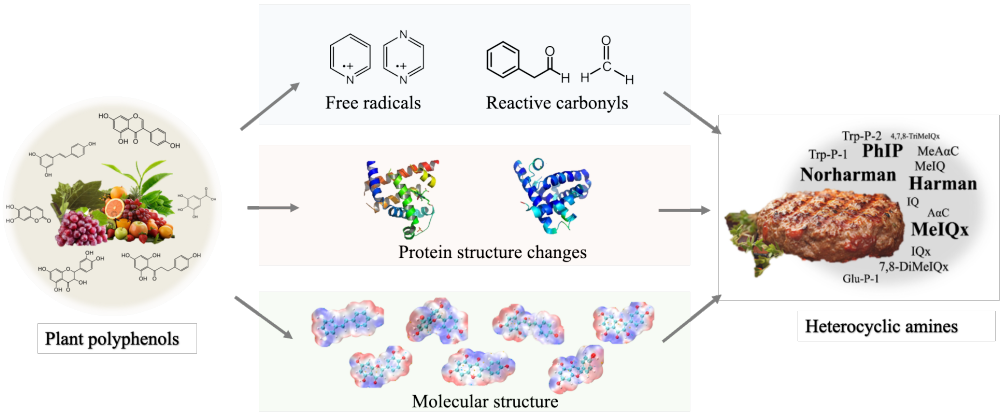
LIÈGE université
Gembloux
Agro-Bio Tech

Molecular mechanisms of plant polyphenols inhibiting the formation of heterocyclic amines in roasted meat



Author: Xiaoyue YANG

Supervisor: Prof. Christophe BLECKER

Co-supervisor: Prof. Zhenyu WANG

Year: 2025

COMMUNAUTÉ FRANÇAISE DE BELGIQUE
UNIVERSITÉ DE LIÈGE – GEMBLOUX AGRO-BIO TECH

Molecular mechanisms of plant polyphenols inhibiting the formation of heterocyclic amines in roasted meat

Xiaoyue Yang

Mécanismes moléculaires d'inhibition de la formation d'amines hétérocycliques dans la viande grillée par les polyphénols végétaux

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Agricultural Science and Biological Engineering

Promoteurs: Prof. Christophe Blecker
Prof. Zhenyu Wang
Année civile: 2025

`

Abstract

Xiaoyue Yang (2025). "Molecular mechanisms of plant polyphenols inhibiting the formation of heterocyclic amines in roasted meat" (PhD Dissertation in English).
Gembloux, Belgium, Gembloux Agro-Bio Tech, University of Liege.

Harmful substances such as heterocyclic amines (HAs) generated during the roasting process seriously affect the food safety of meat products. Polyphenols, as natural antioxidants, have been shown to have significant effects in inhibiting the generation of HAs. However, due to the wide variety of polyphenols and HAs, the inhibition patterns between polyphenols of different structures and specific HAs and their mechanisms of action are still unclear, and in addition, there is a lack of systematic studies on the mechanisms of polyphenols' effects on HAs generated by proteins involved in the production of HAs. This study systematically investigated the inhibitory effects and molecular mechanisms of polyphenols with different structural characteristics on the formation of free and protein-bound HAs by establishing model systems from three perspectives: the grilling process, protein interactions, and the structural features of polyphenols.

Firstly, seven polyphenols with different classes and structural features, including m-hydroxyl and o-hydroxyl groups, were selected and added to lamb patties for roasting to investigate their inhibitory effects on various types of HAs. The inhibitory efficiency of different types of polyphenols on total HAs formation in roasted meat followed the order: stilbenes ≈ coumarins ≈ chalcones > flavonoids > phenolic acids. Cluster analysis and validation using model systems further demonstrated that polyphenols with m-hydroxyl structures have a stronger inhibition of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), 1-methyl-9H-pyrido[3,4-b]-indole (Harman) and 9H-pyrido[3,4-b]indole (Norharman) by capturing the reaction intermediate carbonyl compounds, while those with the o-hydroxyl group had a stronger inhibitory effect on 2-amino-3,8-dimethyl-imidazo[4,5-f]quinoxaline (MeIQx) by scavenging free radicals.

Secondly, the effects of resveratrol and HAs on the conformational changes of sarcoplasmic protein (SP) and myofibrillar protein (MP) were evaluated to investigate the inhibitory patterns and mechanisms of polyphenols on protein-bound HAs. The results showed that resveratrol effectively inhibited both free and bound HAs formation mediated by MP, while for SP, it only suppressed the formation of free HAs. Moreover, the addition of polyphenols promoted the conversion of bound HAs into their free forms. The observed changes in free amino acid content detected only in the SP system suggest that polyphenols mainly inhibit the formation of protein-bound HAs by preventing MP from directly replacing amino acids in the reaction. Moreover, polyphenols not only suppress the generation of free HAs but may also reduce overall HAs content by facilitating the binding of free HAs with SP. Furthermore, analysis of

resveratrol's effects on the secondary structure, fluorescence intensity, and molecular docking with MP revealed strong interactions between resveratrol and MP, primarily through hydrogen bonding and hydrophobic interactions. These interactions reduce the exposure of amino acids involved in HAs formation and increase the exposure of non-precursor amino acids, thereby regulating the formation of MP-bound HAs. Additionally, the effects of resveratrol and free HAs on the particle size, surface hydrophobicity, and intermolecular forces of SP suggest that free HAs promote the formation of bound HAs by altering the surface hydrophobicity of the SP solution. Polyphenols may influence the formation of SP-bound HAs by modulating ionic and disulfide bond interactions.

Finally, a combination of experimental analysis and theoretical calculations was used to investigate the molecular characteristics of various polyphenols containing m-hydroxyl groups and their correlation with the ability to trap reactive carbonyl compounds or inhibit PhIP formation. The results showed that the effectiveness of polyphenols in inhibiting PhIP formation was closely related to the concentration of reactive carbonyl intermediates during the reaction but was not directly associated with the carbonyl-trapping capacity of the polyphenols. Furthermore, analysis of the effects of polyphenols on the thermal degradation of phenylalanine indicated that their inhibitory effect on PhIP formation involves not only the trapping of carbonyl compounds but also the suppression of phenylalanine pyrolysis. In addition, the HOMO–LUMO gap of the polyphenols showed a significant positive correlation with their inhibition rate of major carbonyl compounds derived from phenylalanine degradation, while exhibiting a highly significant negative correlation with the binding energy of polyphenol–phenylacetaldehyde adducts. These findings suggest that the HOMO–LUMO gap of a polyphenol molecule can serve as a predictive indicator of its effectiveness in inhibiting PhIP formation.

In summary, this study systematically investigated the inhibitory patterns and underlying mechanisms of HAs formation in roasted meat by polyphenols with different structural features. The inhibitory effect of polyphenols on HAs formation is closely related to their molecular structure. As exogenous additives, polyphenols can competitively bind to key precursors (such as amino acids or proteins) or intermediates (such as free radicals or carbonyl compounds) involved in HAs formation, thereby effectively suppressing their formation. This study provides a theoretical basis for screening efficient HAs inhibitors and offers scientific support for the development of healthier and safer meat products.

Key words: Heterocyclic amines, Polyphenols, Reactive carbonyls, Muscle proteins, Structural characteristics, Interaction, Density functional theory (DFT)

Résumé

Xiaoyue Yang (2025). "Mécanismes moléculaires d'inhibition de la formation d'amines hétérocycliques dans la viande grillée par les polyphénols végétaux" (Thèse de doctorat en anglais).
Gembloux, Belgique, Gembloux Agro-Bio Tech, Université de Liège.

Des substances nocives telles que les amines hétérocycliques (AHs), générées lors du processus de cuisson à haute température, peuvent gravement affecter la sécurité sanitaire des produits carnés. Les polyphénols, en tant qu'antioxydants naturels, ont démontré une efficacité significative dans l'inhibition de la formation des AHs. Cependant, en raison de la grande diversité des polyphénols et des AHs, les modes d'inhibition spécifiques entre différentes structures de polyphénols et types d'AHs, ainsi que leurs mécanismes d'action, restent encore mal compris. De plus, il existe un manque d'études systématiques sur les mécanismes par lesquels les polyphénols influencent la formation des AHs induite par les protéines. La présente étude a exploré de manière systématique les effets inhibiteurs et les mécanismes moléculaires des polyphénols possédant différentes caractéristiques structurelles sur la formation des AHs libres et liés aux protéines, en établissant des systèmes modèles selon trois approches : le processus de cuisson, les interactions protéiques et les caractéristiques structurelles des polyphénols.

Tout d'abord, sept polyphénols appartenant à différentes classes et présentant des caractéristiques structurelles variées, notamment des groupes hydroxyles en méta et en ortho, ont été sélectionnés et ajoutés à steaks hachés d'agneaux avant cuisson afin d'évaluer leur effet inhibiteur sur différents types d'amines hétérocycliques (AHs). L'efficacité inhibitrice des différents types de polyphénols sur la formation totale des AHs dans la viande rôtie suit l'ordre suivant : stilbènes ≈ coumarines ≈ chalcones > flavonoïdes > acides phénoliques. Une analyse en grappes et une validation par des systèmes modèles ont en outre montré que les polyphénols présentant une structure m-hydroxyle inhibent plus fortement la formation de PhIP, Harman et Norharman en capturant les composés carbonylés intermédiaires, tandis que ceux possédant un groupe o-hydroxyle exercent un effet inhibiteur plus marqué sur MeIQx en neutralisant les radicaux libres.

Deuxièmement,, les effets du resvératrol et des HAs sur les changements conformationnels des protéines sarcoplasmiques (SP) et myofibrillaires (MP), principales protéines de la viande, ont été évalués afin d'étudier les profils inhibiteurs et les mécanismes des polyphénols sur les HAs liées aux protéines. Les résultats ont montré que le resvératrol inhibait efficacement la formation de HAs libres et liés médiée par MP, tandis que pour SP, il a seulement supprimé la formation de HAs libres. En outre, l'addition de polyphénols a favorisé la conversion des ap liées dans leurs formes libres. Les changements observés dans la teneur en acides aminés libres détectés uniquement dans le système SP suggèrent que les polyphénols inhibent

principalement la formation d'HAs liés aux protéines en empêchant les MP Prendre le rôle le rôle directement des acides aminés dans la réaction. En outre, l'analyse des effets du resvératrol sur la structure secondaire, l'intensité de fluorescence, et l'amorce moléculaire avec MP a révélé de fortes interactions entre le resvératrol et MP, principalement par liaison d'hydrogène et interactions hydrophobes. Ces interactions réduisent l'exposition des acides aminés impliqués dans la formation d'as et augmentent l'exposition des acides aminés non précurseurs, régulant ainsi la formation d'as lié au mp. De plus, les effets du resvératrol et des ap libres sur la taille des particules, l'hydrophobicité de surface et les forces intermoléculaires de SP suggèrent que les ap libres favorisent la formation de ap liées en modifiant l'hydrophobicité de surface de la solution de SP. Les polyphénols peuvent influer sur la formation d'as liés à la sp-en modulant les interactions entre les liaisons ionique et disulfure.

Enfin, une combinaison d'analyses expérimentales et de calculs théoriques a été utilisée pour étudier les caractéristiques moléculaires de divers polyphénols contenant des groupes méta-hydroxyle et leur corrélation avec la capacité de piéger des composés carbonyliques réactifs ou d'inhiber la formation de PhIP. Les résultats ont montré que l'efficacité des polyphénols dans l'inhibition de la formation de phips était étroitement liée à la concentration des intermédiaires carbonyliques réactifs pendant la réaction, mais n'était pas directement associée à la capacité de piégeage du carbone des polyphénols. De plus, l'analyse des effets des polyphénols sur la dégradation thermique de la phénylalanine a indiqué que leur effet inhibiteur sur la formation du PhIP implique non seulement le piégeage des composés carbonyliques, mais aussi la suppression de la pyrolyse de la phénylalanine. En outre, l'écart HOMO-LUMO des polyphénols a montré une corrélation positive significative avec leur taux d'inhibition des principaux composés carbonyliques dérivés de la dégradation de la phénylalanine, tout en présentant une corrélation négative très significative avec l'énergie de liaison des adduits polyphénol-phénylacétaldéhyde. Ces résultats suggèrent que l'écart HOMO-LUMO d'une molécule de polyphénol peut servir d'indicateur prédictif de son efficacité à inhiber la formation de phips.

En résumé, cette étude a systématiquement étudié les schémas inhibiteurs et les mécanismes sous-jacents de la formation de HAs dans la viande rôtie par des polyphénols aux caractéristiques structurelles différentes. L'effet inhibiteur des polyphénols sur la formation de HAs est étroitement lié à leur structure moléculaire. En tant qu'additifs exogènes, les polyphénols peuvent se lier de façon concurrentielle aux précurseurs clés (tels que les acides aminés ou les protéines) ou aux intermédiaires (tels que les radicaux libres ou les composés carbonyliques) impliqués dans la formation d'HAs, inhibant ainsi efficacement leur formation. Cette étude fournit une base théorique pour le dépistage efficace des inhibiteurs de HAs et offre un soutien scientifique pour le développement de produits carnés plus sains et plus sûrs.

Mots clés: amines hétérocycliques, polyphénols, carbonyles réactifs, protéines musculaires, caractéristiques structurelles, Interaction, théorie des fonctions de densité (DFT)

Acknowledgment

I once thought the acknowledgements would be the easiest part of my dissertation to write—yet, ironically, it has become the hardest to begin. Looking back on the journey from the college entrance exam to where I am now, especially these past four years of doctoral study, it feels as if life has been running with a loading bar, and the loading becomes increasingly difficult as time goes by. I've lost count of the sleepless nights and the countless obstacles and moments of doubt along the way, but fortunately, I made it through—one step at a time. But I am deeply aware that I could not have made it this far without the help and support of so many people along the way.

Firstly, I would like to express my deepest gratitude to my supervisors: Prof. Christophe Blecker, Prof. Dequan Zhang, and Prof. Zhenyu Wang. Prof. Blecker's encouragement, support, and confidence in me have been a source of warmth and hope throughout my time studying abroad. Without the unwavering support and guidance of Prof. Zhang and Prof. Wang, I would not have been able to achieve what I have today. You have provided invaluable support at every critical point throughout my four years of research, for which I am truly grateful. I would also like to express my sincere thanks to my thesis committee and jury members—Prof. Aurore Richel, Prof. Giorgia Purcaro, Prof. Nicolas Jacquet, and Prof. Mohamed Ali Ayadi—for their dedicated guidance and insightful comments on my research.

Secondly, I would like to thank Chengli Hou, Li Chen, Xin Li, Xiaochun Zheng, Shaobo Li, Shanshan Liu, Fei Fang, Xiangru Wei, Caiyan Huang, Yuqiang Bai, Haonan Shi, Chi Ren, Xiangxiang Sun, Minghui Gu, Simin Fan, Yuqian Xu, Rongmei Gao, Ruotong Nie, Dejuan Meng and Can Xiang for their tremendous support in both my research and academic life at the Institute of Food Science and Technology of Chinese Academy of Agricultural Sciences. I would like to thank Haopeng Lin, Xiaoqian Zhou, and Haiguang Tan for their invaluable assistance and technical support with the use of laboratory instruments. I would like to thank Ziwu Gao, Jinhua He, Haijie Wang, Linggao Liu, Jiale Ma, and Anqi Xu for their companionship, which brought me so much joy throughout this journey. I am also deeply grateful to Majorie Servais, Claire Ndayisenga, Junmei Liu, Jin Xie, Fangzhou Wang, Meng Liu, Jingfan Wang, Xiaoyan Liang, Xiaoxian Liu, Ling Li, Guanmo Li, Mariem Boukraâ, and Alfred Kouassi Kouakou for their help and companionship during my time in the University of Liège.

Lastly, my heartfelt thanks go to my family. Every step of my journey has been accompanied by your love and support, and I am truly grateful for everything you have done for me. I have always considered myself truly fortunate to have met so many kind and supportive people at every stage of my life. It is because of your help and encouragement that I have become who I am today. I sincerely wish you all the very best and a bright, successful future.

Xiaoyue Yang

21/05/2025 in Gembloux, Belgium

List of figures

List of tables

List of abbreviations

4,7,8-TriMeIQx	2-amino-3,4,7,8-tetramethylimidazo[4,5-f]-quinoxaline
4,8-DiMeIQx	2-amino-3,4,8-trimethyl-3H-imidazo[4,5-f]-quinoxaline
7,8-DiMeIQx	2-amino-3,7,8-trimethyl-3H-imidazo[4,5-f]-quinoxaline
AαC	2-amino-9H-pyrido[2,3-b]indole
ABTS	2, 2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
ANS	8-anilinonaphthalene-1-sulfonic acid
AIAs	aminoimidazoazoarenes
Ala	Alanine
DFT	density functional theory
DMIP	2-amino-1,6-dimethylimidazo[4,5-b]-pyridine
DPPH	1,1-Diphenyl-2-picrylhydrazyl radical 2,2-Diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl
ESI	electrospray ionization
ESR	electron spin resonance
FT-IR	Fourier Transform Infrared Spectroscopy
GC	gas chromatography
Glu	Glutamate
Glu-P-1	2-Amino-6-methyldipyrido[1-2-a:3'-2'-d]imidazole
Glu-P-2	2-Aminodipyrido[1,2-a: 3 ', 2'-d]imidazole
HAs	heterocyclic amines
Harman	1-methyl-9H-pyrido[3,4-b]-indole
HPLC	high-performance liquid chromatography
IARC	International Agency for Research on Cancer
IQ	2-amino-3-methylimidazo[4,5-f]quinoline
IQ-type	imidazoquinoline-type
IQx	imidazoquinoxaline
LC	liquid chromatography
Lys	Lysine
MCs	major carbonyl compounds
MeAαC	2-amino-3-methyl-9H-pyrido[2,3-b]indole
MeIQ	2-amino-3,4-dimethyl-imidazo[4,5-f]quinoline
MeIQx	2-amino-3,8-dimethyl-imidazo[4,5-f]quinoxaline
MP	myofibrillar proteins

MS	mass spectrometry
Norharman	9H-pyrido[3,4-b]indole
NTP	National Toxicology Program
Phe	phenylalanine
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine
Pro	Proline
QuEChERS	quick, easy, cheap, effective, and safe extraction
SOD	superoxide dismutase
SP	sarcoplasmic proteins
SPE	solid-phase extraction
TD_{50}	Median toxic dose
THβC	tetrahydro-β-carboline
Trp-P-2	3-amino-1-methyl-5H-pyrido[4,3-b]indole
Trp-P-1	2-amino-1,6-dimethyl-furo[3,2-e]imidazo[4,5-b]-pyridine
Tyr	Tyrosine
VEA	Vertical electron affinity
VIP	Vertical ionization potential

1

Chapter I General introduction

1. Context and objectives

1.1 Context

Heterocyclic amines (HAs) are carcinogenic and mutagenic compounds commonly found in protein-rich foods, such as meat and fish, cooked at high temperatures. To date, over 30 types of HAs have been identified in various foods (Cheng et al., 2006). The concentration of HAs in cooked meat can range from 1 to 500 ng/g depending on the cooking conditions and the type of meat (Alaejos, & Afonso, 2011; Sinha et al., 1995). Epidemiological studies indicate that excessive consumption of cooked red meat increases the risk of colorectal cancer, likely due to HAs exposure (Boada et al., 2016). The total dose required to induce tumor formation (TD_{50}) varies among different HAs and depends on the host species. Reported TD_{50} values in rodents range from 0.1 to 64.6 mg/kg/day for individual HAs (Sugimura et al., 2004). The International Agency for Research on Cancer (IARC) has classified several heterocyclic amines (HAs) as possible human carcinogens (Group 2B), including 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx), 2-amino-3,4-dimethylimidazo[4,5-f]quinoline (MeIQ), and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). Additionally, 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) is categorized as a probable human carcinogen (Group 2A) (Jinap et al., 2013; Barzegar, 2019). The results from the previous researchers found that there were 0.08-127.2ng/g HAs in charcoal grilled lamb patties (Suleman et al., 2019). Control the levels of HAs in roasted meats is important to ensure the safety of the consumers.

According to their presence in food, HAs can be categorized into free and bound states (Kataoka et al., 2010; Szterk, 2013). Creatine, amino acids, as well as reducing sugars are the main precursors for the formation of free HAs (Wang, Hong, Ke, Hu, & Chen, 2017). HAs can be easily formed through Maillard reaction (150–200 °C) and amino acid or protein pyrolysis (above 250 °C) under high temperature treatments (Meurillon, & Engel, 2016). Reducing sugars and amino acids undergo Maillard and Strecker degradation reactions to form pyridines and pyrroles, which can further react with creatinine and aldehydes to produce IQ-type HAs. Carbonyl compounds and free radicals generated during these reactions serve as key intermediates. The discovery of bound HAs has brought into view that proteins also play an important role in the formation of HAs. There are currently two pathways for the formation of bound HAs: (1) Firstly, free HAs are formed by the reaction of free amino acids, produced by protein degradation or originally present in the system, with other precursors. Then the free HAs bind to the protein to form protein-bound HAs; (2) Protein-bound HAs are directly formed by some groups in the protein peptide chain that react with glucose and creatinine (Chen et al., 2021). And the bound HAs will be released after gastrointestinal digestion in humans (Szterk, 2013; Xue et al., 2022).

A lot of studies have shown that reasonable addition of some natural antioxidants, especially phenolic compounds (protocatechuic acid, chlorogenic acid, ferulic acid,

and p-coumaric acid) and flavonoids (naringenin, quercetin, luteolin, and rutin) can effectively inhibit the production of HAs and different types of polyphenols have different inhibitory effects on HAs formation (Zeng et al., 2016; Zhu, Zhang, Wang, Chen, & Zheng, 2016). It has been widely accepted that their capacity to scavenge and trap free radicals and active carbonyl groups involved in the HAs formation pathways contributes their inhibitory effects on the formation of HAs (Meurillon & Engel, 2016; Viegas, Amaro, Ferreira, & Pinho, 2012). These scavenging abilities are closely related to the free radical scavenging and trapping active carbonyl domains that their structures possess (Zamora & Hidalgo,2016). But it is unclear which antioxidant regions in polyphenols has the effect the formation of which heterocyclic amines and it is also not clear the mechanism of molecular interactions between the plant polyphenols and the inducing factors of HAs formation and the mechanism of action of polyphenols on bound HAs involved in the formation of muscle proteins.

1.2 Objectives

The general objective of the present study is to identify the patterns of different structural polyphenols inhibiting the generation of different heterocyclic amines and to systematically investigate the molecular mechanisms of polyphenols inhibiting heterocyclic amines in roasted meat at three levels: in situ meat, protein, and molecular structure. This work aims to provide valuable insights for the screening of polyphenols that can effectively inhibit the production of heterocyclic amines, and to provide an important reference for the control of HAs production during the processing of high-protein foods. In details:

(1) To confirm the inhibition rule of different polyphenols on heterocyclic amines in roasted meat, and verified in the model system;

(2) To clarify the effect of resveratrol on myofibrillar and sarcoplasmic proteins involved in the formation of HAs through a protein-based model system, and to further explore the underlying mechanism by examining changes in the structural and physicochemical properties of proteins induced by resveratrol;

(3) To investigate the correlation between the inhibitory effects of different polyphenols on PhIP and their molecular structures using model system experiments combined with density functional theory (DFT) calculations.

Based on the above objectives, the research roadmap, outline and structure of thesis are completed in the following content.

2. Research roadmap and outline

2.1 Research roadmap

The research roadmap is displayed in **Figure 1-1**.

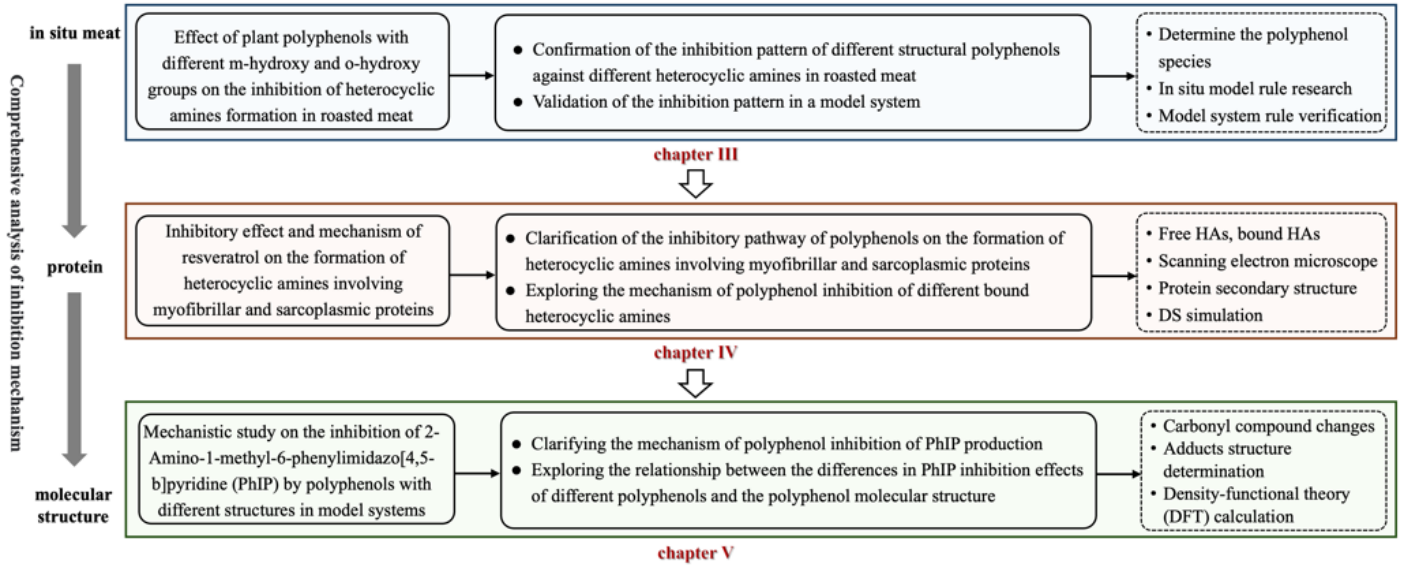


Figure 1-1: Research roadmap

2.2 Outline

In chapter II, the literature review summarizes the recent research progress on the hazards of heterocyclic amines, their formation mechanisms and their control strategies, and focuses on the mechanisms of polyphenols in inhibiting the production of heterocyclic amines as well as their *in vivo* modulation of heterocyclic amine hazards in humans.

In chapter III, the inhibition pattern of different structural polyphenols on the formation of HAs in roasted lamb meat was revealed by adding different structural polyphenols to the roasted lamb patties and determining the changes in the content of heterocyclic amines. Then, polyphenols with strong and regular inhibitory effects were selected to verify the speculated inhibitory ways of polyphenols on different HAs in the model systems.

In chapter IV, based on the chapter III, resveratrol, a polyphenol with good HAs inhibition effect in roast meat, was selected, and the effect of resveratrol-muscle protein interaction on HAs production was systematically explored through the muscle protein model system. Meanwhile, the pathways and mechanisms by which polyphenols inhibit muscle proteins involved in heterocyclic amine production were also elucidated.

In chapter V, on the basis of Chapter III, the mechanism of polyphenol inhibition of PhIP generation was further verified, and the correlation between the quantum chemical parameters of different structural polyphenols and their ability to capture carbonyl compounds was clarified through experiments coupled with density-functional theory (DFT) calculations to further explore the molecular mechanism of action of the inhibition of PhIP by polyphenols of different structures.

In Chapter VI, the relationships between the structural characteristics of polyphenols and proteins and their inhibitory effects on the formation of HAs are comprehensively discussed based on existing studies. Moreover, potential industrial applications are also highlighted. Various mechanisms by which plant polyphenols inhibit HAs formation are summarized. Finally, the limitations of current research are analyzed, and prospects for future investigations are presented.

3. References

Alaejos, M. S., & Afonso, A. M. (2011). Factors that affect the content of heterocyclic aromatic amines in foods. *Comprehensive Reviews in Food Science and Food Safety, 10*, 52e108.

