipping, brushing, foaming, dipping, spraying, casting, rolling, or individual wrapping (O. Fennema & Greener, 1994; Grant & Burns, 1994). Irregular surfaces of food products such as meat can be challenging to apply the coating on them uniformly.

Therefore, it is important to find a way to obtain a uniform coating on a food surface. Emulsions can work in cases of multiple applications where the coating will solidify. This is conducted usually after dipping the product that is being coated, draining the coating to set before applying the next coating layer (O. Fennema & Greener, 1994). Spraying is another appropriate method in which thinner films are formed on the surface of the product (O. Fennema & Greener, 1994). Brushing/rolling the coating onto meat surfaces directly are also a method of edible coatings' application. These edible films need to be applied on the surface of food products and wait till they set or solidify at either ambient temperatures or by using heat (Grant & Burns, 1994).

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Chapter 2 - Effect of whey protein isolate based edible films containing amino acids on the PhIP level and physicochemical properties of pan-fried chicken breasts ^{1,2}

Abstract

This study was conducted to investigate the inhibitory effects of edible films containing amino acids (AAs) on the formation of 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP) in chicken breasts and to evaluate the physicochemical properties of the chicken. Heated whey protein isolate (HWPI) solution (100 g) was made by heating 5 g whey protein isolate (WPI) solution at 90 °C for 30 min (in a water bath). After cooling down the solution, it was mixed with 2.5 g glycerol (GLY), and tryptophan (Trp) or lysine (Lys) were added at 0.25%, 0.5%, and 0.75% concentrations (w/w), subsequently. Unheated whey protein isolate (UHWPI)-based casting solution was prepared with the same method but without heating of WPI solution. Chicken breasts were cut at the same dimensions (4 cm × 3 cm × 1.5 cm) were covered with the prepared edible films and were fried at 195 °C for 7.5 min on each side. For edible films, total soluble matter (TSM%), color (colorimeter), radical scavenging activity (DPPH), and Fourier transform infrared spectroscopy (FTIR) were evaluated. For chicken breasts PhIP level, color

¹ Farshi P, Amamcharla J, Smith JS. Effect of whey protein isolate-based edible films containing amino acids on the PhIP level and physicochemical properties of pan-fried chicken breasts. Journal of Food Science. 2022 Sep 19.

² Abstract presented in 2022 IFT annual meeting.

before and after frying (calorimeter), cooking loss percentage (weight loss before after frying), and tenderness (texture analyzer) were measured.

The average PhIP level decreased from 78.47 ppb to 6.69-8.31 ppb for chicken covered with Lys containing HWPI edible films, and to 16.09-46.80 ppb for chicken covered with Trp containing ones. For chicken covered with UHWPI edible films the PhIP decreased 28.4-56.04 ppb for Trp containing ones and 7.75-40.32 ppb for Lys containing ones. Moreover, chicken breasts covered with HWPI edible films had lower cooking loss and improved tenderness compared to the chicken breasts with no edible film. This study provides a new approach to decrease the PhIP levels in fried chicken breast.

Keywords: 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine, L-lysine, L-tryptophan, edible films, whey protein isolate

Introduction

Heterocyclic amines (HCAs) are a group of extremely carcinogenic and mutagenic substances that form on the surface of cooked or fried muscle-rich meals such as poultry, red meat, fish, and pork. HCAs are formed under high-temperature ($>150^{\circ}\text{C}$) and constant-cooking conditions (Cooper, Jankhaikhot, & Cuskelly, 2014). The carcinogens include 2-amino-3,4,8-trimethylimidazo [4,5-f] quinoxaline (4,8-DiMeIQx), 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx), 2-amino-3,4-dimethylimidazo [4,5-f] quinolone (MeIQ), 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). PhIP which is the most abundant of these heterocyclic amines in fried chicken (Alaejos & Afonso, 2011), has been shown to induce breast, colon, and prostate cancers in rats (N. Ito, et al., 1991; Stuart, Holcroft, de Boer, & Glickman, 2000). The Maillard process produces HCAs as a by-product from creatin(in)e, amino acids, and

reducing sugars (Bordas, et al., 2004). Food components are clearly precursors to HCAs, and once created, these molecules are difficult to eliminate. As a result, it's critical to identify the potential inhibitors of their formation and to decrease their concentrations in foods (Q. Zhu, Zhang, Wang, Chen, & Zheng, 2016). Considerable research has been conducted to reduce the formation of HACs by addition of several additives to the meat products or model systems. Some studies have indicated that additives such as amino acids (Linghu, et al., 2020), vitamins (Ruan, et al., 2014), and natural plant extracts (Damašius, Venskutonis, Ferracane, & Fogliano, 2011) can prevent the formation of HCAs. Amino acids (AAs) along with their derivatives, such as L-lysine (Lys) and L-tryptophan (Trp), have been extensively used in commercial foods as food additives to increase their nutritive value (Belitz, Grosch, & Schieberle, 2009). Free AAs are considered to be essential precursors of Maillard reaction, and are known to significantly ($p < 0.05$) affect the quantity and variety of HCAs (Murkovic, 2004). Heating of various single AA with other HCA precursors such as hexose and creatine/creatinine, has been broadly used in model systems for *in vitro* study of the HCAs formation. Moreover, there are several studies on the addition of certain AAs into model systems or meats which can either increase (Ashoor, Dietrich, Chu, & Pariza, 1980) or inhibit (Linghu, et al., 2017) the formation of mutagens. Researchers (Kataoka, et al., 2012) found that in a dry heat treatment, AAs are able to reduce the amount of PhIP through the formation of PhIP-AA adducts; however, another study (Zamora, et al., 2013) stated that reactive carbonyl compounds which are being produced from AAs can have a significant impact on PhIP formation. Therefore, PhIP inhibition can also be associated to these mechanisms. Formation mechanism of PHIP is through direct condensation reaction of creatinine with phenylacetaldehyde (PheAce), which is an intermediate Maillard product, followed by cyclization and dehydration (Alaejos & Afonso, 2011; Murkovic, 2004). It can be derived that,

scavenging of PheAce can inhibit PhIP formation during the Maillard reaction. However, there is still no clear understanding of the mechanism of inhibitory effect of AAs on PhIP formation.

Recently, it was reported that among the studied AAs, Trp and Lys are the most effective ones in reducing the PhIP levels in Maillard reaction model system (Linghu, et al., 2017).

It is known that edible films are thin layers of material which can be covered around a food product to act as a barrier against the surrounding environment. Moreover, edible films can be used as carriers of antioxidants, several chemical or natural antimicrobial agents, anti-browning agents, natural flavor vitamins, enzymes, nutraceuticals, aroma compounds, or minerals (Arnon-Rips & Poverenov, 2016; Janes & Dai, 2012). This method of encapsulation is more successful than applying bioactive compounds directly to the food surface because edible coatings help to keep these compounds on the surface and delay their migration away from it, resulting in a high concentration of bioactive compounds where they are needed (Quirós-Sauceda, Ayala-Zavala, Olivas, & González-Aguilar, 2014). To our knowledge, there is no study evaluating the effect of edible films on PhIP formation. Therefore, in this study we aimed to prepare edible films based on heated/unheated whey protein isolate (HWPI and UHWPI) by incorporating Trp or Lys in them. The prepared edible films were applied on the chicken breasts to determine their inhibitory effect against the PhIP formation on the chicken breast after pan-frying at 195°C for 7.5 min. For edible films, total soluble matter (TSM%), color, radical scavenging activity (DPPH), and FTIR spectroscopy measurements were conducted. For chicken breasts PhIP, color before and after frying, cooking loss percentage, and tenderness were evaluated.

Material and methods

Chemicals and materials

The 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP) standard was purchased from Cayman Chemical (Ann Arbor, MI, USA) and whey protein isolate (WPI) was supplied by Glanbia Nutritionals (Glanbia Nutritionals, Inc. Twin Falls, ID, USA). L-tryptophan (Trp), L-lysine (Lys), glycerol (GLY), triethylamine, acetic acid (HPLC grade), methanol and acetonitrile (HPLC grade), 2,2-Diphenyl-1-picryl-hydrazyl (DPPH), sodium hydroxide, and ammonium acetate were purchased from MilliporeSigma (Burlington, MA, USA). Deionized water was obtained from a Sybron/Barnstead PCS filtration unit (Barnstead/Thermolyne, Inc, Dubuque, Iowa, U.S.A.). Bond Elut Enhanced Matrix Removal (EMR)-Lipid (QUEChERS dSPE EMR and Lipid-Polish) was obtained from Agilent Technologies (Santa Clara, Ca, U.S.A.). Chicken breasts (SmartChicken®), and canola oil were obtained from a local store.

Edible film preparation

The HWPI and UHWPI edible films were prepared according to the method described, previously (Janjarasskul, et al., 2020). HWPI-based casting solution (100 g) was made by heating 5 g WPI solution at 90 °C for 30 min in a water bath and subsequently mixed (Ultra Turrax homogenizer, 11000 rpm, 2 min) with 2.5 g GLY, and Trp or Lys at 0.25%, 0.5%, and 0.75% concentrations. UHWPI-based casting solution was prepared by dissolving 5 g WPI and 2.5 g GLY in distilled water which was later spiked with either Lys or Trp at 0.25%, 0.5%, and 0.75% (w/w). The UHWPI and HWPI casting solutions were stirred at 200 rpm for 30 min to get homogenous solutions. The casting solution (16.5 g) was pipetted and spread on to a 12 cm × 12 cm polystyrene surface in order to minimize variations in the edible film thickness resulted in a

thickness of 0.19 ± 0.02 mm (Gyros Digi-Science Accumatic Digital Caliper (Monsey, NY, US)). The casted edible films were dried overnight in oven (Fisher Scientific Isotemp Oven 200 Series Model 230G, Pittsburgh, PA, USA) with 30 ± 2 °C, and $35 \pm 5\%$ relative humidity. Control edible films were prepared without addition of AAs in both UHWPI and HWPI edible films. Edible films were stored in polyethylene-low density sealed plastic bags at room temperature (25°C) and 50% relative humidity.

Edible film characterization

Edible film total soluble matter (TSM percent)

Total soluble matter of the edible films was measured according to the previously described method (Janjarasskul, et al., 2020) with slight modifications. The 4 cm × 4 cm edible film was dried in the vacuum oven (Fisher Scientific Isotemp vacuum Oven Model 282A, Pittsburgh, PA, USA) at 70 °C and 10.2 inch Hg pressure for 24 h. The primary dry weight of edible film was recorded as (W_i). Then edible film was placed in a beaker and 100 mL distilled water was added to it under constant magnetic stirring. After 1 min, the remaining intact edible film was poured onto an aluminum foil cup through a strainer and dried in the oven for 24 h. The final dry weight (W_f) was determined and total soluble matter (%TSM) of the edible films was calculated by this equation: $\%TSM = (W_i - W_f) / W_i \times 100$.

Color measurement

The CIE Lab parameters, lightness (L^*), greenness ($a^* < 0$), redness ($a^* > 0$), blueness ($b^* < 0$), and yellowness ($b^* > 0$) of edible film were measured with a HunterLab MiniScan EZ 4500 L (Hunter Associates Laboratory Inc., Reston, VA, USA). The standard black and white

plates were used for calibrating the colorimeter. Color of the edible film was measured against a black surface. Color difference (ΔE) was measured according to this equation:

$\Delta E = \sqrt{\Delta L^2 + \Delta a^2 + \Delta b^2}$ where ΔL , Δa , and Δb for the edible film were the differences between color coordinates of the edible film and the black surface on which the measurements were conducted (with average $L^* = 18.5$, $a^* = -0.21$, and $b^* = -0.04$).

Radical scavenging activity measurement

The 2,2-Diphenyl-1-picryl-hydrazyl (DPPH) reduction method was used to measure the antioxidant activity of the edible film, according to the previously described method (Ruiz-Navajas, Viuda-Martos, Sendra, Perez-Alvarez, & Fernández-López, 2013), with slight modifications. Each edible film (0.25 mg) was dissolved in 3 mL distilled water in a beaker, and stirred for 2 h at 25 °C to get the edible film extract. A methanolic solution of DPPH (1mM) was prepared and then 2.7 mL of edible film extract solution was added to 0.3 mL of 1 mM DPPH solution and mixed well in 15 mL test tubes. After filtering the solution (with a 0.45 μ m syringe filter) and 30 min storage in the dark, the absorbance of the solution was measured at 517 nm by UV/VIS spectrophotometer. DPPH radical scavenging activity percentage was calculated using the formula: %DPPH Activity = $(A_0 - A_s / A_0) \times 100$, where A_0 was the absorbance of control solution (2.7 mL water and 0.3 mL of 1 mM methanolic solution of DPPH) and A_s was the absorbance of edible film solution.

Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) analysis

A Fourier transform infrared spectrometer (Thermo Scientific Nicolet iS20, Waltham, MA, USA) was used to obtain the FTIR spectra of the edible films, WPI, Trp, and Lys powders,

and GLY. Samples were placed on the surface of the ATR crystal and the spectra were obtained in the wavenumber range of 400-4000 cm^{-1} at a 16 cm^{-1} resolution, and the OMNIC software was used for their collection.

Application of edible film on chicken breast

Chicken breasts (about 4 cm $\times$ 3 cm $\times$ 1.5 cm dimension and weight of 20 g $\pm$ 3.4) were fully covered with the edible films and incubated 15 min at room temperature. Chicken breasts with no edible film and chicken breast covered with edible film with no AA were used for comparison. Pan-frying was conducted in Teflon-coated frying pan. Before frying, canola oil was added and the pan was heated to 195°C (surface temperatures were measured by using a digital thermocouple). The frying was conducted for 7.5 min for each side of chicken.

Pan-Fried chicken breast characterization

The CEM Smart 5 microwave moisture and Rapid NMR Fat Analyzer (CEM Corporation, CEM, Matthews, NC, USA) was used for moisture and fat measurements which consisted of a microwave-drying step followed by nuclear magnetic resonance (NMR) analysis according to the official AOAC 2008.06 method. Protein measurement was conducted according to AOAC 993.13 method using LECO Tru-Mac N (St. Joseph, MI, USA). The pH values of fried meat crusts were determined according to (Fan, et al., 2019). One gram of fried chicken breast crust and 9 mL of distilled water were homogenized for 1 min at medium speed using an Omni TH homogenizer (Omni Intl., Marietta, GA, USA). A portable pH meter (Fisher, Bedford, TX, USA) was used for measuring pH of each sample at room temperature.

PhIP determination

PhIP extraction using Enhance Matrix Removal-Lipid method

The PhIP extraction was conducted by Agilent Bond Enhance Matrix Removal-Lipid (EMR-Lipid) method according to a previous study (Linghu, Karim, Taghvaei, & Smith, 2019). The 2% formic acid–acetonitrile (15 mL) was added to 3 g of finely grounded pan-fried chicken breast crust and then it was mixed in a 50 mL polypropylene (PP) tube and shaken for 30 min on a mechanical shaker at room temperature. All of the samples were centrifuged ($10000 \times g$ at 4°C) for 10 min. EMR-Lipid dSPE sorbent (1 g), preactivated with 5 mL of 5 mM ammonium acetate buffer, and then 12 mL of supernatant from centrifuged sample was transferred to the same tube for matrix cleanup. After vigorously shaking the tube for 2 min, the tube centrifuged at $10000 \times g$ and 4°C for 10 min. Subsequently, 10 mL of supernatant was mixed with 2 g EMR-Lipid Polish salts for a salting-out extraction and further cleanup. After another vigorous hand shaking for 2 min, and 10 min centrifugation ($10000 \times g$ at 4°C), 1 mL of the final extract from top layer was pipetted in to HPLC vials and was evaporated under a constant flow of nitrogen until it drying up. The extract was then reconcentrated with 100 μL of methanol and filtered through a 0.2 μm nylon filter prior to HPLC analysis. Recovery of the extraction method was calculated by spiking 50 ng of PhIP standard to a gram of chicken breast meat (obtained from inside, not the crust). After the extraction process, the final solution was injected into HPLC.

Identification and quantification of PhIP

The detection of PhIP was conducted by HPLC (Agilent Technologies, 1100 series) according to a method described by researchers (Persson, Sjöholm, Nyman, & Skog, 2004;

Vangnai, Houser, Hunt, & Smith, 2014) with some modifications. The isocratic mobile phase was consisted of 65% acetonitrile and 35% of 0.01 M triethylamine (adjusted to the pH of 3.6 with acetic acid). The injection volume was 10 μ L and the column temperature was 26 $^{\circ}$ C. The C18 column (TOSOH Bioscience LC, TSKgel ods-80, 4.6 mm ID $\times$ 25 cm, 5 micrometer Millipore Sigma, MA, USA), was eluted with the isocratic mobile phase with the flow rate was 1.0 mL/min. PHIP was detected by fluorescence detector (Hewlett-Packard, Palo Alto, CA, USA) with excitation and emission wavelengths of 306 and 371 nm. The matrix matched standard curve was obtained by adding different amounts of PhIP to the chicken breast matrix and PhIP extraction using the EMR-Lipid method. The concentration of PhIP (ng per g of fried chicken breast crust) was calculated according to the equation from matrix matched standard curve, after obtaining the PhIP area from Chem Station software.

The PhIP percent reduction was calculated based on:

Percent reduction = $(P_0 - P_s / P_0) \times 100$, where P_0 was the PhIP concentration of the control chicken breast without edible film and P_s was the PhIP concentration of chicken breast with edible film.

HPLC detection method were calculated based on the following equations:

$$\text{LOD} = 3.3 \times \text{SD}_y / S$$

$$\text{LOQ} = 10 \times \text{SD}_y / S$$

Where SD_y was standard deviation of the response of the calibration curve and S was the slope of the calibration curve.

Color measurement

The CIE Lab parameters, lightness (L^*), greenness ($a^* < 0$), redness ($a^* > 0$), blueness ($b^* < 0$), and yellowness ($b^* > 0$) of edible film were measured with a HunterLab MiniScan EZ 4500 L (Hunter Associates Laboratory Inc., Reston, VA, USA). The standard black and white

plates were used for calibrating the colorimeter. Color of the chicken breast was measured before and after frying. Color difference (ΔE) was according to this equation:

$\Delta E = \sqrt{\Delta L^2 + \Delta a^2 + \Delta b^2}$, where ΔL , Δa , and Δb for the edible film were the differences between color coordinates of the chicken breasts covered with edible films and with no edible film.

Cooking loss percentage

The edible film covered chicken breast and control chicken breast were weighted before and after frying, and the cooking loss percentage was calculated as follows:

% Cooking Loss = $(W_0 - W_1) / W_0 \times 100$, where W_0 and W_1 are the weights before and after frying, respectively.

Tenderness

Tenderness of fried chicken breast samples was measured using a texture analyzer (Texture Technologies Corp, Stable Micro Systems, Surrey, England) coupled with a Meullenet–Owens razor shear blade (MORS) and a 5-kg load cell. The crosshead speed was set to 10 mm/s and the blade penetration depth was set to 15 mm (Küçüközet & Uslu, 2018). The chicken breasts were cut to in vertical direction to the orientation of muscle fiber (Xiong, Cavitt, Meullenet, & Owens, 2006). Shear force (maximum force (N)) and shear energy (area under force versus displacement curve (N. mm)), were calculated by the texture analyzer software.

Statistical analysis

Measurements were conducted in three replicates and SAS 9.4 software was used for statistically analyzing the results. For each replicate, three measurements were conducted except

for texture analyzer in which ten measurements were conducted per sample. Analysis was done with PROC GLIMMIX procedure with Tukey's test for determination of the significant differences between different treatments at a 95% confidence level ($p < 0.05$), which were shown by different letters in each column on the tables.

Results and discussion

Edible film characterization

Edible films with average thickness of 0.19 ± 0.02 mm were characterized for their application on chicken breasts. No significant differences ($p > 0.05$) was observed between the thicknesses of the edible films, which is an important parameter for comparing different properties of edible films.

Total soluble matter (TSM) of edible film

Total soluble matter of edible films are presented as TSM Percent in Table 6. The TSM percent of control UHWPI edible film with no AA was $51.52 \pm 3.41\%$, which was lower than that of the 0.25% Trp containing edible film ($54.95 \pm 3.74\%$); however, there was no significant differences ($p > 0.05$) between the TSM percent of control and other Trp or Lys containing HWPI edible films. By increase in the concentration of Trp from 0.25% to 0.5% and 0.75%, the average TSM percent has decreased ($p < 0.05$) from 54.95% to 45.60 and 44.59%, respectively. Among UHWPI edible films, the control and 0.25% Trp-containing edible films had the TSM percent of 100%, and by increasing in the Trp concentration to 0.5% and 0.75%, the average TSM percent decreased to 80.25% and 79.19%, respectively. For Lys-containing UHWPI edible films, the average TSM percent was decreased ($p < 0.05$) from 100% to 79.32% and 77.91%, by increasing

the Lys concentration to 0.5% and 0.75%, respectively. All UHWPI edible films showed significantly higher ($p < 0.05$) water solubility compared to the HWPI edible films, which can be attributed to the hydrophilic nature of WPI that provides a lower moisture barrier in UHWPI edible films (Kandasamy, et al., 2021) compared to the HWPI edible films in which denatured WPI provides higher moisture barrier (Schmid, Krimmel, & Noller, 2014). The lower TSM percent for HWPI edible films can be related to the effect of high temperature on protein's structure. Heating at 90 °C caused denaturation in WPI, enhancing the molecular interactions such as disulfide crosslinks and exposure of internal hydrophobic bonds which resulted in the increased insolubility (Mohammed, Hill, & Mitchell, 2000). Heating the peanut protein at 70 °C for 30 min was also found to decrease the soluble matters from 50.0% to 42.7% (C.-C. Liu, Tellez-Garay, & Castell-Perez, 2004). Our results are in accordance to the results from a previous study (Janjarasskul, et al., 2020), in which UHWPI and HWPI edible films were produced by 5% WPI and GLY with different ratios, and TSM percent was found to be more than 99% for UHWPI and around 22% for HWPI, however the higher TSM percent for HWPI; edible films in our study can be attributed to the differences in the preparation of edible films.