Boada, L. D., Henríquez-Hernández, L. A., & Luzardo, O. P. (2016). The impact of red and processed meat consumption on cancer and other health outcomes: Epidemiological evidences. *Food and Chemical Toxicology: an international journal published for the British Industrial Biological Research Association, 92*, 236–244.

Barzegar, F., Kamankesh, M., & Mohammadi, A. (2019). Heterocyclic aromatic amines in cooked food: A review on formation, health risk-toxicology and their analytical techniques. *Food Chemistry, 280*, 240–254.

Chen, Q., Xue, C., He, Z., Wang, Z., Qin, F., Chen, J., & Zeng, M. (2021). Generation of Sarcoplasmic and Myofibrillar Protein-Bound Heterocyclic Amines in Chemical Model Systems under Different Heating Temperatures and Durations. *Journal of Agricultural and Food Chemistry, 69*(10), 3232–3246.

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

Jinap, S., Mohd-Mokhtar, M. S., Farhadian, A., et al. (2013). Effects of varying degrees of doneness on the formation of Heterocyclic Aromatic Amines in chicken and beef satay. *Meat Science, 94*(2), 202–207.

Kataoka, H., Miyake, M., Nishioka, S., Matsumoto, T., Saito, K., & Mitani, K. (2010). Formation of protein adducts of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine in cooked foods. *Molecular Nutrition & Food Research, 54*(7), 1039–1048.

Meurillon, M., & Engel, E. (2016). Mitigation strategies to reduce the impact of heterocyclic aromatic amines in proteinaceous foods. *Trends in Food Science & Technology,50*, 70–84.

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

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

Suleman, R., Hui, T., Wang, Z., Liu, H., & Zhang, D. (2019). Comparative analysis of charcoal grilling, infrared grilling and superheated steam roasting on the colour, textural quality and heterocyclic aromatic amines of lamb patties. *International*

Journal of Food Science and Technology, 55, 1057–1068.

Szterk, A. (2013). Chemical state of heterocyclic aromatic amines in grilled beef: Evaluation by in vitro digestion model and comparison of alkaline hydrolysis and organic solvent for extraction. *Food and Chemical Toxicology: An International Journal Published for the British Industrial Biological Research Association, 62*, 653–660.

Wang, P., Hong, Y., Ke, W., Hu, X., & Chen, F. (2017). Formation of heterocyclic amines in Chinese marinated meat: Effects of animal species and ingredients (rock candy, soy sauce and rice wine). *Journal of the Science of Food and Agriculture, 97*(12), 3967–3978.

Zeng, M., Li, Y., He, Z., et al. (2016). Discrimination and investigation of inhibitory patterns of flavonoids and phenolic acids on heterocyclic amine formation in chemical model systems by UPLC-MS profiling and chemometrics. *European Food Research and Technology, 242*(3), 313–319.

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

Meurillon, M., & Engel, E. (2016). Mitigation strategies to reduce the impact of heterocyclic aromatic amines in proteinaceous foods. *Trends in Food Science & Technology, 50*, 70–84.

Viegas, O., Amaro, L. F., Ferreira, I. M. P. L. V. O., & Pinho, O. (2012). Inhibitory effect of antioxidant-rich marinades on the formation of heterocyclic aromatic amines in panfried beef. *Journal of Agricultural and Food Chemistry, 60*, 6235–6240.

Zamora, R., & Hidalgo, F. J. (2016). The triple defensive barrier of phenolic compounds against the lipid oxidation-induced damage in food products. *Trends in Food Science & Technology, 54*, 165-174.

Xue, C., Chen, Q., He, Z., Qin, F., Wang, Z., Chen, J., & Zeng, M. (2022). Release profiles of beef myofibril protein-bound heterocyclic amines and effects of dietary components on in vitro digestion. *Food Research International, 155*, Article 111006.

2

Chapter II Literature review on the inhibitory effects and mechanism of plant polyphenols on heterocyclic amines

1. Introduction

Heterocyclic amines (HAs) are a class of carcinogenic and mutagenic hazardous compounds that are formed mainly during high-temperature processing of protein-rich foods. Since their first discovery in the 1970s, at least 30 HAs have been identified in various types of foods (Sugimura et al., 1977; Gibis, 2016). According to their chemical structure, HAs can be classified into two major categories: Aminoimidazoazaarenes (AIAs) and Aminocarbolines (ACs). AIAs HAs are usually produced at 100 to 300℃, and thus are also called "thermic HAs"(Jägerstad et al., 1998). ACs are typically generated by the thermal decomposition of proteins or amino acids when heated above 300℃, and are therefore also known as "pyrolytic HAs"(Jägerstad et al., 1998). In addition, according to their presence in food, HAs can be categorized into free and bound states (Kataoka et al., 2010; Szterk, 2013). These HAs are widely present in various dairy products and meat products that have undergone high-temperature heating treatment, such as common processing methods like steaming, boiling, frying, grilling and deep-frying, and have become the main source of HAs intake in people's daily diets (Alaejos & Afonso, 2011; Liao et al., 2010). HAs are carcinogenic and mutagenic, and epidemiologic studies have shown that intake of processed meats can increase the risk of many types of cancer, which is closely related to the presence of HAs (Boada, Henríquez-Hernández， & Luzardo, 2016; Bukowska et al., 2023). Due to the wide distribution, high content and high toxicity of HAs, how to effectively reduce the generation of HAs during thermal processing has become an important issue of widespread concern in the field of food safety.

The formation of HAs is affected by a variety of factors, including processing temperature and time (Balogh et al., 2020; Bjeldanes et al., 1983), processing method (Dundar, Sarıçoban, & Yılmaz, 2012), type and content of precursors (Szterk, 2015), addition of antioxidants (Johansson & Jagerstad, 1996), moisture content (Lee, Lin, & Chan, 1994; Skog, Solvakov, Jgerstad, 2000), and pH changes (Buła et al., 2019). Therefore, the generation of HAs in processed meat products can be effectively reduced by optimizing the processing conditions, rationally selecting raw and auxiliary materials, and adding exogenous functional ingredients (Meurillon, & Engel, 2016). Among them, the addition of polyphenolic compounds is still regarded as one of the most effective and deeply studied strategies for inhibiting the formation of HAs at present (Khan et al., 2022). Besides the direct addition, the active ingredients that play a major role in many exogenous additives are also polyphenols (Khan et al., 2017). Due to the wide variety of polyphenols, their structural characteristics are closely related to the inhibition of HAs, but there is a lack of systematic analysis on the relationship between polyphenol structure and inhibition mechanism. In addition, polyphenols have been shown to reduce the toxicity of HAs *in vivo* by interfering with the binding of HAs to DNA (Lin et al., 2003), but their specific mechanism of action *in vivo* has not yet been fully elucidated, and still needs to be further explored.

This chapter systematically reviews the formation mechanism, potential hazards and regulatory strategies of HAs, with a focus on the inhibitory mechanism of polyphenols on free and bound HAs *in vitro*, as well as the intervention role of polyphenols in the health hazards induced by HAs *in vivo*. Through the in-depth analysis of the mechanism of HAs generation inhibition by polyphenolic compounds, this paper provides a solid theoretical basis for controlling the formation of heterocyclic amines in meat products, as well as scientific support and technical references to enhance the consumption safety and health value of meat products.

2. Hazards, formation mechanism and control strategies of heterocyclic amines

2.1 Hazards of heterocyclic amines

2.1.1 The carcinogenicity and mutagenicity of HAs

HAs have strong mutagenic activity. As early as 1939, the Swedish scientist Widmark found that mammary tumors could be induced in mice by applying a substance extracted from roasted horse meat to their backs (Widmark, 1939), although this did not attract sufficient attention at the time. With the improvement of screening test methods for carcinogenicity, teratogenicity and mutagenicity of food additives, Sigimura (Sugimura et al., 1977; Sugimura et al., 2004) and others found that a highly mutagenic substance, HAs, was detected by the Ames test in grilled fish roasted over an open fire or charcoal. The mutagenicity of heterocyclic amines was shown to be significantly higher than that of aflatoxin B1 and benzo[a]pyrene (Püssa, 2013). Although the Harman and Norharman are not mutagenic per se due to the lack of extracyclic amino structures, studies have shown that they can significantly enhance the mutagenic effects of other HAs (Kawamori et al., 2004; Totsuka et al., 2004), and their potential toxicity should not be ignored.

Heterocyclic amines are also a class of carcinogens that can induce tumors in the liver, prostate, lung, mammary gland, and colon of rodents and nonhuman primates (Esumi et al., 1989; Ito et al., 1991; Sugimura, 1992; Wakabayashi et al., 1992; Takahashi et al., 1993; Tamano et al., 1994). PhIP can increase the risk of prostate cancer in humans (Guo et al., 2022). Sugimura et al. (2004) determined the doses of various HAs required to induce tumors in 50% of rodents under standardized experimental conditions, reporting TD_{50} values ranging from 0.1 to 64.6 mg/kg/day. In 1993, the International Agency for Research on Cancer (IARC) defined IQ as a probable carcinogen (grade 2A), and defined MeIQ, MeIQx, PhIP, AαC, MeAαC, Trp-P-2, Trp-P-1, Glu-P-2 and Glu-P-1 as possible carcinogens (grade 2B) (IARC, 1993). This was followed by the inclusion of IQ, MeIQ, 8-MeIQx, and PhIP in the National Toxicology Program as contributors to human carcinogenesis in 2004 (NTP, 2004).

Table 2-1. Carcinogenicity of main HAs

Compound	Target organs	Species	Reference
PhIP	Mammary gland	Sprague–Dawley rat, CD rat	Ghoshal et al. (1994); EL-Bayoumy et al. (1995)
	Lymphoid tissue	CDF_1 mouse, F344 rat	Rao et al. (1996); Esumi et al. (1989)
	Prostate	F344 rat	Shirai et al. (1997)
	Colon	F344 rat	Ito et al. (1991); Hasegawa et al., (1993)
MeIQx	Prostate	Rats	Archer et al. (2000);
	Colon	F344 rats & humans	Garner et al. (1999); Tanakamaru et al. (2001);
	Liver	F344 rats	Kato et al. (1988); Kushida et al. (1994); Suzuki et al. (2002)
IQ	Liver	F344 rats, monkey, $B6C3F_1$ mouse, CDF_1 mouse, Sprague–Dawley rat	Takayama et al. (1984); Tanaka et al. (1985); Adamson et al. (1990); Ohgaki et al. (1984); Dooley et al. (1992)
	small and large intestine	F344 rats,	Takayama et al. (1984)
	Zymbal gland,	F344 rats, Sprague–Dawley rat	Takayama et al. (1984); Tanaka et al. (1985)
	clitoral gland	F344 rats,	Takayama et al. (1984)
AαC	Colon	A/J mouse	Kim et al. (2016)
	Liver	C57BL/6 mouse	Tang et al. (2013)
MeIQ	Liver	CDF_1 mouse,	Ohgaki et al. (1986); Huang et al. (2003)
	Forestomach	F344 mouse, CDF1mouse	Fujita et al. (1999)

2.1.2 The metabolism of HAs and the toxicity of HAs-DNA adducts

HAs bind to DNA during metabolism in vivo to form adducts, which are the basis for their carcinogenic or mutagenic effects (Turesky, & Vouros, 2004). Studies have shown that most carcinogens do not act directly upon ingestion, but require metabolic enzyme-mediated activation to trigger mutagenic effects in organisms, and HAs follow this mechanism (Felton et al., 1999). HAs are absorbed in the small intestine and transported to the liver, where they are metabolically activated by the cytochrome

P450 enzyme system (Lynchet al., 1995; Yamazo et al., 1988; Sadrieh, & Snyderwine, 1995). Within this enzyme family, CYP1A2 is the primary isoenzyme that catalyzes the molecular N-hydroxylation of HAs to produce N-hydroxylamines metabolites (Edwards et al., 1994; Frandsen et al., 1991; Boobis et al., 1994). Subsequently, N-hydroxylamines are further esterified by acetyltransferases, sulfotransferases, aminoacyl tRNA synthetases, or phosphokinases (Hein et al., 1994), and the resulting ester compounds are unstable and susceptible to degradation, resulting in the N-acetoxy amine intermediate (Sadrieh, Davis, & Snyderwine, 1996). This intermediate is highly reactive and is capable of forming exocyclic amino nitrogen ions (Turesky, & Le Marchand, 2011), which are key metabolites for toxicity and DNA damage, and their binding to DNA can induce base mutations, thereby increasing the risk of tumorigenesis.

Carcinogen-DNA adducts are recognized as biomarkers for assessing potential mutagenic effects and cancer risk (Hemminki, 1993). In mammalian cells, the adducts formed by the combination of HAs and DNA can cause a range of genotoxic responses by triggering sister chromatid exchanges, chromosomal aberrations, point mutations, DNA strand breaks, and inducing DNA repair synthesis (Schut, & Snyderwine, 1999). Among them, the DNA adducts formed by AIA-type HAs mainly cause base substitution mutations (G→T) and frameshift mutations in guanine bases (Morgenthaler, & Holzhäuser, 1995; Yadollahi-Farsani et al., 1996). In addition, short-term exposure to PhIP can induce DNA damage by increasing the phosphorylation level of histone H2AX (γH2AX) in the colon, and trigger oxidative stress by elevating malondialdehyde (MDA) levels while reducing the activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx) (Li et al., 2013; Chen et al., 2017b). MDA, as one of the end products of polyunsaturated fatty acid peroxidation in cells, is generally considered a biomarker of oxidative stress (Huang et al., 2018). The research of Zhao et al. (2021a) indicates that PhIP can disrupt the composition of the intestinal microbiota, interfere with the metabolism of glycerophospholipids and linoleic acid, and simultaneously inhibit the lipid metabolism function of the intestinal microbiota. Further transcriptomic analysis revealed that PhIP significantly inhibited the expression of genes related to metabolic pathways such as steroid hormone biosynthesis, fatty acid extension, fatty acid degradation, and glyceride metabolism.

2.2 Formation mechanism of heterocyclic amines

2.2.1 The formation of imidazoquinoline and imidazoquinoxaline

During the Maillard reaction, reducing sugars and amino acids are degraded by Strecker to form pyridine and pyrazine compounds respectively, which are the key precursors for the formation of IQ-type HAs. The reaction mechanism is shown in **Figure 2-1**a. Under heating conditions, creatine undergoes dehydration and cyclization to form creatinine, which then undergoes aldehyde-alcohol condensation reactions with pyridine or pyrazine derivatives respectively, thereby generating imidazoquinoline and imidazoquinoxaline (Skog, & Jägerstad, 1993). Aldehyde compounds and creatinine also play a significant role in the construction of imidazole

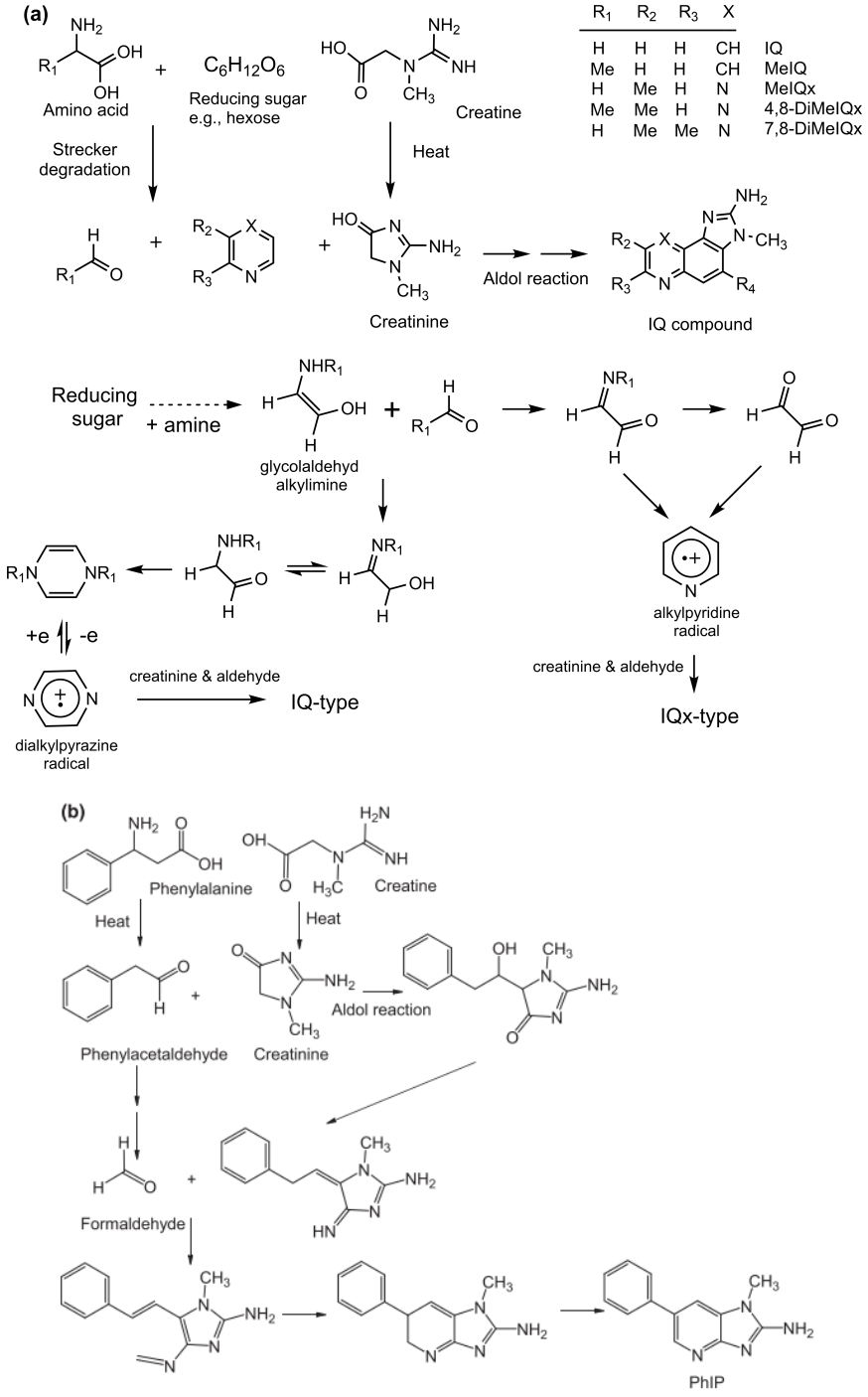
rings in HAs. Through the condensation reaction of aldehydes in the Strecker degradation products or the corresponding Schiff base with butanol aldehydes, the pyridine/pyrazine structure is partially connected to the creatinine part (Jägerstad et al., 1984). This proposed formation pathway has been confirmed through the structural identification of MeIQx and 7,8-DiMeIQx, both of which have been detected in model reaction systems composed of creatine, glycine, and glucose (Negishi et al., 1984). In addition, Pearson et al., (1992) proposed an alternative free radical–involved pathway, in which alkylpyridine and dialkylpyrazine radicals—formed from the reaction of reducing sugars and amine—further react with aldehydes and creatinine to produce quinoline or quinoxaline. However, this mechanism remains controversial due to a lack of experimental validation.

2.2.2 The formation of PhIP

At present, among numerous heterocyclic amines, the one whose formation mechanism has been studied relatively clearly is PhIP. The first step is the thermal degradation of phenylalanine or, in the presence of glucose, the reaction of phenylalanine with creatinine via Strecker degradation to form phenylacetaldehyde and creatinine. Subsequently, phenylacetaldehyde undergoes hydroxyaldol condensation with creatinine and then dehydrates to produce an intermediate product. Formaldehyde is formed during the reaction of phenylacetaldehyde, and ammonia is formed during the degradation of creatinine, and the hydroxyaldehyde condensation product reacts with formaldehyde and ammonia to form PhIP (Murkovic et al., 1999; Zamora, Alcón, & Hidalgo, 2014; Zochling, & Murkovic, 2002). The reaction mechanism is illustrated in **Figure 2-1**b. Subsequently, Deng et al. (2022) also found that benzaldehyde can similarly act as an intermediate carbonyl compound in the formation of PhIP. Unlike phenylacetaldehyde, the pathway involving benzaldehyde requires the participation of two formaldehyde molecules to form PhIP, whereas the phenylacetaldehyde pathway requires only one.

2.2.3 The formation of β-carboline

Amino-carboline HAs are primarily formed through the high-temperature pyrolysis of amino acids or proteins. For β-carboline-type HAs such as Harman and Norharman, a relatively well-defined formation pathway has been proposed by researchers (Finot et al., 1990). In this pathway, tryptophan reacts with glucose to form Amadori rearrangement products, which undergo dehydration. Assisted by the lone pair electrons on the epoxide group, a β-elimination occurs to generate a conjugated oxonium ion. Subsequent C–C bond cleavage and intramolecular nucleophilic substitution ultimately lead to the formation of β-carbolines. In addition, Herraiz (2000) proposed that tryptophan can react with acetaldehyde or α-keto acids via the Pictet–Spengler reaction to produce tetrahydro-β-carboline (THβC), which is then further oxidized and decarboxylated to yield β-carbolines.


(a)
Amino acid
Reducing sugar
e.g., hexose
Creatine
Strecker degradation
Heat
Creatinine
Aldol reaction
IQ compound
R1 R2 R3 X
H H H CH IQ
Me H H CH MeIQ
H Me H N MeIQx
Me Me H N 4,8-DiMeIQx
H Me Me N 7,8-DiMeIQx
Reducing sugar
+ amine
glycolaldehyd alkylimine
alkylpyridine radical
creatinine & aldehyde
IQx-type
+e -e
dialkylpyrazine radical
creatinine & aldehyde
IQ-type
(b)
Phenylalanine
Creatine
Heat
Heat
Phenylacetaldehyde
Creatinine
Aldol reaction
Formaldehyde
PhIP

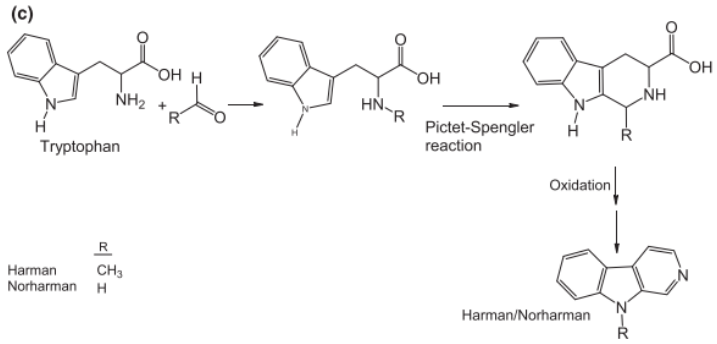


Figure 2-1: The possible generation pathways of different types of HAs like imidazoquinoline and imidazoquinoxaline (a), PhIP (b) and β-carboline (c) (Chen et al., 2019; Meurillon, & Engel, 2016).

2.2.4 The formation of bound heterocyclic amines

With the advancement of research, protein-bound HAs have garnered increasing attention. Martin et al. (2002) found that proteolytic digestion of cooked beef released genotoxic substances, which were speculated to be HAs that had bound to proteins during heating and were subsequently released upon enzymatic digestion. Later, Kataoka et al. (2010) directly heated PhIP with proteins and successfully obtained PhIP-protein adducts, confirming the formation of bound HAs. In addition, myofibrillar proteins can generate carbonyl compounds through the Maillard reaction in the presence of reducing sugars (Villaverde, & Estévez, 2013). Oxidation of amino acid side chains in proteins is a primary pathway for protein carbonylation (Stadtman, & Levine, 2000). Therefore, there are two main pathways for the formation of protein-bound HAs: the first involves free amino acids participating in the formation of free HAs, which then bind to proteins to form bound HAs; the second pathway involves proteins directly replacing free amino acids to react with glucose and creatinine, leading to the direct formation of protein-bound HAs. Chen et al. (2021) investigated the formation of protein-bound HAs in model systems containing myofibrillar and sarcoplasmic proteins. They found that myofibrillar proteins were more prone to forming bound HAs, while sarcoplasmic proteins tended to generate free HAs when involved in the reaction. Moreover, the formation of bound HAs in sarcoplasmic proteins primarily followed pathway 1 (binding of free HAs to proteins), whereas in myofibrillar proteins, bound HAs were mainly formed via pathway 2 (direct reaction of proteins with glucose and creatinine).

2.3 Control strategies of heterocyclic amines

2.3.1 Optimize processing conditions

A variety of thermal processing methods for meat products, such as frying, steaming,

and grilling, can lead to the production of HAs, and different processing methods have different effects on the type and amount of HAs produced (Zhang, Yu, Mei, & Wang, 2013; Dundar, Sarıçoban, & Yılmaz, 2012). Liao et al. (2010; 2012) successively studied the effects of heat treatment methods such as microwave, boiling, steaming, charcoal grilling, roasting, deep-frying, and pan-frying on the formation of HAs in meat products. The results showed that the content of HAs was higher in meat products processed by stronger heat treatments such as frying, charcoal grilling, and roasting, while it was lower in products processed by boiling, microwave and steaming. Different processing methods lead to differences in heat transfer coefficients through the type of heat transfer and its interaction with the surrounding medium, thus affecting the formation of HAs (Gibis, 2016). Processing methods with high water content such as boiling and steaming help to maintain the stability of precursor substances due to lower heating temperatures and the ability to reduce water evaporation during processing, thus inhibiting the formation of HAs. Temperature and time have a significant effect on the formation of HAs. It has been shown that increasing the heating time of beef patties from 6 to 10 min at a constant processing temperature of 225 °C more than doubled the total heterocyclic amine content, and that the HAs content increased about 8-fold when the processing temperature was increased from 175 to 225 °C (Balogh et al., 2000). Bjeldanes et al. (1983) further demonstrated in a simulated system that the effect of processing temperature on the formation of HAs was more pronounced than that of heating time. Moreover, the content of some HAs (e.g., 8-MeIQx and 7,8-DiMeIQx) tends to increase and then decrease during continuous high-temperature processing (Bordas et al., 2004). Therefore, by optimizing the processing temperature, time and method, the generation of HAs can be effectively intervened, thereby enhancing the safety of thermally processed meat products.

2.3.2 Select raw and auxiliary materials reasonably

The varieties and parts of different raw meats can also affect the formation of HAs. Puangsombat et al. (2012) found that the total HAs content in fried pork is higher than that in fried beef and fried chicken, and the HAs content in fried chicken with skin is higher than that in skinless chicken. This may be related to the differences in the contents of HAs precursor substances such as amino acids, glucose and creatinine in different meats, and it is also closely related to the contents of fat and water. The free radicals and lipid oxidation products generated during the fat oxidation process can promote the formation of HAs (Zamora, Alcón, & Hidalgo, 2012), while moisture may play an inhibitory role. Kikugawa et al. (1999) proposed that high moisture content could destroy the free radicals formed in the Maillard reaction, thereby inhibiting the generation of HAs through the free radical pathway.

In the meat products industry, various ingredients are often added to improve texture, flavor and appearance. These ingredients, while enhancing sensory quality, sometimes also have an inhibitory effect on the formation of HAs. Studies have shown that adding starch to meat products can significantly inhibit the formation of HAs in most cases, probably due to the ability of starch to prevent the migration of water-soluble precursors (Skog, Jägerstad, & Reuterswärd, 1992; Vangnai et al., 2014). For

example, potato starch HAs been proven to significantly inhibit the formation of various HAs (Persson et al., 2004). Spices, as common natural excipients, are widely used in meat products to enhance sensory properties attributes and have been shown to inhibit the formation of HAs. Lu et al. (2018) found that after adding equal amounts (0.5%) of onions, garlic, black pepper, red chili pepper and ginger to beef and chicken meatballs, respectively, and deep-frying treatment, resulted in a certain degree of inhibition of HAs formation. Puangsombat et al. (2011) further found that the addition of turmeric, galangal and Astragalus officinalis leaves could reduce the total amount of HAs by 39.2%, 18.4% and 33.5% respectively.

3.2.3 Add exogenous functional components

A large number of studies have shown that edible natural plant extracts, as a healthy and safe HAs inhibitor, have broad application prospects in the meat products industry, and their mechanism of action is mostly closely related to the antioxidant components they are rich in. Salazar et al. (2014) systematically evaluated the inhibitory effects of 25 polyphenolic compounds on PhIP in a model system and found that resveratrol and resorcinol had significant inhibitory effects on the generation of PhIP. Oguri et al. (1998) investigated the intervention effects of different antioxidants on PhIP in a model system. The results showed that flavonoids such as (-) -epigallocatechin gallate (EGCG) and quercetin had an inhibitory rate of up to 80% on PhIP. Cheng et al. (2007) added epicatechin, theaflavin, rosmarinic acid, and naringenin to a model system containing phenylalanine, creatinine, and glucose, as well as to actual beef patties, and found that these polyphenols significantly inhibited the formation of PhIP in the model system, and also had a good inhibitory effect on the generation of PhIP, MeIQx, and 4,8-DiMeIQx in the beef patties. In addition, vitamin compounds, as essential nutrients, also showed some HAs inhibition potential, and Liao et al (2009) demonstrated that the addition of vitamin E could effectively reduce the content of Norharman, PhIP, AαC, and MeAαC. Hydrocolloids have also been applied in meat products to inhibit the formation of heterocyclic amines (HAs). Zhang et al. (2020) investigated the effects of various hydrocolloids—including sodium alginate, sodium carboxymethyl cellulose, chitosan, carrageenan, konjac glucomannan, and xanthan gum—on the formation of PhIP, MeIQx, and 4,8-DiMeIQx in both model systems and actual beef patties. The results showed that all tested hydrocolloids exhibited inhibitory effects on HAs formation to varying degrees, with sodium carboxymethyl cellulose showing the most significant reduction. Yang et al. (2021) reported that the addition of 1% carrageenan significantly reduced the levels of PhIP, MeIQx, and 4,8-DiMeIQx in beef patties, with inhibition rates of 90%, 64%, and 48%, respectively. Further research by Zhao et al. (2024) demonstrated that grafting gallic acid and ferulic acid onto chitosan resulted in hydrocolloids that significantly inhibited the formation of heterocyclic amines in pan-fried golden pompano. These hydrocolloids may serve as a novel coating material to reduce HAs formation in food.

3. Introduction of polyphenols and the inhibition mechanism of polyphenols on heterocyclic amines

3.1 Classification of polyphenols

Plant polyphenols are a class of natural antioxidants widely found in the roots, leaves, fruits and seeds of plants. Structurally, they are characterized by the presence of at least one aromatic ring bearing one or more hydroxyl groups (Crozier, Jaganath, & Clifford, 2009). Based on the number of phenolic rings and the structural elements linking them, polyphenols are mainly classified into phenolic acids, flavonoids, lignans, stilbenes, and other subclasses (Pinto, & Santos, 2017). To date, over 8,000 phenolic compounds have been identified, among which flavonoids account for more than 4,000, making them the most abundant group (Lund, 2021).

Flavonoids are characterized by a basic C6-C3-C6 carbon skeleton, consisting of two aromatic rings (A and B rings) linked by a three-carbon bridge that usually forms a heterocyclic oxygen-containing C-ring (Bravo, 1998). As shown in **Figure 2-2**(1), this structure represents the core flavonoid backbone and its carbon numbering system. The major dietary flavonoid subclasses include flavanols (2), isoflavones (3), flavonols (4), flavanones (5), flavones (6), anthocyanins (7). Although the number of flavonoid aglycones is limited, they give rise to a vast array of derivatives. Substitutions commonly occur at the 3-position of the C-ring, as well as at the 5-, 7-, 4'-, 3'-, and 5'-positions, resulting in various modifications such as hydroxylation, methylation, O- and C-alkylation, and glycosylation. These diverse structural modifications contribute to the broad variety and complexity of plant polyphenols (Vastano et al., 2000).

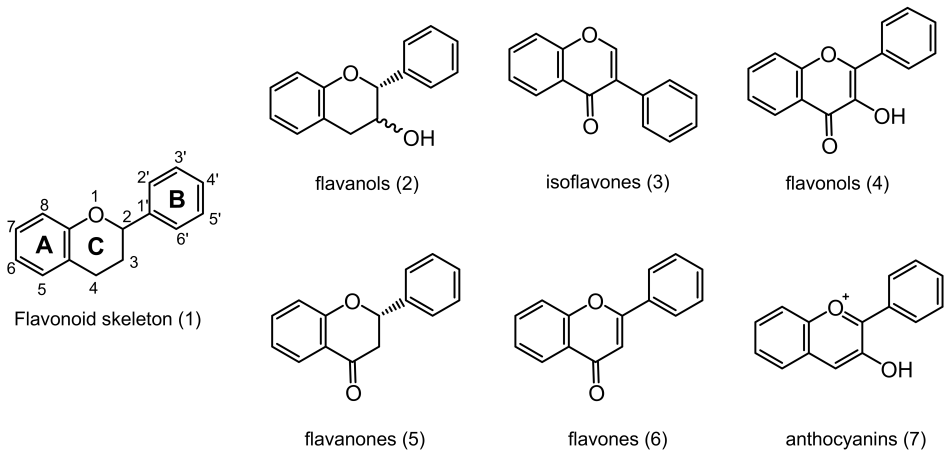


Figure 2-2: The fundamental structure of flavonoids

Phenolic acids are one of the most important classes of non-flavonoid polyphenols and are generally classified into benzoic acid and cinnamic acid derivatives based on their C1–C6 and C3–C6 carbon skeletons (**Figure 2-3**). Gallic acid is the most representative benzoic acid-derived polyphenol and serves as a precursor for the synthesis of hydrolyzable tannins, C3–C6 hydroxycinnamic acids and their conjugated derivatives, as well as C6–C2–C6 stilbenoid polyphenols (Crozier, Jaganath, & Clifford, 2009).

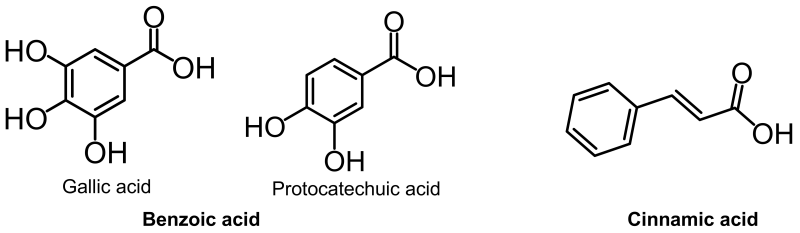


Figure 2-3: Basic structures of common phenolic acids

Stilbenes are a class of polyphenolic compounds with a C6-C2-C6 structure characterized by the presence of a hydroxyl-substituted 1,2-diaryl vinyl nucleus on the aromatic ring. The predominant stilbene polyphenol compound in food is resveratrol, which exists as cis- and trans-isomers (**Figure 2-4**), and the main source plants of naturally occurring resveratrol are the peanuts, and grapes (Teka et al., 2022).

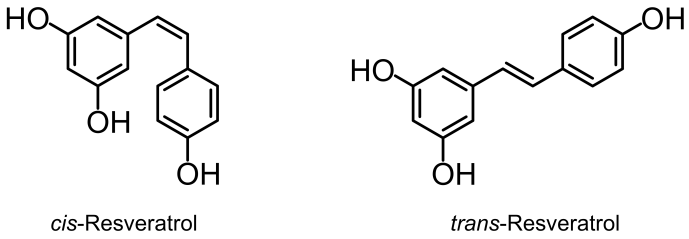


Figure 2-4: Cis and trans resveratrol

Chalcones possess an aromatic ketone structure characterized by a 1,3-diphenyl-1-propenone backbone (**Figure 2-5**). They exist in fruits and vegetables in various forms, including monomers, dimers, oligomers, and Diels–Alder adducts, often conjugated with different functional groups. In certain plants, such as Coreopsis and species from the Asteraceae family, chalcones, dihydrochalcones, and aurones serve as key plant pigments, contributing to their yellow to orange coloration (Aksöz, & Ertan, 2012).

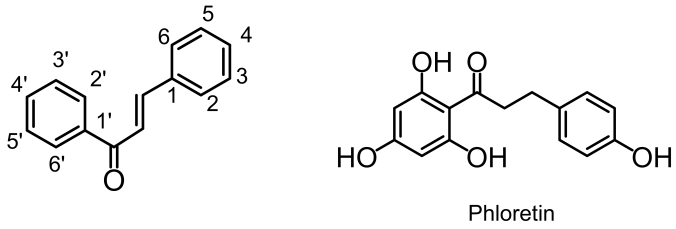


Figure 2-5: Basic structures of chalcones

Coumarins are lactone compounds formed by the cyclization of cis-ortho-hydroxycinnamic acid and belong to a class of phenolic compounds with a basic C6+C3 skeleton (**Figure 2-6**). In plants, coumarins exist both in free form and as glycosides. The main types of coumarin compounds include hydroxycoumarins, furanocoumarins and isofuranocoumarins, pyranocoumarins, and dihydroisocoumarins (Cai et al., 2003; Cai et al., 2006; Surveswaran et al., 2007).

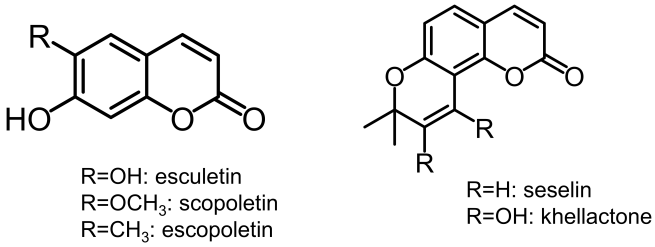


Figure 2-6: Basic structures of common coumarins

Lignans are a class of compounds derived from cis-hydroxycinnamic acid, formed by the tail-to-tail dimerization of two units of coniferyl alcohol or sinapyl alcohol, each containing a C6-C3 structure. They are predominantly found in flaxseed, sesame seeds, and various cereals (Huang, Cai, & Zhang, 2010). Other categories of polyphenolic compounds include phenolic alkaloids, phenolic terpenes (including aromatic essential oils), specialized phenolic glycosides, and benzo-triol derivatives, all of which are structurally classified as phenolic compounds.

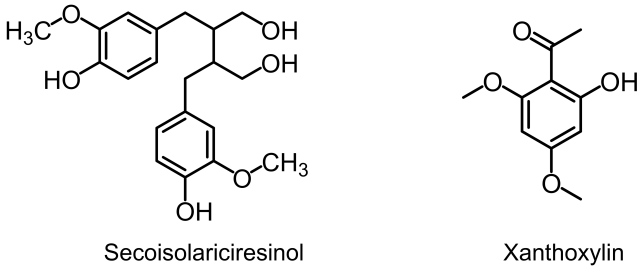


Figure 2-7: Basic structures of common lignans

3.2 Mechanism of free heterocyclic amine inhibition by polyphenols of different structures

Current studies have shown that the quantity and position of hydroxyl groups in polyphenolic compounds play an important role in exerting antioxidant activity and inhibiting the formation of HAs (Falowo, Fayemi, & Muchenje, 2014). The mechanisms by which polyphenols have antioxidant properties and inhibit the formation of HAs are as follows:

(1) Scavenging free radicals

The traditional view holds that the inhibitory effect of polyphenolic flavonoids on PhIP formation is primarily due to their strong free radical scavenging ability. Studies have shown that the ability of certain flavonoids to scavenge alkoxyl radicals is positively correlated with their inhibitory effects on PhIP formation (as indicated by IC_{50} values), suggesting that these compounds can suppress PhIP generation by eliminating alkyl radicals in model systems (Yu et al., 2016). Kikugawa et al. (1999) proposed that free radical intermediates, such as pyrazine cation radicals, are formed during the generation of PhIP. Given the strong radical-scavenging ability of polyphenolic flavonoids, they can react with these intermediates and thereby indirectly inhibit PhIP formation. Different classes of phenolic compounds exhibit significant differences in radical scavenging activity. Tannins show the highest activity, whereas quinones, isoflavones, and lignans display the weakest. The presence of ortho-dihydroxy or ortho-trihydroxy structures (e.g., pyrogallol) is considered a key structural feature contributing to the high radical scavenging activity of phenolic compounds (**Figure 2-8**) (Cai et al., 2006; Sekher Pannala et al., 2001).

With the advancement of research, it has become evident that free radical scavenging alone cannot fully account for the differences in the inhibitory effects of various polyphenols on HAs formation, indicating the limitations of this mechanism. This suggests that mechanisms beyond antioxidant activity may play a predominant role in the inhibition of HAs by polyphenols. Cheng et al. (2007) found that, in a

model system, the free radical scavenging capacities of 12 antioxidant phenolic compounds derived from various food sources showed a very poor correlation with their inhibitory activities against PhIP formation. This suggests that mechanisms beyond antioxidant activity may play a predominant role in the inhibition of HAs by polyphenols.

(2) Trapping active carbonyl compounds

The carbonyl-trapping ability of polyphenols also plays a crucial role in the inhibition of HAs formation, which can be inhibited by the formation of carbonyl-phenol adducts (Hidalgo, & Zamora, 2014). Through a series of studies, Hidalgo and Zamora found that phenolic compounds with two hydroxyl groups in the meta-position on the aromatic ring are the most effective inhibitors of PhIP formation (Hidalgo, Navarro, & Zamora, 2018). These phenolics inhibit the formation of PhIP, MeIQx, IQ, and MeIQ by trapping reactive intermediates such as phenylacetaldehyde, acrolein, and crotonaldehyde (Hidalgo, & Zamora, 2023). Carbonyl compounds can undergo electrophilic aromatic substitution at the C-8 or C-6 position on the A-ring of polyphenol molecules to form adduct (Ghassem Zadeh, & Yaylayan, 2021). To date, a variety of polyphenol–phenylacetaldehyde adducts have been identified, including mangiferin, kaempferol, naringenin, hyperoside and quercitrin (Wang et al., 2021; Dong et al., 2024; Wang et al., 2022). In addition, the structures of the adducts formed between quercetin and acrolein or crotonaldehyde have also been elucidated (Zamora et al., 2016).

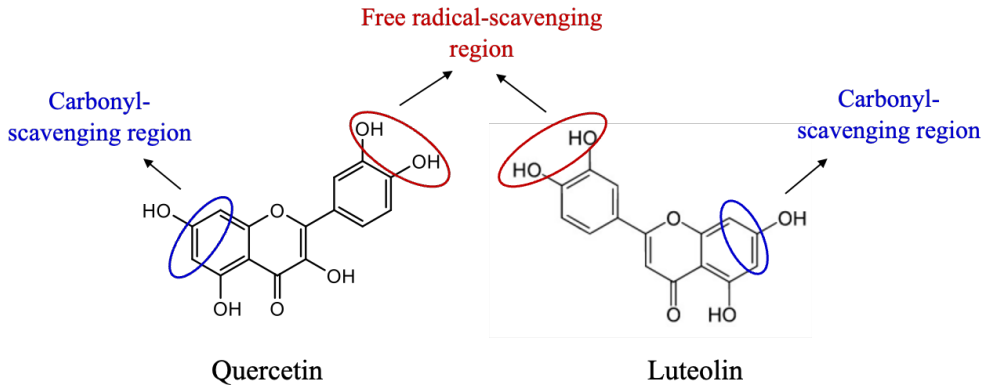


Figure 2-8: Structural regions in which polyphenols exert free radical scavenging and capture of reactive carbonyl groups

3.3 Effect and mechanism of bound heterocyclic amine inhibition by polyphenols

In recent years, the inhibitory effects of polyphenols on bound HAs have attracted increasing attention, although related studies remain limited. Xu et al. (2023)

investigated the effects of different concentrations of mulberry leaf extract on HAs in Muscovy duck (*Cairina moschata*) meat and found that a 0.03% addition exhibited the strongest inhibitory effect on both free and bound HAs. Analysis revealed that the extract contained 15 phenolic compounds, primarily flavonoids. Shen et al. (2024) found that *Angelica dahurica* (AD) and imperatorin exhibited a dose-dependent inhibitory effect on bound HAs in roasted beef. Zeng's team found that adding chili pepper, Sichuan pepper, and black pepper could effectively inhibit the bound HAs in roast beef (Chen et al., 2017a). They also found that *Kaempferia galanga* L. (KG) and kaempferol exhibited dose-dependent inhibitory effects on bound HAs in roasted beef, showing significant suppression of the most abundant compounds, norharman and harman (Xue et al., 2022a). What's more, it has been found that SP-bound HAs can be released as free HAs during *in vitro* digestion under the action of trypsin, and the addition of polyphenol-rich ingredients such as onions, pepper powder, and apples can further promote this conversion (Xue et al., 2022b). Chen and Xi (2022) found that the addition of curcumin, tea polyphenols, grape seed proanthocyanidins, and dihydromyricetin could alter the structure of soy protein isolate through non-covalent interactions, thereby affecting the formation of HAs. Although extensive research has been conducted on polyphenol–protein interactions and polyphenolic compounds are known to effectively inhibit the formation of bound HAs, research on the underlying mechanisms of this inhibition remains limited.

3.4 Mechanisms of polyphenol inhibition of heterocyclic amines toxicity

The addition of polyphenols not only inhibits the formation of HAs but also suppresses the various toxicities induced by them, exerting protective effects both *in vivo* and *in vitro*. By evaluating the mutagenic effects of IQ in *Salmonella typhimurium* TA98 with 64 different flavonoids, it was found that the majority of these flavonoid compounds exhibited antimutagenic activity (Edenharder, von Petersdorff, & Rauscher, 1993). Resveratrol can inhibit the expression of the *umu* gene induced by the SOS response induced by Trp-P-1 in *Salmonella typhimurium* TA1535/pSK1002 (Uenobe, Nakamura, & Miyazawa, 1997). Quercetin and epicatechin can significantly inhibit DNA damage and oxidative stress responses in lymphocytes of patients with IQ induced inflammatory bowel disease (IBD) (Najafzadeh et al., 2009). It was also found that polyphenols in black tea and green tea can effectively inhibit the mutagenicity of PhIP (Apostolides et al., 1996; Apostolides et al., 1997; Weisburger, Dolan, & Pittman, 1998). In addition, Lin et al. showed that tea polyphenols can inhibit the binding of N-acetoxy-PhIP to DNA through direct reaction with N-acetoxy-PhIP, thus effectively blocking the carcinogenicity and genotoxicity of PhIP in vivo and playing an important chemopreventive role (Lin et al., 2003). Curcumin can effectively inhibit the cytotoxicity of PhIP on normal breast epithelial cells (MCF-10A) by significantly suppressing the formation of DNA adducts induced by PhIP and the reduction of reactive oxygen species (ROS) production accompanying DNA double-strand breaks

(Jain et al., 2015). Zhao et al. (2021b; 2021c) demonstrated that the metabolic product of cyanidin-3-O-glucoside (C3G), protocatechuic acid (PCA), inhibits IQ-induced cytotoxicity in HepG2 cells by interfering with the binding of IQ to the BIR3 domain of the XIAP protein, thereby disrupting the apoptosis signaling pathway. Additionally, both C3G and PCA were found to modulate the crosstalk between p62-mediated autophagy and the Keap1-Nrf2 pathway, effectively alleviating PhIP-induced cellular damage.

4. Conclusion and future trends

Meat products are an important component of the human diet, however, the formation of carcinogenic HAs during high-temperature cooking is an undesirable consequence. Due to their excellent antioxidant properties, plant polyphenols have been extensively studied for their ability to inhibit HAs formation. This paper reviews the formation mechanisms of HAs and current mitigation strategies, outlines the structure and classification of polyphenols, and explores their mechanisms of action in reducing HAs formation *in vitro* and toxicity *in vivo* from three aspects: First, polyphenols effectively inhibit the formation of free HAs by scavenging free radicals and capturing active carbonyl groups; Second, polyphenols inhibit the formation of bound HAs by regulating proteins to participate in reactions. The third is the mitigating effect of polyphenols on the cytotoxicity and carcinogenicity induced by HAs.

Polyphenols exhibit great structural diversity, and their structural characteristics are closely related to their inhibitory effects on HAs. Therefore, future research should systematically investigate the relationship between polyphenol structures and their inhibitory activity, in order to efficiently screen for polyphenols with strong anti-HAs potential. At present, most studies on the inhibitory mechanisms of polyphenols focus on free HAs, while the mechanisms related to the inhibition of protein-bound HAs remain poorly understood. Future studies should explore the interactions between polyphenols and protein-bound HAs to further elucidate their potential roles in modulating HAs formation. Moreover, the impact of polyphenols on the metabolic pathways of HAs in the human body, as well as the safety of polyphenol–HAs adducts, requires further clarification.

In-depth exploration of the mechanisms by which polyphenols inhibit HAs formation not only contributes to enhancing food safety but also provides a theoretical basis for the development of natural, safe, and efficient inhibitors, holding significant scientific and industrial value. In the meat processing industry, the rational use of polyphenol additives may effectively reduce the risk of carcinogen formation, extend product shelf life, and improve consumer health protection.

5. References

Adamson, R. H., Thorgeirsson, U. P., Snyderwine, E. G., Thorgeirsson, S. S., Reeves, J., Dalgard, D. W., Takayama, S., & Sugimura, T. (1990). Carcinogenicity of 2-

amino-3-methylimidazo[4,5-f]quinoline in nonhuman primates: induction of tumors in three macaques. *Japanese Journal of Cancer Research : Gann, 81*(1), 10–14.

Aksöz, B.E., & Ertan, R., (2012). Spectral properties of chalcones II. *Fabad Journal of Pharmaceutical Sciences, 37*(4), pp.205-216.

Alaejos, M. S., & Afonso, A. M. (2011). Factors that affect the content of heterocyclic aromatic amines in foods. *Comprehensive Reviews in Food Science and Food Safety, 10*, 52e108.

Apostolides, Z., Balentine, D. A., Harbowy, M. E., & Weisburger, J. H. (1996). Inhibition of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) mutagenicity by black and green tea extracts and polyphenols. *Mutation Research, 359*(3), 159–163.

Apostolides, Z., Balentine, D. A., Harbowy, M. E., Hara, Y., & Weisburger, J. H. (1997). Inhibition of PhIP mutagenicity by catechins, and by theaflavins and gallate esters. *Mutation Research, 389*(2-3), 167–172.

Archer, C.L., Morse, P., Jones, R.F., Shirai, T., Haas, G.P. and Wang, C.Y. (2000). Carcinogenicity of the N-hydroxy derivative of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine, 2-amino-3,8-dimethyl-imidazo[4,5-f]quinoxaline and 3,2'-dimethyl-4-aminobiphenyl in the rat. *Cancer Letters, 155*(1), 55-60.

Balogh, Z., Gray, J. I., Gomaa, E. A., & Booren, A. M. (2000). Formation and inhibition of heterocyclic aromatic amines in fried ground beef patties. *Food and chemical toxicology: an international journal published for the British Industrial Biological Research Association, 38*(5), 395–401.

Bjeldanes, L. F., Morris, M. M., Timourian, H., & Hatch, F. T. (1983). Effects of meat composition and cooking conditions on mutagen formation in fried ground beef. *Journal of Agricultural and Food Chemistry, 31*(1), 18–21.

Boada, L. D., Henríquez-Hernández, L. A., & Luzardo, O. P. (2016). The impact of red and processed meat consumption on cancer and other health outcomes: Epidemiological evidences. *Food and Chemical Toxicology: an international journal published for the British Industrial Biological Research Association, 92*, 236–244.

Bordas, M., Moyano, E., Puignou, L., & Galceran, M. T. (2004). Formation and stability of heterocyclic amines in a meat flavour model system. Effect of temperature, time and precursors. *Journal of Chromatography. B, Analytical technologies in the biomedical and life sciences, 802*(1), 11–17.

Boobis, A. R., Lynch, A. M., Murray, S., de la Torre, R., Solans, A., Farré, M., Segura, J., Gooderham, N. J., & Davies, D. S. (1994). CYP1A2-catalyzed conversion of dietary heterocyclic amines to their proximate carcinogens is their major route of metabolism in humans. *Cancer Research, 54*(1), 89–94.

Bravo L. (1998). Polyphenols: chemistry, dietary sources, metabolism, and nutritional significance. *Nutrition Reviews, 56*(11), 317–333.

Bukowska, B., Duchnowicz, P., Tumer, T. B., Michalowicz, J., & Krokosz, A. (2023). Precarcinogens in food-mechanism of action, formation of DNA adducts and preventive measures. *Food Control, 152*, Article 109884.

Buła, M., Przybylski, W., Jaworska, D., & Kajak-Siemaszko, K. (2019). Formation of heterocyclic aromatic amines in relation to pork quality and heat treatment parameters. *Food Chemistry, 276*, 511–519.

Cai, Y., Sun, M., & Corke, H. (2003). Antioxidant activity of betalains from plants of the amaranthaceae. *Journal of agricultural and food chemistry, 51*(8), 2288–2294.

Cai, Y. Z., Mei Sun, Jie Xing, Luo, Q., & Corke, H. (2006). Structure-radical scavenging activity relationships of phenolic compounds from traditional Chinese medicinal plants. *Life Sciences, 78*(25), 2872–2888.

Chen, J., He, Z., Qin, F., Chen, J., Cao, D., Guo, F., & Zeng, M. (2017a). Inhibitory profiles of spices against free and protein-bound heterocyclic amines of roast beef patties as revealed by ultra-performance liquid chromatography-tandem mass spectrometry and principal component analysis. *Food & Function, 8*(11), 3938–3950.

Chen, J. X., Liu, A., Lee, M. J., Wang, H., Yu, S., Chi, E., Reuhl, K., Suh, N., & Yang, C. S. (2017b). δ- and γ-tocopherols inhibit PhIP/DSS-induced colon carcinogenesis by protection against early cellular and DNA damages. *Molecular Carcinogenesis, 56*(1), 172–183.

Chen, Q., Xue, C., He, Z., Wang, Z., Qin, F., Chen, J., & Zeng, M. (2021). Generation of Sarcoplasmic and Myofibrillar Protein-Bound Heterocyclic Amines in Chemical Model Systems under Different Heating Temperatures and Durations. *Journal of Agricultural and Food Chemistry, 69*(10), 3232–3246.

Chen, X., Jia, W., Zhu, L., Mao, L., & Zhang, Y. (2020). Recent advances in heterocyclic aromatic amines: An update on food safety and hazardous control from food processing to dietary intake. *Comprehensive Reviews in Food Science and Food Safety, 19*(1), 124–148.

Chen, Y., & Xi, J. (2022). Effects of the non-covalent interactions between polyphenols and proteins on the formations of the heterocyclic amines in dry heated soybean protein isolate. *Food Chemistry, 373*(Pt B), 131557.

Cheng, K. W., Chen, F., & Wang, M. (2007). Inhibitory activities of dietary phenolic compounds on heterocyclic amine formation in both chemical model system and beef patties. *Molecular Nutrition & Food Research, 51*(8), 969–976.

Crozier, A., Jaganath, I. B., & Clifford, M. N. (2009). Dietary phenolics: chemistry, bioavailability and effects on health. *Natural Product Reports, 26*(8), 1001–1043.

Deng, P., Xue, C., He, Z., Wang, Z., Qin, F., Oz, E., Chen, J., El Sheikha, A. F., Proestos, C., Oz, F., & Zeng, M. (2022). Synergistic Inhibitory Effects of Selected Amino Acids on the Formation of 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in both Benzaldehyde- and Phenylacetaldehyde-Creatinine Model Systems. *Journal of Agricultural and Food Chemistry, 70*(35), 10858–10871.

Dong, H., Chen, Q., Xu, Y., Li, C., Bai, W., Zeng, X., Wu, Q., Xu, H., & Deng, J. (2024). Effect and mechanism of polyphenols containing m-dihydroxyl structure on 2-amino-1-methyl-6-phenylimidazole [4, 5-b] pyridine (PhIP) formation in chemical models and roast pork patties. *Food Chemistry: X, 23,* 101672.