Edible film color

Color parameters of edible films were measured against a black background and results are presented in Table 6. Results showed that the control HWPI edible films with no AA had the highest L^* (lightness) value compared to all other edible films. There was no significant differences ($p > 0.05$) between the L^* values of edible films with increase in the AA concentrations in both HWPI and UHWPI edible films. All of the HWPI and UHWPI edible films had negative a^* value showing the presence of green color in them. There was no

significant difference ($p>0.05$) between the a^* values of control and AA containing HWPI edible films. However, among UHWPI edible films 0.5% and 0.75% Lys-containing edible films had significantly higher ($p<0.05$) greenness, compared to the control and other UHWPI edible films. For both HWPI and UHWPI edible films, the b^* value which showed yellowness was significantly higher ($p<0.05$) in 0.5% and 0.75% Lys-containing edible films compared to the Trp containing and control edible films, which can be attributed to the yellowish color of Lys powder. The decrease in L^* of the HWPI edible films after addition of AAs can be attributed to the off-white color of Trp and yellowish color of Lys powder, which decreases the whiteness of the edible films. These results were in accordance with the previous study (J. Lin, et al., 2018) in which addition of Lys at 0.5% and 1% concentrations to the gelatin-based films significantly ($p<0.05$) decreased the average L^* value from 90.38 to 89.84 and 89.86, and increased the average b^* values of the edible films from -0.36 to 2.33, and 3.54 compared to the control edible films, respectively. The ΔE of the edible films, which was determined as the difference between the color parameters of the edible films and a black surface, was the better indication of color of the edible films. The ΔE value for HWPI edible films was significantly ($p<0.05$) higher than that of the UHWPI edible films, which can be attributed to the opaque appearance of HWPI edible films, resulting in a higher color difference against the background.

Edible film radical scavenging activity

Radical scavenging activity percentage of edible films is presented in Table 6. Among HWPI edible films, the control with no AA edible film had average radical scavenging activity of 28.44%, which was not significantly ($p>0.05$) different than that of the 0.25% Trp-containing edible film (29.44%). By increasing the Trp concentration to 0.5% and 0.75%, the average

radical scavenging activity increased ($p < 0.05$) to 38.89% and 38.36%, respectively. Lys addition at 0.25%, 0.5%, and 0.75% increased the average radical scavenging activity to 39.29%, 40.03%, and 42.53%, respectively. These results indicated that Trp or Lys addition to the edible films increased the radical scavenging activity, especially at higher concentrations, which showed the antioxidant activity of Trp and Lys. Among the UHWPI edible films, the control edible film with no AA had average radical scavenging activity of 30.25%, which significantly increased by addition of Trp and Lys at all concentrations. The 0.75% Trp, and 0.5% and 0.75% Lys-containing edible films had significantly higher ($p < 0.05$) radical scavenging activities compared to other edible films with the average of 50.62%, 49.41%, and 48.98%, respectively. The higher radical scavenging activity in most of the UHWPI edible films, compared to the HWPI edible films with the same concentrations of Trp and Lys, can be related to the higher solubility of UHWPI edible films which can expose more AA for the reaction. There are several studies on the antioxidant activities of AAs. It was shown that AAs containing an extra amine group, a thioether, or a thiol, such as lysine, tryptophan, methionine, cysteine, and arginine had the strongest antioxidant activities among the studied AAs (Hwang & Winkler-Moser, 2017). The strong antioxidant activity of alkaline amino acids including Lys was also reported in a study on the antioxidative peptides in which Lys-containing peptides indicated 38.9% - 80.6% antioxidant activity (Guo, Kouzuma, & Yonekura, 2009). In another study, three tyrosyl dipeptides, Arg-Tyr, Lys-Tyr, and Tyr-Tyr were shown to have free radical scavenging activity due to their phenolic hydroxyl groups (H.-Y. Zhang, 2005). In a previous study, it was shown that the effective inhibition of the oxidation of linoleic acid was related to the presence of ~33% of hydrophobic amino acids and ~2.5% histidine in the protein hydrolysate composition and peptide fractions (Girgih, Udenigwe, & Aluko, 2011).

Table 6. Color, water solubility (TSM percent), color parameters, and radical scavenging activity of HWPI and UHWPI edible films.

Sample		AA	TSM (%) ¹	Color ¹				Radical
				Conc.	L*	a*	b*	ΔE
		(% w/w)						
HWPI edible film	AA	0	51.52 ± 3.41 ^D	27.74 ± 1.00 ^A	-1.02 ± 0.02 ^{DEC}	-1.20 ± 0.10 ^E	25.54 ± 1.00 ^A	28.44 ± 0.7 ^D
	Trp	0.25	54.95 ± 3.74 ^C	40.12 ± 0.61 ^B	-0.65 ± 0.09 ^{BAC}	-1.12 ± 0.35 ^{ED}	21.65 ± 0.60 ^B	29.44 ± 1.77 ^D
		0.5	45.60 ± 1.55 ^D	38.09 ± 0.37 ^{CB}	-0.91 ± 0.04 ^{BDEC}	-1.33 ± 0.19 ^{ED}	19.65 ± 0.37 ^{CB}	38.89 ± 0.89 ^C
		0.75	44.59 ± 2.31 ^D	40.66 ± 1.39 ^B	-0.70 ± 0.05 ^{BDAC}	-1.14 ± 0.15 ^E	22.19 ± 1.39 ^B	38.36 ± 0.93 ^C
	Lys	0.25	44.00 ± 2.04 ^D	38.83 ± 0.12 ^{CB}	-0.60 ± 0.09 ^{BAC}	0.12 ± 0.09 ^{CD}	20.33 ± 0.12 ^{CB}	39.29 ± 0.78 ^C
		0.5	50.53 ± 3.77 ^D	37.92 ± 0.23 ^{CB}	-1.14 ± 0.02 ^{FDE}	0.64 ± 0.18 ^{CB}	19.45 ± 0.23 ^{CB}	40.03 ± 1.00 ^{CB}
		0.75	50.00 ± 2.94 ^D	36.25 ± 0.58 ^C	-1.33 ± 0.19 ^{FE}	0.62 ± 0.18 ^{CB}	17.80 ± 0.59 ^C	42.53 ± 0.79 ^B
UHWPI edible film	AA	0	100 ± 0.00 ^A	27.74 ± 1.36 ^D	-0.32 ± 0.02 ^A	0.77 ± 0.09 ^{CB}	9.27 ± 1.43 ^D	30.25 ± 1.28 ^D
	Trp	0.25	100 ± 0.00 ^A	27.93 ± 0.86 ^D	-0.35 ± 0.03 ^{BA}	1.24 ± 0.27 ^{CB}	9.52 ± 0.88 ^D	42.41 ± 0.19 ^B
		0.5	80.25 ± 1.09 ^A	27.54 ± 0.51 ^D	-0.44 ± 0.02 ^A	1.53 ± 0.17 ^B	9.18 ± 0.47 ^D	40.62 ± 1.00 ^{CB}
		0.75	79.19 ± 1.05 ^B	26.80 ± 2.25 ^D	-0.37 ± 0.04 ^A	1.09 ± 0.12 ^{CB}	8.39 ± 2.21 ^D	50.62 ± 1.20 ^A
	Lys	0.25	100 ± 0.00 ^A	27.36 ± 1.08 ^D	-0.60 ± 0.16 ^{BAC}	1.69 ± 0.15 ^B	11.02 ± 1.22 ^D	42.65 ± 0.88 ^B
		0.5	79.32 ± 0.59 ^B	25.80 ± 0.88 ^D	-1.56 ± 0.48 ^F	5.02 ± 0.88 ^A	8.99 ± 1.27 ^D	49.41 ± 0.79 ^A

0.75	77.91 ± 1.16 ^B	28.36 ± 0.66 ^D	-1.54 ± 0.30 ^F	4.18 ± 0.75 ^A	10.83 ± 0.46 ^D	48.98 ± 0.70 ^A
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¹Values (means ± SD from Excel, n=3) in the same column with different letters are significantly different at P<0.05.

Abbreviations: WPI: Whey protein isolate, HWPI: Heated whey protein isolate, UHWPI: Unheated whey protein isolate, Conc.: Concentration, AA: Amino acid, Lys: L- Lysine, Trp: L-Tryptophan, TSM%: Total soluble matter, L* = measure of lightness to darkness (larger number indicates a lighter color); a* = measure of redness (larger number indicates a more intense red color); and b* = measure of yellowness (larger number indicates more yellow color, ΔE: Difference between color parameters of edible films and black surface with L*=18.5, a*=-0.21, and b*=-0.04)

Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) analysis

FTIR transmittance percentage spectra of all of the edible films (Figure 3. a and 3.b) showed characteristic broad absorption O-H stretch band about 3250 cm^{-1} , C-H stretch band at about 2900 cm^{-1} , and alkenyl C=O stretch band at around 1625 cm^{-1} . Moreover, there are =C-H aromatics between $1450\text{--}1600\text{ cm}^{-1}$ (Nandiyanto, Oktiani, & Ragadhita, 2019). The amide I (1630 cm^{-1}) and amide II (1530 cm^{-1}) region can be found in all of the edible films, which are related to peptide bonds (CO–NH) of WPI. The secondary derivative spectra of the edible films in amide I region ($1600\text{--}1700\text{ cm}^{-1}$) was also obtained (spectra not provided). It was shown that the intensity of bands in the region of $1660\text{--}1620\text{ cm}^{-1}$ were higher in UHWPI edible films, which are attributed to β -sheets, showing that WPI denaturation in HWPI edible films affects this region. Lys-containing edible films showed slightly different bands at 1620 cm^{-1} , 1672 cm^{-1} , and 1690 cm^{-1} areas compared to the control edible film with no AA, showing that Lys addition can have effect on β -sheet and β -turn structures (Usoltsev, Sitnikova, Kajava, & Uspenskaya, 2019). Trp-containing edible films also showed differences in 1636 cm^{-1} bands (β -sheet) compared to other edible films. The absorption bands observed in the region between 1150 cm^{-1} and 800 cm^{-1} in all edible films could be attributed to GLY. The FTIR spectrum of the GLY (Figure 3.c) showed O-H stretching at 3265 cm^{-1} and C-H stretching at $2810\text{--}2950\text{ cm}^{-1}$. Also $1500\text{--}700\text{ cm}^{-1}$ bands are associated to C-H bending of $1500\text{--}1200\text{ cm}^{-1}$ and C–O stretching $1200\text{--}800\text{ cm}^{-1}$ modes of GLY (Khalique Ahmed, McLeod, Nézivar, & Giuliani, 2010). For Trp spectra (Figure 3. c), the N–H stretching in the amine group bands located at $\sim 3414\text{ cm}^{-1}$, C-H stretching bands at $3100\text{--}2800\text{ cm}^{-1}$, and C=O group at $\sim 1600\text{ cm}^{-1}$ were observed (Aziz, 2019). The C=O

stretching was also observed for Lys at 1575 cm^{-1} . FTIR-ATR spectrum of WPI showed a band at 3268.6 cm^{-1} , which was corresponded to the stretching vibrations of -OH linked to -NH_2 . The bands at 2915 cm^{-1} and 2850 cm^{-1} , which were observed in both WPI and Lys, corresponded to the -CH_2 groups. Moreover, the bands at 1620 cm^{-1} and 1517 cm^{-1} of WPI spectra were characteristic bands of primary amide group (-CO-NH_2) and the secondary amide group (-CO-NH) of protein, respectively.

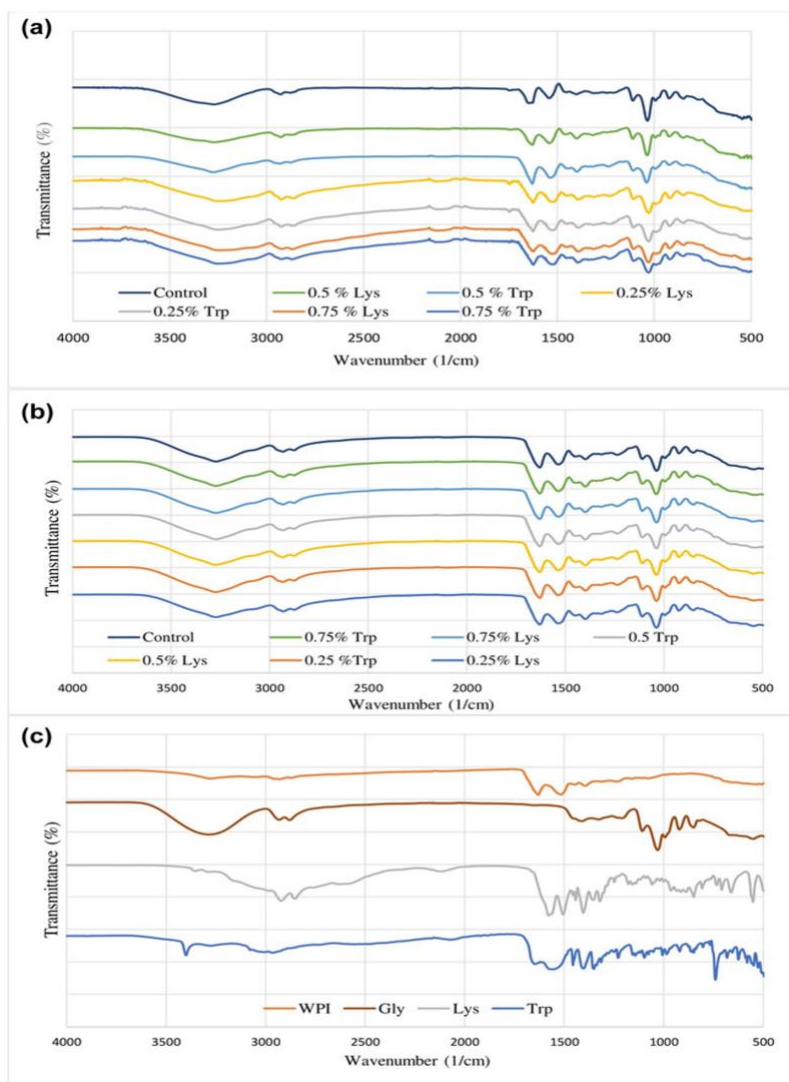


Figure 3. FTIR spectra of unheated whey protein isolate/glycerol edible films (UHWPI/GLY) (a), heated whey protein isolate/glycerol (HWP/GLY) edible films (b), and edible film components (c).

Chicken breast characterization

Chicken breast cuts with the size of $4 \times 3 \text{ cm} \times 1.5 \text{ cm}$ thickness with final weight of $20 \text{ g} \pm 3.4$ were used for further analysis. The raw chicken breast had the moisture, fat, and protein percentages of $76 \pm 2\%$, $4.5 \pm 0.5\%$, and $22 \pm 1.5\%$, respectively. After frying, chicken breast had the water content of $31.75 \pm 4.20\%$ to $48.59 \pm 4.50\%$, fat percentage of $3.00 \pm 0.31\%$ to $7.00 \pm 1.02\%$, and the protein percentage of 27.95 to 50.63%. Moreover, the pH of the all chicken breast crusts after frying was in the range of 5.97-6.1. All fried chicken breast along with the controls were used for further experiments.

PhIP detection

PhIP concentration and percent reduction (Table 7), was calculated based on the control chicken breast with no edible film. The LOD and LOQ HPLC detection method were 0.71 ppb and 2.14 ppb, respectively, and the average recovery of the extraction method was 81 % which showed the high efficiency of this extraction method for PhIP. These values were in the range of the values calculated for the heterocyclic amines method of analysis in previous study (Xian, et al., 2019) which were in the range of 0.3-1.5 ppb and 1.0-2.5 ppb for LOD and LOQ, respectively.

Results showed that the average PhIP concentration decreased from 78.47 ppb for the control chicken breast without edible film to 38.65 ppb for the chicken breast covered with control HWPI edible film with no AA, which accounted for 50.75% average reduction. In HWPI edible films the addition of 0.25% and 0.5% Trp significantly decreased ($p < 0.05$) the PhIP concentration to 25.82 ppb and 16.09 ppb, which accounted for 68.14% and 79.50% reduction, respectively. However, PhIP concentration increased to 46.80 ppb by increase in the Trp

concentration of HWPI edible film to 0.75%. Addition of Lys at 0.25%, 0.5%, and 0.75% (w/w) concentrations decreased the PhIP concentration to 6.69 ppb, 7.29 ppb, and 8.32 ppb, respectively, which accounted for more than 89% reduction compared to the control chicken breast with no edible film. The Lys-containing HWPI edible films were more effective than Trp-containing ones in PhIP reduction when compared at each concentration. The control chicken breast covered with edible film with no AA decreased the average PhIP concentration to 33.43 ppb, which was an average reduction of 57.39% compared to the control chicken breast with no edible film. Among the Trp-containing UHWPI edible film, the 0.5% Trp-containing ones had the lowest PhIP concentration, with 63% average reduction percentage. Moreover, the Lys addition at 0.5% Lys-containing edible films resulted in a significantly lower ($p < 0.05$) PhIP concentration (7.75 ppb) compared to other UHWPI edible films which accounted for a 90.13% average PhIP reduction, followed by the 0.75% Lys-containing UHWPI edible film with 74.93% average PhIP percent reduction compared to the control chicken breast with no edible film. Previously, it was reported that Lys seems to be more reactive than Trp in impeding the Maillard browning reaction in model systems (Linghu, et al., 2017). The significantly lower PhIP level in chicken breast covered with no AA edible films, in comparison to the control chicken breast with no edible film, may be related to the composition of edible film itself which contains whey protein isolate in globular and denatured form. Moreover, edible film provides a layer on the chicken breast which may protect the chicken breast crust from the direct exposure to excess heat and also the moisture loss that result in their higher inhibitory effect on the formation of PhIP during heating of the chicken breast.

Among all chicken breasts covered with both HWPI and UHWPI edible films, certain concentrations of Trp or Lys showed the most significant PhIP reduction, showing the

importance of AA concentration is PhIP formation. Moreover, no significant differences ($p>0.05$) was observed between PhIP levels of chicken breasts covered with control HWPI and UHWPI edible films with no AA, which showed that addition of AA determines the differences between the PhIP inhibitory effect of edible films. Results of previous studies have indicated that the incorporation of bioactive compounds into edible coatings can protect them from the effect of food matrices or the environment, while providing controlled release (Küçüközet & Uslu, 2018). In our study, the differences between the interaction of AAs with WPI and denatured WPI in the structure of edible film is the reason for different PhIP inhibitory activities of Trp and Lys-containing HWPI and UHWPI edible films. It was mentioned that the spice-incorporated WPI film or edible wrapping can be applied as a suitable delivery system of the spices which are pre-measured for instant marination, which can modify the food flavor and offer various novel products (Ket-On, Pongmongkol, Somwangthanaroj, Janjarasskul, & Tananuwong, 2016). There are several studies on PhIP prevention associated to the antioxidant activity of a bioactive compound (Viegas, Amaro, Ferreira, & Pinho, 2012; Y. Zhang, Luo, Shao, Yu, & Wang, 2014). Results of radical scavenging activity of edible films showed significantly higher antioxidant activities for all Lys-containing and 0.75% Trp-containing UHWPI edible films compared to other edible film; however, no trend could be obtained for the antioxidant activities of edible films and their PhIP reduction effect, showing that there are additional mechanisms of PhIP reduction by AAs. Our results were in accordance with a study in which direct addition of AAs at concentrations of 0.05%, 0.2%, and 0.5% (w/w) was investigated on PhIP level of beef patties fried at 180 °C for 1 h, and it was observed that Lys and Trp were more effective in decreasing PhIP levels at 0.5% (w/w) concentration compared to the lower concentrations (Linghu, et al., 2020). However, our results showed that the most effective concentration of AA against PhIP

differs based on the edible film preparation method. Moreover, in another study (Linghu, et al., 2017), the direct addition of AAs were investigated in Maillard model system, and Lys and Trp were shown to be effective in reducing the PhIP level. The PhIP reduction was attributed to phenylacetaldehyde (PheAce) scavenging activity of the amino acids, and a correlation was observed between PhIP inhibition and PheAce-scavenging activity of AAs when AAs and PheAce were heated. The process of PhIP formation is known to involve a direct condensation reaction of creatinine with PheAce, an intermediate Maillard product, rather than free radical intermediates (pyridine or pyrazine cation radicals) followed by cyclization and dehydration (Alaejos & Afonso, 2011; Murkovic, 2004). The Trp can form an adduct with PhIP in animal products (beef, fish, poultry, and pork) after cooking, as previously described (Kataoka, et al., 2010). Furthermore, another study found that the production of the PhIP–amino acid adduct can help the removal of PhIP at high temperatures > 200 °C with a heating time of more than 5 minutes (Kataoka, et al., 2012). Thus, the creation of PhIP- and PheAce-AA adducts can both contribute to the decrease of PhIP in cooked meat, and a substance containing these adducts can reduce PhIP in cooked meat. In this study, the AAs are incorporated in the edible films so the mechanism of PhIP reduction by edible films may be consisted of several pathways.

Table 7. PhIP concentrations in fried chicken breasts covered with HWPI and UHWPI edible films.