Dundar, A., Sarıçoban, C., & Yılmaz, M. T. (2012). Response surface optimization of effects of some processing variables on carcinogenic/mutagenic heterocyclic aromatic amine (HAA) content in cooked patties. *Meat Science, 91*(3), 325–333.

Edenharder, R., von Petersdorff, I., & Rauscher, R. (1993). Antimutagenic effects of flavonoids, chalcones and structurally related compounds on the activity of 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) and other heterocyclic amine mutagens from cooked food. *Mutation Research, 287*(2), 261–274.

Edwards, R. J., Murray, B. P., Murray, S., Schulz, T., Neubert, D., Gant, T. W., Thorgeirsson, S. S., Boobis, A. R., & Davies, D. S. (1994). Contribution of CYP1A1 and CYP1A2 to the activation of heterocyclic amines in monkeys and human. *Carcinogenesis, 15*(5), 829–836.

EL-Bayoumy, K., Chae, Y. H., Upadhyaya, P., Rivenson, A., Kurtzke, C., Reddy, B., & Hecht, S. S. (1995). Comparative tumorigenicity of benzo[a]pyrene, 1-nitropyrene and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine administered by gavage to female CD rats. *Carcinogenesis, 16*(2), 431–434.

Esumi, H., Ohgaki, H., Kohzen, E., Takayama, S., & Sugimura, T. (1989). Induction of lymphoma in CDF1 mice by the food mutagen, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine. *Japanese Journal of Cancer Research: Gann, 80*(12), 1176–1178.

Falowo, A. B., Fayemi, P. O., & Muchenje, V. (2014). Natural antioxidants against lipid-protein oxidative deterioration in meat and meat products: A review. *Food research international (Ottawa, Ont.), 64*, 171–181.

Felton, J. S., Knize, M. G., Hatch, F. T., Tanga, M. J., & Colvin, M. E. (1999). Heterocyclic amine formation and the impact of structure on their mutagenicity. *Cancer Letters, 143*(2), 127–134.

Finot, P. A., Aeschbacher, H. U., Hurrell, R. F., Liardon, R. (1990). The Maillard Reaction in Food Processing, Human Nutrition and Physiology. Basel: Birkhäuser.

Frandsen, H., Rasmussen, E. S., Nielsen, P. A., Farmer, P., Dragsted, L., & Larsen, J. C. (1991). Metabolic formation, synthesis and genotoxicity of the N-hydroxy derivative of the food mutagen 2-amino-1-methyl-6-phenylimidazo (4,5-b) pyridine (PhIP). *Mutagenesis, 6*(1), 93–98.

Fujita, H., Nagano, K., Ochiai, M., Ushijima, T., Sugimura, T., Nagao, M., & Matsushima, T. (1999). Difference in target organs in carcinogenesis with a heterocyclic amine, 2-amino-3,4-dimethylimidazo[4,5-f]quinoline, in different strains of mice. *Japanese Journal of Cancer Research : Gann, 90*(11), 1203–1206.

Garner, R.C., Lightfoot, T.J., Cupid, B.C., Russell, D., Coxhead, J.M., Kutschera, W., Priller, A., Rom, W., Steier, P., Alexander, D.J., Leveson, S.H., Dingley, K.H.,

Mauthe, R.J. and Turteltaub, K.W. (1999). Comparative biotransformation studies of MeIQx and PhIP in animal models and humans. *Cancer Letters, 143*(2), 161-165.

Ghassem Zadeh, R., & Yaylayan, V. (2021). Interaction pattern of histidine, carnosine and histamine with methylglyoxal and other carbonyl compounds. *Food Chemistry, 358*, 129884.

Ghoshal, A., Preisegger, K. H., Takayama, S., Thorgeirsson, S. S., & Snyderwine, E. G. (1994). Induction of mammary tumors in female Sprague-Dawley rats by the food-derived carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine and effect of dietary fat. *Carcinogenesis, 15*(11), 2429–2433.

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

Guo, J., Koopmeiners, J. S., Walmsley, S. J., Villalta, P. W., Yao, L., Murugan, P., Tejpaul, R., Weight, C. J., & Turesky, R. J. (2022). The Cooked Meat Carcinogen 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine Hair Dosimeter, DNA Adductomics Discovery, and Associations with Prostate Cancer Pathology Biomarkers. *Chemical Research in Toxicology, 35*(5), 703–730.

Hasegawa, R., Sano, M., Tamano, S., Imaida, K., Shirai, T., Nagao, M., Sugimura, T., & Ito, N. (1993). Dose-dependence of 2-amino-1-methyl-6-phenylimidazo[4,5-b]-pyridine (PhIP) carcinogenicity in rats. *Carcinogenesis, 14*(12), 2553–2557.

Hein, D. W., Rustan, T. D., Ferguson, R. J., Doll, M. A., & Gray, K. (1994). Metabolic activation of aromatic and heterocyclic N-hydroxyarylamines by wild-type and mutant recombinant human NAT1 and NAT2 acetyltransferases. *Archives of Toxicology, 68*(2), 129–133.

Hemminki K. (1993). DNA adducts, mutations and cancer. *Carcinogenesis, 14*(10), 2007–2012.

Herraiz T. (2000). Analysis of the bioactive alkaloids tetrahydro-beta-carboline and beta-carboline in food. *Journal of chromatography. A, 881*(1-2), 483–499.

Hidalgo, F. J., & Zamora, R. (2014). 2-Alkenal-scavenging ability of m-diphenols. *Food Chemistry, 160*, 118–126.

Hidalgo, F. J., Navarro, J. L., & Zamora, R. (2018). Structure-Activity Relationship (SAR) of Phenolics for 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) Formation in Phenylalanine/Creatinine Reaction Mixtures Including (or Not) Oxygen and Lipid Hydroperoxides. *Journal of Agricultural and Food Chemistry, 66*(1), 255–264.

Hidalgo, F. J., & Zamora, R. (2023). Carbonyl-trapping by phenolics and the inhibition of the formation of carcinogenic heterocyclic aromatic amines with the structure of aminoimidazoazaarene in beef patties. *Food Chemistry, 425*, 136505.

Huang, H., Ushijima, T., Nagao, M., Sugimura, T., & Ohgaki, H. (2003). Beta-catenin mutations in liver tumors induced by 2-amino-3,4-dimethylimidazo[4,5-f]quinoline in CDF1 mice. *Cancer Letters, 198*(1), 29–35.

Huang, W. Y., Cai, Y. Z., & Zhang, Y. (2010). Natural phenolic compounds from medicinal herbs and dietary plants: potential use for cancer prevention. *Nutrition and Cancer, 62*(1), 1–20.

Huang, W. Y., Wu, H., Li, D. J., Song, J. F., Xiao, Y. D., Liu, C. Q., Zhou, J. Z., & Sui, Z. Q. (2018). Protective Effects of Blueberry Anthocyanins against H2O2-Induced Oxidative Injuries in Human Retinal Pigment Epithelial Cells. *Journal of Agricultural and Food Chemistry, 66*(7), 1638–1648.

International Agency for Research on Cancer (IARC). (1993). Some naturally occurring substance, food items and constituents, heterocyclic aromatic amines and mycotoxins. In *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans* (Vol. 56, pp. 165–244). Lyon, France: WHO Press.

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

Jägerstad, M., Olsson, K., Grivas, S., Negishi, C., Wakabayashi, K., Tsuda, M., Sato, S., & Sugimura, T. (1984). Formation of 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline in a model system by heating creatinine, glycine and glucose. *Mutation Research, 126*(3), 239–244.

Jägerstad M, Skog K, Arvidsson P, Solyakov A. (1998). Chemistry, formation, and occurrence of genotoxic heterocyclic amines identified in model systems and cooked foods. *Z Lebensm-Unters Forsch A, 207*: 419–27.

Jain, A., Samykutty, A., Jackson, C., Browning, D., Bollag, W. B., Thangaraju, M., Takahashi, S., & Singh, S. R. (2015). Curcumin inhibits PhIP induced cytotoxicity in breast epithelial cells through multiple molecular targets. *Cancer Letters, 365*(1), 122–131.

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

Kataoka, H., Miyake, M., Nishioka, S., Matsumoto, T., Saito, K., & Mitani, K. (2010). Formation of protein adducts of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine in cooked foods. *Molecular Nutrition & Food Research, 54*(7), 1039–1048.

Kato, T., Ohgaki, H., Hasegawa, H., Sato, S., Takayama, S. and Sugimura, T. (1988). Carcinogenicity in rats of a mutagenic compound, 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline. *Carcinogenesis, 9*(1), 71-73.

Kawamori, T., Totsuka, Y., Uchiya, N., Kitamura, T., Shibata, H., Sugimura, T., & Wakabayashi, K. (2004). Carcinogenicity of aminophenylnorharman, a possible novel endogenous mutagen, formed from norharman and aniline, in F344 rats. *Carcinogenesis, 25*(10), 1967–1972.

Khan, I. A., Khan, A., Zou, Y., Zongshuai, Z., Xu, W., Wang, D., & Huang, M. (2022).

Heterocyclic amines in cooked meat products, shortcomings during evaluation, factors influencing formation, risk assessment and mitigation strategies. *Meat Science, 184,* 108693.

Khan, M. R., Naushad, M., Alothman, Z. A., Algamdi, M. S., Alsohaimi, I. H., & Ghfar, A. A. (2017). Effect of natural food condiments on carcinogenic/mutagenic heterocyclic amines formation in thermally processed camel meat. *Journal of Food Processing and Preservation, 41*(1), Article e12819.

Kikugawa, K., Kato, T., Hiramoto, K., Takada, C., Tanaka, M., Maeda, Y., & Ishihara, T. (1999). Participation of the pyrazine cation radical in the formation of mutagens in the reaction of glucose/glycine/creatinine. *Mutation Research, 444*(1), 133–144.

Kim, S., Guo, J., O'Sullivan, M. G., Gallaher, D. D., & Turesky, R. J. (2016). Comparative DNA adduct formation and induction of colonic aberrant crypt foci in mice exposed to 2-amino-9H-pyrido[2,3-b]indole, 2-amino-3,4-dimethylimidazo[4,5-f]quinoline, and azoxymethane. *Environmental and Molecular Mutagenesis, 57*(2), 125–136.

Kushida, H., Wakabayashi, K., Sato, H., Katami, M., Kurosaka, R. and Nagao, M. (1994). Dose-response study of MeIQx carcinogenicity in F344 male rats. *Cancer Letters, 83*(1-2), 31-35.

Lee, H., Lin, M. Y., & Chan, S. C. (1994). Formation and identification of carcinogenic heterocyclic aromatic amines in boiled pork juice. *MutationResearch, 308*(1), 77–88.

Liao, G. Z., Xu, X. L., & Zhou, G. H. (2009). Effects of Cooked Temperatures and Addition of Antioxidants on Formation of Heterocyclic Aromatic Amines in Pork Floss. *Journal of Food Processing and Preservation, 33*(2), 159–175.

Liao, G. Z., Wang, G. Y., Xu, X. L., & Zhou, G. H. (2010). Effect of cooking methods on the formation of heterocyclic aromatic amines in chicken and duck breast. *Meat Science, 85*(1), 149–154.

Liao, G. Z., Wang, G. Y., Zhang, Y. J., Xu, X. L., & Zhou, G. H. (2012). Formation of heterocyclic amines during cooking of duck meat. *Food Additives & Contaminants. Part A, Chemistry, analysis, control, exposure & risk assessment, 29*(11), 1668–1678.

Li, R., Tian, J., Li, W., & Xie, J. (2013). Effects of 2-amino-1-methyl-6-phenylimidazo [4, 5-b] pyridine (PhIP) on histopathology, oxidative stress, and expression of c-fos, c-jun and p16 in rat stomachs. *Food and Chemical Toxicology: an international journal published for the British Industrial Biological Research Association, 55*, 182–191.

Lin, D. X., Thompson, P. A., Teitel, C., Chen, J. S., & Kadlubar, F. F. (2003). Direct reduction of N-acetoxy-PhIP by tea polyphenols: A possible mechanism for chemoprevention against PhIP–DNA adduct formation. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 523*, 193–200.

Lu F, Kuhnle G K, & Cheng Q. (2018) The effect of common spices and meat type on the formation of heterocyclic amines and polycyclic aromatic hydrocarbons in

deep-fried meatballs. *Food Control, 92*: 399-411.

Lund, M. N. (2021). Reactions of plant polyphenols in foods: Impact of molecular structure. *Trends in Food Science & Technology, 112*, 241–251.

Lynch, A. M., Murray, S., Gooderham, N. J., & Boobis, A. R. (1995). Exposure to and activation of dietary heterocyclic amines in humans. *Critical Reviews in Oncology/Hematology, 21*(1-3), 19–31.

Martin, F. L., Cole, K. J., Phillips, D. H., & Grover, P. L. (2002). The proteolytic release of genotoxins from cooked beef. *Biochemical and Biophysical Research Communications, 293*(5), 1497–1501.

Meurillon, M., & Engel, E. (2016). Mitigation strategies to reduce the impact of heterocyclic aromatic amines in proteinaceous foods. *Trends in Food Science & Technology,50*, 70–84.

Morgenthaler, P. M., & Holzhäuser, D. (1995). Analysis of mutations induced by 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in human lymphoblastoid cells. *Carcinogenesis, 16*(4), 713–718.

Murkovic, M., Weber, H.-J., Geiszler, S., Fröhlich, K., & Pfannhauser, W. (1999). Formation of the food associated carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in model systems. *Food Chemistry, 65*, 233–237.

Najafzadeh, M., Reynolds, P. D., Baumgartner, A., & Anderson, D. (2009). Flavonoids inhibit the genotoxicity of hydrogen peroxide (H2O2) and of the food mutagen 2-amino-3-methylimadazo[4,5-f]-quinoline (IQ) in lymphocytes from patients with inflammatory bowel disease (IBD). *Mutagenesis, 24*(5), 405–411.

National Toxicology Program (NTP). (2004). NTP 11th report on carcinogens. *Report on Carcinogens: Carcinogen Profiles, 11*, 1–A32.

Negishi, C., Wakabayashi, K., Tsuda, M., Sato, S., Sugimura, T., Saitô, H., Maeda, M., & Jägerstad, M. (1984). Formation of 2-amino-3,7,8-trimethylimidazo [4,5-f]quinoxaline, a new mutagen, by heating a mixture of creatinine, glucose and glycine. *Mutation Research, 140*(2-3), 55–59.

Oguri, A., Suda, M., Totsuka, Y., Sugimura, T., & Wakabayashi, K. (1998). Inhibitory effects of antioxidants on formation of heterocyclic amines. *Mutation Research, 402*(1-2), 237–245.

Ohgaki, H., Kusama, K., Matsukura, N., Morino, K., Hasegawa, H., Sato, S., Takayama, S., & Sugimura, T. (1984). Carcinogenicity in mice of a mutagenic compound, 2-amino-3-methylimidazo[4,5-f]quinoline, from broiled sardine, cooked beef and beef extract. *Carcinogenesis, 5*(7), 921–924.

Ohgaki, H., Hasegawa, H., Suenaga, M., Kato, T., Sato, S., Takayama, S., & Sugimura, T. (1986). Induction of hepatocellular carcinoma and highly metastatic squamous cell carcinomas in the forestomach of mice by feeding 2-amino-3,4-dimethylimidazo[4,5-f]quinoline. *Carcinogenesis, 7*(11), 1889–1893.

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

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

Pinto, P., & Santos, C. N. (2017). Worldwide (poly)phenol intake: assessment methods and identified gaps. *European Journal of Nutrition, 56*(4), 1393–1408.

Puangsombat, K., Jirapakkul, W., & Smith, J. S. (2011). Inhibitory activity of Asian spices on heterocyclic amines formation in cooked beef patties. *Journal of Food Science, 76*(8), T174–T180.

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

Püssa T. (2013). Toxicological issues associated with production and processing of meat. *Meat Science, 95*(4), 844–853.

Rao, C. V., Rivenson, A., Zang, E., Steele, V., Kelloff, G., & Reddy, B. S. (1996). Inhibition of 2-Amino-1-methyl-6-phenylimidazo[4,5]pyridine-induced lymphoma formation by oltipraz. *Cancer Research, 56*(15), 3395–3398.

Sadrieh, N., & Snyderwine, E. G. (1995). Cytochromes P450 in cynomolgus monkeys mutagenically activate 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) but not 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx). *Carcinogenesis, 16*(7), 1549–1555.

Sadrieh, N., Davis, C. D., & Snyderwine, E. G. (1996). N-acetyltransferase expression and metabolic activation of the food-derived heterocyclic amines in the human mammary gland. *Cancer Research, 56*(12), 2683–2687.

Salazar, R., Arámbula-Villa, G., Hidalgo, F. J., & Zamora, R. (2014). Structural characteristics that determine the inhibitory role of phenolic compounds on 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) formation. *Food Chemistry, 151*, 480–486.

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

Sekher Pannala, A., Chan, T. S., O'Brien, P. J., & Rice-Evans, C. A. (2001). Flavonoid B-ring chemistry and antioxidant activity: fast reaction kinetics. *Biochemical and Biophysical Research Communications, 282*(5), 1161–1168.

Shen, X., Liu, X., Wang, X., Xue, C., Chai, Z., Zeng, M., & Chen, J. (2024). Effect of Angelica dahurica, Angelica dahurica polysaccharides, and imperatorin on free and bound heterocyclic amine generation in roasted beef patties and release profiles of bound heterocyclic amines during in vitro digestion. *Food Research International (Ottawa, Ont.), 175*, 113639.

Shirai, T., Sano, M., Tamano, S., Takahashi, S., Hirose, M., Futakuchi, M., Hasegawa,

R., Imaida, K., Matsumoto, K., Wakabayashi, K., Sugimura, T., & Ito, N. (1997). The prostate: a target for carcinogenicity of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) derived from cooked foods. *Cancer Research, 57*(2), 195–198.

Skog, K., Jägerstad, M., & Reuterswärd, A. L. (1992). Inhibitory effect of carbohydrates on the formation of mutagens in fried beef patties. *Food and Chemical Toxicology: an international journal published for the British Industrial Biological Research Association, 30*(8), 681–688.

Skog, K., & Jägerstad, M. (1993). Incorporation of carbon atoms from glucose into the food mutagens MeIQx and 4,8-DiMeIQx using 14C-labelled glucose in a model system. *Carcinogenesis, 14*(10), 2027–2031.

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

Stadtman, E. R. & Levine, R. L. (2000) Protein oxidation. *Annals of the New York Academy of Sciences, 899*, 191– 208.

Sugimura, T., Nagao, N., Kawachi, T., Honda, M., Yahagi, T., Seino, Y., et al. (1977). Mutagen-carcinogens in food, with special reference to highly mutagenic pyrolytic products in broiled foods. Cold Spring Harbour, New York: Cold Spring Harbour Laboratory.

Sugimura T. (1992). Multistep carcinogenesis: a 1992 perspective. *Science (New York, N.Y.), 258*(5082), 603–607.

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

Surveswaran, S., Cai, Y. Z., Corke, H., & Sun, M. (2007). Systematic evaluation of natural phenolic antioxidants from 133 Indian medicinal plants. *Food Chemistry, 102*(3), 938–953.

Suzuki, S., Takahashi, S., Asamoto, M. and Inaguma, S. (2002). Lack of modification of 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx)-induced hepatocarcinogenesis in rats by fenbendazole - a CYP1A2 inducer. *Cancer Letters, 185*(1), 39-45.

Szterk A. (2013). Chemical state of heterocyclic aromatic amines in grilled beef: evaluation by in vitro digestion model and comparison of alkaline hydrolysis and organic solvent for extraction. *Food and chemical toxicology: an international journal published for the British Industrial Biological Research Association, 62*, 653–660.

Szterk A. (2015). Heterocyclic aromatic amines in grilled beef: the influence of free amino acids，nitrogenous bases，nucleosides，protein and glucose on HAAs content. *Journal of Food Composition and Analysis, 40*: 39-46.

Takayama, S., Nakatsuru, Y., Masuda, M., Ohgaki, H., Sato, S., & Sugimura, T. (1984).

Demonstration of carcinogenicity in F344 rats of 2-amino-3-methyl-imidazo[4,5-f]quinoline from broiled sardine, fried beef and beef extract. *Japanese Journal of Cancer Research: Gann, 75*(6), 467–470.

Takahashi, M., Toyoda, K., Aze, Y., Furuta, K., Mitsumori, K., & Hayashi, Y. (1993). The rat urinary bladder as a new target of heterocyclic amine carcinogenicity: tumor induction by 3-amino-1-methyl-5H-pyrido[4,3-b]indole acetate. *Japanese Journal of Cancer Research: Gann, 84*(8), 852–858.

Tamano, S., Hasegawa, R., Hagiwara, A., Nagao, M., Sugimura, T., & Ito, N. (1994). Carcinogenicity of a mutagenic compound from food, 2-amino-3-methyl-9H-pyrido[2,3-b]indole (MeAαC), in male F344 rats. *Carcinogenesis, 15*(9), 2009–2015.

Tanaka, T., Barnes, W. S., Williams, G. M., & Weisburger, J. H. (1985). Multipotential carcinogenicity of the fried food mutagen 2-amino-3-methylimidazo[4,5-f]quinoline in rats. *Japanese Journal of Cancer Research : Gann, 76*(7), 570–576.

Tanakamaru, Z., Mori, I., Nishikawa, A. and Furukawa, F. (2001). Essential similarities between spontaneous and MeIQx-promoted aberrant crypt foci in the F344 rat colon. *Cancer Letters, 172*(2), 143-149.

Tang, Y., Kassie, F., Qian, X., Ansha, B., & Turesky, R. J. (2013). DNA adduct formation of 2-amino-9H-pyrido[2,3-b]indole and 2-amino-3,4-dimethylimidazo[4,5-f]quinoline in mouse liver and extrahepatic tissues during a subchronic feeding study. *Toxicological Sciences: an official journal of the Society of Toxicology, 133*(2), 248–258.

Teka, T., Zhang, L., Ge, X., Li, Y., Han, L., & Yan, X. (2022). Stilbenes: Source plants, chemistry, biosynthesis, pharmacology, application and problems related to their clinical Application-A comprehensive review. *Phytochemistry, 197*, 113128.

Totsuka, Y., Takamura-Enya, T., Nishigaki, R., Sugimura, T., & Wakabayashi, K. (2004). Mutagens formed from beta-carbolines with aromatic amines. *Journal of Chromatography. B, Analytical technologies in the biomedical and life sciences, 802*(1), 135–141.

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

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

Uenobe, F., Nakamura, S., & Miyazawa, M. (1997). Antimutagenic effect of resveratrol against Trp-P-1. *Mutation Research, 373*(2), 197–200.

Vangnai, K., Houser, T. A., Hunt, M. C., & Smith, J. S. (2014). Effect of enhancement on the formation of heterocyclic amines in cooked pork loins: preliminary studies. *Meat Science, 98*(2), 88–93.

Vastano, B. C., Chen, Y., Zhu, N., Ho, C. T., Zhou, Z., & Rosen, R. T. (2000). Isolation and identification of stilbenes in two varieties of Polygonum cuspidatum. *Journal*

of Agricultural and Food Chemistry, 48(2), 253–256.

Villaverde, A., & Estévez, M. (2013). Carbonylation of myofibrillar proteins through the maillard pathway: effect of reducing sugars and reaction temperature. *Journal of Agricultural and Food Chemistry, 61*(12), 3140–3147.

Wakabayashi, K., Nagao, M., Esumi, H., & Sugimura, T. (1992). Food-derived mutagens and carcinogens. *Cancer Research, 52*(7 Suppl), 2092s–2098s.

Wang, W., Ren, X. P., Bao, Y. J., Zhu, Y. X., Zhang, Y. W., Li, J. K., & Peng, Z. Q. (2022). Inhibitory effects of hyperoside and quercitrin from Zanthoxylum bungeanum Maxim. leaf on 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine formation by trapping phenylacetaldehyde. *European Food Research and Technology, 248*, 25–34.

Wang, Q., Cheng, W., Zhang, Y., Kang, Q., Gowd, V., Ren, Y., Chen, F., & Cheng, K. W. (2021). A novel potent inhibitor of 2-amino-1-methyl-6-phenylimidazo[4,5-b] pyridine (PhIP) formation from Chinese chive: Identification, inhibitory effect and action mechanism. *Food Chemistry, 345*, 128753.

Weisburger, J. H., Dolan, L., & Pittman, B. (1998). Inhibition of PhIP mutagenicity by caffeine, lycopene, daidzein, and genistein. *Mutation Research, 416*(1-2), 125–128.

Widmark E. (1939). Presence of cancer-producing substances in roasted food. *Nature, 143* (3632): 984.

Xu, Y., Cheng, Y., Zhu, Z., Guo, H., Bassey, A. P., Huang, T., Huang, Y., & Huang, M. (2023). Inhibitory effect of mulberry leaf (Morus alba L.) extract on the formation of free and bound heterocyclic amines in pan-fried muscovy duck (Cairina moschata) patties. *Food Control, 144,* Article 109359.

Xue, C., Quan, W., Li, Y., He, Z., Qin, F., Wang, Z., Chen, J., & Zeng, M. (2022a). Mitigative capacity of Kaempferia galanga L. and kaempferol on heterocyclic amines and advanced glycation end products in roasted beef patties and related mechanistic analysis by density functional theory. *Food Chemistry, 385*, 132660.

Xue, C., Chen, Q., He, Z., Qin, F., Wang, Z., Chen, J., & Zeng, M. (2022b). Release mechanism between sarcoplasmic protein-bound and free heterocyclic amines and the effects of dietary additives using an in-vitro digestion model. *Food Chemistry, 377*, 131993.

Yadollahi-Farsani, M., Gooderham, N. J., Davies, D. S., & Boobis, A. R. (1996). Mutational spectra of the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine(PhIP) at the Chinese hamsters hprt locus. *Carcinogenesis, 17*(4), 617–624.

Yamazoe, Y., Abu-Zeid, M., Manabe, S., Toyama, S., & Kato, R. (1988). Metabolic activation of a protein pyrolysate promutagen 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline by rat liver microsomes and purified cytochrome P-450. *Carcinogenesis, 9*(1), 105–109.

Yang, H., Ji, Z., Wang, R., Fan, D., Zhao, Y., & Wang, M. (2021). Inhibitory effect of

selected hydrocolloids on 2-amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP) formation in chemical models and beef patties. *Journal of Hazardous Materials, 402*, 123486.

Yu, C., Shao, Z., Liu, B., Zhang, Y., & Wang, S. (2016). Inhibition of 2-Amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP) Formation by Alkoxy Radical Scavenging of Flavonoids and Their Quantitative Structure-Activity Relationship in a Model System. *Journal of Food Science, 81*(8), C1908–C1913.

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

Zamora, R., Alcón, E., & Hidalgo, F. J. (2014). Ammonia and formaldehyde participate in the formation of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in addition to creati(ni)ne and phenylacetaldehyde. *Food Chemistry, 155*, 74–80.

Zamora, R., Aguilar, I., Granvogl, M., & Hidalgo, F. J. (2016). Toxicologically Relevant Aldehydes Produced during the Frying Process Are Trapped by Food Phenolics. *Journal of Agricultural and Food Chemistry, 64*(27), 5583–5589.

Zhang, N., Zhao, Y., Fan, D., Xiao, J., Cheng, K.-W., & Wang, M. (2020). Inhibitory effects of some hydrocolloids on the formation of heterocyclic amines in roast beef. *Food Hydrocolloid, 108,* Article 106073.

Zhang, Y., Yu, C., Mei, J., & Wang, S. (2013). Formation and mitigation of heterocyclic aromatic amines in fried pork. *Food Additives & Contaminants. Part A, Chemistry, analysis, control, exposure & risk assessment, 30*(9), 1501–1507

Zhao, M., Liu, Z., Sun, Y., Shi, H., Yun, Y., Zhao, M., Xia, G., & Shen, X. (2024). Novel hydrocolloids synthesized by polyphenols grafted onto chitosan: A promising coating to inhibit PhIP formation during pan-frying of golden pompano fillets. *Food Chemistry, 447*, 139029.

Zhao, X., Liu, H., Zhou, X., Chen, X., Hu, N., Zhang, Y., & Wang, S. (2021a). 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine Induced Colon Injury by Disrupting the Intestinal Bacterial Composition and Lipid Metabolic Pathways in Rats. *Journal of Agricultural and Food Chemistry, 69*(1), 437–446.

Zhao, L., Zhou, N., Zhang, H., Pan, F., Ai, X., Wang, Y., Hao, S., & Wang, C. (2021b). Cyanidin-3-O-glucoside and its metabolite protocatechuic acid ameliorate 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) induced cytotoxicity in HepG2 cells by regulating apoptotic and Nrf2/p62 pathways. *Food and Chemical Toxicology : an international journal published for the British Industrial Biological Research Association, 157*, 112582.

Zhao, L., Pan, F., Zhou, N., Zhang, H., Wang, Y., Hao, S., & Wang, C. (2021c). Quantitative proteomics and bioinformatics analyses reveal the protective effects of cyanidin-3-O-glucoside and its metabolite protocatechuic acid against 2-amino-3-methylimidazo[4,5-f]quinoline (IQ)-induced cytotoxicity in HepG2 cells via apoptosis-related pathways. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association, 153*,

112256.

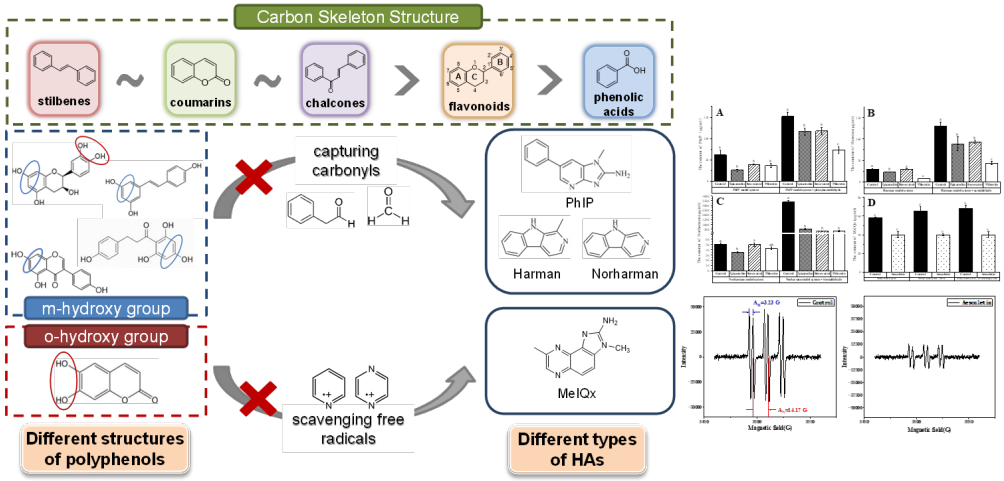
Zochling, S., & Murkovic, M. (2002). Formation of the heterocyclic aromatic amine PhIP: Identification of precursors and intermediates. *Food Chemistry, 79*(1), 125–134.

3

Chapter III Effect of plant polyphenols with different m-hydroxy and o-hydroxy groups on the inhibition of heterocyclic amines formation in roasted meat

Short overview of chapter III

Based on the literature review in chapter II, there are many types of polyphenols, which inhibit HAs generation mainly through the scavenging of free radicals and capture of reactive carbonyls, and the number and location of hydroxyl groups on the polyphenols play an important role. However, the inhibition patterns of polyphenols with different structures on different types of HAs are not clear, which is of great significance for the effective identification and screening of polyphenols on inhibition of HAs. Therefore, in this study, the inhibition effects of nine polyphenols with different structures on various HAs in roasted meat were investigated and further verified in a model system, and the patterns of inhibition of different HAs by polyphenols with different structures were clarified.



Graphical abstract: Clarify the inhibitory patterns of polyphenols with different structures on various types of heterocyclic amines.

The work from this chapter was published on *Food Control*, as can be retrieved by: Yang, X. Y., Blecker, C., Liu, H., Zhang, D. Q., Wang, Z. Y. (2023). Effect of plant polyphenols with different m-hydroxy and o-hydroxy groups on the inhibition of heterocyclic amines formation in roasted meat. Food Control, 153, 109960. https://doi.org/10.1016/j.foodcont.2023.109960.

Abstract

To elucidate the regulation of different structures of polyphenolic compounds with m-hydroxy and o-hydroxy groups inhibiting the formation of different heterocyclic amines (HAs), the reactions between classified polyphenols (cover flavonoids, phenolic acids, stilbenes, coumarins, chalcones, and with different hydroxyl groups) and roasted lamb patties were studied, and then validated in model systems in vitro. The results indicated that the chalcone, coumarin, and stilbene compounds were more efficient to inhibit the HAs generation in roasted lamb patties in comparison with the flavonoids, while phenolic acid had the worst effect. Besides, polyphenols inhibit the formation of HAs through a synergistic action of scavenging free radicals and trapping active carbonyls. Polyphenols with m-hydroxyl structures have a stronger inhibition of PhIP, Harman and Norharman by capturing the reaction intermediate carbonyl compounds. Polyphenols with the o-hydroxyl group had a stronger inhibitory effect on MeIQx by scavenging free radicals. It was concluded that the ability of polyphenols to inhibit different HAs can be predicted by their m-hydroxyl and o-hydroxyl groups.

Keywords: Heterocyclic amines, Polyphenols, Reactive carbonyls, Free radicals, M-hydroxy, O-hydroxy

1. Introduction

Heterocyclic amines (HAs), a group of carcinogenic and mutagenic substances, are usually found in high-protein foods (e. g. meat and fish) undergone high-temperature treatment. Up to now, more than 30 different HAs have been discovered in different foods (Barzegar, Kamankesh, & Mohammadi, 2019). Different HAs share similar chemical structures, including imidazole rings, pyridine rings and pyrazine rings (Fig. S2). Maillard and pyrolytic reactions play an important role in the formation of HAs. HAs may be formed by further conversion of heterocyclic pyridine/pyrazine from Strecker aldehyde resulting from Maillard reaction and creatinine (Alaejos, & Afonso, 2011). In addition, the involvement of free radicals also has been considered by many researchers as a potential mechanism for the formation of HAs (Pearson et al., 1992). Since the reaction to form HAs involves free radicals and active carbonyl groups, there are currently two views of the HAs production pathway: the free radical and the carbonyl formation pathway respectively (Zamora, & Hidalgo, 2020). The radical generation pathway mainly appears in some Aminoimidazoazaarenes (AIAs) HAs. The possible mechanism is that pyrazine and pyridine radicals from the Maillard reaction react with creatinine and aldehydes to form heterocyclic amines to form imidazolquinoline and imidazolquinoxaline (Xiong, 2017). The carbonyl pathway has been found in the formation of multiple HAs. The formation mechanism of PhIP is relatively clear, phenylalanine and creatinine are degraded by Strecker to generate phenylacetaldehyde and creatinine, and then the aldol condensation product generated by the aldol condensation reaction is then reacted with formaldehyde and ammonia to generate PhIP (Zöchling, Murkovic, 2002). Tryptophan Amadori rearrangement product under the existence of carbonyl compound after Pictet Spengler cyclization reaction to form tetrahydro-β-carbolines, then β-carbolines are formed by an oxidation process (Rönner et al., 2000). However, neither of the HAs production pathways has been fully elucidated. Epidemiological studies have shown that eating too much cooked red meat will increase the risk of cancer, which is strongly linked to the presence of HAs (Boada, Henríquez-Hernández, & Luzardo, 2016). Therefore, it is significant to find out potent inhibitors in the formation pathways of HAs to reduce their amount in foods.

The addition of polyphenols has been widely considered to be a remarkably effective method to control the production of HAs (Santhakumar, Battino, & Alvarez-Suarez, 2018; Vauzour et al., 2010). More than 8,000 phenolic substances have been found in nature, which can be divided into flavonoids, stilbenes, chalcones, phenolic acids, and so on according to their carbon skeleton structure (Lund, 2021). Many of the existing studies have shown that the main mechanisms of polyphenols, especially flavonoids, to inhibit the HAs, are due to the scavenging of free radicals and trapping active carbonyl compounds (Yu et al., 2016; Vitaglione, & Fogliano, 2004; Cheng et al., 2009). Moreover, polyphenolic compounds are closely related to the quantity and

location of hydroxyl structures in exerting antioxidant activity and inhibiting the formation of HAs. (Falowo, Fayemi, & Muchenje, 2014; Hidalgo, Aguilar, & Zamora, 2017). Flavonoids with two or three hydroxyl structures on the ortho site of the B-ring (on the 3,4 position) are the most effective radical scavengers (Seyoum, Asres, & El-Fiky, 2006) (**Figure 2-8**). Phenolic antioxidants inhibit the formation of free radical intermediates, pyrazine cations and carbon center radicals produced in the Maillard reaction, and effectively inhibit the formation of HAs. (Kikugawa, 1999). Phenolic compounds with two hydroxyl structures on the meta site of the aromatic nucleus had a remarkable inhibition effect on PhIP formation with high carbonyl scavenging ability (**Figure 2-8**). The introduction of additional hydroxy and amino groups into the ortho or para positions in relation to the other two hydroxy groups, such as in pyrogallol and 1,2,4-trihydroxybenzene, significantly reduces the ability to remove active carbonyl compounds and thus reduce the inhibition of HAs (Salazar, Arámbula-Villa, Hidalgo, & Zamora, 2014). Polyphenols with different structures have different inhibitory effects on HAs, but there is still a lack of systematic research on which structural polyphenols have a strong effect on the inhibition of which HAs, and also a lack of the study of the inhibitory mechanism of other structural polyphenols except for flavonoids against different kinds of HAs.

Therefore, this study investigated the effects of polyphenol compounds from five different classes (flavonoids, phenolic acids, stilbenes, coumarins, chalcones) and covering three different structures (only with o-hydroxyl structure, only with m-hydroxyl structure, both o-hydroxyl and m-hydroxyl) on the type and content of HAs in roasted lamb meat. By summarizing the inhibition of the formation of different types of HAs by different structural polyphenols and validating in a model system, we propose to offer a theoretical foundation for the subsequent in-depth study of the molecular mechanism of phenolic compounds of different structures inhibiting the formation of different types of HAs. This new knowledge could also be useful for the research and development of HAs control technology in meat processing.

2. Materials and methods

2.1 Materials

HAs standards (99.9%): IQ, Harman, Trp-P-1, Norharman, MeIQ, AαC, Glu-P-2, MeAαC, MeIQx, 7,8-DiMeIQx, Trp-P-2, Glu-P-1, IQx, PhIP, 4,8-DiMeIQx, 4,7,8-DiMeIQx, were source from TRC (Toronto, Canada). 97% purity of tryptophan and phenylalanine were sourced from Sinopharm Chemical Reagent. Glycine was purchased from Amresco of America. Creatinine was sourced from Solarbio (Beijing, China). Ammonium formate (99.9%), sodium hydroxide, diethylene glycol (99%), ethyl acetate (99.5%), glucose, and hydrochloric acid were sourced from Merck of Germany, Aladdin Company, Macklin Company of China, and Sinopharm company of China. The chromatographic grade of acetonitrile, methanol and acetic acid were supplied by Thermo Fisher Company of USA, respectively. Bond Elut QuEChERS extraction packet and Bond Elut QuEChERS kit (Agilent, USA). Quercetin (98%), naringenin (98%), epicatechin (97%), luteolin (96%), genistein (98%), gallic acid

(99%), resveratrol (98%), aesculetin (98%) and phloretin (98%) were from Yuanye (Shanghai, China).

2.2 Samples collection and preparation

The Longissimuss lumborum (LL) muscles of the nine small-tailed Han lambs (approximately 8 months of age and net weighed 27.39 ± 0.88 kg on average) were purchased from Hebei Jinhong Halal Meat Co. Ltd. in the P.R. China. After the carcass was chilled in a cooling chamber for 24 h (3 ± 0.5 °C, 95% humidity) and Longissimus lumborum (LL) muscles were collected, all samples were transported to the laboratory by cold chain truck and stored in cold storage at -20 °C.

2.3 Roasted lamb patties preparation

After the sample was thawed at 4 ± 1℃ in a constant temperature incubator, all connective tissue and redundant fat were removed. Next, 20 ± 0.1 g meat slurries with 0.1 mmol polyphenols powder was prepared into a round patty with a thickness of 0.8 cm and a diameter of 5.5 cm. Selected nine polyphenols are quercetin, naringenin, epicatechin, luteolin, genistein, gallic acid, resveratrol, aesculetin and phloretin (The structures of nine polyphenols are shown in **Figure 3-1**.). The group without polyphenols was the control. Each group had 3 meat patties, and the experiment was repeated twice. According to Ding et al. (Ding et al., 2022), all patties were left to marinate for 8 h at 4°C, then roasted on a 250-300℃ electric oven for 10 min (each side takes 5 min). After roasting, the patties were cooled naturally to room temperature, ensuring that all samples were handled in a consistent manner. All the roasted meat was ground and finally stored at −30 °C by vacuum-packed until used.

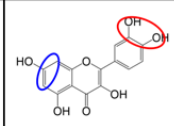
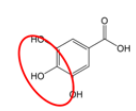
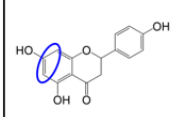
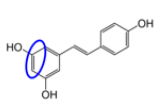
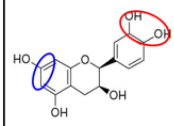
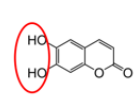
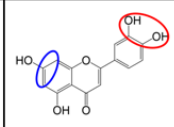
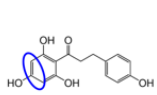
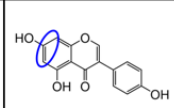
classification		polyphenol	structural formula	classification	polyphenol	structural formula
flavonoid	flavonol	Quercetin		phenolic acid	Gallic acid	
	flavanone	Naringenin		stilbenes	Resveratrol	
	flavanol	Epicatechin		coumarin	Aesculetin	
	flavone	Luteolin		chalcone	Phloretin	
	isoflavone	Genistein				

Figure 3-1: The selected five classes of polyphenols with different structures in this study. The red circles highlight the free radical scavenging structures, while the blue circles indicate the moieties responsible for capturing reactive carbonyl groups.

2.4 Analysis of HAs

The protocol for extraction and analysis of HAs of the roasted lamb patties was adopted from our previous method with some modifications (Suleman et al., 2019; Ding, Zhang, Liu, Wang, & Hui, 2022). The QuEChERS method was used to extract HAs. Typically, 10 mL of deionized water, 2 g of sample meat and one homogeneous stone were placed in a centrifuge tube of 50 mL, then shaken at room temperature for 20 min. Next, 10 mL of acetonitrile solution containing 1% acetic acid was added and shaken for 15 min. Then, a bag of Bond Elut QuEChERS extraction packet was added and shaken for 60s and centrifuged for 600 s (13,020 × g). Six milliliters of the supersolution were taken to a Bond Elut QuEChERS tube, then homogenized for 60 s and centrifuged for 5 min (4 °C, 10,000 r/min). 1 mL of the supersolution was taken and blown dry with N_2, and 0.20 mL methanol was poured to the blow-dry solution and shaken to fully dissolve. After filtering through a membrane of 0.22 μm, the dissolved solution was prepared for analysis.

The extraction method of HAs in model systems was slightly modified by the previous study. (Özdestan, Kaçar, Keşkekoğlu, Üren, 2013). All the reaction solution (4 mL) was poured into a 150 mL conical flask containing 50 mL ethyl acetate. After a 15 min ultrasonication, 10 mL of ethyl acetate extraction solution was collected as a sample. Then, the Waters Oasis MCX column was excited by both 6 mL chromatographic methanol, ultra-pure water and ethyl acetate, respectively. Next, 10 mL of the extract was loaded to the MCX column in batches, followed by washing the column with 6 mL of hydrochloric acid (0.1 mol/L) and chromatographic methanol, respectively. Finally, 6 mL of ammoniated methanol with a volume ratio of 19:1 was added to elute the adsorbed HAs in the column packing. The collected ammoniated methanol elution solution was blown dry using N_2 to nearly dry. After that, the dry matter was reconstituted with 0.5 mL of chromatographic methanol and then diluted with methanol to a suitable multiple. Finally, the complex solution was filtered with an organic filter (0.22 μm) before the analysis of UHPLC–MS/MS.

The analysis of HAs was conducted by UHPLC-MS/MS with triple quadrupole (6495, Agilent, USA). The mass spectrometer parameters: the ionization mode was ESI; the needle voltage was 4 kV; the temperature and flow of dry gas (N_2) were 300°C and 11 L/min, respectively; the capillary temperature was 260 °C; the detection mode was positive multiple-reaction mode (MRM), positive product-ion, and positive parent-ion. Column (1.8 μm, 2.1 mm × 50 mm): Zorbax SB-C18; mobile phase A: acetic acid - ammonium acetate buffer (10 mmol, pH 2.9); mobile phase B: 100 % acetonitrile. The flow rate, sample injection volume, and column temperature were 0.4 mL/min, 2 μL, and 30 °C respectively. Mobile phase gradient was set as following: 0–0.5 min, 5 % B; 0.5–5 min, 15 % B; 5–7 min, 27 % B; 7–8 min, 55 % B; 8–8.5 min, 27 % B; 8.5–9.0 min, 5 % B; 9.0–10.0 min, 5 % B. The gradient elution procedure was also based on Ding et al (2022). Identification and quantification of HAs in roasted lamb meat were performed by comparing the retention times and peak areas with those of HAs standards.

Table 3-1. Mass spectrpmetric parameters for 16 HAs

HAs	Retention Time/min	m/z			Cone voltage/V	Collision energy/eV
		Precursor ion	Quantifier ion	Qualifier ion		
Glu-P-2	1.066	185.10	78.05	78.05, 158.10	120	25, 37
IQ	1.294	199.10	184.12	131.09, 184.12	120	21, 30
IQx	1.588	200.08	185.12	132.14, 185.12	120	30, 30
MeIQ	1.862	213.11	198.09	145.15, 198.09	120	29, 27
Glu-P-1	2.189	199.10	92.10	172.15, 92.10	120	30, 25
MeIQx	2.942	214.10	131.07	173.18, 131.07	120	25, 41

Continued Table 3-1. Mass spectrpmetric parameters for 16 HAs

HAs	Retention Time/min	m/z			Cone voltage/ V	Collision energy/eV
		Precursor ion	Quantifier ion	Qualifier ion		
Norharman	3.912	169.06	115.09	89.05, 115.09	120	48, 33
4,8-DiMeIQx	4.015	228.10	213.09	187.09, 213.09	120	30, 30
7,8-DiMeIQx	4.015	228.10	187.15	187.15, 131.13	120	30, 30
Harman	4.659	183.09	115.15	89.09, 115.15	120	49, 34
4,7,8-DiMeIQx	5.133	242.13	145.09	201.21, 145.09	120	26, 42
PhIP	5.724	225.10	210.05	140.08, 210.05	120	30, 30
AαC	6.283	184.07	167.07	167.07, 140.13	120	24, 33
Trp-P-1	6.382	212.12	195.14	168.09, 195.14	120	30, 25
MeAαC	7.400	198.10	181.13	127.13, 181.13	120	38, 25
Trp-P-2	7.400	198.10	154.14	181.08, 154.14	120	24, 30

2.5 Assessment of radical scavenging activity (ABTS•+ and DPPH assays)

The DPPH radical scavenging activity was determined based on the procedure of the previous study with little modifications (Musa, Abdullah, & Al-Haiqi, 2016). 1g of each meat patty before and after roasting was added with 20 mL 60% ethanol in a 37°C constant temperature water bath for 24 h and then centrifuged at 293 × g for 10 min to collect the supernatant as samples. 180 μL of DPPH solution (0.4 mM) and 20 μL of the sample were added to each of the 96 wells of the microtiter plate (Absolute ethanol was used as a control). Plates were mixed, covered, and stored for 30 min away from light at room temperature. The light absorption value at 517 nm was determined using a microplate reader. The calculation method of DPPH radical scavenging activity (DRSA%) was as follows:

DRSA%= (1-(Absorbance of sample)/ (Absorbance of control))×100

The ABTS radical scavenging activity was determined based on the procedure of the previous study with little modifications (Chen, & Kang, 2014). The concentration of 2.45 mM potassium persulfate was mixed with 7 mM ABTS (1:1, v/v). The mixture was incubated for 12h away from light at room temperature. After that, the absorbance was adjusted to 0.70 ± 0.05 by adding absolute ethanol (at 734 nm) to obtain a usable ABTS working solution. Then, 180 μL of ABTS reagent and 20 μL of the sample were added to each of the 96 wells of the microtiter plate (Trolox was used as control), and stored for 6 min at room temperature of the reaction. The calculation method of ABTS free radical scavenging rate (ARSA%) was as follows:

ARSA%= (1-(Absorbance of sample)/ (Absorbance of control))×100

2.6 Glucose/amino acid/creatinine model system production

The construction of model response systems of Norharman, Harman, PhIP, and MeIQx was performed as described previously with little modifications (Hui et al., 2021; Oguri et al., 1998). (i) PhIP formation: Creatinine (0.4 mmol), glucose (0.2 mmol), and phenylalanine (0.4 mmol) were added in 4 mL of pH 7.0 diethylene glycol (20% citric acid-phosphate buffer, w/w), with or without (control) the polyphenols (0.2 mmol), and heated at 150 °C in closed reaction bottles for 1 h. What's more, phenylacetaldehyde (0.2 mmol) was employed in the model system to verify the rule of inhibiting PhIP formation by polyphenols containing m-dihydroxy groups. Three polyphenols were: epicatechin, resveratrol, and phloretin. (ii) Harman/Norharman formation: Tryptophan (0.4 mmol), and glucose (0.2 mmol) were added in 4 mL of pH 7.0 diethylene glycol (20% citric acid-phosphate buffer, w/w), with or without (control) the polyphenols (0.2 mmol), and heated at 150 °C in closed reaction bottles for 1 h. Formaldehyde and acetaldehyde (0.08 mmol), two kinds of reactive carbonyls were employed in the model system respectively to verify the rule of inhibiting Harman and Norharman formation by polyphenols containing m-dihydroxy groups. Three polyphenols were selected: epicatechin, resveratrol, and phloretin. (iii) MeIQx formation: Creatinine (0.4 mmol), glucose (0.2 mmol), and glycine (0.4 mmol) were added in 4 mL of pH 7.0 diethylene glycol (20% citric acid-phosphate buffer, w/w), with or without (control) the aesculetin (0.2 mmol), and heated at 150 °C in closed reaction bottles for 1 h. Methylglyoxal and 2, 5-dimethylpyrazine (0.08 mmol), two compounds related to the carbonyl formation pathway were employed respectively to verify the rule of inhibiting MeIQx formation by polyphenols containing ortho dihydroxy groups.

2.7 Quantification of free radical formation by spin trap ESR

200 μL of spin trap N-tert-butyl-α-phenylnitrone (PBN) (100 mM) was added to the 200 μL of creatinine/glucose/glycine model system containing 0.2mmol of aesculetin (control group without aesculetin). Samples were taken by capillary after the mixture was homogeneous. PBN adducts were monitored on a Bruker ESR spectrometer (A300-10/12, Germany) at 150 °C after 10 min. The ESR conditions were as follows: static field, 3460 G; modulation amplitude 1.00 G; microwave bridge frequency, 9.85 GHz; microwave bridge power, 19.72 mW.

2.8 Statistical analysis

The statistical analysis was presented using the SPSS 20 (IBM Corp., New York, USA). The Duncan test and one-way ANOVA analysis were used to determine the multi-group comparison hypothesis test. Values were mean ± SD from at least three independent repeated experiments. The correlation between free radical scavenging and HAs inhibition by polyphenols with different structures were expressed by the Pearson correlation coefficient.

3. Results and discussion

3.1 Effect of different structure polyphenols on HAs in roasted lamb meat

The variation of the types and levels of HAs after polyphenols addition are exhibited in **Table 3-1**. Six different HAs including two polar HAs (PhIP and MeIQx) and four non-polar HAs (Norharman, MeAαC, Trp-P-2, and Harman) were totally detected. The contents of Harman, Norharman, PhIP, MeIQ, Trp-P-2, and MeAαC in the normal group were 3.62, 7.42, 5.83, 11.55, 0.58, and 0.60 ng/g respectively. Previous studies have shown that IQx, MeIQx, Harman, Norharman, and PhIP can be detected in charcoal-grilled lamb patties with a content ranging from 0.83 to 6.70 ng/g (Ding, Zhang, Liu, Wang, & Hui, 2022). PhIP, MeIQx, Harman, IQx, MeAαC, Norharman, Glu-P-2, IQ (4, 5-B), and IQ with the contents ranged 0.13-15.60 ng/g were detected by superheating steam roasting (Suleman et al., 2019). The results showed that the different processing method used has a great influence on the formation of HAs. These changes may be due to the different heat transfer methods leading to different heat transfer coefficients, the influence of different cooking media (such as water), temperature differences associated with different cooking techniques, as well as, in the case of barbecue, the influence of some compounds from charcoal and the smoke produced during grilling (Gibis, 2016).

The variation in the total HAs content showed that polyphenols can effectively inhibit the production of HAs and there are differences in the inhibition effect of different types of polyphenols. Overall, phenolic acid compounds were the least effective in inhibiting the production of HAs, with an inhibition rate of only 10.88%. Flavonoids inhibited HAs production by 44.05%-64.87%. Chalcone, coumarin, and stilbene compounds had higher inhibition rates, of 69.59%, 70.41%, and 72.30% respectively. Among the flavonoids, quercetin and genistein had stronger inhibitory effects than luteolin. It may be due to the fact that flavonoids with a substituent on the 3' site of the C-ring have stronger antioxidant activity (Cai et al., 2006). In addition, the inhibitory effect of naringenin was stronger than that of luteolin under similar structural conditions, which is related to a double bond between the 2 and 3 sites in the C ring that may affect the antioxidant activity. Therefore, the order of inhibiting the production of HAs in flavonoids can be inferred as follows: isoflavones > flavonols > flavanones > flavanols > flavones.

Table 3-2. Effects of different structure polyphenols on the formation of HAs in roasted lamb patties

Groups		Harman (ng/g)	Norharman (ng/g)	Trp-P-2 (ng/g)	MeAαC (ng/g)	PhIP (ng/g)	MeIQx (ng/g)	Total HAs (ng/g)
	Control	3.62±0.18^{a}	7.42±0.97^{a}	0.58±0.09^{a}	0.60±0.08^{a}	5.83±0.14^{a}	11.55±1.18^{a}	29.60±0.27^{a}
	Quercetin	2.00±0.45^{bc}	2.89±0.53^{bc}	0.47±0.06^{ab}	0.48±0.06^{ab}	4.35±0.79^{abc}	4.23±0.42^{bc}	14.41±0.55^{e}
	Naringenin	1.87±0.08^{bc}	3.46±0.29^{c}	0.31±0.02^{cd}	0.31±0.02^{cde}	4.10±0.14^{bcd}	5.42±0.05^{b}	15.47±0.29^{de}
flavonoid	Epicatechin	0.84±0.03^{e}	2.12±0.15^{bc}	0.30±0.02^{d}	0.30±0.01^{de}	2.68±0.51^{cd}	9.97±1.06^{a}	16.22±0.25^{d}
	Luteolin	2.15±0.02^{bc}	5.64±0.91^{b}	0.44±0.04^{bc}	0.45±0.04^{bc}	5.83±0.89^{a}	3.40±0.48^{cd}	17.89±0.50^{c}
	Genistein	1.57±0.08^{cd}	2.22±0.11^{bc}	0.39±0.04^{bcd}	0.43±0.06^{bcd}	2.65±0.12^{cd}	3.14±0.23^{cd}	10.40±0.24^{f}
phenolic acid	Gallic acid	2.52±0.13^{b}	6.69±0.67^{ab}	0.42±0.00^{bcd}	0.41±0.02^{bcd}	4.99±0.51^{ab}	11.36±0.47^{a}	26.38±0.58^{b}
stilbenes	Resveratrol	0.84±0.06^{e}	1.59±0.26^{d}	0.33±0.02^{cd}	0.25±0.02^{e}	2.91±0.48^{cd}	2.27±0.38^{de}	8.20±0.17^{g}
coumarin	Aesculetin	1.82±0.50^{bc}	2.03±0.55^{c}	0.29±0.01^{d}	0.30±0.01^{de}	3.31±0.55^{cd}	1.00±0.12^{e}	8.76±0.39^{g}
chalcone	Phloretin	1.01±0.02^{de}	1.41±0.16^{d}	0.34±0.03^{bcd}	0.34±0.03^{bcde}	2.59±0.41^{d}	3.30±0.09^{cd}	9.00±0.15^{g}

Note: Results are represented as the mean values ± standard deviations (n = 3). Lowercase letters (a-e) indicate significant differences in the content of HAs in roasted lamb patties in the same column ($p < 0.05$).