Sample		AA Conc. (%) w/w)	PHIP concentration (ppb) ¹	Percent reduction (%) ¹
No edible film		0	78.47 ± 7.35 ^A	-
HWPI edible film	No AA	0	38.65 ± 3.82 ^{CD}	50.75 ± 4.87 ^D
	Trp	0.25	25.82 ± 0.82 ^{EF}	68.14 ± 1.05 ^C
		0.5	16.09 ± 0.94 ^{HGF}	79.5 ± 1.20 ^B
		0.75	46.80 ± 4.58 ^{CB}	40.37 ± 5.83 ^E
	Lys	0.25	6.69 ± 0.43 ^H	91.47 ± 0.55 ^A
		0.5	7.29 ± 1.95 ^{HG}	90.71 ± 2.48 ^A
		0.75	8.31 ± 0.39 ^{HG}	89.41 ± 0.49 ^A
	UHWPI edible film	No AA	0	33.43 ± 3.57 ^{ED}
Trp		0.25	56.04 ± 1.10 ^B	28.59 ± 0.81 ^F
		0.5	28.41 ± 3.21 ^{EDF}	63.79 ± 4.10 ^{DC}
		0.75	54.26 ± 4.03 ^B	30.85 ± 5.13 ^F
Lys		0.25	40.32 ± 2.80 ^{CD}	48.61 ± 3.57 ^E
		0.5	7.75 ± 0.95 ^{HG}	90.13 ± 1.21 ^A
		0.75	19.67± 4.90 ^{GF}	74.93 ± 6.25 ^B

¹ Values (means ± SD from Excel, n=3) in the same column with different letters are significantly different at p<0.05. Abbreviations: WPI: Whey protein isolate, HWPI: Heated whey protein isolate, UHWPI: Unheated whey protein isolate, AA: Amino acid, Lys: L-Lysine, Trp: L-Tryptophan, PhIP: 2-Amino-1-methyl-6-phenylimidazo(4,5-b)pyridine, Conc.: Concentration.

Chicken breast color measurement

Surface color of chicken breast was measured before and after frying (Table 8 and Table 9). Before frying, among chicken breast covered with HWPI, there was no significant ($p>0.05$) differences in the L^* and a^* values of the chicken breast with no edible film and the Trp- and Lys-containing edible films. The b^* values were significantly lower ($p<0.05$) in all chicken breasts covered with HWPI edible films compared to the control chicken breast with no edible film. For chicken breast covered with UHWPI edible films, the L^* values were significantly lower compared to the control with no edible film except, for chicken breast covered with no AA and 0.25% Trp-containing edible films, in which the lightness did not differ significantly ($p>0.05$) compared to the control chicken breast with no edible film. In addition, there was no significant differences ($p>0.05$) between the a^* values of the UHWPI-covered chicken breasts and compared to the control chicken breast covered with no edible film, before frying. Moreover, the b^* values of the chicken breast covered with UHWPI edible films were significantly ($p<0.05$) lower than the control chicken breast with no edible film, except for the chicken breast covered with 0.5% and 0.75% Lys-containing edible film which showed significantly higher yellowness than that of the control chicken breast. The L^* and b^* values were significantly ($p<0.05$) lower in chicken breast covered with the UHWPI edible films compared to the chicken breast covered with HWPI edible films, which was due to the denaturation of WPI in HWPI edible films. Moreover, ΔE values before frying, showed that the chicken breast covered with UHWPI edible films had significantly ($p<0.05$) lower ΔE value compared to the HWPI-covered edible films, indicating that covering chicken breast with UHWPI edible films provides less detectable difference in color compared to the chicken breast covered with HWPI edible film.

Color parameters after frying showed that there were no significant differences in L*, a*, and b* values of the chicken breasts covered with both HWPI and UHWPI edible film compared to the control (no edible film) chicken breast. Moreover, for both HWPI and UHWPI edible films no significant differences ($p>0.05$) was observed between the L*, a*, and b* values of chicken breast covered with Trp- or Lys-containing edible films based on the differences in AA concentration. The ΔE values after frying, which show the differences in color parameters of chicken breast covered with edible films and the control chicken breast with no edible film, were in the range of 2.84 ± 0.43 to 8.42 ± 2.42 , and there was not a significant differences ($p>0.05$) between the ΔE of all the chicken breasts. It can be mentioned that the average human eye cannot detect any color differences with a ΔE value of 3 or less (Francis & Clydesdale, 1975).

Table 8. Color parameters of chicken breast covered with HWPI and UHWPI edible films before frying

Sample		AA Conc. (% w/w)	Surface Color before frying ¹			
			L*	a*	b*	ΔE
No edible film		0	63.25 ± 2.93 ^{AB}	4.76 ± 0.39 ^{BDAC}	21.63 ± 0.92 ^{BA}	-
HWPI edible film	No AA	0	69.83 ± 2.00 ^A	2.71 ± 0.35 ^D	7.80 ± 0.57 ^D	13.08 ± 1.36 ^{BC}
	Trp	0.25	69.56 ± 2.03 ^A	2.77 ± 0.32 ^D	2.67 ± 1.41 ^E	21.39 ± 0.85 ^A
		0.5	69.18 ± 2.22 ^A	3.52 ± 0.50 ^{BDAC}	3.19 ± 0.47 ^D	19.51 ± 1.34 ^{BA}
		0.75	68.71 ± 1.01 ^A	2.82 ± 0.02 ^{BDC}	3.51 ± 0.02 ^D	19.03 ± 2.45 ^{BA}
	Lys	0.25	69.56 ± 2.03 ^A	2.74± 0.27 ^{DC}	3.70 ± 1.05 ^E	19.20 ± 0.70 ^{BA}
		0.5	69.20 ± 1.03 ^A	3.99 ± 0.02 ^{BDAC}	7.88 ± 1.38 ^E	15.06 ± 0.95 ^{BCD}
		0.75	66.51 ± 2.07 ^A	3.26 ± 0.04 ^{DC}	8.91 ± 0.85 ^E	13.30 ± 1.06 ^{ECD}
UHWPI edible film	No AA	0	58.55 ± 1.02 ^{BC}	5.61 ± 0.21 ^A	23.27 ± 0.92 ^A	7.89 ± 0.33 ^G
	Trp	0.25	57.73 ± 2.72 ^{BC}	4.96 ± 1.08 ^A	16.60 ± 1.72 ^C	7.89 ± 1.18 ^{FG}
		0.5	53.55 ± 2.04 ^C	5.10 ± 1.58 ^A	17.11 ± 1.46 ^C	10.89 ± 1.36 ^{EFGD}
		0.75	55.13 ± 1.01 ^C	4.57 ± 0.93 ^{BDAC}	17.75 ± 0.90 ^C	5.14 ± 1.16 ^{EFG}
	Lys	0.25	53.80 ± 1.13 ^C	5.37 ± 1.26 ^{BAC}	17.45 ± 0.60 ^C	9.98 ± 1.01 ^{EFG}
		0.5	53.81 ± 4.75 ^C	5.52 ± 0.66 ^{BA}	18.12 ± 0.40 ^{BC}	10.50 ± 4.24 ^{EFD}
		0.75	54.68 ± 3.67 ^C	4.58 ± 0.82 ^{BDAC}	24.15 ± 1.44 ^A	6.49 ± 2.89 ^{FG}

¹ Values (means $\pm$ SD from Excel, n=3) in the same column with different letters are significantly different at $p < 0.05$. Abbreviations: WPI: Whey protein isolate, HWPI: Heated whey protein isolate, UHWPI: Unheated whey protein isolate, AA: Amino acid, Lys: L-Lysine, Trp: L- Tryptophan, Conc.: Concentration, L^* = measure of lightness to darkness (larger number indicates a lighter color); a^* = measure of redness (larger number indicates a more intense red color); and b^* = measure of yellowness (larger number indicates more yellow color), ΔE : Difference between color parameters of chicken breast covered with edible film and raw chicken breast, before frying.

Table 9. Color parameters of chicken breasts covered with HWPI and UHWPI edible films, after frying.¹ Values

Sample		AA Conc. (%) w/w)	Surface Color after frying ¹			
			L*	a*	b*	ΔE
No edible film		0	42.15 ± 1.34 ^{BAC}	20.50 ± 0.50 ^{BAC}	28.77 ± 0.97 ^A	-
HWPI edible film	No AA	0	40.63 ± 1.28 ^{BAC}	18.80 ± 0.84 ^{BC}	28.87 ± 0.58 ^A	2.81 ± 0.43 ^A
	Trp	0.25	45.96 ± 4.12 ^A	22.02 ± 2.49 ^{BAC}	33.64 ± 0.26 ^A	7.40 ± 1.43 ^A
		0.5	41.73 ± 2.62 ^{BAC}	20.96 ± 1.00 ^{BAC}	30.93 ± 0.49 ^A	3.17 ± 0.72 ^A
		0.75	42.03 ± 3.26 ^{BAC}	24.71 ± 0.54 ^A	31.52 ± 0.70 ^A	5.67 ± 1.08 ^A
	Lys	0.25	45.72 ± 3.60 ^{BA}	22.40 ± 0.82 ^{BA}	29.86 ± 3.38 ^A	5.72 ± 1.38 ^A
		0.5	37.40 ± 1.02 ^{BC}	22.25 ± 3.15 ^{BA}	30.27 ± 3.09 ^A	7.30 ± 1.87 ^A
		0.75	43.10 ± 4.21 ^{BAC}	20.92 ± 0.63 ^{BAC}	33.50 ± 2.32 ^A	5.97 ± 2.59 ^A
UHWPI edible film	No AA	0	44.27 ± 1.53 ^{BAC}	18.30 ± 1.36 ^C	33.60 ± 2.08 ^A	6.04 ± 1.68 ^A
	Trp	0.25	38.31 ± 2.23 ^{BAC}	18.67 ± 0.42 ^{BC}	34.47 ± 1.52 ^A	7.22 ± 2.31 ^A
		0.5	41.34 ± 1.02 ^{BAC}	18.72 ± 1.24 ^{BC}	32.73 ± 1.09 ^A	4.50 ± 1.59 ^A
		0.75	39.94 ± 1.51 ^{BAC}	19.54 ± 1.25 ^{BC}	33.85 ± 1.01 ^A	5.83 ± 1.09 ^A
	Lys	0.25	39.10 ± 1.02 ^C	22.11 ± 0.78 ^{BAC}	29.95 ± 1.38 ^A	3.75 ± 2.08 ^A
		0.5	39.55 ± 1.51 ^{BAC}	18.11 ± 1.14 ^C	32.24 ± 0.71 ^A	5.15 ± 2.42 ^A
		0.75	38.68 ± 1.62 ^{BAC}	21.07 ± 1.40 ^{BAC}	34.80 ± 1.51 ^A	7.10 ± 1.95 ^A

(means $\pm$ SD from Excel, n=3) in the same column with different letters are significantly different at $p < 0.05$.

Abbreviations: WPI: Whey protein isolate, HWPI: Heated whey protein isolate, UHWPI: Unheated whey protein isolate, AA: Amino acid, Lys: L-Lysine, Trp: L- Tryptophan, Conc.: Concentration, TSM%: Total soluble matter, L^* = measure of lightness to darkness (larger number indicates a lighter color); a^* = measure of redness (larger number indicates a more intense red color); and b^* = measure of yellowness (larger number indicates more yellow color), ΔE : Difference between color parameters of chicken breast covered with edible film and the chicken breast after fr

Cooking loss percentage

The average cooking loss (Table 10) of chicken breast with no edible film (45.10%) was significantly ($p < 0.05$) decreased by applying the HWPI edible films (27.55% to 36.05%). However, no significant differences ($p > 0.05$) was shown between the cooking loss percentages of chicken breast covered with Trp- or Lys- containing HWPI edible films with the change in concentration of AAs. Higher cooking loss in chicken breast without edible film was related to the higher rate of water evaporation, fat dripping, and protein shrinkage (Haghighi, et al., 2021). These results were in accordance with the results from a study in which cooking loss significantly ($p < 0.05$) decreased for the chicken breasts wrapped with sodium caseinate and sodium caseinate–starch edible films (Küçüközet & Uslu, 2018). Chicken breast covered with UHWPI edible films didn't show significant ($p > 0.05$) differences in cooking loss percentages compared to the control chicken breast with no edible film. Moreover, there were no significant differences ($p > 0.05$) between the cooking loss percentages of UHWPI edible films with and without Trp or Lys. This can be attributed to the higher solubility of UHWPI films on the surface of chicken breast, which can decrease their ability to decrease the cooking loss.

The cooking loss percentage of chicken breast with no edible film in this study was higher than the study by Barbanti & Pasquini in 2005 in which the cooking loss percentage was found to be 26.42% after frying of chicken breast with $38.72 \text{ g} \pm 5.60$ weight at 170°C for 8 min for (Barbanti & Pasquini, 2005). This difference in results can be due to the higher temperature of frying (195°C), and lower samples weight ($20 \text{ g} \pm 3.40$) in current study. Cooking loss is highly affected by cooking time and temperature, and it goes up by the increase in cooking time and temperature. The increase in temperature increases moisture evaporation and

results in the denaturation of the actomyosin complex and myofibrillar proteins, which cause the shrinkage of the muscle fiber (Haghighi, et al., 2021).

Table 10. Cooking loss percentage of chicken breasts covered with HWPI and UHWPI edible films.

Sample		AA Conc. (% w/w)	Cooking loss (%) ¹
No edible film		0	45.10 ± 1.80 ^A
HWPI edible film	No AA	0	36.05 ± 3.68 ^{BDC}
	Trp	0.25	32.19 ± 2.90 ^{DEC}
		0.5	34.80 ± 3.78 ^{BDEC}
		0.75	36.85 ± 1.65 ^{BDC}
	Lys	0.25	30.16 ± 2.08 ^{DE}
		0.5	33.95 ± 0.49 ^{DEC}
		0.75	27.55 ± 3.78 ^E
UHWPI edible film	No AA	0	37.09 ± 1.81 ^{BDAC}
	Trp	0.25	43.26 ± 1.12 ^A
		0.5	43.03 ± 2.62 ^{BA}
		0.75	41.71 ± 3.76 ^{BAC}
	Lys	0.25	37.86 ± 1.37 ^{BDAC}
		0.5	37.59 ± 1.55 ^{BDAC}
		0.75	37.06 ± 0.50 ^{BDAC}

¹ Values (means ± SD from Excel, n=3) in the same column with different letters are significantly different at p<0.05. Abbreviations: WPI: Whey protein isolate, HWPI: Heated whey protein isolate, UHWPI: Unheated whey protein isolate, AA: Amino acid, Lys: L-Lysine, Trp: L-Tryptophan, Conc.: Concentration.

Chicken breast tenderness

As it is shown in Table 11, the control chicken breasts with no edible film showed 20.35 N mm average shear energy, which is significantly higher than that of the chicken breasts covered with HWPI edible films; however, the average shear force, which was 9.81 N for the chicken breast with no edible film, did not significantly differ ($p>0.05$) by application of HWPI edible films. The lower shear energy showed the higher tenderness of the chicken breasts covered with HWPI edible films (Therkildsen, Larsen, Bang, & Vestergaard, 2002). Moreover, the UHWPI-covered chicken breast showed no significant differences ($p>0.05$) in the shear force and shear energy compared to the control chicken breast with no edible film, which can be due to the higher solubility of UHWPI edible films which dissolved on the surface of chicken breast during incubation time.

The improved tenderness of chicken breasts covered with HWPI edible films was consistent with the findings of a previous study (Küçüközet & Uslu, 2018), in which edible films based on sodium caseinate and a sodium caseinate–starch mixture improved the tenderness of chicken breasts after roasting, and the average shear energy and shear force values of edible film-wrapped chicken breasts were about 30% and 40% lower than that of the control chicken, respectively. Meat softness is determined by the proteolysis of important myofibrillar proteins, which are responsible for maintaining structural integrity in muscle fibers, according to a prior study (Koohmaraie, Kent, Shackelford, Veiseth, & Wheeler, 2002). Post mortem proteolytic degradation results in the weak muscle fibers and thus contributes to the meat tenderization. The lower shear energy which showed higher tenderness in chicken breast covered with HWPI edible film, was also in consistent with our cooking loss results, showing that HWPI edible films can improve the tenderness along with decreasing the cooking loss.

Table 11. Shear force and shear energy of chicken breasts covered with HWPI and UHWPI edible films., after frying.

Sample		AA Conc. (%) w/w)	Shear force (N) ¹	Shear energy (N mm)
No edible film		0	9.81 ± 2.05 ^{BDAC}	20.35 ± 2.10 ^{BA}
HWPI edible film	No AA	0	7.97 ± 1.08 ^{BDC}	13.98 ± 4.19 ^{EDC}
	Trp	0.25	8.21 ± 0.73 ^{BDC}	13.67 ± 1.46 ^{ED}
		0.5	8.12 ± 1.21 ^{BDC}	12.59 ± 1.97 ^{ED}
		0.75	6.48 ± 0.55 ^D	11.23 ± 2.48 ^E
	Lys	0.25	7.38 ± 1.07 ^{BDC}	13.47 ± 2.81 ^{EDC}
		0.5	7.26 ± 1.46 ^{DC}	11.20 ± 2.99 ^{ED}
		0.75	7.66 ± 0.89 ^{DC}	13.14 ± 2.13 ^{EDC}
	UHWPI edible film	No AA	0	12.09 ± 1.58 ^{BA}
Trp	0.25	12.73 ± 1.90 ^{BA}	25.90 ± 4.06 ^A	
	0.5	9.72 ± 0.30 ^{BDAC}	20.59 ± 2.13 ^{BA}	
	0.75	13.41 ± 2.81 ^A	25.30 ± 4.10 ^{BA}	
	Lys	0.25	7.83 ± 1.90 ^{BDC}	25.56 ± 2.74 ^{BA}
		0.5	9.04 ± 1.21 ^{BDAC}	19.50 ± 1.57 ^{EBDAC}
		0.75	7.77 ± 1.47 ^{BDC}	17.27 ± 1.55 ^{EBDC}

¹ Values (means ± SD from Excel, n=10) in the same column with different letters are significantly different at p<0.05. Abbreviations: HWPI: Heated whey protein isolate, UHWPI: Unheated whey protein isolate, AA: Amino acid, Lys: L-Lysine, Trp: L-Tryptophan, Conc.: Concentration.

Conclusion

This study provides a new approach to decrease the PhIP level in fried chicken breast by WPI edible film containing lysine and tryptophan. Results showed that both HWPI and UHWPI edible films, with or without amino acid, could significantly decrease the PhIP level compared to the chicken breast with no edible film, and lysine-containing edible films had higher PhIP percent reduction, compared to the tryptophan-containing edible film with the same concentrations. HWPI edible films had lower antioxidant activity and lower water solubility, and were more effective in PhIP reduction. Moreover, chicken breast covered with HWPI edible films had lower cooking loss and improved tenderness after frying compared to the chicken breast with no edible film. These results showed that WPI-based edible films can be applied on chicken breast to decrease PhIP level without having negative effects on the tenderness, cooking loss, and physicochemical properties of chicken breast.

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Chapter 3 - Effect of potato starch coating containing amino acids to prevent the formation of 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP) in pan-fried chicken breast

Abstract

The inhibitory effects of potato starch (PS) coating containing amino acids (AAs) on the formation of 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP) in chicken breasts, and physicochemical properties of chicken was evaluated. The PS coating solution (100 g) was made by heating 5 g of PS in water solution at 80 °C for 30 min (in a water bath). After cooling down, glycerol (GLY) (2 g/100 g w/w of PS), and tryptophan (Trp) or lysine (Lys) were added to the solution at 0.25%, 0.5%, and 0.75% (w/w of the coating solution). Chicken breasts cuts with the same dimensions (5 cm x 2 cm x 1.5 cm) were dipped in the PS coating solution for 15 min before frying. Chicken coated with PS coating with no AA and chicken with no coating were used for comparison. After frying the chicken at 195 °C for 7.5 min on each side, PhIP levels, color, cooking loss, tenderness, and texture profile assay were evaluated. Viscosity of the PS coating solution and chicken pickup percentage were also measured and a triangle test was conducted. The average PhIP concentration was decreased from 92.62 ppb for the control chicken breast without coating to 6.30 ppb (0.25% Lys), 6.76 ppb (0.5% Lys), and 11.98 ppb (0.75% Lys), accounting for a 89-92% reduction in PhIP level compared to the chicken breast without coating. However, dipping in Trp containing PS coating had significantly lower ($p < 0.05$) PhIP reduction effect (34-67%). There wasn't a significant difference in cooking loss percentage, tenderness, texture profile parameters, color parameters of PS coated chicken compared to the

chicken breasts with no coating, after frying. Triangle test results showed that consumers did not detect a significant difference between the PS coated and uncoated chicken breasts ($p < 0.001$).

Keywords: Potato starch, coatings, L-tryptophan, L-lysine, 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine

Introduction

Diet is one of the main factors that can have effect on cancer development (Doll & Peto, 1981). It was shown that consumption of meat has a positive correlation with human cancer and the cooking of meat is known to produce chemical carcinogens including a family of heterocyclic amines (Aune, et al., 2013; Zheng & Lee, 2009). The most abundant of these heterocyclic amines, 2-amino-1-methyl-6-phenylimidazo[4-5-*b*] pyridine (PhIP), is known to stimulate tumors of the prostate, breast, and colon in rats (N. Ito, et al., 1991; Shirai, et al., 1997). It has been also shown that there is a positive dose-dependent response association between PhIP intake and Colorectal Adenoma Risk (Martínez Góngora, Matthes, Castaño, Linseisen, & Rohrmann, 2019).

Due to some drawbacks about direct application of bioactive compounds in food, including changes of organoleptic properties, fast release of active compounds, and their interaction with other food ingredients, researchers have adopted coating as a method of incorporating natural and active compounds (Quirós-Sauceda, et al., 2014). Addition of amino acids (AA) directly (Linghu, et al., 2017) or incorporated in WPI-based edible films has been shown to reduce the formation of PhIP in beef patties and chicken breasts in previous studies . Coatings have been used on seafood products (Dursun & Erkan, 2014), red meat (Ahmad, Benjakul, Sumpavapol, & Nirmal, 2012), and chicken (Higueras, López-Carballo, Hernández-Muñoz, Gava, & Rollini, 2013) for various purposes such as maintaining the quality, delaying spoilage, color stability, antimicrobial and antioxidant effect. Of the many polysaccharides used to develop edible coatings, potato starch (PS) showed Excellent film-making properties being a biocompatible, biodegradable, and easily accessible compound with low cost (Sapper & Chiralt, 2018). Starch films are tasteless, transparent, and odorless with little oxygen permeability,

making this biopolymer a good candidate to be used as coating for different food items (Sanchez-Gonzalez, Arab-Tehrany, Cháfer, González-Martínez, & Chiralt, 2015). Moreover, starch-based coatings were shown as appropriate compounds to be used for new applications through the addition of various ingredients. For instance, starch edible coating incorporated with several antifungal compounds such as essential oils (Sapper, Palou, Pérez-Gago, & Chiralt, 2019), natamycin-cyclodextrin complex (Yang, et al., 2019), and lactic acid bacteria (Marín, Plotto, Atarés, & Chiralt, 2019) were shown to significantly control the postharvest decay of persimmon, apple, cherry tomato, grapes among others. However, there is no study on incorporation of AAs into PS coating solutions. In this study we aimed to incorporate different concentrations (0.25%, 0.5%, and 0.75% (w/w)) of L-Lysine (Lys) and L-Tryptophane (Trp) into PS solutions, which were used as a dipping coating for chicken breast before pan-frying. The effect of coating was investigated on PhIP level, color, cooking loss, tenderness, and texture properties (TPA) of the chicken breasts, and consumer acceptance of the chicken breasts. Viscosity of the coating solution and pick up percentage of the chicken breast after dipping was also evaluated.

Materials and Methods

Chemicals and materials

The 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP) standard was purchased from Cayman Chemical (Ann Arbor, MI, USA). L-tryptophan (Trp), L-lysine (Lys), glycerol (GLY), triethylamine, acetic acid (HPLC grade), methanol and acetonitrile (HPLC grade), ammonium acetate were purchased from MilliporeSigma (Burlington, MA, USA). Deionized water was obtained from a Sybron/Barnstead PCS filtration unit (Barnstead/Thermolyne, Inc, Dubuque, Iowa, U.S.A.). Bond Elut Enhanced Matrix Removal (EMR)-Lipid (QUEChERS

dSPE EMR and Lipid-Polish) was obtained from Agilent Technologies (Santa Clara, Ca, U.S.A.). Chicken breasts (SmartChicken®), potato starch (PS), peanut oil, and bamboo skewers were obtained from a local store.

Edible coating preparation

The coating solution was prepared based on a previous study with minor modifications (Ghanbarzadeh & Almasi, 2011). An aqueous solution of PS was prepared by dissolving 5 g of starch powder in 100 ml of distilled deionized H₂O, and after stirring at room temperature, it was heated to 80 °C for 30 min for gelatinization. After cooling the solution at room temperature, GLY was added as a plasticizer at a concentration of 2 g/100 g (w/w, on dry basis of the weight of starch) and the resulting dispersion was subsequently mixed for 5 min. Then Lys or Trp were incorporated into the coating solution at several concentrations of 0.25%, 0.5%, and 0.75% (w/w, on the basis of neat film solution). A coating solution without AA served as the control. The coating solution was then homogenized at 11000 rpm for 5 min using a laboratory homogenizer (Homogenizer, OMNI International, Kenneswa, GA, USA) and was used as a dipping coating for the chicken breasts.

Viscosity measurement

A stress-strain-controlled rheometer (MCR-92 Anton Paar, Vernon Hills, IL) was used to collect the continuous rotational flow data. The rheometer was coupled with a 50-mm diameter stainless steel cone with an angle of 1° and 101- μ m gap (Loveday, Su, Rao, Anema, & Singh, 2012). The shear rate range of 0.01 s⁻¹ to 200 s⁻¹ was used at 20 °C and viscosity was reported at shear rate of 100 s⁻¹.

Treatment of chicken breasts

Fresh chicken breasts were kept at refrigerator temperature (4 °C) before application of the coating solutions. Chicken breasts cuts with the same dimensions (5 cm × 2 cm × 1.5 cm) were dipped in the 0.25%, 0.5%, and 0.75% Lys or Trp containing coating solutions and were kept at 4 °C for 15 min. Chicken breasts dipped in PS coating without AA and chicken breast with no coating were used as controls. The chicken breasts were swirled in the coating solution using a glass rod to ensure complete surface contact of them with the coating solution. Chicken were removed and were put on bamboo skewers under a biological containment hood to drain the excess coating and dry the remaining. Subsequently, after adding a small amount of peanut oil, chicken breasts were fried on an electrical frying pan with adjustable heat on each side (surface temperature of 195 °C) for 7.5 min. After removing, the chicken breast were cooled at room temperature and were used for further analysis.

Coating pickup percentage

Coating pickup percentage was determined as the amount of coating adhering to the chicken breast after dipping. In this experiment the pickup percentage was calculated after the coated chicken was drained for 10 or 60 s according to the following formula:

$$\text{Coating pickup percentage} = \frac{C_1 - C_0}{C_0} \times 100$$

Where, C_1 and C_0 was the weights of the PS coated chicken and chicken before dipping, respectively.

Determination of PhIP

Extraction of PhIP by Enhance Matrix Removal-Lipid method

The Agilent Bond Enhance Matrix Removal-Lipid (EMR-Lipid) method was used for PhIP extraction according to a previous study (Linghu, et al., 2019). Finely grounded fried chicken breast crust (3 g) was weighted to 50 ml polypropylene (PP) centrifuge tube and then 15 mL of 2% formic acid–acetonitrile solution was added. The tube was mixed on a mechanical shaker at room temperature for 30 min. After mixing, the tube was centrifuged ($10000 \times g$ at 4 °C) for 10 min (Fisher Scientific, Marathon 21000 R, MA, USA). For matrix cleanup, 12 mL of the supernatant was taken and added to 1 g of EMR-Lipid dSPE sorbent which was preactivated with 5 mL of 5 mM ammonium acetate buffer. After 2 min of vigorous shaking, the tube was centrifuged at RDF of $10000 \times g$ and 4 °C for 10 min. Afterward, 10 mL of supernatant was added to a 15 ml PP centrifuge tube containing 2 g EMR-Lipid Polish salts for salting-out extraction and further cleanup. After 2 min of vigorous hand shaking, the tube was centrifuged for 10 min ($10000 \times g$ at 4 °C). Then, 1 mL of the final extract from top layer was transferred to a conical vial and was dried up under a constant flow of nitrogen. At the end, 100 µL of methanol was added to the vial for reconcentration and the solution was filtered through a 0.2 µm nylon filter prior to HPLC analysis.

Identification and quantification of PhIP

HPLC (Agilent Technologies, 1100 series) was used for detection of PhIP according to a previously described method (Jinap, et al., 2013) with some modifications. The mobile phase started with 20% acetonitrile (A) and 80% 0.01 M triethylamine solution (made in water and adjusted to the pH= 3.6 using acetic acid) (B), and it was programmed to 25% A/75% B for 4

min, and 55% A/45% B for 20 min. The flow rate was 1.0 mL/min during the run time. The injection volume was 10 μ L and the column temperature was 26 °C. The C18 column (TOSOH Bioscience LC, TSKgel ods-80, 4.6 mm ID $\times$ 25 cm, 5 micrometer Millipore Sigma, MA, USA) and fluorescence detector (Hewlett-Packard, Palo Alto, CA, USA) with excitation and emission wavelengths of 306 and 371 nm were used for PhIP detection. The matrix matched standard curve was used for calculation of PhIP concentration to account for the matrix effect on its concentration. The concentration of PhIP was reported as ng/g of fried chicken breast crust. The PhIP percent reduction was calculated based on the following equation:

%Reduction = $(P_1 - P_s / P_1) \times 100$, where P_1 was the PhIP concentration of the control chicken breast without coating and P_s was the PhIP concentration of chicken breast dipped in PS coating.

Color analysis

HunterLab MiniScan EZ 4500 L (Hunter Associates Laboratory Inc., Reston, VA, USA) was used to measure the CIE Lab parameters, lightness (L^*), greenness/redness (a^*), blueness/yellowness (b^*) of chicken breasts after frying. The colorimeter was calibrated by white and black plates. Color of the chicken breast was measured before and after frying. The ΔE which showed the color difference of the PS coated chicken compared to the chicken with no coating was calculated according to the following equation:

$\Delta E = \sqrt{\Delta L^2 + \Delta a^2 + \Delta b^2}$ where ΔL , Δa , and Δb were the differences between color coordinates of the chicken breasts dipped the PS coating and chicken breasts with no coating.

Cooking loss percentage

The weights of chicken breasts dipped in PS coating and control chicken breast were recorded before and after frying, and the cooking loss percentage was calculated as follows:

% Cooking Loss = $(W_1 - W_2) / W_1 \times 100$, where W_1 and W_2 were the weights before and after frying, respectively.

Tenderness analysis

A texture analyzer (Texture Technologies Corp, Stable Micro Systems, Surrey, England) coupled with a Meullenet–Owens razor shear blade (MORS) was used for tenderness analysis of PS coated and control chicken breasts. The crosshead speed of 10 mm/s was set and the blade penetration depth was set to 15 mm (Küçüközet & Uslu, 2018). The fried chicken breast cuts were put in a direction vertical to the orientation of muscle fiber under the razor shear blade (Xiong, et al., 2006). Shear force which was maximum force (N) and shear energy which was area under maximum force vs. displacement curve (N. mm), were calculated by the texture analyzer software.

Texture profile analysis (TPA)

Texture profile analysis was performed as suggested by (Khanal, et al., 2019) and (Rathod & Khamrui, 2015). Sample was cut into 2 x 2 x 2 cm diameters using a sharp knife. TPA was conducted with a TAXT2i texture analyzer (Stable Micro Systems, Godalming, UK) with 30 kg load cell. A 50 mm cylindrical flat prob and double bite compression method (with 2 seconds rest period between the two bites) was used. The samples were compressed to 80% (1.6 cm compression) of their original height (20 cm) by the probe with a crosshead speed of 0.8

mm/s. The TPA curve of force (N) v/s time (S) was plotted and used to derive the instrumental texture attributes such as hardness, cohesiveness, springiness, and chewiness. The force-time plot was used to determine and calculate the TPA parameters as follows (Barbut, 2016; Meilgaard, Carr, & Civille, 1999): Two areas were taken from the measurement. Area 1 was recorded as the area of the curve (mm^2) during the first downstroke. Area 2 was the area of the curve (in mm^2) during the second downstroke. Hardness from TPA was calculated as the resistance (N) at maximum compression during the first compression. Springiness was the force (g) at maximum compression during the second compression cycle or hardness of the sample at second bite. Cohesiveness was the ratio of positive force of the second compression to that of the first compression cycle (only downward strokes). Chewiness was the energy required to chew a solid sample until swallowing it ($\text{hardness} \times \text{springiness} \times \text{cohesiveness}$). TPA measurement was conducted 6 to 10 times for each sample.

Consumer discrimination test²

A triangle test ($n = 62$) was conducted to see whether consumers can identify the difference between chicken breast with and without PS coating. Fried chicken with $2 \times 2 \times 2$ cm dimensions were kept warm ($60\text{--}70^\circ\text{C}$) for less than 30 min on aluminum trays to be evaluated by panelists. Random 3-digit numbers were allocated to each of the 3 sample that were presented to the panelists (2 of which were the same and 1 was different). For each panelist, the presentation order of samples was randomized to account for bias. Panelists were asked to choose the different sample among the three presented samples. Water and unsalted crackers were provided, and panelists were asked to rinse their mouth between each sample. The result

² IRB number: 8790

was analyzed according to the table of the minimum required number of correct responses to conclude that based on the triangle test a significant difference exists (Sinkinson, 2017). The result was reported at significance level of $p \leq 0.001$ with panel number of 62 people.

Statistical analysis

All of the experiments were conducted in triplicates and SAS 9.4 software was used to determine whether there is a significant difference between samples at a 95% confidence level ($p < 0.05$). For texture analysis 6 measurements were conducted per sample to account for variations in measurements. Analysis were done with PROC GLIMMIX procedure with Tukey's test. The significant differences were shown by different letters in each column on the tables.

Results and discussions

Viscosity and pickup percentage

The viscosity results are reported in Table 12. The average viscosity of the PS solutions was in the range of 104.73-128.91 mPa.s. There was not a significant difference ($p < 0.05$) among the viscosity values of the PS coating solution with Lys/Trp and the coating without AA showing that addition of Lys and Trp at different concentrations didn't alter the viscosity of the PS coatings. The viscosity results were in contrary with findings of previous studies in which a decrease in viscosity of PS paste was shown by addition of Lys at different levels (0.3 and 1.7% (dwb)) (Gałkowska & Juszczak, 2019). However, in that study after oxidizing the PS, Lys was mixed with PS solution for 5 h and starch was recovered for further experiments (J. Li, Vasanthan, Bressler, & Tyler, 2010), which is different than our preparation method. In another study in which Lys was added to PS solution before gelatinization, the peak viscosity of the

gelatinized starch was decreased by addition of Lys at 0.4, 2 and 10% levels (dry weight base) (A. Ito, Hattori, Yoshida, & Takahashi, 2006). The difference in preparation methods due to different approaches in studies would be the reason for the observed differences. Moreover, coating pickup percentage of chicken was in the range of (8.55%-11.72%) there was not a significant difference ($p>0.05$) between the pickup percentages of PS coatings by chicken breasts which was in accordance with the viscosity results. Previous studies showed that there is a correlation between the viscosity of the coating solution and its pick up percentage by the sample (Lane, Abdel-ghany, & Jones, 1986). Due to the similar viscosity values for PS coating solutions, similar coating pickup percentages were also expected.

Table 12. Viscosity of PS coatings and coating pickup percentages of chickens.

Coating	AA Conc. (% w/w)	Viscosity (mPa.s) ¹	Coating pickup (%) ¹
PS	0	104.73 ± 5.05 ^A	8.87 ± 1.90 ^A
PS-Trp	0.25	128.91 ± 14.44 ^A	8.55 ± 0.22 ^A
	0.5	153.37 ± 24.74 ^A	9.50 ± 0.37 ^A
	0.75	126.11 ± 9.08 ^A	8.93 ± 0.90 ^A
PS-Lys	0.25	128.35 ± 16.59 ^A	9.74 ± 1.49 ^A
	0.5	119.10 ± 21.82 ^A	9.96 ± 1.12 ^A
	0.75	121.10 ± 20.20 ^A	11.72 ± 1.89 ^A

¹ Values (means ± SD from Excel, n=3) in the same column with different letters are significantly different at $p<0.05$. Abbreviations: PS: Potato starch, AA; Amino acid Lys: L-Lysine, Trp: L-Tryptophan, Conc.: concentration.

PhIP quantification

The concentration of PhIP and percent reduction which was calculated based on the control chicken breast with no PS coating, are presented in Table 13. Results showed that dipping chicken breast in Lys containing PS coating solution could significantly ($p < 0.05$) decrease the average PhIP concentration from 92.62 ppb for the control chicken breast without coating to 12.81 ppb (0.25% Lys), 7.00 ppb (0.5% Lys), and 12.01 ppb (0.75% Lys), accounting for a 89-92% reduction in PhIP level compared to the chicken breast without coating. However, dipping in Trp containing PS coating had significantly lower ($p < 0.05$) PhIP reduction effect 34-67% with average PhIP concentrations of 55.63 ppb (0.25% Trp), 23.70 ppb (0.5% Trp), and 97.70 ppb (0.75% Trp). These results are in accordance with our previous chapter on the effect of WPI-based edible films in decreasing PhIP level in which Lys containing edible films were more effective in PhIP reduction, compared to the Trp-containing ones (Farshi, et al., 2022). Moreover, Lys was reported to be more reactive in inhibiting the Maillard browning reaction in model systems compared to Trp (Linghu, et al., 2017). Our results were in accordance with the previous study in which the effect of direct addition of AAs (0.05%-0.5% (w/w)) was investigated on PhIP level of pan-fried beef patties (180 °C for 1 h), and it was observed that Lys and Trp were more effective in decreasing PhIP levels at 0.5% (w/w) concentration compared to the lower concentrations (Linghu, et al., 2020). Results showed that there was no significant differences ($p > 0.05$) in PhIP level of chicken dipped in coating with no AA and chicken with no edible film showing that AA was responsible for PhIP reduction in the tested chicken breasts. In current study the control chicken had an average of 89.07 ng/g PhIP which was higher than reported values for chicken fried in similar condition. Chicken average PhIP levels has been estimated to be between 0.6 ng/g and 38 ng/g for pan-fried chicken with highest amount for

chicken fried at 190 °C, and up to average of 327 ng/g for grilled chicken (340 °C) (Keating & Bogen, 2004). There are many factors affecting the PhIP level in chicken such as extraction and detection method (M. G. Knize, Felton, & Gross, 1992), AA, creatinine, moisture, fat content and type of meat (Lu, Kuhnle, & Cheng, 2018; ur Rahman, Sahar, Khan, & Nadeem, 2014) therefore, higher PhIP levels in the current study can be attributed to the different samples used.

Table 13. PhIP concentration in PS coated and uncoated chicken breasts.

Chicken breast	AA Conc. (% w/w)	PhIP concentration (ng/g)	PhIP percent reduction ¹
1			
Without coating	-	89.07 ± 7.76 ^A	-
PS coated	0	91.30 ± 4.18 ^A	3.29 ± 0.67 ^D
PS-Trp coated	0.25	55.63 ± 1.62 ^B	66.96 ± 6.85 ^B
	0.5	23.70 ± 3.56 ^C	82.87 ± 2.25 ^A
	0.75	97.70 ± 6.76 ^A	34.01 ± 5.16 ^C
PS-Lys coated	0.25	12.81 ± 4.21 ^D	92.31 ± 2.78 ^A
	0.5	7.00 ± 1.21 ^D	93.33 ± 0.70 ^A
	0.75	12.01 ± 3.45 ^D	89.56 ± 2.17 ^A

¹ Values (means ± SD from Excel, n=6) in the same column with different letters are significantly different at p<0.05. Abbreviations: PS: Potato starch, AA; Amino acid Lys: L-Lysine, Trp: L-Tryptophan, Conc.: concentration.

Texture profile analysis (TPA)

The TPA analysis results showed that depending on the measurement conditions, both the measurement values and significant differences between samples can vary. Therefore, the resulting values from the six measurement conditions were collected and presented as average mean values. Texture profile analysis results (Table 14) showed that there were not any significant differences ($p < 0.05$) in the hardness of the chicken breasts dipped in PS coating solution and the chicken breast without coating. The average hardness of chicken breasts were in the range of 192.81 N to 238.13 N. Results for hardness were consistent with the finding of the study by (Purcell, Wang, & Seo, 2014) in which oxidized PS was used as a batter for chicken nuggets and no significant differences ($p > 0.05$) was observed in the hardness value of battered chicken compared to the control. However, in another study in which quinoa starch was blended into ground chicken meat the hardness value after frying decreased compared to the samples with no starch, which can be due to the differences in preparation method (J.-H. Park, Lee, Lim, Jeon, & Yoon, 2021). Previously it was reported (Devadason, Anjaneyulu, & Babji, 2010) that the score of gumminess, and chewiness of buffalo meat nuggets treated with corn starch as filler was higher than the ones treated with wheat flour. The average chewiness of the chicken breast which is energy that needed to chew the chicken till it's ready to swallow was in the range of 33.79-64.41 and there was no significant differences ($p > 0.05$) between the chicken breast. Moreover, there was no significant differences observed in other texture profile parameters such as cohesiveness (average 0.37-0.44), and springiness (average 31.51%-61.95%) of chicken breasts with no coating and the ones with PS coatings. Springiness is the ability of sample to recover its original form after deforming force removed and cohesiveness is ratio of energy for second and first compression. The results were consistent with the previous study in which the use of corn

starch in buffalo meat nuggets and sago in duck sausages did not have significant effect on the springiness and cohesiveness (Devadason, et al., 2010; Muthia, Nurul, & Noryati, 2010).

Table 14. Texture profile analyses of PS-AA coated and uncoated chicken breasts.

Chicken					
breast	AA Conc. (% w/w)¹	Hardness ¹	Cohesiveness¹	Springiness¹(%)	Chewiness¹
Without coating	0	188.32 ± 28.89 ^A	0.40 ± 0.04 ^A	52.35 ± 3.40 ^A	33.79 ± 7.94 ^A
PS coated	0	192.81 ± 27.80 ^A	0.38 ± 0.02 ^A	56.39 ± 1.66 ^A	41.28 ± 9.37 ^A
PS-Trp coated	0.25	237.79 ± 32.08 ^A	0.44 ± 0.04 ^A	47.54 ± 2.64 ^A	59.19 ± 19.21 ^A
	0.5	218.22 ± 26.38 ^A	0.46 ± 0.03 ^A	49.24 ± 4.23 ^A	50.87 ± 9.58 ^A
	0.75	195.44 ± 41.84 ^A	0.46 ± 0.05 ^A	31.51 ± 0.52 ^A	64.41 ± 16.71 ^A
PS-Lys coated	0.25	189.22 ± 44.05 ^A	0.37 ± 0.04 ^A	52.09 ± 4.64 ^A	46.65 ± 12.02 ^A
	0.5	174.27 ± 47.48 ^A	0.44 ± 0.06 ^A	61.95 ± 8.28 ^A	50.86 ± 9.58 ^A
	0.75	238.13 ± 25.50 ^A	0.41 ± 0.02 ^A	52.41 ± 3.42 ^A	55.61 ± 8.29 ^A

¹ Values (means ± SD from Excel, n=6) in the same column with different letters are significantly different at p<0.05. Abbreviations: PS: Potato starch, AA; Amino acid Lys: L-Lysine, Trp: L-Tryptophan, Conc.: concentration.