3.2 Changes in antioxidant activities of polyphenols with different structures before and after roasting

The radical scavenging activities of polyphenols presented as percent of inhibition of DPPH and ABTS were determined before and after roasting are illustrated in **Figure 3-2**. As can be observed, after 10 min of roasting, the radical scavenging activity of all samples experienced a decrease, although the radical-scavenging profiles did not change. Polyphenols with only o-hydroxyl groups (gallic acid, aesculetin) showed the highest radical-scavenging activity, followed by polyphenols with both o-hydroxyl groups and m-dihydroxy groups (epicatechin, quercetin, and luteolin), and much stronger than that with only m-dihydroxy groups (phloretin, resveratrol, naringenin, and genistein). Similar to the findings of previous studies, the antioxidant capacity of flavonoids is related to the o-dihydroxy on the B ring (3 ', 4 '-dihydroxy), the double bond on the 2 and 3 sites is closely related to the conjugation of the 4-keto bond on the C ring and the 3- and 5-hydroxy structures (Amić et al., 2007; Lucić, Amić, & Trinajstić, 2008; Vauzour et al., 2010). The O - dihydroxyl group is the main structure of phenolic scavenging free radicals (Cai et al., 2006). Phloretin, naringenin, and genistein with only m-dihydroxy structures had relatively high ABTS scavenging ability compared with DPPH scavenging ability, which suggests that antioxidant activity of polyphenols with the m-dihydroxy structure is reflected in the scavenging of cationic free radicals.

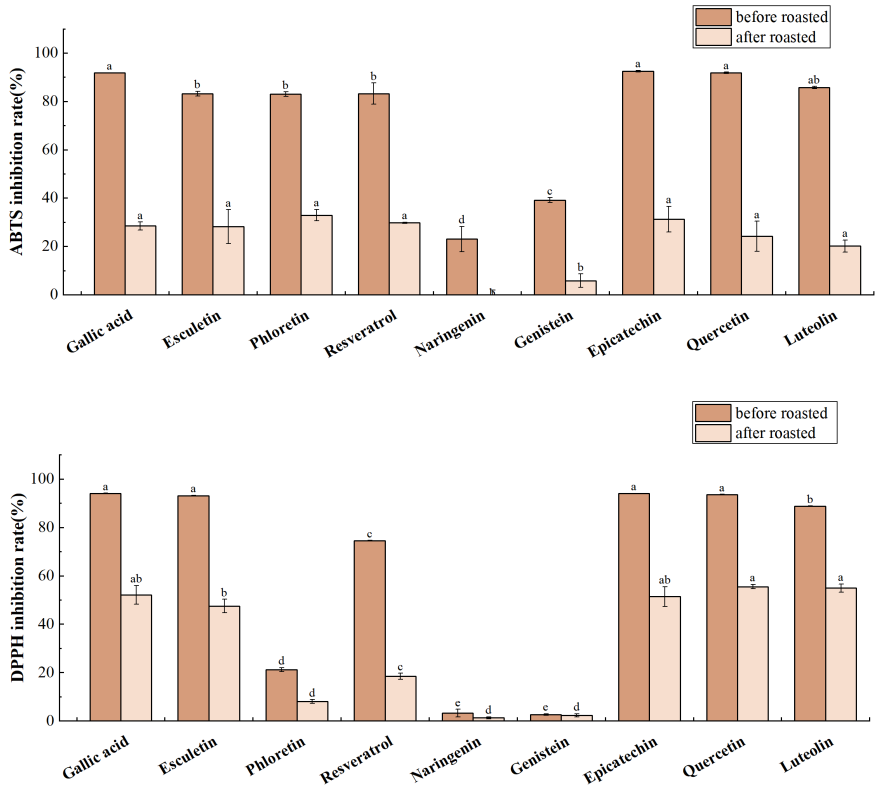


Figure 3-2: Changes of DPPH and ABTS free radical scavenging rates of polyphenols with different structures before and after roasting. Different letters indicate significant differences among groups ($P < 0.05$).

We further analyze the correlation between ABTS or DPPH scavenging activities and HAs inhibition rate which is shown in **Figure 3-3**. There was no correlation between ABTS or DPPH clearance rates and HAs inhibition rates for polyphenols containing o-hydroxyl structures (gallic acid, aesculetin, quercetin, epicatechin, luteolin) (**Figure 3-3**A and **Figure 3-3**C). This more clearly indicates that the o-hydroxyl structure of polyphenols is not the main structure for the inhibition of HAs. In contrast, there was a positive correlation between ABTS or DPPH clearance and HAs inhibition for polyphenols containing only m-dihydroxy groups (resveratrol, phloretin, genistein, naringenin) with correlation coefficients of 0.8 and 0.72, respectively (**Figure 3-3** and **Figure 3-3**D). Consistently, it further illustrates the m-dihydroxyl structure in polyphenols is an effective structural basis for inhibiting the formation of HAs. The results of other studies have also shown that polyphenols indirectly inhibit the formation of HAs by binding to the carbonyl compounds in the

process of HAs through the high-density carbon between the two hydroxyl structures in the aromatic ring interposition (Cheng et al., 2008; Teng, Hu, Tao, Wang, 2018). In conclusion, polyphenols inhibit the formation of HAs through the synergistic action of scavenging free radicals and capturing carbonyl compounds, and capturing carbonyl compounds is the main way, and this is consistent with previous reports (Alaejos, Afonso, 2011).

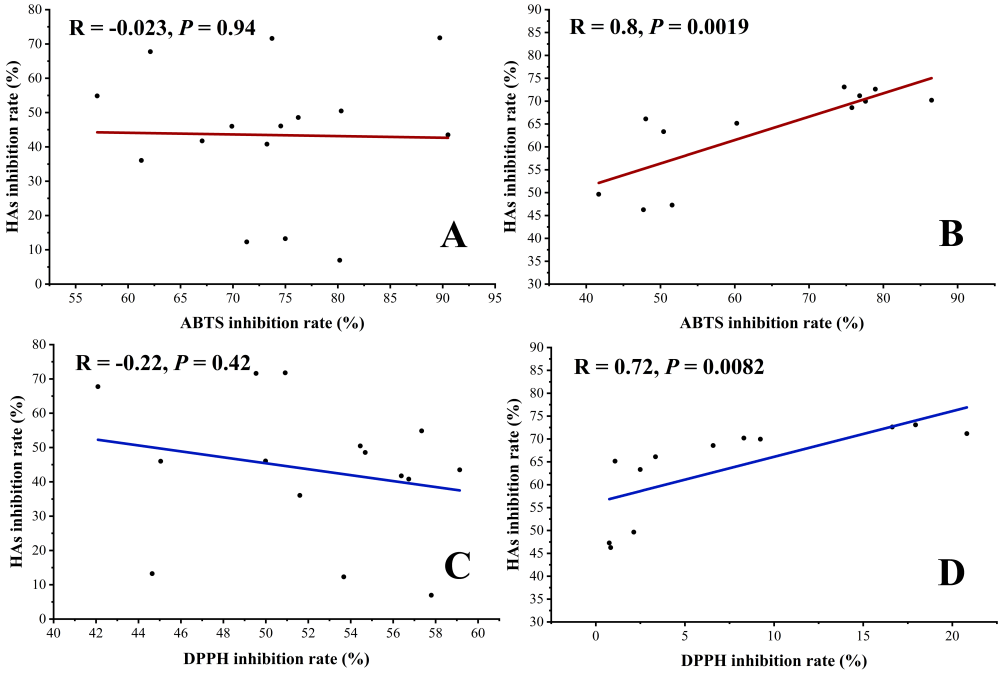


Figure 3-3: Correlation between ABTS (A) and DPPH (C) scavenging activities of polyphenols containing o-hydroxyl groups and HAs inhibition rate; Correlation between ABTS (B), DPPH (D) scavenging activity and inhibition rate of HAs in polyphenols containing only meso-dihydroxy structures

3.3 Regularity of polyphenols with different structures inhibiting the major detected HAs

Harman, Norharman, PhIP and MeIQx are not only the main HAs determined in roasted lamb meat but also the focus of researchers. Therefore, further cluster analysis was conducted on the results of different structural polyphenols inhibition of major HAs and shown in **Figure 3-4**. The results indicated that there is a similar inhibitory condition on PhIP, Norharman, and Harman by polyphenols. What's more, it can be seen that epicatechin, resveratrol, and phloretin with strong inhibition effects on these three HAs all contain meso-dihydroxy structure, indicating that active carbonyl capturing might be the main way for the inhibition of Harman, Norharman and PhIP

production by polyphenolic compounds.

The inhibitory effect of polyphenolic compounds on MeIQx was significantly different from that of the other three HAs. Among the flavonoids, epicatechin was the least effective in inhibiting MeIQx production while the best of the other three HAs. A similar result was shown in the non-flavonoid, aesculetin had the best inhibitory effect on MeIQx compared to resveratrol and phloretin which was just the opposite of the other three HAs. This suggests that polyphenolic compounds may inhibit MeIQx production mainly by scavenging free radicals. In particular, it was noted that the weak inhibition of MeIQx by gallic acid, which also contains only the o-hydroxy structure like aesculetin, suggesting that the inhibition of HAs production by polyphenols may be related to the synergistic effect of other groups on the same molecular weight in addition to the o-2 (3) hydroxyl and m-dihydroxyl structures.

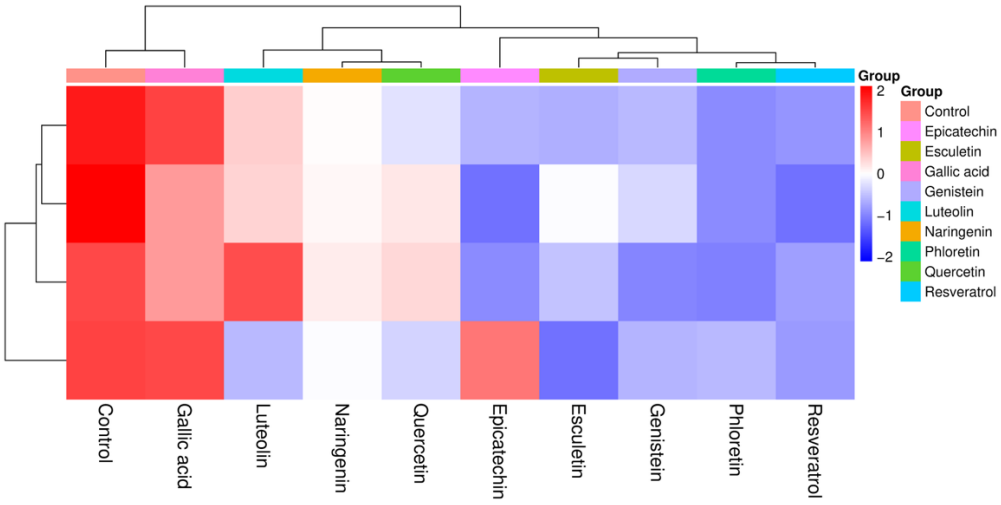


Figure 3-4: Heatmap analysis of the inhibitory effects of polyphenols with different structures on different major HAs

3.4 Validation of inhibition of HAs formation by polyphenols with different structures in the model systems

Further, for purpose of verifying the hypothesis that polyphenols inhibit Harman, Norharman, and PhIP mainly by trapping the reactive carbonyls through the m-hydroxyl structure and inhibit MeIQx mainly by scavenging free radicals through the o-hydroxyl structure, validation experiments were designed. Through the validation experiments, the effects of the addition of different structural polyphenols and intermediate reactive carbonyl compounds on the production of different HAs were

carried out in different model systems.

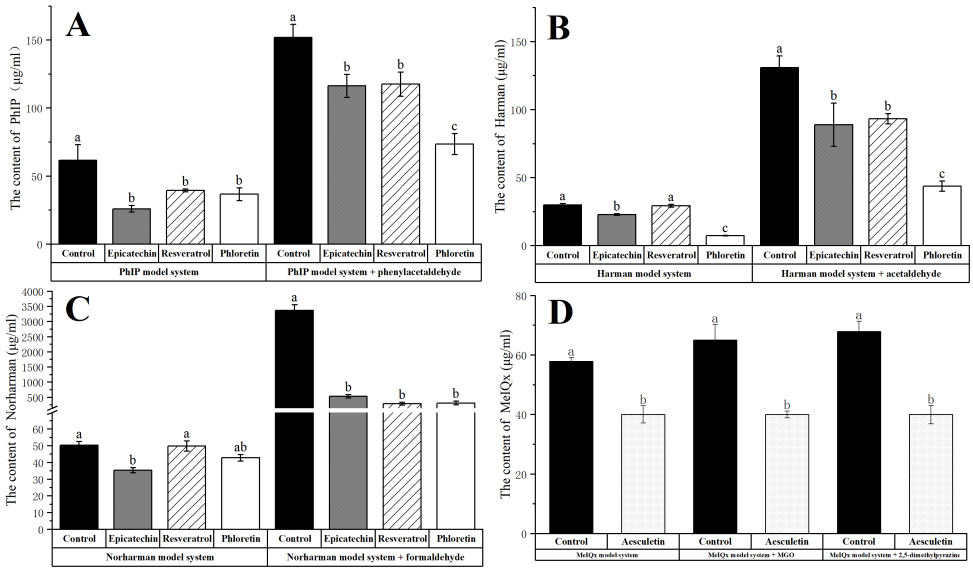


Figure 3-5: Effects of polyphenols and intermediate carbonyl compounds on the formation of HAs in PhIP (A), Harman (B), Norharman (C) and MeIQx (D) model systems. Different letters indicate significant differences among groups ($P < 0.05$).

The formation of HAs involves reactive carbonyls, phenylacetaldehyde is considered to be the pivotal carbonyl compound involved in the synthesis of PhIP (Zöchling, Murkovic, 2002; Murkovic et al., 1999; Cheng et al., 2008). The effect of polyphenols with m-dihydroxyl structure in the model system on the effect of PhIP formation was displayed in **Figure 3-5**A. The content of PhIP was prominently inhibited by the supplementation of epicatechin, resveratrol and phloretin ($P < 0.05$). It is obvious that the addition of phenylacetaldehyde promoted the production of PhIP from 61.7 μg/mL to 151.9 μg/mL, indicating that phenylacetaldehyde is a key control point for PhIP production. While the addition of polyphenols significantly reduced the production of PhIP, indicating that polyphenols containing m-hydroxyl structures do inhibit the production of PhIP mainly by trapping the reaction intermediate reactive carbonyl (**Figure 3-6**b). And it also shows that the main generation pathway of PhIP formation is the carbonyl pathway. Moreover, the formation of Harman and Norharman depends primarily on tryptophan. Tryptophan reacts with the mono-carbonyl compounds formaldehyde and acetaldehyde to form end products via the Pictet–Spengler reaction (Chen et al., 2020). Similarly, the addition of formaldehyde and acetaldehyde remarkably increased the content of Harman and Norharman (**Figure 3-5**B and **Figure 3-5**C). Epicatechin, resveratrol, and phloretin all had significant inhibitory effects on Harman and Norharman, obviously ($P < 0.05$). This verifies that polyphenols mainly inhibit the generation of Norharman and Harman by capturing active carbonyls and **Figure 3-6**a exhibits the possible inhibitory pathways.

And it also confirmed that the main generation pathway of Harman and Norharman is the carbonyl pathway.

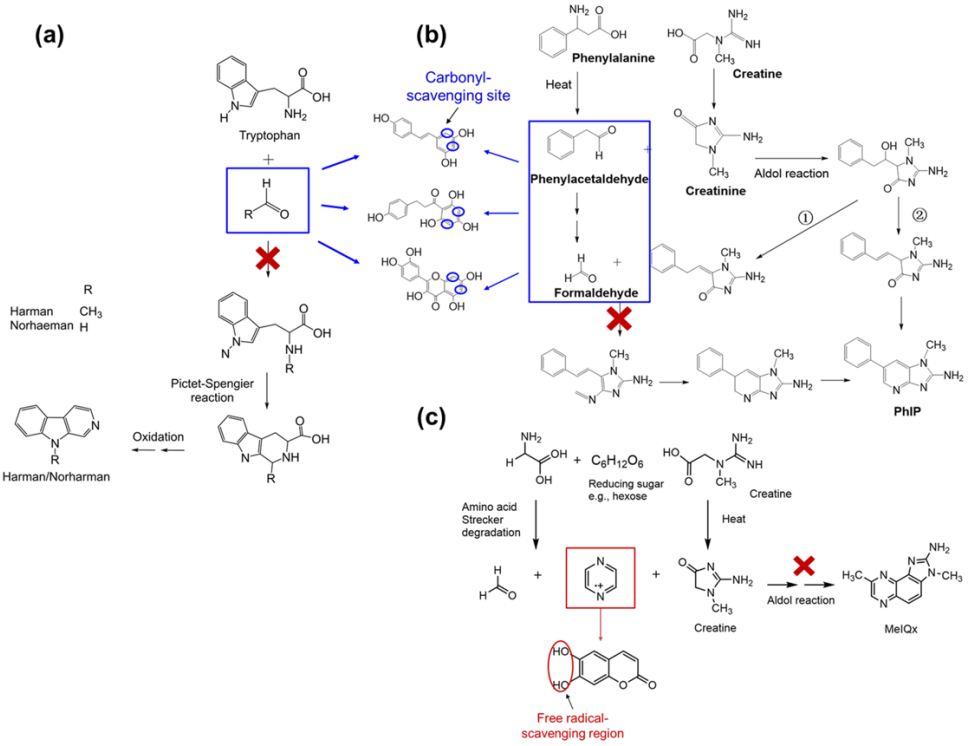


Figure 3-6: Possible inhibitory pathways of resveratrol, phloretin, epicatechin on Harman/ Norharman (a) or PhIP (b), and esculetin on MeIQx (c)

The reaction pathways for aminoimidazoazarene HAs formation suggested two main general proposals. On the one hand, the pyridine and pyrazine radicals, produced from the Maillard reaction, would react with Streker aldehyde and creatinine to form IQ-derivatives or IQx-derivatives HAs (Stoesser et al., 2007). But the exact mechanism of this proposed reaction is still unclear. On the other hand, 2,5-dimethylpyrazine or 2-methylpyridine reacts with aldehydes produced by Strecker degradation reactions and creatinine to synthesize aminoimidazoazarene HAs (Milic, Djilas, 1993). And these proposed reaction pathways have been further confirmed in the formation of MeIQ and MeIQx (Skog & Jägerstad, 2005). In this study, it can be observed that the content of MeIQx was significantly reduced by 37.75% in the model system after the addition of aesculetin compared with the control in **Figure 3-5**D ($P < 0.05$). After adding the carbonyl pathway reaction intermediates methylglyoxal and 2, 5-dimethylpyrazine (Bao, Miao, Huang, & Lai, 2020), MeIQx production increased

by 14.03% and 17.40%, respectively (P > 0.05). The result indicates that the carbonyl formation pathway plays little role in MeIQx generation. A noticeable inhibition of MeIQx with the rate of 38.43% and 41.13% by the addition of aesculetin in the model system containing aldehyde compound, but there is little difference with the group without added methylglyoxal and 2,5-dimethylpyrazine (P > 0.05), which indicates that MeIQx was not mainly inhibited by aesculetin through the capture of reactive carbonyls. The characteristic of aesculetin only has an o-hydroxy structure indicating that it acts as a MeIQx inhibitor mainly by scavenging free radicals (**Figure 3-6**c).

3.5 Characterization of formed radicals in the MeIQx model system

As an important tool for studying the unpaired electronic state in compounds or minerals, Electron spin resonance (ESR) is used to determine free radicals (Barba et al., 2020). The radical formation at 10 min was investigated by using the model system to further verify the mechanism of the inhibition of MeIQx production by aesculetin. **Figure 3-7** displays the ESR results of free radicals in the MeIQx chemical reaction system treated with aesculetin. The spectral analysis showed the presence of hyperfine couplings (AN =14.17 G, AH = 3.23 G) with coupling constants that could be arising from PBN adducts. It can be observed that six signal peaks can be seen in the model system with and without the addition of aesculetin, indicating that the reaction generated free radicals and was captured by PBN traps. It is clear that aesculetin decreased PBN adduct signal intensity compared with the control group. This decreasing phenomenon indicated that the free radicals produced by the reaction could be effectively scavenged by aesculetin, and then inhibit the production of MeIQx by scavenging free radicals. It further indicated that the main generation pathway of MeIQx is the free radical pathway, which needs to be further analyzed.

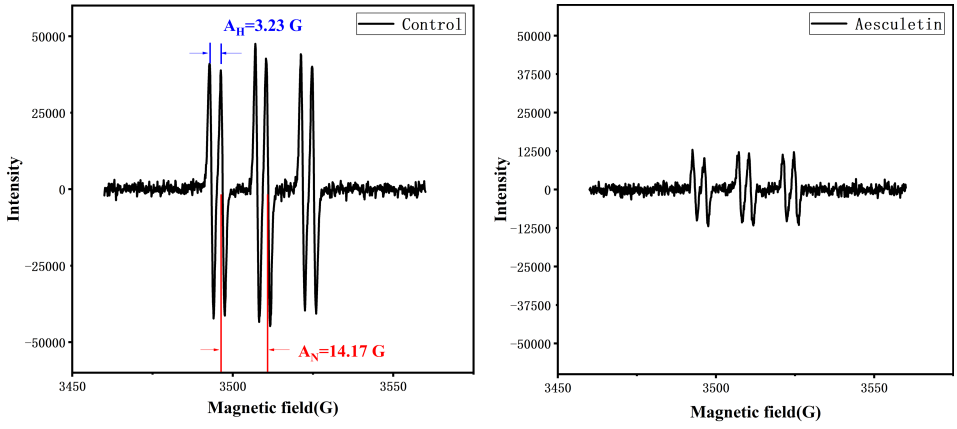


Figure 3-7: ESR signal intensities of PBN adduct when esculetin was added or not in the MeIQx model system

4.Conclusions

In conclusion, our results showed a new and systematic law of the polyphenols with different structures on the inhibition of HAs formation. Stilbene, coumarin and chalcone had a better inhibitory effect on HAs than flavonoids. These four polyphenolic classes above presented a stronger effect than phenolic acids. Besides, polyphenols with m-hydroxyl structures have a stronger inhibition of PhIP, Harman and Norharman by capturing the reaction intermediate carbonyl compounds. Polyphenols with the o-hydroxyl group had a stronger inhibitory effect on MeIQx by scavenging free radicals. Moreover, it can be further indicated the PhIP, Harman and Norharman are mainly generated by the carbonyl pathway and indirectly confirmed that MeIQx is mainly generated by the free radical pathway. Therefore, this study can not only speculate the inhibition mechanism of different HAs through the structure of unknown polyphenols, but also provides the basis for exploring the formation pathway of these HAs. It further provides a theoretical basis for effectively controlling the formation of HAs and reducing their harm to human beings.

5. References

Alaejos, M.S., & Afonso, A.M. (2011), Factors that affect the content of heterocyclic aromatic amines in foods. *Comprehensive Reviews in Food Science and Food Safety, 10*: 52-108.

Amić, D., Davidović-Amić, D., Beslo, D., Rastija, V., Lucić, B., & Trinajstić, N. (2007). SAR and QSAR of the antioxidant activity of flavonoids. *Current Medicinal Chemistry, 14*(7), 827–845.

Bao, X., Miao, J., Huang, Y., & Lai, K. (2020). Revealing a key inhibitory mechanism of 2-amino-3,8-dimethylimidazo[4,5-f] quinoxaline via trapping of methylglyoxal. *Journal of Food Science, 85*(7), 2090–2097.

Barba, F. J., Roohinejad, S., Ishikawa, K., Leong, S. Y., Bekhit, A. E. D. A., Saraiva, J. A. (2020). Electron spin resonance as a tool to monitor the influence of novel processing technologies on food properties. *Trends in Food Science & Technology, 100*, 77–87.

Barzegar, F., Kamankesh, M., & Mohammadi, A. (2019). Heterocyclic aromatic amines in cooked food: A review on formation, health risk-toxicology and their analytical techniques. *Food Chemistry, 280*, 240–254.

Boada, L. D., Henríquez-Hernández, L. A., & Luzardo, O. P. (2016). The impact of red and processed meat consumption on cancer and other health outcomes: Epidemiological evidences. *Food and chemical toxicology: an international journal published for the British Industrial Biological Research Association, 92*, 236–244.

Cai, Y. Z., Mei Sun, Jie Xing, Luo, Q., & Corke, H. (2006). Structure-radical

scavenging activity relationships of phenolic compounds from traditional Chinese medicinal plants. *Life Sciences, 78*(25), 2872–2888.

Chen, L., & Kang, Y. H. (2014). Antioxidant and enzyme inhibitory activities of Plebeian herba (Salvia plebeia R. Br.) under different cultivation conditions. *Journal of Agricultural and Food Chemistry, 62*(10), 2190–2197.

Chen, X., Jia, W., Zhu, L., Mao, L., & Zhang, Y. (2020). Recent advances in heterocyclic aromatic amines: An update on food safety and hazardous control from food processing to dietary intake. *Comprehensive Reviews in Food Science and Food Safety, 19*(1), 124–148.

Cheng, K. W., Wong, C. C., Cho, C. K., Chu, I. K., Sze, K. H., Lo, C., Chen, F., & Wang, M. (2008). Trapping of phenylacetaldehyde as a key mechanism responsible for naringenin's inhibitory activity in mutagenic 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine formation. *Chemical Research in Toxicology, 21*(10), 2026–2034.

Cheng, K. W., Wong, C. C., Chao, J., Lo, C., Chen, F., Chu, I. K., Che, C. M., Ho, C. T., & Wang, M. (2009). Inhibition of mutagenic PhIP formation by epigallocatechin gallate via scavenging of phenylacetaldehyde. *Molecular Nutrition & Food Research, 53*(6), 716–725.

Ding, X., Zhang, D., Liu, H., Wang, Z., & Hui, T. (2022). Chlorogenic acid and Epicatechin: An efficient inhibitor of heterocyclic amines in charcoal roasted lamb meats. *Food Chemistry, 368*, 130865.

Falowo, A. B., Fayemi, P. O., & Muchenje, V. (2014). Natural antioxidants against lipid-protein oxidative deterioration in meat and meat products: A review. *Food Research International, 64*, 171–181.

Gibis M. (2016). Heterocyclic aromatic amines in cooked meat products: causes, formation, occurrence, and risk assessment. *Comprehensive Reviews in Food Science and Food Safety, 15*(2), 269–302.

Hidalgo, F. J., Aguilar, I., & Zamora, R. (2017). Model studies on the effect of aldehyde structure on their selective trapping by phenolic compounds. *Journal of Agricultural and Food Chemistry, 65*(23), 4736–4743.

Hui, T., Li, Y., Chen, R., Yang, X., Liu, H., Wang, Z., & Zhang, D. (2021). Formation and prediction of PhIP, Harman, and Norharman in chemical model systems containing Epicatechin under various reaction conditions. *Journal of Agricultural and Food Chemistry, 69*(49), 14975–14984.