Tenderness of chicken

To evaluate the average tenderness of samples, the average of values from the six measurements of shear force and shear energy were reported as average mean values (Table 15). The average shear force of the chicken was in the range of 11.56 N to 19.33 N and the average shear energy was in the range of 48.73 Nmm to 565.73 Nmm. There was no significant differences ($p>0.05$) in the shear force and shear energy of the PS coated chicken and chicken without coating. Previously sensory evaluation of coated chicken has shown that the tenderness of the coated ones was significantly ($p<0.05$) higher than the uncoated counterparts which was attributed to the lower cooking loss in them. However, it was reported that application of oxidized PS on the chicken does not significantly ($p>0.05$) alter the tenderness of the chicken (Kristam, Eswarapragada, Bandi, & Tumati, 2016). In another study in which amylo maize starch was included in the nuggets, it was shown due to release of more amylose during the frying process and forming a film on the surface, that the nuggets had higher crispiness (Altunakar, Sahin, & Sumnu, 2004).

Table 15. Tenderness analyses of PS-AA coated and uncoated chicken breasts.

Chicken breast	AA concentration (%) w/w) ¹	Shear force (N) ¹	Shear energy (N.mm) ¹
Without coating	0	13.02 ± 4.26 ^A	65.73 ± 13.55 ^A
PS coated	0	19.33 ± 2.02 ^A	48.73 ± 21.67 ^A
PS-Trp coated	0.25	15.31 ± 4.24 ^A	71.93 ± 11.28 ^A
	0.5	17.35 ± 6.49 ^A	58.61 ± 20.38 ^A
	0.75	12.20 ± 2.89 ^A	50.17 ± 17.48 ^A
PS-Lys coated	0.25	13.71 ± 1.05 ^A	60.81 ± 19.92 ^A
	0.5	14.12 ± 0.79 ^A	50.01 ± 14.83 ^A
	0.75	11.56 ± 2.36 ^A	59.05 ± 19.57 ^A

¹ Values (means ± SD from Excel, n=6) in the same column with different letters are significantly different at p<0.05. Abbreviations: PS: Potato starch, AA; Amino acid Lys: L-Lysine, Trp: L-Tryptophan.

Cooking loss percentage

Results of cooking loss percentage are shown in Table 16. The average cooking loss for the chicken breasts were in the range of 47.54%- 54.16%, and no significant differences (p>0.05) was seen between the cooking loss percentages of coated chickens compared to the control chicken without coating. Our finding was not consistent with the finding of the study by (Küçüközet & Uslu, 2018) in which casted PS edible film was used to wrap chicken and cooking loss was significantly lower than the control chicken with no film. This differences in reported results may be due to the differences in coating preparation method. In our study chicken were dipped in the coating solution; however in the mentioned study, chicken was wrapped in the casted and dried coating, before frying.

Table 16. Cooking loss percentages of PS-AA coated and uncoated chicken breasts.

Chicken breast	AA Conc. (% w/w)	Cooking loss Percentage ¹
Without coating	0	47.47 ± 2.84 ^A
PS coated	0	46.78 ± 4.23 ^A
PS-Trp coated	0.25	57.01 ± 1.58 ^A
	0.5	51.35 ± 2.04 ^A
	0.75	54.21 ± 4.71 ^A
PS-Lys coated	0.25	47.74 ± 5.94 ^A
	0.5	49.14 ± 4.31 ^A
	0.75	54.16 ± 4.07 ^A

¹ Values (means ± SD from Excel, n=3) in the same column with different letters are significantly different at p<0.05. Abbreviations: PS: Potato starch, AA; Amino acid Lys: L-Lysine, Trp: L-Tryptophan, Conc: concentration.

Color

Color parameters of chicken breasts after frying are showed in Table 17. Results indicated that there is no significant differences ($p>0.05$) between the lightness (L^*) of the chicken dipped in coatings (average $L^*=36.40-41.69$) and that of the chicken with no coating (average $L^*=41.71$). In a study by (Adrah, Ananey-Obiri, & Tahergorabi, 2021) in which sweet potato starch was used in the batter of chicken, no significant differences ($p>0.05$) was observed in color parameters of chicken after frying compared to the chicken with no coating. Furthermore, in other study by (Küçüközet & Uslu, 2018) it was shown that the L^* value was increased in chicken wrapped with sodium caseinate-starch coating compared to the control chicken with no coating, however the values were very close. No significant differences ($p>0.05$) was shown between the a^* value of chicken dipped in PS coatings (average $a^*=16.96-20.30$)

and the chicken with no coating (average $a^*=18.28$) indicating that the redness of chicken does not change by dipping them in the PS starch coatings. The average b^* values of PS coated chicken was in the range of 22.60-30.81 with no significant differences compared to the control chicken without coating (25.89). Also, addition of Trp and Lys at different concentrations did not have a significant effect ($p<0.05$) on the color parameters of the chicken. The average ΔE values were in the range of 2.69-5.78 with no significance differences ($p>0.05$) between them, showing that ΔE of the chicken may not be detected by average human eye (ΔE value of 3 or less cannot be detected by the average human eye (Francis & Clydesdale, 1975)).

Table 17. Color parameters of PS-AA coated and uncoated chicken breasts.

Chicken breast	AA Conc. (% w/w)	L^*^1	a^*^1	b^*^1	ΔE^1
Without coating					
	0	41.71 ± 2.97^A	18.28 ± 0.84^{BA}	25.89 ± 1.92^A	-
PS coated	0	41.69 ± 4.46^A	15.53 ± 1.53^B	24.60 ± 2.95^A	5.27 ± 0.76^A
PS-Trp coated	0.25	40.71 ± 3.60^A	18.16 ± 1.59^{BA}	27.86 ± 3.93^A	5.15 ± 1.30^A
	0.5	39.98 ± 1.77^A	18.70 ± 2.21^{BA}	29.54 ± 2.52^A	2.69 ± 0.95^A
	0.75	40.16 ± 1.88^A	17.99 ± 0.51^{BA}	27.02 ± 3.04^A	3.31 ± 1.02^A
PS-Lys coated	0.25	41.34 ± 3.33^A	18.89 ± 0.86^{BA}	23.70 ± 2.73^A	5.56 ± 2.1^A
	0.5	36.68 ± 3.94^A	16.01 ± 0.97^{BA}	22.60 ± 0.53^A	5.78 ± 2.86^A
	0.75	36.40 ± 2.51^A	19.21 ± 1.74^A	25.40 ± 1.11^A	5.01 ± 1.05^A

¹ Values (means $\pm$ SD from Excel, n=3) in the same column with different letters are significantly different at $p<0.05$. Abbreviations: PS: Potato starch, AA; Amino acid Lys: L-Lysine, Trp: L-Tryptophan, Conc.: concentration.

Consumer discrimination test

The 0.25% Lys containing PS coating was selected for conducting the triangle test. In each plate one 0.25% Lys-PS coated chicken and two chicken without coating were served with 3 random digit codes. After presenting the chicken breasts to the panelists at the same environmental conditions, 29 of 62 people could differentiate the PS coated chicken and uncoated chicken. According to the reference table (Sinkinson, 2017), with 62 panelists and p_d of 50% which shows the large numbers of allowable proportion of distinguishers, significant level of 0.001 can be used and at this significance level, minimum of 34 people should be able to differentiate the samples to conclude that a significant difference exists. Therefore, considering 29 people that were able to select the different sample correctly in triangle test, it can be concluded that statistically ($p < 0.001$), there is not a significant difference between the consumer ability to differentiate the PS coated and uncoated chicken breasts.

Conclusion

This study showed that potato starch (PS) coating containing Lys and Trp were effective in decreasing the PhIP level of chicken breasts dipped in the coating solution. Lys containing PS coating showed the highest PhIP reduction percentage in all concentrations, therefore even including 0.25% Lys in the PS coating solution resulted in 92% average PhIP reduction compared to the chicken without coating. Moreover, there was no significant differences ($p>0.05$) between the cooking loss percentage, texture properties, and color parameters of coated chicken. Therefore, it can be stated that 0.25% Lys-PS coating is an appropriate coating with low Lys amount and high effect in PhIP reduction without having negative effect on other physical properties of chicken. Moreover, triangle test showed that consumers are not able to differentiate the PS-0.25% Lys coated and uncoated chicken at ($p<0.001$).

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Chapter 4 - Effect of dipping time of chicken breast in potato starch coating containing lysine on PhIP level

Abstract

This study investigated the effect of dipping time of chicken breast in potato starch (PS) coating containing amino acids (AAs) on the formation of 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP) and to evaluate the possible inhibition mechanism of PhIP in chicken breast. Lysine (Lys) 0.25% (w/w of PS solution) was added to the gelatinized 5% (w/w) PS solution. Chicken breasts (5 cm x 2 cm x 1.5 cm) were dipped in the coating solution for different times (15 min, 30 min, 1 h, 3 h, and 6 h) and after draining the excess coating chicken were kept under a hood for 20 min to dry up the coating. Samples were subsequently pan-fried at 195 °C for 7.5 min on each side. The PhIP level, tenderness, texture profile, color and cooking loss were evaluated for chicken breasts after pan-frying. Phenylacetaldehyde (PheAce), and volatile compounds were determined using GC-MS for a selected samples. Chicken with no coating was used for comparison. Results showed that dipping time does not significantly decrease ($p < 0.05$) the PhIP level suggesting that the 15 min dipping time is enough for PhIP reduction compared to the control chicken. Moreover, phenylacetaldehyde (PheAce) was shown to increase in chicken breast coated with PS-0.25% Lys, suggesting that there may be another pathway to prevent PhIP by PS-0.25% Lys. Volatile compounds analysis also confirmed this and showed increases in several aroma compounds such as pyrazines in the coated chicken. Moreover, no significant differences ($p > 0.05$) was shown between cooking loss percentage, color parameters, texture profile and tenderness of chicken with PS-0.25% coating and chicken without coating.

Keyword: Chicken, coating, starch, lysine, PhIP

Introduction

Heterocyclic amines (HCAs) are known as genotoxic carcinogens which are associated with important types of human cancer such as breast, colon or pancreas cancers among people consuming meat (Layton, et al., 1995). The type of the meat, temperature and time of cooking, and muscle quality has been known to affect the formation of HCAs formed in meats (Janoszka, Błaszczuk, Damasiewicz-Bodzek, & Sajewicz, 2009; Jautz, Gibis, & Morlock, 2008; Melo, et al., 2008). The most abundant of these compounds is 2-Amino-1-methyl-6-phenylimidazo[4,5-b]-pyridine (PhIP) with usual range of 0.3 to 182 ng/g, specially in poultry (J. Felton, et al., 2000; Nakagama, Nakanishi, & Ochiai, 2005). Due to strong mutagenic properties of PhIP, this HCA is regarded as a possible class 2B human carcinogen by the International Agency for Research on Cancer (IARC), and was anticipated to be a human carcinogen by the National Toxicology Program (NTP) in its 15th report (NTP, 2021). Therefore, investigating prevention methods of PhIP is critical. Amino acids (AAs) have been extensively used in commercial foods as nutrition supplements and were shown to significantly reduce the level of HCAs (Kataoka, et al., 2012; Linghu, et al., 2020). Among polysaccharides used to develop edible coatings, potato starch (PS) is an Excellent film-former, biocompatible, biodegradable, easily accessible, and cost effective compound (Sapper & Chiralt, 2018). Moreover, coatings based on starch are appropriate to be used for new applications through the incorporation of additional ingredients into their formulation (Sapper, et al., 2019). Lys was previously shown to decrease the PhIP either by direct addition to beef patties (Linghu, et al., 2020) or incorporation in edible films that were applied to chicken surface (Farshi, et al., 2022). It was shown that even low concentrations of Lys (0.25%) was able to decrease the PhIP level up to 90% compared to the control chicken without coating (Farshi, et al., 2022); However, there is no study on the effect of AA

incorporation time with meat surface on PhIP or in general HCAS level. Moreover, the possible PhIP prevention mechanisms of AAs in model systems were reported to be the formation of AA-PhIP (Kataoka, et al., 2012), AA- phenylacetaldehyde (PheAce) adducts or AA-benzaldehyde adducts (Deng, et al., 2022). PheAce (the Strecker aldehyde of phenylalanine) was reported to be an important intermediate in PhIP formation and was reported to condense with creatinine to form an adduct, with subsequent addition of nucleophilic and dehydration to form PhIP (Deng, et al., 2022). However, there is no study on the mentioned possible prevention mechanisms in real chicken. In this study we aimed to investigate the effect of dipping chicken breast in PS coating containing 0.25% Lys (PS-0.25% Lys) for different times (15 min, 30 min, 1h, and 6h) and its effect on the PhIP level, PheAce content, volatile compounds, color, and texture of the chicken compared to the control chicken breast.

Materials and methods

Chemicals and materials

The 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP) standard was purchased from Cayman Chemical (Ann Arbor, MI, USA). L-lysine (Lys), glycerol (GLY), triethylamine, acetic acid (HPLC grade), methanol and acetonitrile (HPLC grade), ammonium acetate were purchased from MilliporeSigma (Burlington, MA, USA). n-decanal and ethyl acetate ($\geq 98\%$, GC grade) was purchased from the Sigma-Aldrich (St. Louis, MO, USA). Syringe filters (nylon, 0.2 μm) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Deionized water was obtained from a Sybron/Barnstead PCS filtration unit (Barnstead/Thermolyne, Inc, Dubuque, Iowa, U.S.A.). Bond Elut Enhanced Matrix Removal (EMR)-Lipid (QUEChERS dSPE EMR and Lipid-Polish) was obtained from Agilent Technologies (Santa Clara, Ca, U.S.A.). Chicken

breasts (SmartChicken®), potato starch (PS), peanut oil, and bamboo skewers were obtained from a local store.

Edible coating preparation

The PS coating solution was prepared based on a previous study with minor modifications (Ghanbarzadeh & Almasi, 2011). Five g of PS powder was dissolved in distilled deionized H₂O (100 g), and subsequently the solution was heated to 80 °C for 30 min for gelatinization. After cooling the solution at room temperature, 2 g/100 g (w/w, on dry basis of the weight of starch) GLY (plasticizer) and 0.25 g Lys (w/w of the solution) were added to solution and the resulting dispersion was mixed for 5 min. A coating solution without AA was used for comparison. The coating solution was then homogenized at 11000 rpm for 5 min using a laboratory homogenizer (Homogenizer, OMNI International, Kennesaw, GA, USA) and was used for dipping chicken breasts before frying.

Treatment of chicken breasts

Fresh chicken breasts were kept at refrigerator temperature (4 °C) before application of the coating solutions. Chicken breasts cuts with the same dimensions (5 × 2 × 1.5 cm) were dipped in the 0.25% Lys containing PS coating solutions and were kept at 4 °C for 15 min, 30 min, 1 h, 3 h, and 6 h. Chicken breast with no coating was used as control for comparison. Samples were removed at the mentioned times and after draining the excess coating they were put on bamboo skewers under a biological containment hood for 20 min. After adding a small amount of peanut frying oil to the electrical frying pan with heat adjustment, chicken breasts

were pan-fried on with surface temperature of 195 °C for 7.5 min on each side. The chicken breast were cooled at room temperature and were used for further analysis.

PhIP determination

PhIP extraction- Enhance Matrix Removal-Lipid method

PhIP was extracted through the Agilent Bond Enhance Matrix Removal-Lipid (EMR-Lipid) method according a previous study (Linghu, et al., 2019). Finely grounded (3 g) sample of pan-fried chicken breast crust was transferred in a 50 ml polypropylene (PP) centrifuge tube and was mixed with 15 mL of formic acid (2%) in acetonitrile solution on a mechanical shaker at room temperature for 30 min. The tube was centrifuged (10 min, $10000 \times g$ at 4 °C) (Fisher Scientific, Marathon 21000 R, MA, USA). Subsequently, 1 g of EMR-Lipid dSPE sorbent which was preactivated with 5 mL of 5 mM ammonium acetate buffer was added to 12 mL of the supernatant from centrifuge and were centrifuged at the same condition after shaking for 2 min. Later, for a salting-out extraction and further cleanup, 10 mL of supernatant was added to the tubes containing 2 g EMR-Lipid Polish salts and the tubes were shaken for 2 min and centrifuged with the same condition. Then, from the final supernatant 1 mL was taken to a conical vial and was dried up under a constant flow of nitrogen gas. After drying out, 100 μ L of methanol was added to the vial (for reconcentration) and the final solution was filtered through a 0.2 μ m nylon filter before HPLC analysis. The average recovery of this extraction method was reported to be 81 % according to the previously published paper (Farshi, et al., 2022).

Identification and quantification of PhIP

The PhIP was detected by HPLC (Agilent Technologies, 1100 series) coupled with fluorescence detector (Hewlett-Packard, Palo Alto, CA, USA) according to a method described by (Jinap, et al., 2013) with some modifications. The excitation and emission wavelengths of detector were 306 and 371 nm and the column was C18 (TOSOH Bioscience LC, TSKgel ods-80, 4.6 mm ID × 25 cm, 5 micrometer Millipore Sigma, MA, USA). The gradient mobile phase was consisted of acetonitrile (A) and 0.01 M triethylamine solution (B) which was adjusted to the pH= 3.6 using acetic acid. The mobile phase started with 20% A: 80% B, and it was programmed to 25% A: 75% B for 4 min, and 55% A: 45% B for 20 min. The flow rate was 1.0 mL/min during the run time. The injection volume was 10 µL and the column temperature was 26 °C. The matrix matched standard curve was used for calculation of PhIP concentration (ng per g of pan-fried chicken breast crust). The PhIP percent reduction was calculated based on the following equation:

%Reduction = $(P_c - P_s / P_c) \times 100$, where P_c was the PhIP concentration of the control chicken breast without coating and P_s was the PhIP concentration of chicken breast dipped in PS coating.

Extraction PheAce

PheAce extraction was conducted according to the previously described method by (Cheng et al., 2009). The grounded chicken crust (5 g) was mixed with ethyl acetate (20 mL) and was subjected to ultrasonic extraction for 10 min. After static stratification, the ethyl acetate phase was collected and the extraction was repeated twice. The ethyl acetate extraction layers were combined and dried up under nitrogen gas flow. Then, the extract residue was

reconcentrated with 25 mL ethyl acetate. To 10 ml of the ethyl acetate extract, 50 μ l of n-decanal solution was added as an internal standard.

Extraction of Volatile compounds

Chicken crust (1.5 g) was transferred to screw cap GC vials (20 ml thread vial and 1.3 mm caps) and sealed with PTFE/red rubber septum. A 75 μ m carboxen/polydimethylsiloxane (CAR/PDMS) SPME, fused silica, 23 Ga fiber from Supelco Inc (Bellefonte, Pa., U.S.A) was used. The samples were heated at 70 °C for 20 min in an aluminum heating block to pre-equilibrate the headspace. The CAR/PDMS SPME fiber was exposed in the upper space of vial for 40 min to adsorb volatiles in the headspace. Then, the fiber was withdrawn into a manual holder and manually injected into the GC injector. The fiber was kept 5 min in the injector for desorption.

GC–MS analysis

The GC–MS chromatograph system (HP 5890–5972 MS, Agilent Technology Inc., Santa Clara, CA, USA) was equipped with an HP-5MS (60 m $\times$ 0.25 mm $\times$ 0.25 μ m, Agilent Technologies) column. For PheAce, 1 μ L of sample was injected in a splitless mode with the inlet temperature of 280 °C. The oven program was started at 50 °C for 2 min, ramped up at a rate of 10 °C/min to 180 °C, and then to 280 °C at a rate of 25 °C/min, and held for 5 min. The MS detector temperature was 180 °C and transfer line temperature was 280 °C. Selective ion monitoring (SIM) mode (65, 91, 92, 120) was used to quantify PheAce. The n-decanal solution was used as internal standard (IS) and was detected with ions (57, 70, 82, 95, 112). The concentration of PheAce in the sample was calculated according to the standard curve equation

of PheAce/IS by injecting different concentrations of PheAce (0.1 – 10 µg/mL) spiked with fixed amount of IS (50 µl to 10 ml of each concentration).

For volatile compounds: The analyte was desorbed from the SPME fiber at the injector port at 280 °C for 5 min in a splitless mode. The carrier gas (flow rate of 1 mL/min) was ultra-high pure helium. The oven temperature program was set as follows: 40 °C for 2 min, increased (rate of 3 °C/min) to 80 °C, and increased (rate of 5 °C/min) again to 150 °C, and finally to 250 °C (at a rate of 10 °C/min) and held for 1 min. The MS detector temperature was 150 °C and transfer line temperatures was 250 °C. A 3 min solvent delay was used to process MS data with total ion scanning in the 40 to 550 m/z mass range (rate of 1 scan/s). NIST 2014 Mass Spectral Library was used to identify the volatile compounds.

Color measurement

CIE Lab parameters (L^* , a^* , and b^*) of the pan-fried chicken breast were measured with HunterLab MiniScan EZ 4500 L (Hunter Associates Laboratory Inc., Reston, VA, USA). The colorimeter was calibrated with white and black plates. The ΔE showed the color difference of the PS coated chicken compared to the control chicken with no coating according to equation:

$\Delta E = \sqrt{\Delta L^2 + \Delta a^2 + \Delta b^2}$, where ΔL , Δa , and Δb were the differences between color coordinates of the PS coated and uncoated chicken breasts.