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

Lucić, B., Amić, D., & Trinajstić, N. (2008). Antioxidant QSAR modeling as exemplified on polyphenols. *Methods in Molecular Biology (Clifton, N.J.), 477*, 207–218.

Lund, M. (2021). Reactions of plant polyphenols in foods: Impact of molecular structure. *Trends in Food Science & Technology, 112*, 241-251.

Milic, B. L., Djilas, S. M. (1993). Canadanovic-Brunet, J. M. Synthesis of some

heterocyclic aminoimidazoazarenes. *Food Chemistry, 46*, 273–276.

Murkovic, M., Weber, H.-J., Geiszler, S., Fröhlich, K., & Pfannhauser, W. (1999). Formation of the food associated carcinogen 2-amino-1-methyl-6phenylimidazo[4,5-b]pyridine (PhIP) in model systems. *Food Chemistry, 65*, 233–237.

Musa, K. H., Abdullah, A., & Al-Haiqi, A. (2016). Determination of DPPH free radical scavenging activity: application of artificial neural networks. *Food Chemistry, 194*, 705–711.

Oguri, A., Suda, M., Totsuka, Y., Sugimura, T., & Wakabayashi, K. (1998). Inhibitory effects of antioxidants on formation of heterocyclic amines. *Mutation Research, 402*(1-2), 237–245.

Özdestan, Ö., Kaçar, E., Keşkekoğlu, H., Üren, A. (2013). Development of a new extraction method for heterocyclic aromatic amines determination in cooked meatballs. *Food Analytical Methods, 7*, 116– 126.

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

Rönner, B., Lerche, H., Bergmüller, W., Freilinger, C., Severin, T., & Pischetsrieder, M. (2000). Formation of tetrahydro-β-carbolines and β-carbolines during the reaction of L-tryptophan with D-glucose. *Journal of Agricultural and Food Chemistry, 48*(6), 2111–2116.

Salazar, R., Arámbula-Villa, G., Hidalgo, F. J., & Zamora, R. (2014). Structural characteristics that determine the inhibitory role of phenolic compounds on 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) formation. *Food Chemistry, 151*, 480–486.

Santhakumar, A. B., Battino, M., & Alvarez-Suarez, J. M. (2018). Dietary polyphenols: Structures, bioavailability and protective effects against atherosclerosis. *Food and Chemical Toxicology: An International Journal Published for the British Industrial Biological Research Association, 113*, 49–65.

Seyoum, A., Asres, K., & El-Fiky, F. K. (2006). Structure-radical scavenging activity relationships of flavonoids. *Phytochemistry, 67*(18), 2058–2070.

Skog, K., & Jägerstad, M. (2005). Incorporation of 14C-glucose into mutagenic heterocyclic amines. In T. P. Labuza, G. A. Reineccius, V. M. Monnie, J. O'Brien, & J. W. Baynes (Eds.), Maillard reactions in chemistry, food and health (pp. 147–152). Cambridge, UK:Woodhead Publishing.

Stoesser, R., Klein, J., Peschke, S., Zehl, A., Cämmerer, B., & Kroh, L. W. (2007). On the time behaviour of the concentration of pyrazinium radical cations in the early stage of the Maillard reaction. *Spectrochimica acta. Part A, Molecular and Biomolecular Spectroscopy, 67*(5), 1161–1168.

Suleman, R., Wang, Z., Hui, T., Pan, T., Liu, H., Zhang, D. (2019) Utilization of Asian spices as a mitigation strategy to control heterocyclic aromatic amines in charcoal grilled lamb patties. *Journal Food Processing Preservation, 43*(11), e14182.

Teng, J., Hu, X., Tao, N., Wang, M. (2018). Impact and inhibitory mechanism of phenolic compounds on the formation of toxic Maillard reaction products in food. *Frontiers of Agricultural Science and Engineering, 5*(3):321–9.

Vauzour, D., Rodriguez-Mateos, A., Corona, G., Oruna-Concha, M. J., & Spencer, J. P. (2010). Polyphenols and human health: prevention of disease and mechanisms of action. *Nutrients, 2*(11), 1106–1131.

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

Xiong Y. L. (2017). Inhibition of hazardous compound formation in muscle foods by antioxidative phytophenols. Annals of the New York Academy of Sciences, 1398(1), 37–46.

Yu, C., Shao, Z., Liu, B., Zhang, Y., & Wang, S. (2016). Inhibition of 2-Amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP) formation by alkoxy radical scavenging of flavonoids and their quantitative structure-activity relationship in a model system. *Journal of Food Science, 81*(8), C1908–C1913.

Zamora, R., & Hidalgo, F. J. (2020). Formation of heterocyclic aromatic amines with the structure of aminoimidazoazarenes in food products. *Food chemistry, 313*, 126128.

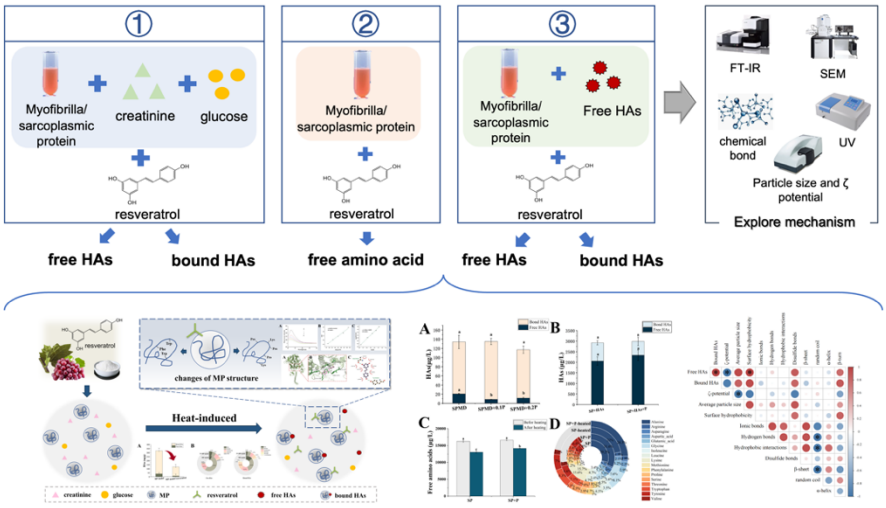
Zöchling, S., Murkovic, M. (2002) Formation of the heterocyclic aromatic amine PhIP: Identification of precursors and intermediates. *Food Chemistry, 79*, 125–134.

4

Chapter IV Inhibitory effect and mechanism of resveratrol on the formation of heterocyclic amines involving myofibrillar and sarcoplasmic proteins

Short overview of chapter IV

Based on the chapter II and III, current studies on the mechanism of inhibition of HAs by polyphenolic compounds are mainly through the inhibition of amino acids as precursors involved in the reaction, while proteins can also be involved in the formation of HAs, especially in roasted meat. However, the mechanism by which polyphenols inhibit proteins involved in the generation of HAs is still unclear. So, in this study, by choosing resveratrol, which had a better effect in inhibiting HAs in roasted meat in previous studies, we investigated its effect in influencing the generation of HAs involved in myofibrillar proteins and sarcoplasmic proteins in a model system, and further explored the mechanism.



Graphical abstract: Elucidate the mechanism by which polyphenols inhibit muscle protein-bound heterocyclic amines.

The work from this chapter was published on Food Bioscience and LWT, as can be retrieved by:

Yang, X., Zhang, D., Blecker, C., Zhang, C., Sun, X., Wang, Z. (2024). Effect of interaction between resveratrol and myofibrillar protein on the production of bound heterocyclic amines. Food Bioscience, 59, 104116. https://doi.org/10.1016/j.fbio.2024.104166

Yang, X., Blecker, C., Zhang, D., Wang, Z. (2025). Mechanistic insights into the inhibitory effect of resveratrol on heterocyclic amine formation involving sarcoplasmic proteins. LWT, 230, 118281. https://doi.org/10.1016/j.lwt.2025.118281.

Abstract

Polyphenols are effective inhibitors of heterocyclic amines (HAs), harmful compounds formed during the heating of high-protein foods such as meat. However, the mechanisms by which polyphenols affect the formation of protein-involved HAs have been rarely studied. The inhibitory pathways and mechanisms of resveratrol on heterocyclic amines (HAs) formed with the participation of myofibrillar proteins (MP) and sarcoplasmic proteins (SP) were investigated in both a model system and a reaction system involving only free HAs and muscle proteins. The results demonstrate that resveratrol significantly reduced bound and free HAs in the MP reaction systems ($P < 0.05$) while only decreased free HAs in the SP reaction models, and facilitates the transition from bound to free HAs. The findings show that resveratrol significantly decreased free HAs in the SP reaction models, promoted the formation of bound HAs, and increased the content of free amino acids after heating ($P < 0.05$). In addition, resveratrol altered the secondary structure of MP and had a static quenching effect on MP fluorescence. The strong interactions between resveratrol and MP are mainly hydrogen bonding and hydrophobic interactions. Furthermore, the results of molecular docking demonstrated that resveratrol exposed more hydrophobic amino acids (Ala, Lys and Pro) at the 556 ~ 578 amino acid sites of myosin. These results indicate that resveratrol controls the formation of MP-bound HAs by reducing exposure to amino acids involved in the HAs reaction and promoting exposure to non-precursor amino acids. Moreover, the addition of resveratrol increased the particle size and surface hydrophobicity of SP, decreased the ζ-potential, and promoted the binding and aggregation of SP and HAs. Correlation and intermolecular force results indicated that free HAs formed bound HAs by changing the surface hydrophobicity of SP solution, and polyphenols could affect the formation of SP-bound HAs by affecting ions and disulfide bonds. This work provides a new screening perspective and basis for HAs mitigation strategies.

Keywords: Bound heterocyclic amines, Resveratrol, Myofibrillar proteins, Sarcoplasmic proteins, Structural characteristics, Interaction

1. Introduction

Heterocyclic amines (HAs) are easily produced when high-protein foods such as fish and meat are heated at high temperatures and have been widely concerned for their carcinogenic and mutagenic effects (Barzegar, Kamankesh, & Mohammadi, 2019). The formation mechanisms of HAs have been extensively studied. It is mainly produced by the dehydration condensation of the reaction precursors (free amino acids, creatinine, glucose) through the Maillard reaction or the pyrolysis of amino acids such as tryptophan (Alaejos, & Afonso, 2011), involving reactive carbonyls and free radicals. Epidemiological studies suggest that always intake of heat-treated foods with HAs may increase the risk of several cancers (Gibis, 2016; Le Marchand et al., 2002; Nowell et al., 2002). Therefore, it is meaningful and necessary to study its mitigation strategies. In addition to the regulation of processing conditions (temperature, time, processing method), the addition of exogenous substances is a more effective means of inhibition (Dong et al., 2020; Lu, Kuhnle & Cheng, 2018). Numerous studies have shown that natural plant extracts, as a healthy and safe HAs inhibitor, are widely used in the meat industry and are inseparable from the polyphenol compounds they contain (Cheng, Chen, & Wang, 2007; Oguri et al., 1998). At present, the mechanism of inhibiting HAs by polyphenols is mainly focused on scavenging and capturing intermediate free radicals and reactive carbonyls formed in the reaction (Vitaglione & Fogliano, 2004). The ability of some flavonoids to scavenge alkoxyl radicals was proportional to their ability to inhibit the formation of PhIP (IC50), indicating that scavenging of alkyl radicals is a pathway to inhibit PhIP generation (Yu et al.,2016). It was subsequently discovered that some of the polyphenols could trap or combine the intermediate active carbonyl compound phenylacetaldehyde produced by PhIP. Polyphenols indirectly inhibit the formation of PhIP by preventing further reactions between phenylacetaldehyde and creatinine. (Cheng et al., 2008; Cheng et al., 2009).

The discovery of bound HAs has brought into view that proteins also play an important role in the formation of HAs (Deng et al., 2024). There are currently two pathways for the formation of bound HAs: (1) Firstly, free HAs are formed by the reaction of free amino acids, produced by protein degradation or originally present in the system, with other precursors. Then the free HAs bind to the protein to form protein-bound HAs; (2) Protein-bound HAs are directly formed by some groups in the protein peptide chain that react with glucose and creatinine (Kataoka et al., 2010, Chen et al., 2021). Induction of protein carbonylation in the presence of reducing sugars via the Maillard reaction pathway, mainly through oxidative deamination of amino acid side chains in proteins, which may further involve in the formation of HAs (Villaverde & Estévez, 2013). And the bound HAs will be released after gastrointestinal digestion in humans (Szterk, 2013; Xue et al., 2022). The plant extracts piperine, sanshoamide and capsaicin have a significant impact on the inhibition of bound HAs (Xue et al., 2020). However, it is not clear what role polyphenols play in the formation of muscle proteins involved in the bound HAs. And

the mechanism by which polyphenols affect protein involvement in the formation of HAs is also unclear. Therefore, three hypotheses were proposed: (i) The binding of polyphenols and proteins affects the direct involvement of proteins in the Maillard reaction to form bound HAs; (ii) polyphenols reduce the formation of free HAs by inhibiting the breakdown of proteins into free amino acids; (iii) Polyphenols promote the formation of bound HAs between proteins and free HAs, reducing the content of free HAs.

Resveratrol, as a natural plant polyphenol mainly present in grapes and red wine, has been widely studied for its antioxidative effect. It can prevent and treat a variety of cancers, including breast cancer induced by PhIP (Dubuisson, Dyess, & Gaubatz, 2002; Wu et al., 2022). In addition, it has been shown to have a significant inhibitory effect on HAs in a wide range of roasted meat and inhibits the formation of free HAs mainly by scavenging intermediate carbonyl compounds (Meurillon et al., 2020; Yang et al., 2023). Therefore, we chose resveratrol as a representative to explore the effect of polyphenols on the muscle protein-bound HAs and the mechanism was identified. The effects of resveratrol on myofibrillar proteins (MP) and sarcoplasmic proteins (SP) participation in the HAs formation were investigated by establishing a Glucose/protein/creatinine model system and a direct reaction system of protein and HAs, and the intervention pathway was clarified. The mechanism of resveratrol's influence on the formation of bound HAs was further explored through its effects on the structure and physicochemical properties of MP. This study provides a reference and theoretical basis for supplementing and perfecting the mechanism of HAs formation inhibition by polyphenols and is of great significance for controlling the formation of HAs during meat processing.

2. Materials and methods

2.1 Materials

Resveratrol (98%) was from Yuanye (Shanghai, China). HAs standards (99.9%): Harman, Norharman, IQx, MeAαC, MeIQx, 7,8-DiMeIQx, PhIP, were purchased from TRC (Toronto, ONT, Canada). Ammonium acetate (99.9%), diethylene glycol (99%), sodium hydroxide, ethyl acetate (99.5%), creatinine and glucose, were purchased from Merck KGaA (Darmstadt, Hessian, Germany), Aladdin (Shanghai, China), and Macklin (Shanghai, China) and Solarbio (Beijing, China), respectively. Acetonitrile, acetic acid and methanol use chromatographic grades purchased from Thermo Fisher (Waltham, MA, USA). The analytical grade was used for all other reagents from Sinopharm Chemical Reagent (Shanghai, China). The Longissimus dorsi (LD) muscles were obtained from 8 months of age of small-tailed Han lambs, which were bought from Hebei Jinhong Halal Meat Company Limited and stored at -20 °C until use.

2.2 Preparation of MP, SP and establishment of reaction systems

MP was prepared based on the method reported by Sun et al. (2023). Ground 60 g

of meat with fat and connective tissue removed. One meat sample and 10 volumes of (w/v) buffer A (MgCl2, EGTA, 0.002 mol/L; KCl, 0.1 mol/L; K2HPO4, 0.02 mol/L; pH 6.8) were homogenized and centrifuged. The sediment was mixed in 8 volumes (w/v) of buffer B (MgCl2, EGTA, 0.002 mol/L; KCl, 0.1 mol/L; 10% Triton X-100; K2HPO4, 0.02 mol/L; pH 6.8) and centrifuged, the operation was repeated twice. Then the precipitate was dissolved in 8 volumes (w/v) 0.1 mol/L KCl and centrifuged, repeated twice. All the conditions of centrifugation were 2000 × g for 15 min at 4 °C (R10A3 rotor, CR21N High-Speed Refrigerated Centrifuge, Hitachi, Ltd., Tokyo, Japan). The final precipitate was myofibrillar proteins and the concentration was determined by a BCA kit.

SP was prepared following the method described by Tadpitchayangkoon (2010). The lamb meat, after removing fat and connective tissue, was initially ground. The meat sample was then homogenized with 10 volumes (w/v) of potassium phosphate buffer solution (0.022M, pH 7.2) and centrifuged at 3000 × g for 30 min and 4 °C. Sarcoplasmic protein was obtained by filtration of supernatant and its concentration was measured using a BCA kit.

The model reaction systems were constructed similarly to the previous research (Yang et al., 2023). (i) Glucose/MP/creatinine model system (MPMD+P): Myofibrillar protein (20 mg/mL,10 mL), glucose and resveratrol (both 0.2 mmol), creatinine (0.4 mmol) were mixture in closed reaction bottles and heated at 150 °C for 1 h. (ii) MP/HAs reaction system (MP+HAs+P): Myofibrillar protein (20 mg/mL,10 mL), HAs mixture standard solution (5 mg/L,100 μL) and resveratrol (0.2 mmol) were mixture in closed reaction bottles and heated at 150 °C for 1 h. The systems without resveratrol were control groups (MPMD and MP+HAs). Similarly, the SP-associated model system is to replace MP with SP. Moreover, the two groups with only HAs and HAs with resveratrol (HAs+P) were also heated at 150 °C for 1 h and served as controls.

2.3 Determination of free and bound HAs in reaction systems

The methods of extraction and determination of two types of HAs refer to previous methods (Yang et al., 2023). (i) Free HAs extraction: All simulated reaction solutions were homogeneously mixed with ethyl acetate (20 mL) and extracted for 30 min by ultrasonication, then centrifuged (1170 × g, 10 min). Collected the supernatant ethyl acetate extraction phase and repeated the operation twice. The collected extract was pooled and nitrogen blasted to about 20 mL as a sample. Samples were separated, enriched and purified with a fully automated solid-phase extractor and MCX solid-phase extraction cartridge. (ii) Bound HAs extraction: The same volume of concentrated HCl was added to the lower liquid obtained by centrifugation in (i) to make the final concentration about 6 mol/L, and hydrolysis at 110 °C for a day. Before hydrolysis, ethyl acetate is completely volatilized. Filtered the hydrolysate and volume to 100 mL and 10 mL was taken for purification. The remaining steps were the same as for free HAs except that when activating the MCX column, 0.1 mol/L HCl (6 mL) was used instead of ethyl acetate (6 mL). After extraction, all the samples

were analyzed by UHPLC-MS/MS (6495, Agilent, USA).

2.4 Determination of free amino acids, FT-IR spectroscopy, microstructural observation using scanning electron microscope (SEM) and chemical interaction

The sample solution was ultrasonicated for 10 minutes and centrifuged for 15 minutes at 2800 × g, then the volume was determined to be 10 mL. Mixed 1 mL of the constant volume solution with an equal volume of sulfosalicylic acid and centrifuged (10000 × g, 15 min). After centrifugation, dried 1 mL of supernatant with nitrogen, and 1 mL 0.02 mol/L HCl were added to redissolve. Then an L-8900 amino acid analyzer (Hitachi, Tokyo, Japan) was used for analysis.

The before and after heating reaction liquids of the Glucose/MP/creatinine model system and SP/HAs reaction system (SP+HAs and SP+HAs+P) in Section 2.2, MP, and MP with resveratrol (MP+P) were freeze-dried to obtain the samples for SEM and FTIR. The samples were observed by SEM-450 (FEI, Hillsboro, OR, USA) at 10 kV and 1500× magnification after spraying gold on the bonded plates. FT-IR operations were performed according to Sun et al. (2023), with slight modifications. KBr and the sample (100:1, w/w) were milled uniformly and pressed into thin pieces before measurements with the instrument (TENSOR 27, Bruker, Ettlingen, Germany). The spectra were recorded between 400 and 4000 cm^{-1}. Changes in the relative content of protein secondary structure of different samples were calculated by Peakfit v4.12 (Chicago, Illinois, USA).

The chemical interactions were measured as protein solubility according to the previous research (Mi et al, 2022). Prepare the solution S1-S5 required for the reaction: S1, 0.05 mol/L NaCl; S2, 0.6 mol/L NaCl; S3, 0.6 mol/L NaCl + 1.5 mol/L Urea; S4, 0.6 mol/L NaCl + 8 mol/L Urea; S5, 0.6 mol/L NaCl + 8 mol/L Urea + 0.5 mol/L 2-β-mercaptoethanol. Ten milliliters of the MP/SP and MP/SP with resveratrol samples were homogenized with 10 mL of S1-S5 solutions at 4 ℃, respectively. After centrifugation, the supernatant was obtained and the protein content was quantified by BCA kit. The chemical forces were calculated from the differences in solubilized proteins in these solutions, such as ionic bonds (S2−S1), hydrogen bonds (S3−S2), hydrophobic interactions (S4−S3), and disulfide bonds (S5−S4).

2.5 Fluorescence spectroscopy

MP and resveratrol were diluted with PBS solution to final concentrations of 0.4 mg/mL and 4-12 μg/mL, respectively, and mixed and left for 2 hours. Fluorescence spectra were then determined with a Spark multifunctional microplate enzyme marker (Tecan, Männedorf, Switzerland). The test conditions of the enzyme-label instrument are excitation wavelength, 280 nm; emission spectra, 300-500 nm at 298 K; slit widths, 5 nm.

2.6 Molecular docking

MP as a mixture is not amenable to direct molecular docking. Therefore, we selected

myosin, which accounts for 55-60% of myofibrillar components (Xia et al., 2019), for further analysis. The amino acid sequences of sheep myosin-1 (MYH-1, ID: W5PPG6) were acquired from UniProt (https://www.uniprot.org/). A suitable template for the construction of sheep MYH-1 fragment (XP_004012755.2) was found by NCBI BLAST analysis searching for sequence similarity. The three-dimensional structure of resveratrol (CID: 445154) was from the PubChem. The molecular docking studies refer to the methods of Morris et al. (2009) with the help of Autodock 4.2.6 and AutoDuck tools 1.5.7. The binding interaction of myosin-heavy chains with resveratrol was demonstrated using LigPlot+ software (Laskowski & Swindells, 2011). The visualization was displayed using PyMoL (Schrödinger & DeLano, 2020).

2.7 Analysis of Ultraviolet–visible (UV–VIS)

Various concentrations of HAs (0.25, 0.5, 1, 1.5 μg/mL), resveratrol (5, 10, 20, 40 μg/mL) and HAs+ resveratrol (0.25+5, 0.5+10, 0.75+15, 1+20 μg/mL) mixture solutions with a protein concentration of 0.5 mg/mL were used for UV–VIS spectrum scanning and with the scanning wavelength range from 370 to 500 nm (Chen, et al., 2019).

2.8 Particle size, ζ- potential and surface hydrophobicity (H0)

The polyphenol-protein (SP+P), HAs-protein mixture (SP+HAs) and polyphenol-HAs-protein (SP+HAs+P) mixtures (The concentrations of SP, resveratrol and HAs were maintained at 1 mg/mL, 1 μg/mL and 1 μg/mL, respectively) were configured, and 1 mL of the homogenized mixture was placed in a cuvette to measure the average particle size and ζ- potential of each sample. The particle size and ζ- potential of sample were measured using a Nano ZS90 zeta sizer (Malvern Instruments Ltd., UK). H0 was determined using an 8-anilinonaphthalene-1-sulfonic acid (ANS). 20 μL of ANS (2.394 g/L) was added to 2 mL of resveratrol-protein, HAs-protein or resveratrol-HAs-protein mixture solutions, with soluble protein concentrations ranging of 0.05–2 mg/mL. H0 was calculated by determining the initial slope of the fluorescence index of samples with different protein concentrations. Set the excitation wavelength 390 nm, the emission wavelength 470 nm, and adjust the slit 5 nm to measure the burst intensity. The fluorescence intensity is used to curve the protein concentration, and the slope of the initial portion of the curve represents the H0 of the protein.

2.9 Statistical analysis

Each experiment was replicated at least in triplicate. Values were presented as mean ± SD. Analysis of ANOVA and Duncan's multiple range tests were performed by SPSS 20 (IBM Corp., New York, USA), where results of $P < 0.05$ were considered significant among samples.

3. Results and discussion

3.1 Inhibitory mechanism analysis of resveratrol on the participation of MP in HAs formation

In the model of the MP/creatinine/glucose system, the bound HAs produced by heating were much higher than free HAs. Resveratrol significantly reduced both free and bound HAs ($P < 0.05$), while increasing the proportion of bound HAs from 89% to 92% (**Figure 4-1**A, **Figure 4-1**D). A total of 5 free HAs (IQx, MeIQx, Norharman, Harman and PhIP) and 6 bound HAs (IQx, MeIQx, Norharman, Harman, MeAαC and 7,8-DiMeIQx) were detected in glucose/MP/creatinine model systems (**Figure 4-1**B). Among them, PhIP mainly exists in the free state, MeAαC and 7,8-DiMeIQx are more inclined to exist in the combined state. In addition, MeIQx, Norharman, Harman and PhIP had higher percentages, similar to previous results in roasted meat (Yang et al., 2023), and the addition of resveratrol also mainly reduced the contents of these HAs. In addition, no free amino acids were detected in the MP solution before and after the addition of resveratrol, which suggests that MP does act as a precursor directly involved in the production of HAs and that polyphenols have an inhibitory effect on this process.

To further clarify whether polyphenols interact with muscle proteins to promote protein and HAs binding, the changes of two types of HAs in the direct response system of free HAs and MP were determined. The results showed that the bound HAs could be formed by the direct binding of MP and free HAs. In addition, resveratrol was effective in converting bound HAs to free HAs (**Figure 4-1**C, **Figure 4-1**D), indicating that the combination may be formed primarily by intermolecular interactions and is easily broken down. Xue et al. (2022) also showed that the addition of pepper, onion and apple, rich in polyphenols, promotes the transition from bound to free HAs during in vitro digestion. This further suggests that polyphenols reduce the production of bound HAs primarily by influencing MP to participate directly in the Maillard reaction.

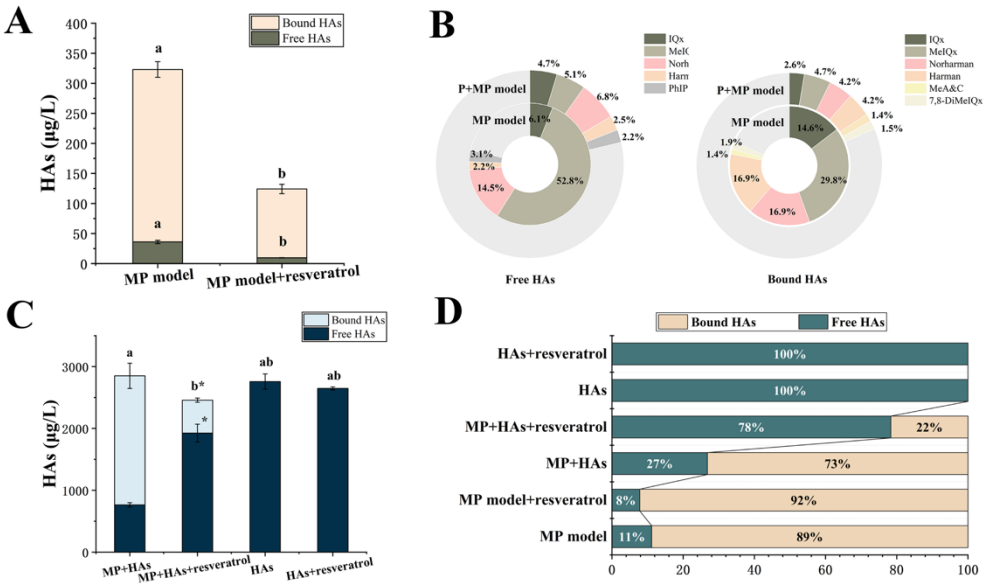


Figure 4-1: Effects of resveratrol on free and bound HAs in the MP/creatinine/glucose model system (A) and the MP/HAs direct reaction system (C); on different types of free and bound HAs in the MP/creatinine/glucose model system (B); and on the proportions of bound and free HAs in each group (D). Different letters indicate significant differences among groups ($P < 0.05$). * indicates a significant difference in the content of free and bound HAs as compared to no resveratrol added.