Cooking loss percentage

The difference between the weights of chicken breasts before and after frying was used to calculate the cooking loss percentage according to:

% Cooking Loss= $(W_b - W_a / W_b) \times 100$, where W_b and W_a were the weights before and after frying, respectively.

Tenderness

A texture analyzer (Texture Technologies Corp, Stable Micro Systems, Surrey, England) coupled with a Meullenet–Owens razor shear blade (MORS) was used for tenderness analysis of PS coated and control chicken breasts. The crosshead speed of 10 mm/s and the blade penetration depth of 15 mm was set (Küçüközet & Uslu, 2018). The pan-fried chicken breast cuts were put in vertical direction to the orientation of muscle fiber under the razor shear blade (Xiong, et al., 2006). Shear force (maximum force (N)) and shear energy (area under maximum force versus displacement curve (N. mm)), were calculated by the texture analyzer software.

Texture profile analysis (TPA)

Texture profile analysis was performed as suggested by (Khanal, et al., 2019) and (Rathod & Khamrui, 2015). Sample was cut into 2 x 2 x 2 cm diameters using a sharp knife. TPA was conducted with a TAXT2i texture analyzer (Stable Micro Systems, Godalming, UK) with 30 kg load cell. A 50 mm cylindrical flat prob and double bite compression method (with 2 seconds rest period between the two bites) was used. The samples were compressed to 80% (1.6 cm compression) of their original height (20 cm) by the probe with a crosshead speed of 0.8 mm/s. The TPA curve of force (N) v/s time (S) was plotted and used to derive the instrumental texture attributes such as hardness, cohesiveness, springiness, and chewiness. Hardness (N) was noted as the maximum force during the first compression cycle. Springiness was calculated as the height recovered after the first compression. Cohesiveness was reported as the ratio of

positive force area under the second and first compression cycle and chewiness was calculated by multiplying gumminess and springiness (Khanal, et al., 2019).

Statistical analysis

SAS 9.4 software was used to determine a significant difference ($p < 0.05$) between samples at a 95% confidence level. Experiments were done in triplicates. For texture studies, and PhIP analysis six measurements were conducted per sample to account for variations in measurements. Analysis were done with the PROC GLIMMIX procedure with Tukey's test. Different letters in each column on the tables show the significant differences.

Results and discussion

Effect of chicken breast dipping time in PS-Lys coating on PhIP level and possible mechanism

The results of the PhIP reduction are listed in Table 18. Results showed that PS-0.25% Lys coated chicken had significantly ($p < 0.05$) lower PhIP level compared to the control chicken. The control chicken had average PhIP level of 97.10 ng/g and the level was decreased to 18.79 - 34.76 ng/g. The average PhIP level for 15 min and 30 min dipped chicken was 24.01 ng/g and 26.14 ng/g, respectively, with no significant differences ($p > 0.05$) between them. For 1 h and 3 h dipped chicken the PhIP level was 33.51 ng/g and 34.49 ng/g with no significant difference ($p > 0.05$) and after 6 h of dipping the PhIP level was decreased to 18.79 ng/g. However, there was no significant differences ($p > 0.05$) between the PhIP levels of 15 min, 30 min and 6 h dipped chicken. Therefore, it was found that with 15 min of dipping chicken in PS-Lys coating, the PhIP level decreases to the level comparable to the 6 h dipping. However, in a study by

(Busquets, et al., 2006) it was shown that chicken marinated with red wine for 24 h had significantly ($p < 0.05$) lower PhIP level (8.8-12 ng/g) compared to 30 min and 3 h marination time which was attributed to antioxidant compounds and AAs such as proline which are present in the wine and may be effective at longer time of treatment. In another study in which beef meat was marinated with green tea, it was shown that PhIP levels decreased by increasing the marinating time up to 6 h and Pearson correlation showed that the PhIP reduction in marinated meat was highly significant with the increase of marinating time (Quelhas, et al., 2010). This can be attributed to the more complex composition of marinating compound in the mentioned studies compared to the current study in which only Lys is incorporated into the PS solution. However, with 15 min of dipping chicken breast in this coating solution, the PhIP level could be decreased to a concentration which can be obtained with longer marinating time by green tea and wine. Lys was previously shown to decrease the PhIP either by direct addition to beef patties (Linghu, et al., 2017) or incorporation in edible films applied to chicken surfaces 15 min prior to frying (Farshi, et al., 2022). The exact mechanism of PhIP prevention by amino acids is still unclear; however, there are some proposed mechanism in the literature such as scavenging the PhIP intermediates. One possible mechanism for prevention of PhIP was stated to be PheAce scavenging capability of a compound (K. W. Cheng, et al., 2009; Linghu, et al., 2017; Yu, Chen, & Yu, 2016). Therefore we aimed to study the PheAce content of the chicken with no coating and chicken dipped in PS-25% Lys coating solution for 15 min.

Table 18. PhIP concentration in chicken breasts treated with PS-0.25% Lys coating solution at different times

Chicken breast	Sample treatment time	PhIP Cons ¹ (ng/g)	PhIP Percent reduction ¹ (%)
Without coating	0	97.10 ± 5.91 ^A	-
	15 min	24.01 ± 5.57 ^D	75.46 ± 5.45 ^{BA}
PS-0.25%-Lys coated	30 min	26.14 ± 5.77 ^{CD}	74.41 ± 5.65 ^B
	1 h	33.51 ± 2.75 ^{CB}	65.96 ± 1.23 ^C
	3 h	34.49 ± 1.48 ^B	66.23 ± 1.45 ^C
	6 h	18.79 ± 3.62 ^D	81.61 ± 3.55 ^A

¹Values (means ± SD from Excel, n=6) in the same column with different letters are significantly different at p<0.05. Lys: lysine, PS: potato starch, Cons: concentration, PhIP: 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine.

The LOD and LOQ values were 0.07 µg/mL and 0.20 µg/mL, respectively. Results of GC-MS showed that the PheAce content in coated chicken breast (0.38 ± 0.08 µg/g chicken crust) was significantly ($p<0.05$) higher than that of the uncoated chicken breast (0.04 ± 0.01 µg/g chicken crust). It was previously shown that in a model system containing all of the precursors for PhIP formation and non-precursor amino acids (including Lys) at different concentrations (0.125% to 2%), PheAce concentration was significantly lower ($P<0.05$) compared to the model systems with no non-precursor amino acids (Linghu, et al., 2017). In another study it was shown that benzaldehyde and PheAce are key intermediates for the formation of PhIP. Their findings showed that histidine–proline combinations were capable of scavenging PheAce and benzaldehyde simultaneously, which increases the suppression of PhIP

formation (Deng, et al., 2022). In this study, the results from volatile compound analysis by GC-MS which was conducted on chicken crust with SPME method, showed that there is no significant differences ($p>0.05$) between benzaldehyde content of chicken breast crust with and without PS-0.25% Lys coating. This phenomenon can be attributed to the complex PhIP formation pathways and its intermediates thus, to inhibit the PhIP formation, all these pathways and intermediates should be blocked. However, there is no information about other potential key ketone or aldehyde intermediates which can be responsible to generate PhIP and whether blocking these intermediates could reduce PhIP (Deng, et al., 2022). The mentioned studies were conducted on the model systems, therefore this inconsistency in results can be attributed to the complexity of real food (chicken) and the interactions due to the matrix effect. It was previously suggested that AAs can contribute to the PhIP removal by forming PhIP–amino acid adducts (Kataoka, et al., 2012), therefore this can be a possible mechanism of PhIP prevention by dipping the chicken in the PS-Lys coating solution.

Volatile compounds chicken breast dipped in PS-0.25% Lys coating solution and uncoated chicken

The volatile compounds in chicken breast dipped in PS-0.25% Lys for 15 min and chicken breast with no coating was evaluated. Different volatile compounds were released from chicken during cooking which mainly through the Maillard reaction, lipid oxidation, and degradation, as well as the interaction between lipid-oxidation products with Maillard intermediates (Brunton, Cronin, & Monahan, 2002). As Figures 4 illustrates, the differences in the volatile compound profile of pan-fried chicken crust (195 °C/ 7.5 min) dipped in PS-0.25% Lys coating solution and control chicken crust without coating were characterized with GC-MS (their GC-MS chromatogram are presented in Appendix A). With the SPME-GC-MS method, 41 volatile compounds (Table A-1 Appendix and Figure 4) were identified: including 13 aldehydes (pentanal, 3-methyl butanal, 2-methyl butanal, hexanal, nonanal, octanal, heptanal, 2-heptenal, dodecanal, (E)-2-decenal, (E)-tetradec-2-enal, benzaldehyde, and phenylacetaldehyde), three pyrazines (3,5-diethyl-2-methylpyrazine, 3-ethyl-2,5-dimethylpyrazines, and 2,5-dimethyl pyrazine), four alcohols (phenoxy ethanol, 2-ethylhexanol, 1-octanol, 2-pentanol), a sulfur compound (benzothiazole), and two furan (2-pentyl furan, 2n Octylfuran), three hydrocarbon (1-methyl-4-(1-methylethenyl)-benzene, 1-dodecane, cyclododecane), one ketone (d-carvone), three pyridines (2-butyl pyridine, dimethylpyridine, 3-Amino-4,6-dimethylpyridine-2(1H)) a pyrimidine (2-Chloro-4-(4-methoxyphenyl)-6-(4-nitrophenyl)pyrimidine), one ester (2-methyl butyl ester-hexanoic acid), a fatty ester (dihydro-5-pentyl-2(3H)-Furanone), 4-[1,1-dimethylpropyl]-phenol, oxime-methoxy phenyl, and some compounds possibly from column or contamination such as di-tms 3-hydroxymandelic acid ethyl ester, arsenous acid tris(trimethylsilyl) ester, 2-hydroxy-iso-butyrophenone, 2-ethyl-salicylate, octamethyl

cyclotetrasiloxane, and homosalate. The most abundant of these volatile compounds were benzothiazole, followed by octanal, 2-pentyl furan, benzaldehyde and other mentioned compounds. As it is illustrated at Figure 4, PS-0.25% Lys coated chicken crust had significantly higher ($p < 0.05$) levels of 2-heptenal, PheAce, d-carvone, dodecanal, cyclododecane, 2-methyl butanal, and pyrazines; however, it had significantly lower levels of benzothiazole level. Moreover, no significant differences ($p > 0.05$) was shown in the levels of other volatile compounds between the control and coated chicken. Dipping the chicken in PS-0.25% Lys coating solution significantly enhanced ($p < 0.05$) the formation of roast odor 3-ethyl-2,5-dimethylpyrazine, and 3,5-diethyl-2-methylpyrazine after frying compared to controls. Therefore findings suggested that dipping in PS-0.25% Lys coating solution can promote the formation of roasted and nutty odors in pan-fried chicken. Pyrazines, furan, and thioethers were detected in the Maillard reaction products which contribute to which contributed to roasted, meat and coffee odors (Chiang, Yeo, Ong, & Henry, 2022). Benzothiazole was reported to be the major nitrogen- and/or sulfur-containing compounds in chicken byproducts (Wettasinghe, Vasanthan, Temelli, & Swallow, 2000). Thiazoles were shown to be present in grilled, fried, or roasted meats, in high amounts. These compounds have low odor threshold values and are considered as important contributors to meat-like aroma (Wei, et al., 2017). The significant increase in PheAce (green and floral odor) in PS-0.25% Lys dipped chicken, confirmed the result from PheAce concentration results from previous section. This indicates that the PS-Lys coating was involved in the Maillard reaction during cooking and influenced formation of different Strecker aldehydes. Researchers (Linghu, et al., 2017) have shown that addition of AAs including Lys could significantly decrease the PheAce level in a beef model system. The inconsistencies in the results could be due to the more complex matrix of real chicken compared to the model system.

Moreover, the coating did not significantly ($p>0.05$) affect formation of alcohols and furans which are mainly originated from Maillard reactions or lipid oxidation and are responsible for unpleasant odor (Kamili, et al., 2010; Mottram, 1998).

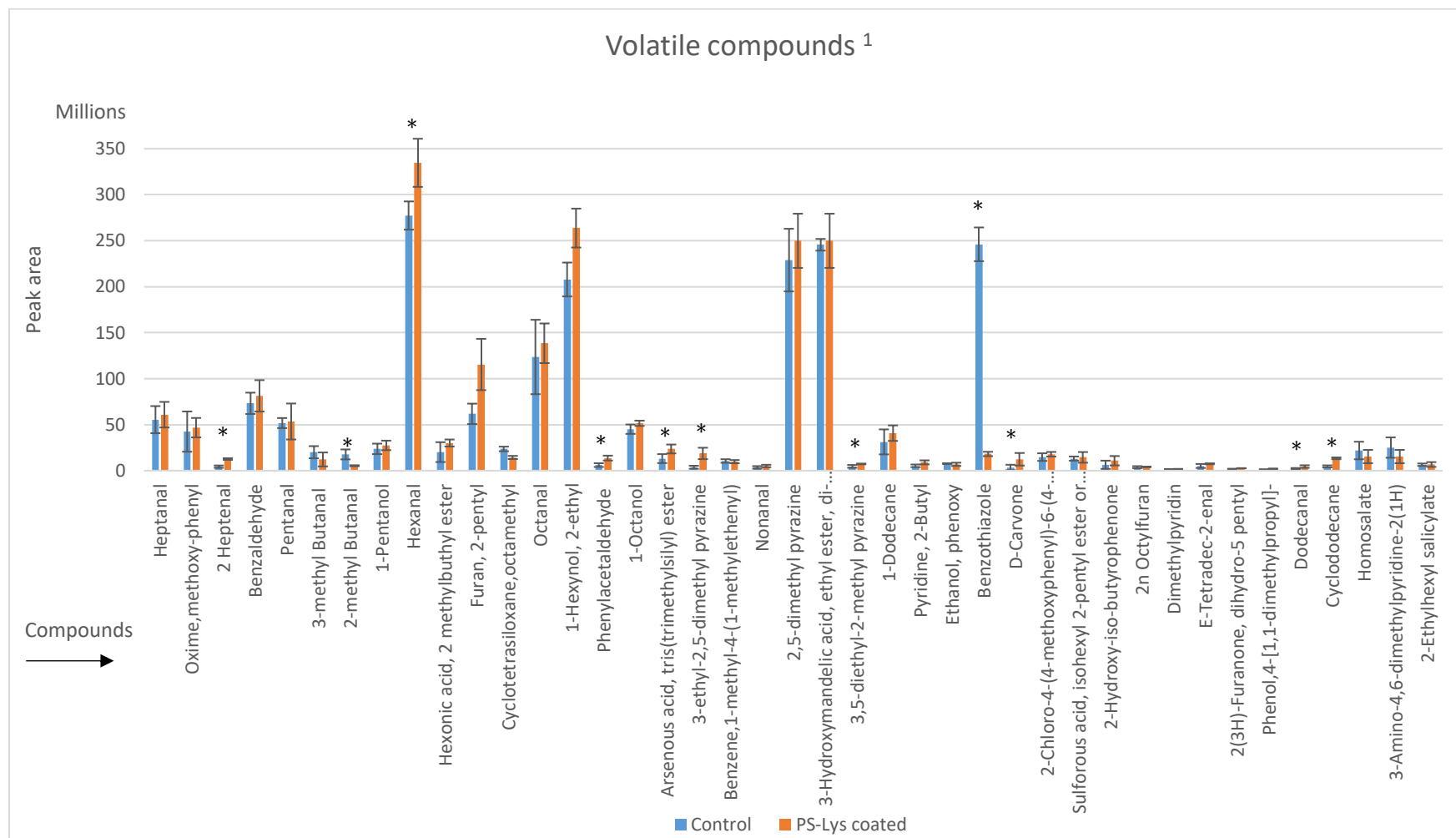


Figure 4. SPME analysis of volatile compounds of pan-fried chicken breast crust with PS-0.25% lysine coating and without coating. The oven temperature program was set as follows: 40 °C for 2 min, increased (at a rate of 3 °C/min) to 80 °C, and increased (at a rate of 5 °C/min) again to 150 °C, and finally to 250 °C (at a rate of 10 °C/min) and held for 1 min. ¹ Compounds with “*” on the top are significantly different at (p<0.05).

It was stated that various volatiles such as aliphatic hydrocarbons, alcohols, aldehydes, ketones, esters, carboxylic acids, and some aromatic hydrocarbons are being formed through the lipid degradation (such as fatty acid oxidation of lipids). Esters mostly do not affect odor of meat products except lactone and thioester (Wei, et al., 2017).

Effect of dipping in PS-0.25% Lys coating solution on physical characteristic of chicken breasts

Cooking loss percentage

The results for cooking loss percentage of chicken breasts are presented in Table 19. The average cooking loss percentages were in the range of 45.75% to 53.34% and there were no significant differences ($p>0.05$) between the values, showing that dipping time in PS-0.25% Lys coating solution didn't have significant effect on cooking loss percentages of chicken breasts and the control chicken without coating. It was previously shown (S. M. Kim, Kim, & Park, 2021) that the cooking loss of the 3D printed crab meat coated with 12% PS gel was decreased compared to the uncoated crab which can be attributed to the higher concentration of PS compared to the current study (5%) on chicken .

Table 19. Cooking loss percentage of chicken breasts dipped in PS-0.25% lysine coating solution and the chicken without coating

Chicken breast	Dipping time	Cooking loss ¹ (%)
Without coating	0	47.47 ± 2.84 ^A
	15 min	47.74 ± 5.94 ^A
PS-0.25%-Lys coated	30 min	53.34 ± 3.81 ^A
	1 h	47.39 ± 2.04 ^A
	3 h	45.75 ± 4.08 ^A
	6 h	48.57 ± 3.55 ^A

¹Values (means ± SD from Excel, n=6) in the same column with different letters are significantly different at p<0.05. Lys: lysine, PS: potato starch

Color

Color parameters are listed in Table 20. During the frying process, several reactions such as Millard reactions occur which is due to interactions between sugar–protein, lipid oxidation reactions, and the formation of primary and secondary oxidation products, which can cause color changes in the fried samples (Jouki & Khazaei, 2022). The average ΔE which shows the color difference between the chicken dipped in the coating solution and control chicken with no coating was in the range of 3.45-6.50 with no significant differences ($p>0.05$) between them. It was stated that $\Delta E>3$ is perceivable by human eye (Quek, Yap, Rosa, Tan, & Teoh, 2018) therefore, due to no significant differences ($p>0.05$) between the values around 3 and higher values, it can be stated that dipping the chicken breast in PS-0.25% Lys coating solution at different times, does not affect the surface color after frying.

Table 20. Color parameters of chicken breasts dipped in PS-0.25% lysine coating solution and the chicken without coating

Chicken breast	Sample	L* ¹	a* ¹	b* ¹	ΔE ¹
treatment time					
Without coating	0	42.15 ± 1.34 ^A	20.50 ± 0.5 ^A	28.77 ± 0.97 ^A	-
PS-0.25% Lys	15 min	41.34 ± 3.33 ^A	18.89 ± 0.86 ^A	26.70 ± 1.03 ^A	3.45 ± 0.20 ^A
coated	30 min	39.76 ± 2.60 ^A	21.53 ± 1.70 ^A	28.09 ± 2.04 ^A	6.50 ± 2.44 ^A
	1 h	40.58 ± 0.70 ^A	21.24 ± 2.20 ^A	28.37 ± 2.27 ^A	3.55 ± 0.59 ^A
	3 h	40.65 ± 2.45 ^A	21.33 ± 2.53 ^A	28.08 ± 1.75 ^A	4.22 ± 3.03 ^A
	6 h	40.77 ± 3.45 ^A	20.36 ± 3.15 ^A	27.44 ± 2.63 ^A	5.20 ± 1.85 ^A

¹Values (means ± SD from Excel, n=6) in the same column with different letters are significantly different at p<0.05. Lys: lysine, PS: potato starch, L*: lightness, a*: redness, b*: yellowness , ΔE: difference in color

Tenderness and Texture profile analysis (TPA)

Table 21 shows the shear force and shear energy of chicken breasts. It was stated that these parameters can be an estimation of tenderness of the chicken. The average shear force of the chickens was in the range of 8.32-12.10 N and shear energy was in the range of 34.3-55.46 Nmm. There were no significant difference (p>0.05) between the shear force or shear energy values of control and PS-0.25% Lys coated chicken (with different dipping times). Texture profile analysis of chicken (Table 22) also showed that there was no significant differences (p>0.05) in cohesiveness, springiness (%), and chewiness values of chicken breasts (dipped in PS- 0.25% Lys coating for different times and uncoated). However compared to the control, the hardness value was significantly higher in 6 h dipped chicken breast with average hardness of 271 N. It was previously shown that coated chicken with starch nanocrystals had higher hardness

compared to the control before frying (S. M. Kim, et al., 2021). There was no significant differences ($p>0.05$) between hardness of chicken breasts dipped in PS-0.25% Lys for different times. The higher hardness was attributed to the higher moisture loss of the samples during the frying process (S. M. Kim, et al., 2021).

Table 21. Shear force and shear energy values of chicken breasts dipped in PS-0.25% lysine coating solution and the chicken without coating.

Chicken breast	Sample treatment time	Shear force ¹ (N)	Shear energy ¹ (Nmm)
Without coating	0	12.10 ± 0.85 ^A	43.10 ± 10.35 ^A
PS-0.25% Lys	15 min	10.74 ± 2.60 ^A	53.06 ± 7.90 ^A
coated	30 min	10.79 ± 2.08 ^A	34.3 ± 9.82 ^A
	1 h	10.77 ± 3.05 ^A	43.05 ± 6.09 ^A
	3 h	8.32 ± 2.57 ^A	47.25 ± 8.62 ^A
	6 h	10.46 ± 1.09 ^A	55.46 ± 12.40 ^A

¹Values (means ± SD from Excel, n=6) in the same column with different letters are significantly different at $p<0.05$. Lys: lysine, PS: potato starch

Table 22. Texture profile of chicken breasts dipped in PS-0.25% lysine coating solution and the chicken without coating.

Chicken breast	Sample treatment time	Hardness ¹ (N)	Cohesiveness ¹	Springiness ¹ (%)	Chewiness ¹
Without coating	0	184.05 ± 16.55 ^B	0.41 ± 1.93 ^A	57.04 ± 0.05 ^A	49.29 ± 4.83 ^A
PS-0.25%	15 min	245.78 ± 11.08 ^{BA}	0.46 ± 0.05 ^A	60.11 ± 8.80 ^A	65.56 ± 5.66 ^A
Lys coated	30 min	231.17 ± 41.08 ^{BA}	0.46 ± 2.23 ^A	58.375 ± 0.05 ^A	62.03 ± 14.15 ^A
	1 h	243.27 ± 27.65 ^{BA}	0.46 ± 2.18 ^A	58.542 ± 0.04 ^A	67.54 ± 10.89 ^A
	3 h	249.40 ± 33.12 ^{BA}	0.44 ± 2.37 ^A	52.477 ± 0.06 ^A	61.13 ± 9.69 ^A
	6 h	257.10 ± 25.26 ^A	0.45 ± 3.16 ^A	59.458 ± 0.06 ^A	64.78 ± 13.48 ^A

¹Values (means ± SD from Excel, n=6) in the same column with different letters are significantly different at p<0.05. Lys: lysine, PS: potato starch

Conclusion

This study showed that extending the dipping time of chicken breasts in potato starch (PS) coating solution containing 0.25% Lys more than 15 min (30 min, 1h, 3 h, and 6 h) does not have a significant ($P>0.05$) effect on decreasing the PhIP level of pan-fried chicken breasts. Therefore, even the dipping time of 15 min in PS-Lys coating solution would be enough to reach to the same average level of PhIP as dipping time of 6 h. Moreover, there was no significant differences ($p>0.05$) between the cooking loss percentage, texture properties, and color parameters of coated chicken and that of uncoated ones. Significantly lower PheAce amount in chicken without coating compared to 0.25% Lys-PS coated chicken (15 min) showed that there may be another pathway of PhIP prevention by this coating. Volatile compound analysis showed the formation of higher levels of some aroma compounds such as pyrazines in the PS-Lys coated chicken which are characteristic roasted aroma of the chicken.

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Figure A- 1. Heated whey protein isolate (HWPI)- and unheated whey protein isolate (UHWPI)-based edible films

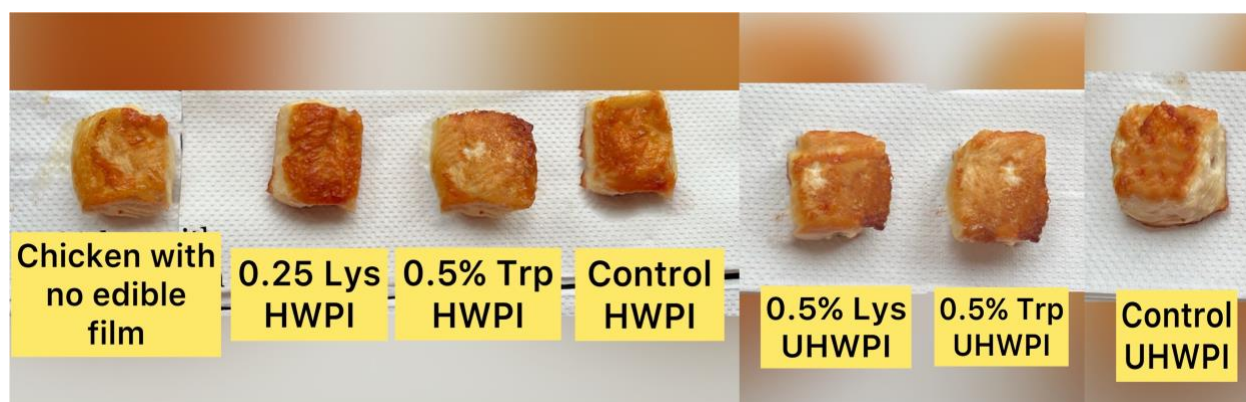


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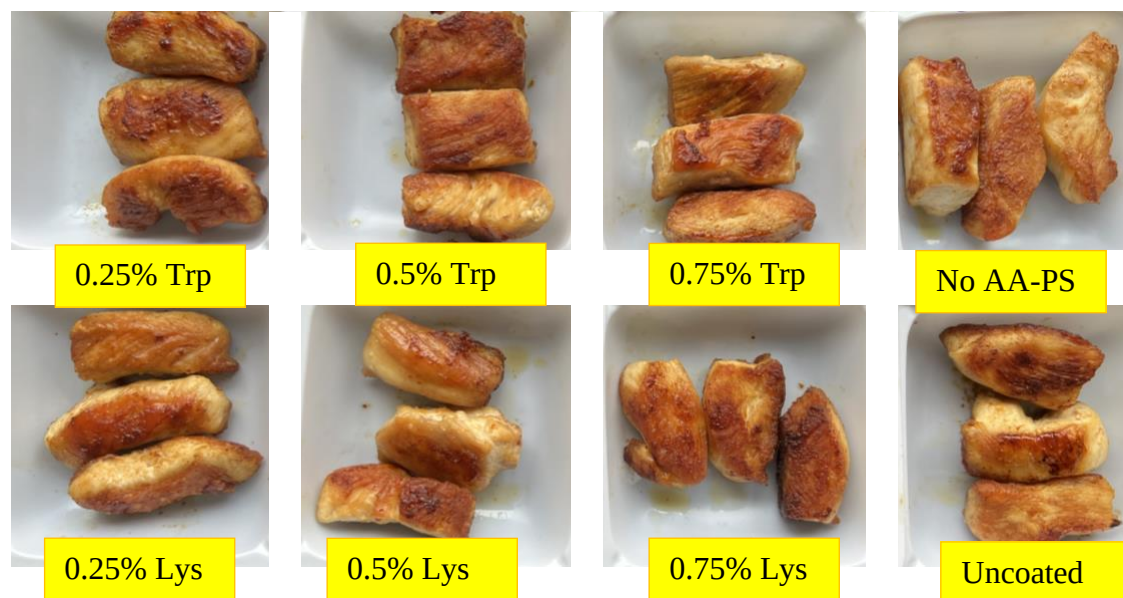


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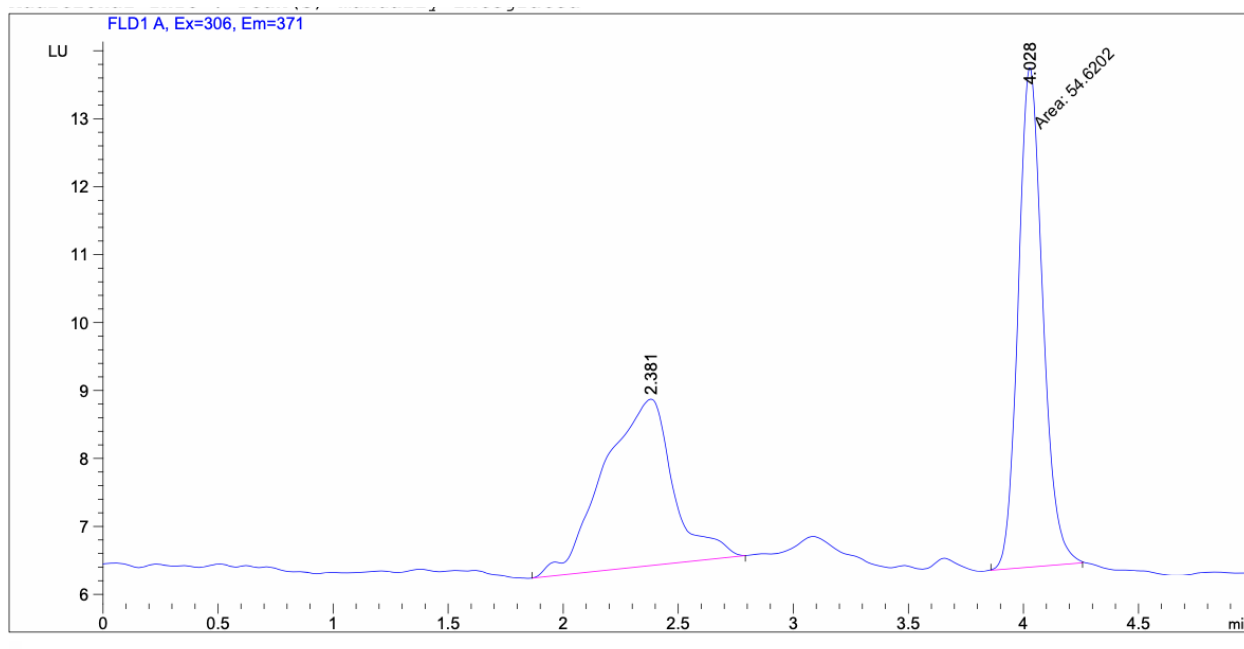


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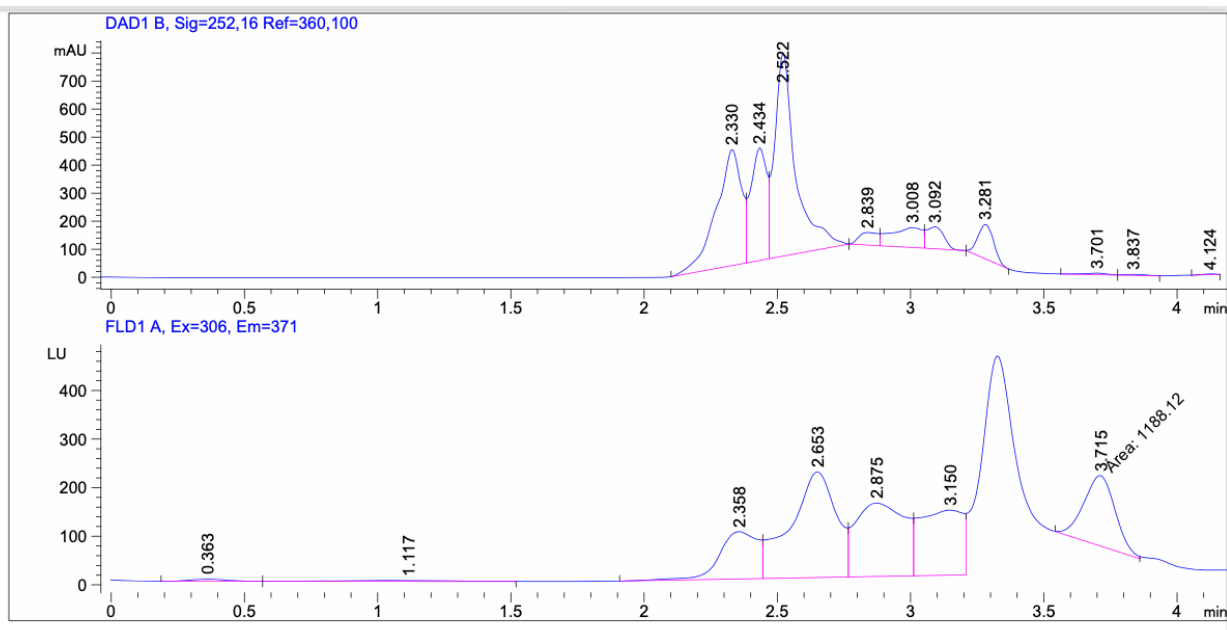


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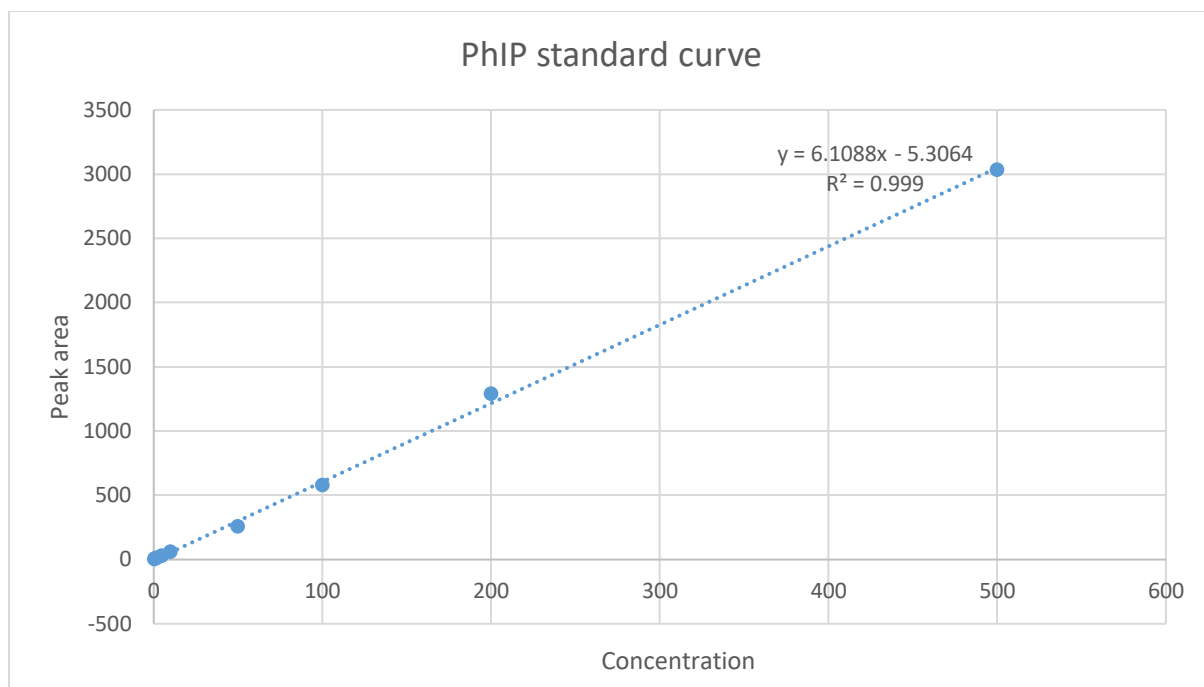


Figure A- 6. Matrix matched standard curve of 0.5-500 ng of PhIP in 3 g of blank chicken with no PhIP (results were reported as ng/g in manuscript).

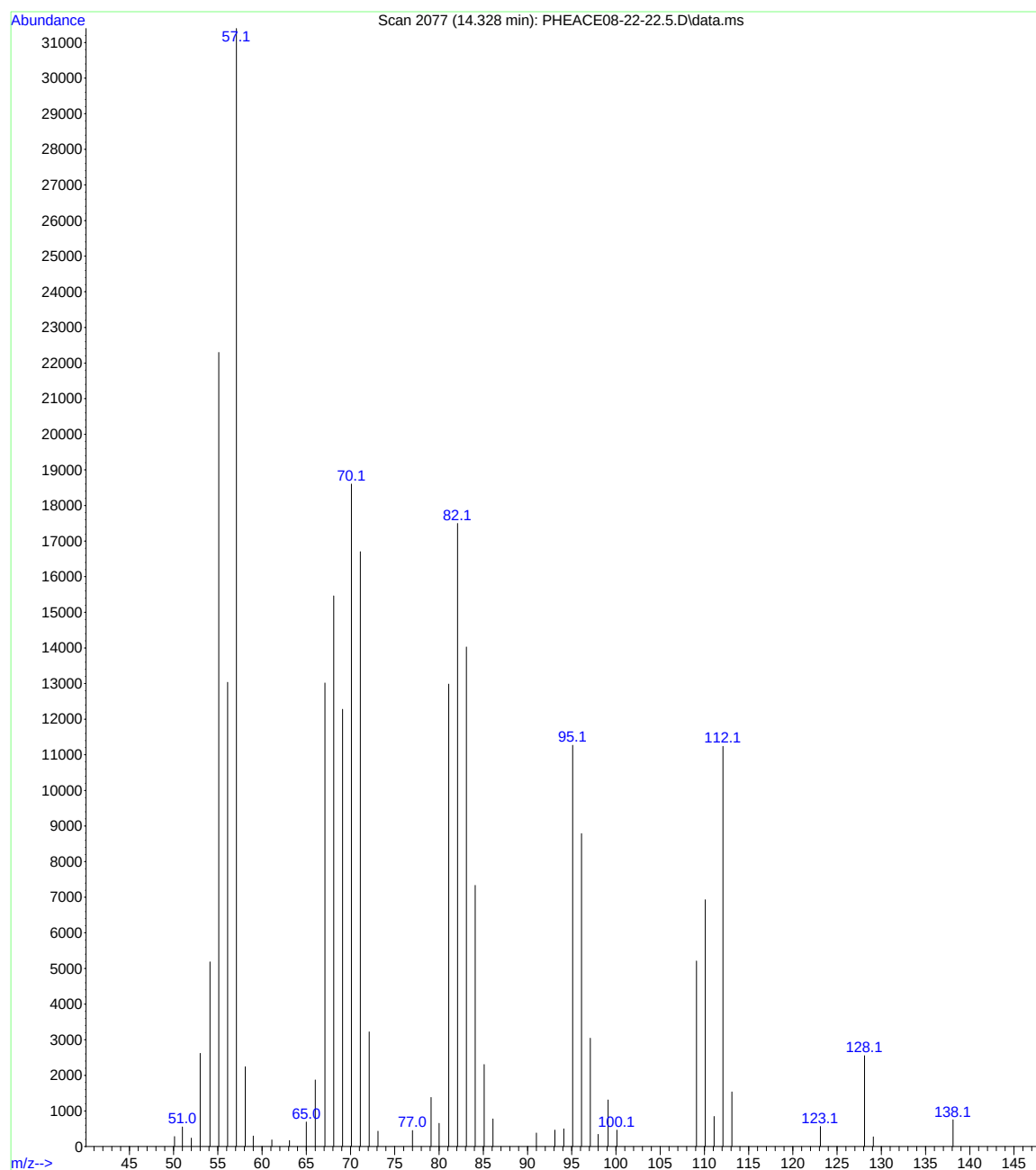


Figure A- 7. Mass spectrum of n-decanal (IS) obtained in full scan mode of GC-MS.

HP-5MS column (60 m x 0.25mm I.D. x 0.25 μ m) with a flow rate at 1 mL/min. The oven temperature program: 50 $^{\circ}$ C for 2 min, ramped to 180 $^{\circ}$ C at a rate of 10 $^{\circ}$ C/min, and increased to 280 $^{\circ}$ C at a rate of 25 $^{\circ}$ C/min, and held for 5 min.

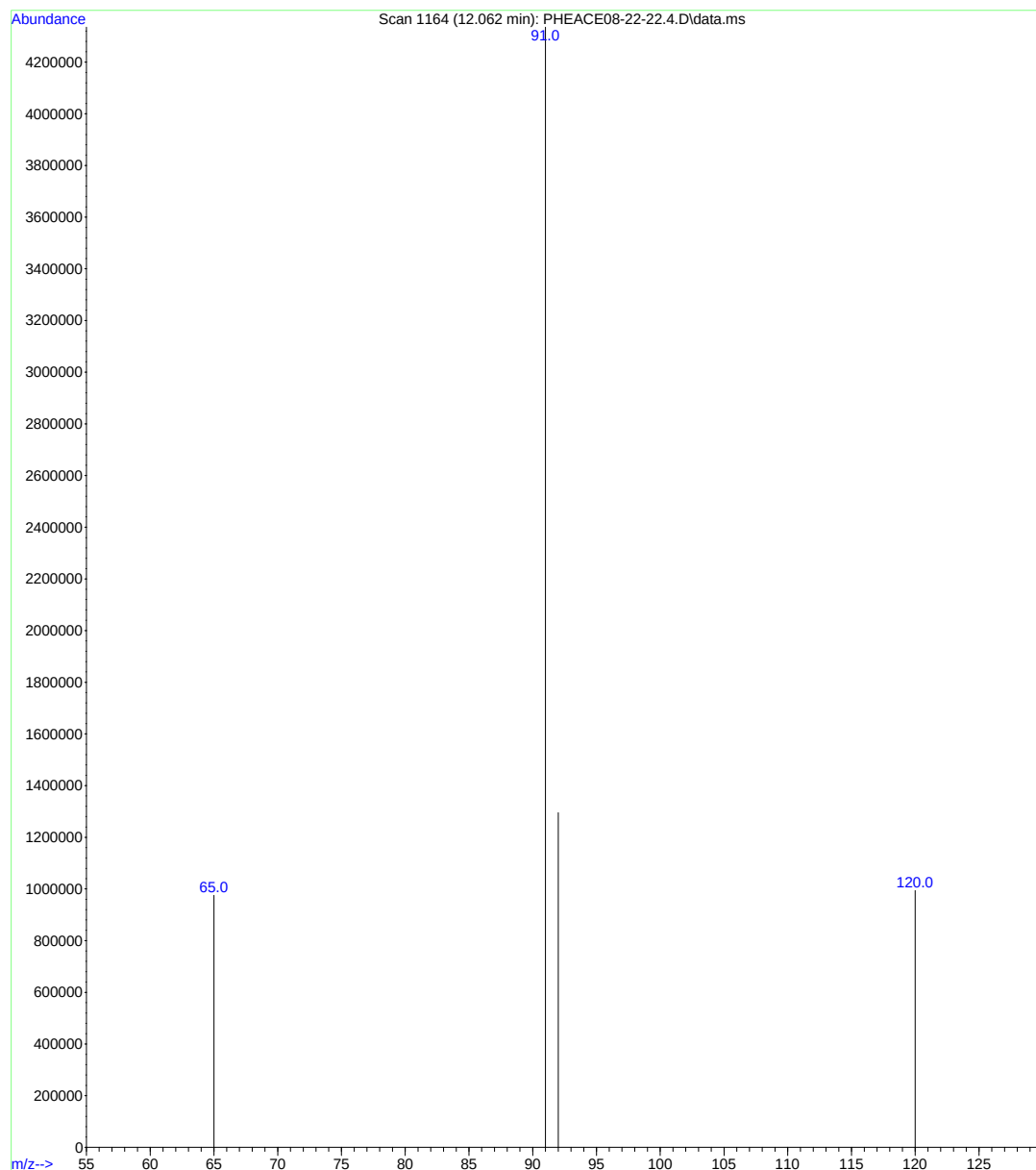


Figure A- 8. Mass spectrum of PheAce obtained in full scan mode of GC-MS.

HP-5MS column (60 m x 0.25mm I.D. x 0.25 μ m) with a flow rate at 1 mL/min. The oven temperature program: 50 $^{\circ}$ C for 2 min, ramped to 180 $^{\circ}$ C at a rate of 10 $^{\circ}$ C/min, and increased to 280 $^{\circ}$ C at a rate of 25 $^{\circ}$ C/min, and held for 5 min.

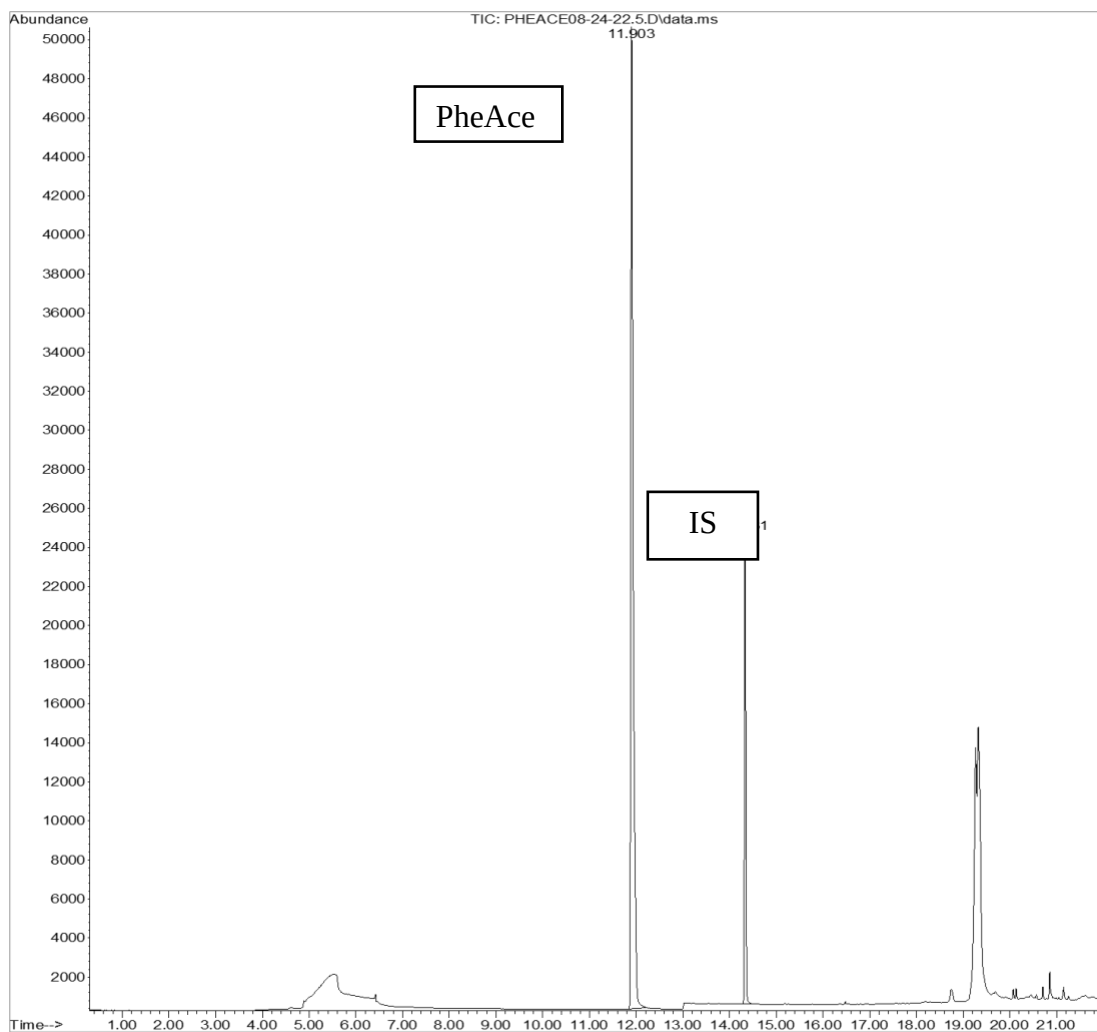


Figure A- 9. GC-MS chromatogram of 50 ppm PheAce for ions m/z 120, 91, 92, 65 and n-decanal (IS) for ions m/z 112, 95, 82, 70, 57 separated on an HP-5MS column (60 m x 0.25mm I.D. x 0.25 μ m) with a flow rate at 1 mL/min. The oven temperature program: 50 °C for 2 min, ramped to 180 °C at a rate of 10 °C/min, and increased to 280 °C at a rate of 25 °C/min, and held for 5 min.

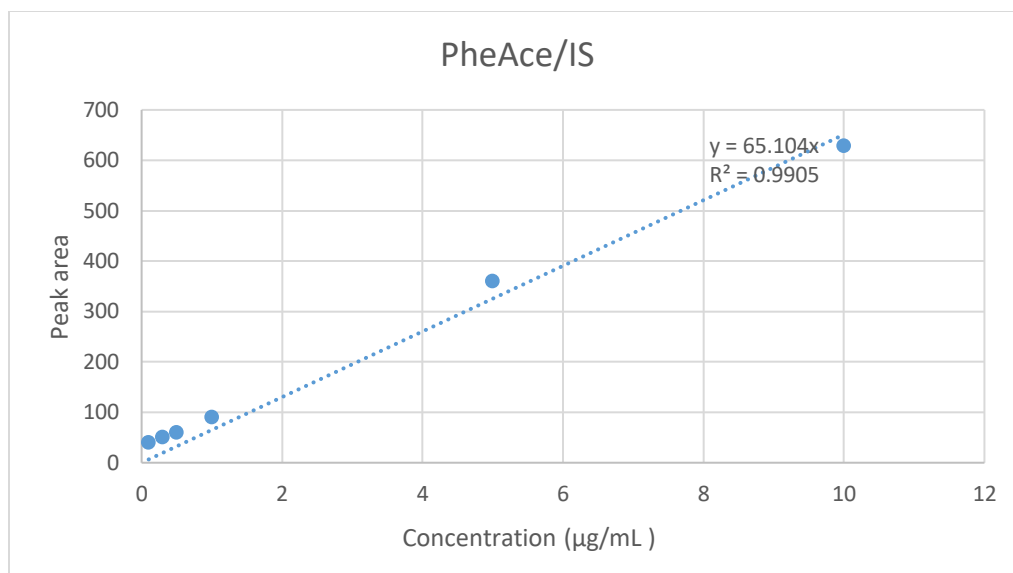


Figure A- 10. Standard curve of 0.1 – 10 µg/mL PheAce/IS.

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.

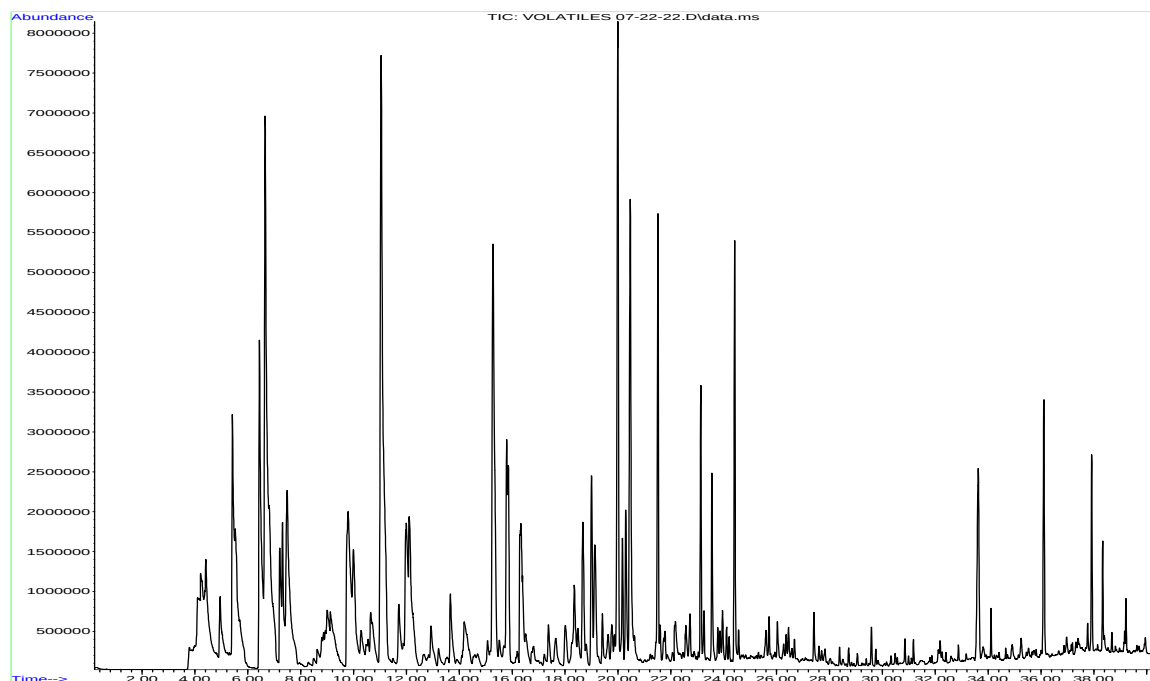


Figure A- 11. An example chromatogram of volatile compounds from pan-fried chicken crust. The carrier gas (flow rate of 1 mL/min) was ultra-high pure helium. The oven temperature program was set as follows: 40 °C for 2 min, increased (rate of 3 °C/min) to 80 °C, and increased (rate of 5 °C/min) again to 150 °C, and finally to 250 °C (at a rate of 10 °C/min) and held for 1 min. The MS detector temperature was 150 °C and transfer line temperatures was 250 °C. A 3 min solvent delay was used to process MS data with total ion scanning in the 40 to 550 m/z mass range (rate of 1 scan/s).

Table A-1. Volatile compounds form pan-fried chicken crust without coating and PS-0.25% Lys coated chicken crust.

Volatile compounds	Control (average peak area)	Ps-0.25% Lys coated (average peak area)	Time (min)	Qual
Pentanal	51781425	53474012	7.467	80
3-methyl Butanal	20098051.7	12203032.67	6.43	91
2-methyl Butanal	17703579.7	5398726.667	6.62	90
1-Pentanol	23698309	27497310.33	9.7	72
2,5-dimethyl pyrazine	228950532	249946235.7	12.56	92
Heptanal	55464782	60882770	15.82	97
Oxime,methoxy-phenyl	42487753.3	46738575.67	16.293	91
2 Heptenal	4366712.33	12665744.67	18.447	52
Benzaldehyde	73237767.7	81355280.33	18.727	97
Hexanal	277395158	334718448.3	11.11	95
Hexonic acid, 2 methylbutyl ester	20194211	30073095.67	19.641	56
Furan, 2-pentyl	61791911.3	115460236.3	20.037	94
Cyclotetrasiloxane,octamethyl	23632129.3	14351688.67	20.191	91
Octanal	123623322	138517546	20.499	98
1-Hexynol, 2-ethyl	207873558	263782738	21.609	64
Phenylacetaldehyde	6072441.67	13699854.33	22.255	93
1-Octanol	45112041.3	51532644.33	23.163	87
Arsenous acid, tris(trimethylsilyl) ester	13112580	23608368	23.425	64
Pyrazine,3-ethyl-2,5-dimethyl	3699352.67	18753311.33	23.582	83
Benzene,1-methyl-4-(1-methylethenyl)	10540661.7	9821596	23.996	96
Nonanal	3582389	5082313.667	24.267	91
3-Hydroxymandelic acid, ethyl ester, di-TMS	245617199	249946235.7	24.595	53
Pyrazine,3,5-diethyl-2-methyl	4685218	7314181	26.389	41
1-Dodecane	31315966.7	40788765.33	27.39	96
Pyridine, 2-Butyl	5254395.67	9039639	27.728	87
Ethanol, phenoxy	7671781	6924037.667	28.496	90
Benzothiazole	246087153	18157997.33	28.805	91
D-Carvone	3538495.33	12392784.67	29.276	96
2-Chloro-4-(4-methoxyphenyl)-6-(4-nitrophenyl) pyrimidine	14739506	17882631.5	29.428	43
Sulforous acid, isohexyl 2-pentyl ester or 2-Decenal, (E)	13141281.3	14451404.67	29.626	86
2-Hydroxy-iso-butyrophenone	6400929.33	10818169.67	30.394	56
2n Octylfuran	3825391.33	4108139.667	30.522	93
Dimethylpyridin	1394319	1714872.5	31.828	30
E-Tetradec-2-enal	5098933.67	7487143	32.216	91
2(3H)-Furanone, dihydro-5 pentyl	1845947.67	2659569.667	32.292	72
Phenol,4-[1,1-dimethylpropyl]-	1084755.67	2054120	33.08	70
Dodecanal	2067531	4475783.667	33.145	95
Cyclododecane	4661380	13438220.33	35.983	94
Homosalate	21939242.3	15354381	40.23	83
3-Amino-4,6-dimethylpyridine-2(1H)	25114866.5	15354381	40.23	43
2-Ethylhexyl salicylate	6497587	6714596	39.189	99

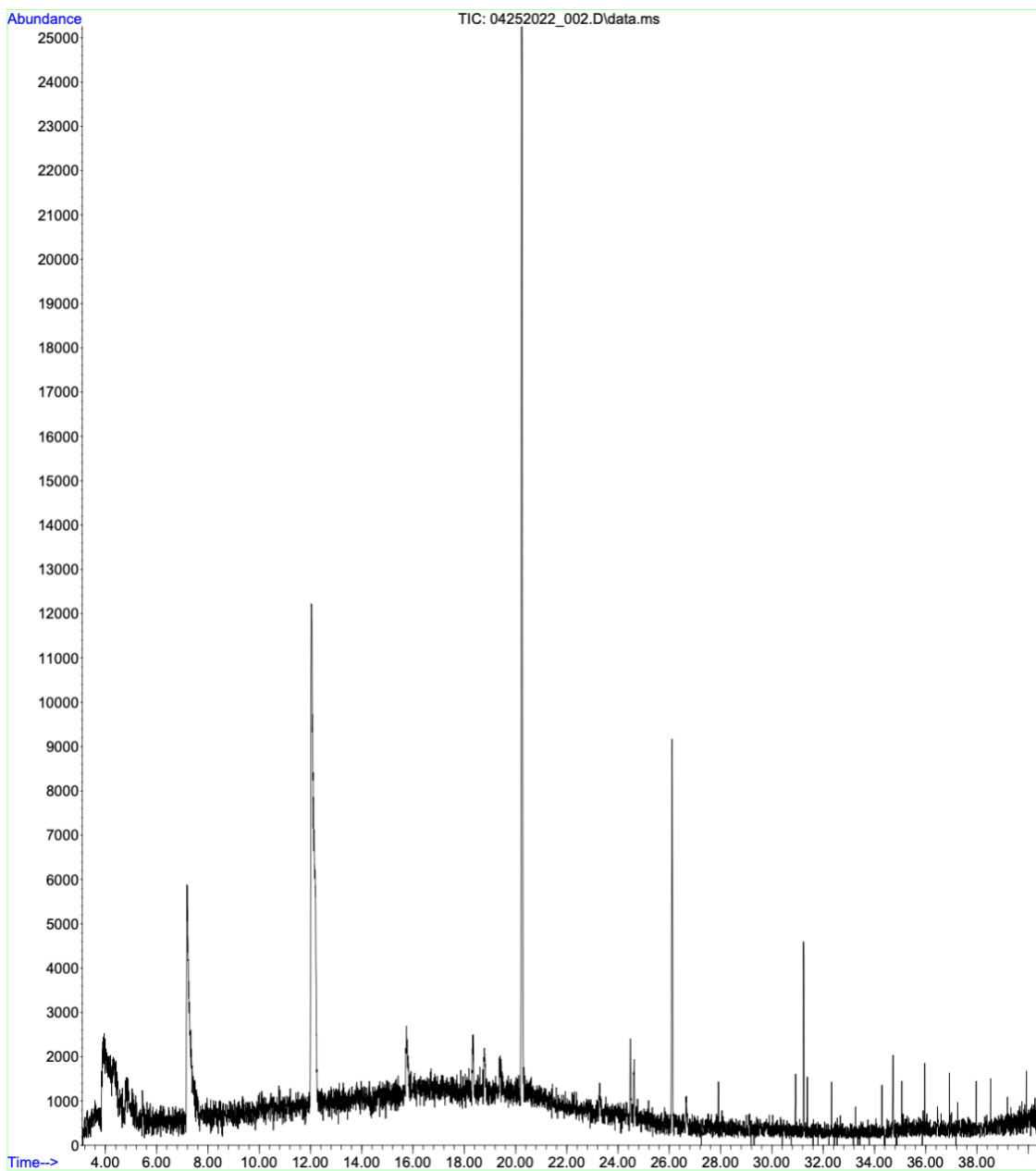


Figure A- 12. Chromatogram of blank SPME injection of 75 μm carboxen/polydimethylsiloxane (CAR/PDMS) SPME, fused silica, 23 Ga fiber from Supelco Inc (Bellefonte, Pa., U.S.A).

GC-MS conditions: 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 $^{\circ}\text{C}$ for 2 min, ramped to 180 $^{\circ}\text{C}$ at a rate of 10 $^{\circ}\text{C}/\text{min}$, and increased to 280 $^{\circ}\text{C}$ at a rate of 25 $^{\circ}\text{C}/\text{min}$, and held for 5 min.

Triangle test on chicken

Evaluate 3 samples in the plate labeled as 641, 305, and 509.

You may evaluate by taste, smell, color, chewiness and any other sensory parameter.

From these three identify which sample is different.

Record your answer in the box:

 top → right → left

Figure A- 13. Triangle test evaluation guide example.

Data chicken;
input aa \$ concentration rep trt \$ Process Delta E;
datalines;

Trp	0.25	1	1	Heated	32.08
Trp	0.25	2	1	Heated	32.61
Trp	0.25	3	1	Heated	34.21
Trp	0.25	1	1	Unheated	13.16
Trp	0.25	2	1	Unheated	11.65
Trp	0.25	3	1	Unheated	14.06
Trp	0.5	1	2	Heated	34.00
Trp	0.5	2	2	Heated	36.54
Trp	0.5	3	2	Heated	36.82
Trp	0.5	1	2	Unheated	16.31
Trp	0.5	2	2	Unheated	13.92
Trp	0.5	3	2	Unheated	14.75
Trp	0.75	1	3	Heated	29.06
Trp	0.75	2	3	Heated	29.23
Trp	0.75	3	3	Heated	29.23
Trp	0.75	1	3	Unheated	13.20
Trp	0.75	2	3	Unheated	14.41
Trp	0.75	3	3	Unheated	12.37
Lys	0.25	1	4	Heated	38.99
Lys	0.25	2	4	Heated	40.04
Lys	0.25	3	4	Heated	40.15
Lys	0.25	1	4	Unheated	12.43
Lys	0.25	2	4	Unheated	11.41
Lys	0.25	3	4	Unheated	11.82
Lys	0.5	1	5	Heated	32.93
Lys	0.5	2	5	Heated	33.60
Lys	0.5	3	5	Heated	27.24
Lys	0.5	1	5	Unheated	13.30
Lys	0.5	2	5	Unheated	13.94
Lys	0.5	3	5	Unheated	12.33
Lys	0.75	1	6	Heated	28.33
Lys	0.75	2	6	Heated	29.42
Lys	0.75	3	6	Heated	27.21
Lys	0.75	1	6	Unheated	14.30
Lys	0.75	2	6	Unheated	14.69
Lys	0.75	3	6	Unheated	15.73
Trp	0	1	7	Heated	41.29
Trp	0	2	7	Heated	41.21

Lys	0	3	7	Heated	41.74
Trp	0	1	7	Unheated	19.22
Trp	0	2	7	Unheated	19.38
Trp	0	3	7	Unheated	18.57

```

;
proc print data=chicken; run;
proc glimmix data=chicken;
class trt process;
model Delta E=trt process trt*process;
random rep;
lsmeans trt/ pdiff lines adjust=tukey;
lsmeans trt*process/ pdiff lines adjust=tukey;
run;

```

Table A- 2. SAS code example used in chapter 2.

```

Data chicken;
input aa $ concentration rep trt $ PhIP;
datalines;

```

Lys	15	1	6	23.22
Lys	15	2	6	29.77
Lys	15	3	6	18.51
Lys	30	1	1	30.95
Lys	30	2	1	33.18
Lys	30	3	1	27.28
Lys	1	1	2	32.26
Lys	1	2	2	35.53
Lys	1	3	2	35.40
Lys	3	1	3	32.91
Lys	3	2	3	35.53
Lys	3	3	3	32.26
Lys	6	1	4	18.24
Lys	6	2	4	15.89
Lys	6	3	4	23.09
Control	0	1	5	87.93
Control	0	2	5	104.30
Control	0	3	5	99.06

```

;
proc print
data=chicken;
run;
proc glimmix
data=chicken;
class trt;
model
PhIP=trt;
random rep;
lsmeans trt/
pdiff lines
adjust=tukey;
run;

```

Table A- 3. SAS code example used in chapter 3 and 4.