3.2 Mechanism analysis of resveratrol's influence on SP participation in HAs formation

The effect of polyphenols on the participation of sarcoplasmic proteins in the formation of HAs is shown in **Figure 4-3**. A total of 5 free HAs and 7 bound HAs were detected in the SP/creatinine/glucose model system (**Figure 4-2**), among which IQx, MeIQx and Norharman were the most abundant. The types of HAs produced are consistent with those produced when myofibrillar proteins participate in the reaction, with MeAαC and 7, 8-DiMeIQx primarily existing in the bound form (Yang et al., 2024). The determination of both free and protein-bound HAs after resveratrol supplementation showed that resveratrol could significantly decrease the content of free HAs ($P < 0.05$) (**Figure 4-3**A). It has been shown that SP form bound HAs mainly through direct binding to free HAs (Chen et al., 2021). Theoretically, the decrease of free HAs after the reaction should lead to the decrease of bound HAs. However, the experimental results (**Figure 4-3**A) revealed that the addition of low doses of resveratrol increased the level of bound HAs without significantly affecting the total HAs, which suggests that polyphenols may facilitate the binding of free HAs to

proteins. This could be attributed to structural changes in SP induced by polyphenols, potentially exposing more binding sites. In addition, at higher concentrations (0.2 mmol), resveratrol reduced total HAs (not significantly) but increased the content of free HAs (**Figure 4-3**A). This suggests that excessive polyphenols inhibited the formation of free HAs, leading to a reduction in total HAs, and high-dose polyphenols caused the conversion of bound HAs into free HAs. It may be that the high dose of polyphenols alters the protein structure and protects the stability of the SP structure, freeing the HAs (Cheng et al., 2020). This was also confirmed in the direct reaction system between SP and HAs (**Figure 4-3**B), as well as in previous studies (Xue et al., 2022b; Yang et al., 2024). These results suggest that polyphenols may reduce free HAs by either promoting the binding of SP to HAs or inhibiting the production of free amino acids.

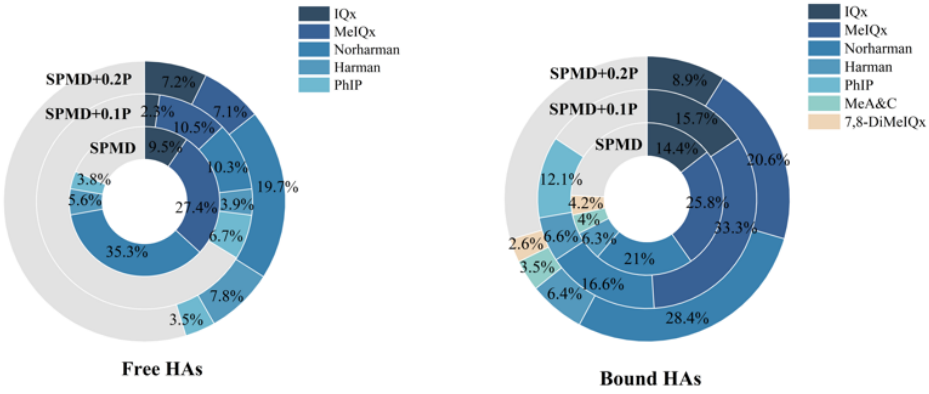


Figure 4-2: Effect of resveratrol on different free and bound HAs in SP/creatinine/glucose model system (SPMD)

To investigate whether the reduction of free HAs is related to changes in free amino acid content due to polyphenols, we measured the variation in free amino acid levels of SP with and without resveratrol. As shown in **Figure 4-3**C, the free amino acid content of SP decreased significantly after heat treatment. During high-temperature treatment, proteins undergo denaturation and oxidation reactions, which may lead to structural changes and depletion of certain amino acids (Nawaz et al., 2022). However, the content of some free amino acids, such as asparagine, alanine, proline, tryptophan, and glycine, increase with the addition of resveratrol, and the addition of resveratrol mitigates the reduction of most of the amino acids caused by heating and participation in the reaction (**Figure 4-3**D). Previous studies have demonstrated that tryptophan, phenylalanine, glycine and so on are the precursor amino acids for the formation of free HAs (Chen et al., 2020). Resveratrol is a potent inhibitor of various free HAs by scavenging free radicals or capturing reactive carbonyls (Oz et al., 2023), which is the reason why the free amino acids increased but the total HAs content did not increase or even decreased. These results suggest that resveratrol reduces free HAs on the one hand, by interacting with SP and promoting the direct binding of SP to free HAs to

form SP-HAs-binding compounds, and on the other hand, excess polyphenols inhibit the production of free HA through the mechanism of scavenging free radicals and capturing carbonyls.

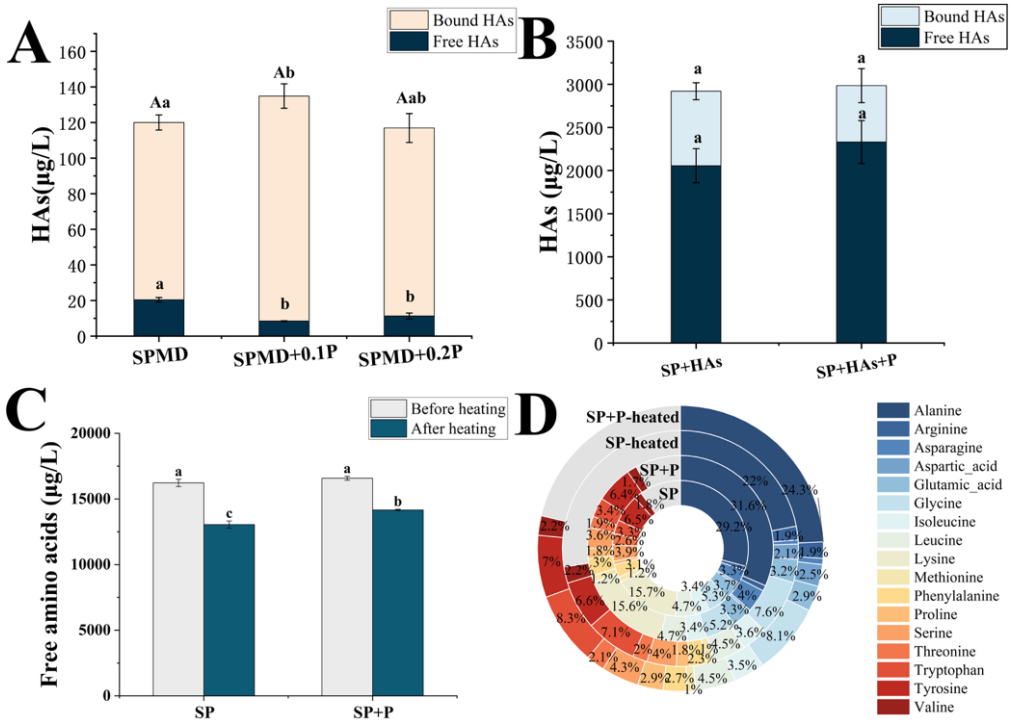


Figure 4-3: Effect of resveratrol on free and bound HAs in SP/creatinine/glucose model system (A) and SP/HAs direct reaction system (B); Lowercase letters indicate significant differences in the contents of free or bound HAs among different treatment groups, while uppercase letters indicate significant differences in total HAs among groups. Effects of resveratrol (0.2mmol) on the content (C) and types (D) of free amino acids in SP before and after heating; Different lowercase letters denote significant differences in free amino acid contents among the treatment groups. Different letters indicate significant differences ($P < 0.05$).

3.3 Effect of resveratrol on morphologic changes in MP and SP involved in HAs formation

To further clarify the structural changes of resveratrol on MP and SP involved in the process of HAs production, the microstructure changes of proteins were observed by scanning electron microscopy with or without resveratrol in the model of MP/glucose/creatinine system and the direct reaction system between SP and HAs before and after heating. In the control group, MP exhibited an orderly arrangement

and compact structure, whereas heating disrupted this organization, leading to rough surfaces and irregular clumps (**Figure 4-4**A, a), consistent with heat-induced denaturation (Liu et al., 2019). Upon the addition of resveratrol, the surface morphology changed further, showing dense spherical aggregates (**Figure 4-4**B, b, D), suggesting direct interaction between resveratrol and MP that altered protein structure. In the MP/glucose/creatinine model system, MP appeared unfolded and loosely clustered, and larger voids were observed between protein clusters (**Figure 4-4**C), likely because excess glucose and amino acids covered the protein surface and inhibited intermolecular interactions (Wang et al., 2023). Heating promoted the formation of large smooth aggregates, which may correspond to bound HAs (**Figure 4-4**c). Interestingly, with resveratrol supplementation, these large aggregates disappeared, and MP exhibited a rougher surface with denser aggregation than MP alone with resveratrol (**Figure 4-4**d), indicating that resveratrol reduced the formation of bound HAs. These results indicated that resveratrol alters the structure of MP involved in the formation of bound HAs.

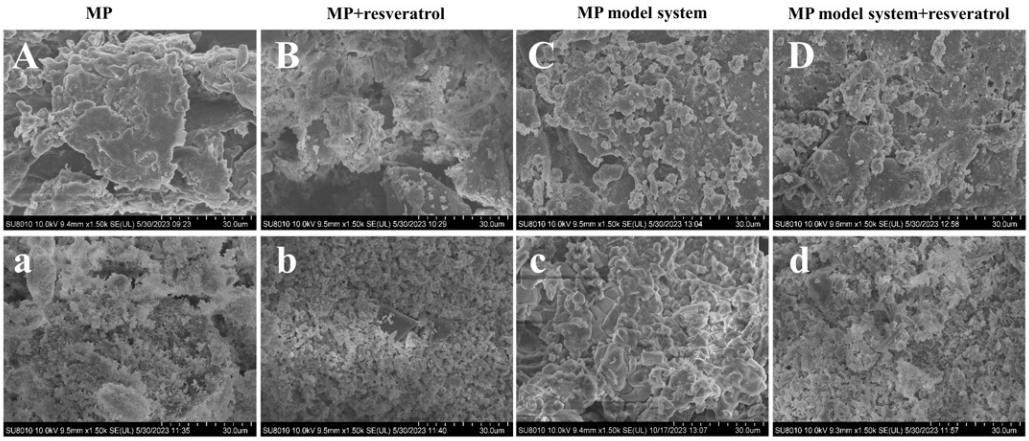


Figure 4-4: Effect of resveratrol on the microstructure of MP before (A-D) and after (a-d) heating in the process of HAs formation. Images were taken at a magnification of 1500×.

The result of SEM observation on the morphologic changes of SP involved in the formation of HAs before and after heating was shown in **Figure 4-5**. Before heating, SP exhibited a smooth surface, which was disrupted by resveratrol to form aggregates (**Figure 4-5**B). By contrast, HAs alone had little effect on SP morphology (**Figure 4-5**C). When both resveratrol and HAs were present, larger aggregates formed (**Figure 4-5**D), likely because HAs were adsorbed and bound to the protein surface while resveratrol interacted with proteins to induce conformational changes, thereby promoting aggregation. After heating, SP formed irregular clumps (**Figure 4-5**a). Addition of resveratrol further enlarged aggregates (**Figure 4-5**b), and combined treatment with HAs and resveratrol produced even larger and denser aggregates

(**Figure 4-5**d) compared with heating with HAs alone (**Figure 4-5**c). These findings are consistent with particle size analysis and indicate that resveratrol facilitates the binding of SP to HAs, enhancing protein aggregation.

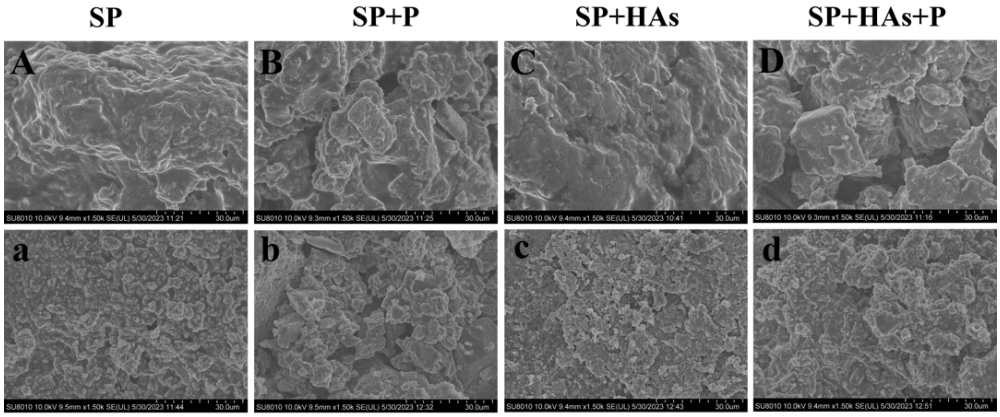


Figure 4-5: Effect of resveratrol and HAs on the microstructure of SP before (A-D) and after (a-d) heating in the process of HAs formation. Images were taken at a magnification of 1500×.

3.4 Effect of resveratrol on FT-IR of MP and SP before and after heating

FT-IR is one of the methods to observe the relative content changes of protein secondary structures by deconvolution, second derivative and fitting analysis of protein amide absorption bands (Wang et al., 2023). The amide I in the Raman bands with the range of 1600-1700 cm-1 reflects the changes in protein secondary structure (Herrero, 2008). Among them, 1615-1637 cm-1, 1646-1664 cm-1, 1637-1645 cm-1, and 1664-1681 cm-1 are characteristic bands of β-sheet, α-helix, random coil and β-turn, respectively (Wu et al., 2023). **Figure 4-6** shows the percentage changes in the secondary structure of proteins in different treatments. After high-temperature heating, the proportion of random coil in MP increased while the β-turn and β-sheet decreased. The addition of resveratrol increased the α-helix in MP from 34% and 33% before and after heating to 42% and 40%, respectively. β-turn was also observed to increase from 27% and 25% to 33% and 41%, respectively, whereas the random coil was down from 16% and 21% to 9% and 7%, respectively. In general, the α-helix structure is maintained primarily by hydrogen bonding between the carbonyl and amino group in the polypeptide chain and is closely related to the stability of proteins (Kašička, 2013). The increase in its content suggests that resveratrol promotes tight binding and aggregation of proteins. In the Glucose/MP/creatinine model system, the decrease in α-helix and the increase in β-turn indicate that glucose and creatinine make MP more

flexible and disordered. After heating, the generated HAs further converted the α-helix and β-sheet of MP to β-turn, indicating that the formation of bound HAs changes the structure of MP. In addition, the binding between HAs and MP may further weaken the intermolecular interaction of MP and reduce the number of hydrogen bonds.

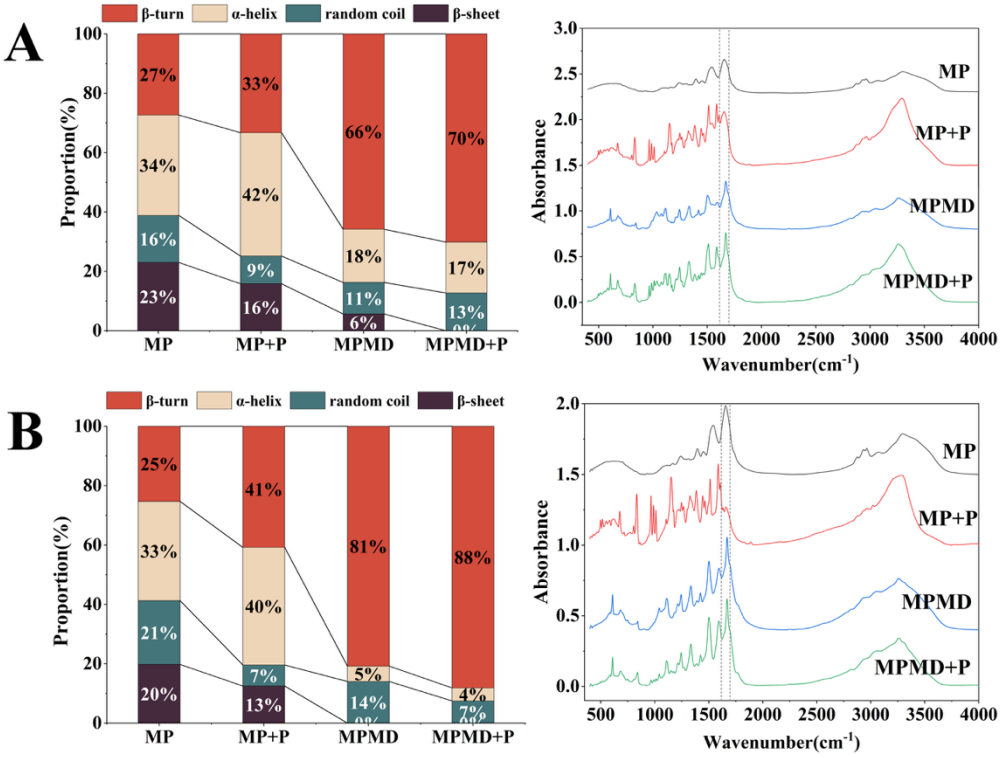


Figure 4-6: Effects of resveratrol on the spectrum of FTIR and the relative content of secondary structure in MP before (A) and after (B) heating.

Figure 4-7 shows the FT-IR results of resveratrol and HAs on sarcoplasmic proteins in the reaction system before and after heating. Under unheated conditions, α-helix is the predominant secondary structure in sarcoplasmic proteins (Wang et al., 2023). Heating transformed the α-helix and random coil in SP to β-turn, β-sheet. The increase of β-sheet and the reduction of α-helix are mainly due to the decrease in intramolecular hydrogen bonds after heating, which leads to an increase in β-sheet structures through hydrophobic interactions and disulfide bond aggregation (Herrero et al., 2008). In the absence of heat treatment, resveratrol led to an increase in α-helix content and a reduction in the other three secondary structures, suggesting that it stabilized the protein and promoted a more ordered conformation. However, this effect was completely disrupted upon heating. The observed decrease in α-helix, along with increases in β-sheet and β-turn structures, indicates a loss of structural stability

and a higher tendency for protein aggregation (Wang et al., 2018). Under non-heated conditions, the addition of HAs induced a transition from random coil to β-sheet structures in the protein, suggesting an ordered interaction between HAs and the protein. In contrast, heat treatment increased the content of random coil and β-turn structures, while decreasing α-helix and β-sheet content. These changes indicate that the formation of bound HAs causes the protein to undergo folding and conformational loosening, the overall structure tends to be disordered, and the structural stability decreases (Yan et al., 2024). In the group treated with both resveratrol and HAs, α-helix content decreased further, while β-sheet and β-turn structures increased compared to the group treated with HAs alone. This suggests that the addition of resveratrol further promotes protein destabilization and aggregation (Wang, & Smith, 1994). These findings are consistent with the SEM results.

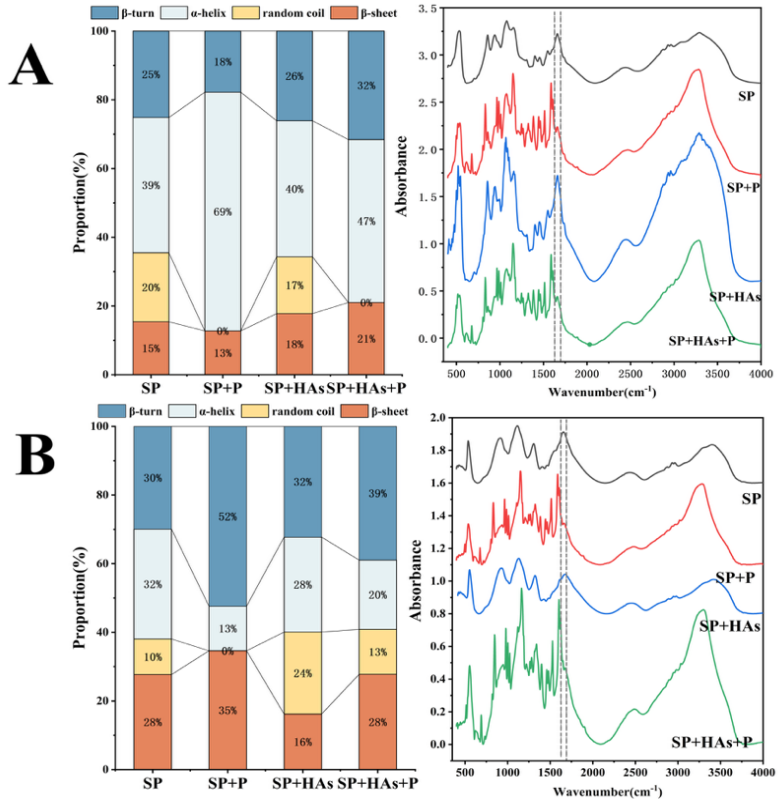


Figure 4-7: Effects of resveratrol on the spectrum of FTIR and the relative content of secondary structure in SP before (A) and after (B) heating.

3.5 Mechanism analysis of resveratrol inhibiting MP participation in HAs formation

3.5.1 Effect of resveratrol on intermolecular interaction forces of MP

The intermolecular forces were analyzed to further clarify how resveratrol affects MP participation in the formation of bound HAs. The difference in protein solubility of MP with and without resveratrol in different solutions reflects changes in specific chemical bonds, the results of which are shown in **Figure 4-8.** It can be seen that the main interactions of MP are hydrophobic interaction and hydrogen bond, and the ionic and disulfide bonds are relatively less. The addition of resveratrol significantly increased hydrophobic interactions, ionic bonds, and disulfide bonds ($P < 0.05$), suggesting that the interaction between resveratrol and MP unfolds the protein, exposing more residues and increasing ionic and disulfide bonds (Yi et al., 2023).

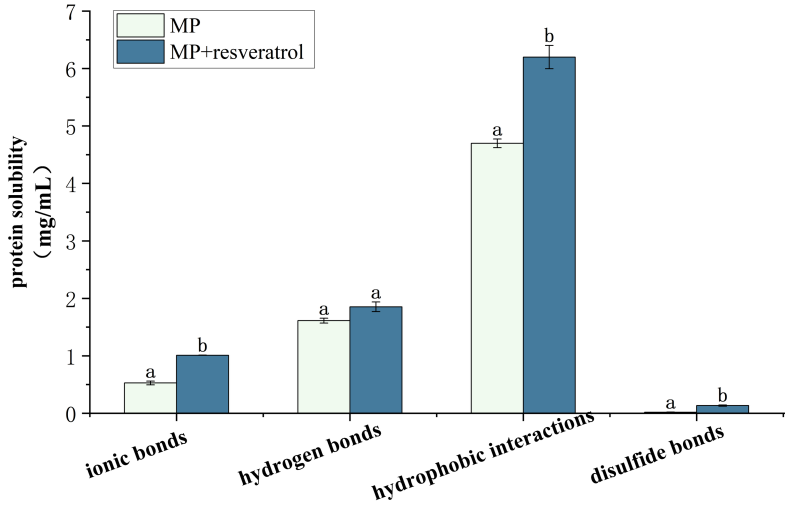


Figure 4-8: Effects of resveratrol on the protein solubility of MP. Different letters indicate significant differences among groups ($P < 0.05$).

3.5.2 Fluorescence spectroscopy of resveratrol-MP systems

Proteins containing residues of aromatic amino acids (e.g. Tyr and Trp) will produce endogenous fluorescence under excitation light at 280 nm (Zhang et al., 2020). **Figure 4-9**A shows the changes in MP fluorescence intensity with the addition of different concentrations of resveratrol. MP has a maximum fluorescence emission wavelength of 320 nm. With the concentration of resveratrol increased, the fluorescence intensity of MP decreased gradually and the maximum emission wavelength was slightly blue-shifted. The changes in fluorescence intensity and maximum emission wavelength indicate the changes in MP conformation and the surrounding environment of chromophore molecules (Klajnert & Bryszewska, 2002). Chromophore groups are stayed in a more hydrophobic environment with less exposure to solvents (Miriani et

al., 2011). Tryptophan is also known to be a precursor for the formation of HAs, primarily Harman and Norharman. These results show that resveratrol may alter the structure of MP to reduce the exposure of residues directly involved in the production of HAs, such as tryptophan and phenylalanine, to inhibit their participation in the production of bound HAs (Fig. 7). The reduced fluorescence intensity is associated to the reduced exposure of Trp and Tyr residues, and may also be due to a shortening of the quenching distance of the quenching group, which leads to the increase in fluorescence quenching (Han et al., 2022). The mechanism of fluorescence quenching was further investigated.

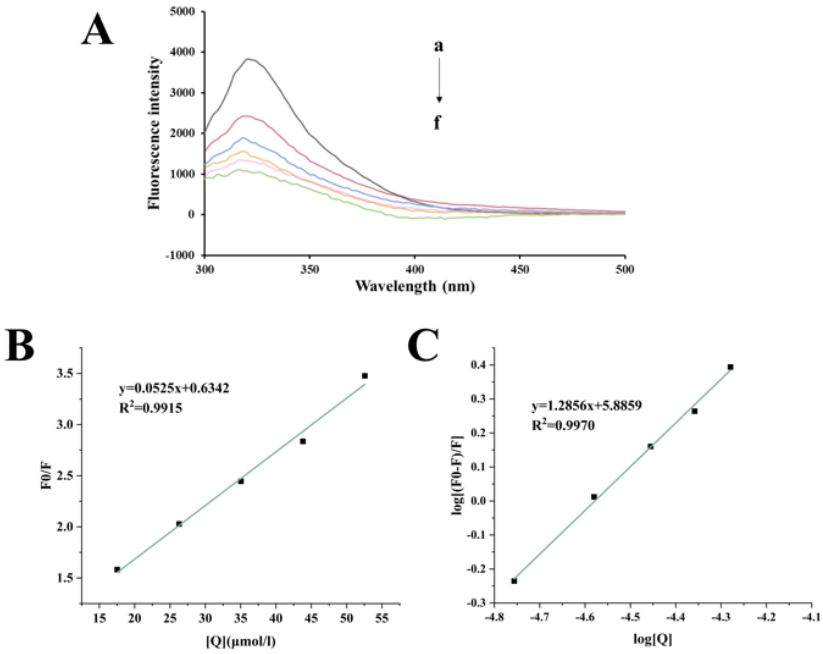


Figure 4-9: Analysis of fluorescence quenching mechanism of MP by resveratrol. (A) Fluorescence emission spectrum (λex = 280 nm) of MP (0.5 mg/mL) with various concentrations of resveratrol (a-f: 0, 4, 6, 8, 10, 12 μM); (B) Stern-Volmer plots of MP with resveratrol added; (C) The plots for the static quenching of MP by resveratrol.

There are two types of fluorescence quenching mechanisms, dynamic and static quenching, which are usually identified by Stern-Volmer quenching constants (Sung, Shin, & Lee, 1992). The Stern-Volmer equation is expressed as follows:

$$F_0/F=1+Ksv[Q]=1+K_q\tau_0[Q] \quad (1)$$

Ksv is the quenching constant; F is the maximum fluorescence intensity with the quencher added, while F0 is without; [Q] is the quencher concentration; τ0 is typically

10-8 s, which is the average lifetime of the fluorescent substance without of the quencher. **Figure 4-9**B shows a Stern-Volmer plot of fluorescence quenching of MP by resveratrol. According to the equation and the linear relationship, the calculated quenching constant Kq is 5.25×1012 M-1 s-1, which is much stronger than the maximum scattering collision quenching constant (2.0×1010 M−1s−1) between different quenchers and biopolymers (Lakowicz & Weber, 1973). These results demonstrate that resveratrol has a static quenching effect on MP, mainly due to the formation of non-luminous substances between quenchers and fluorescence molecules (Joye et al., 2015). Further calculation of the number of binding sites (n) and binding constant (Ka):

$$\log[(F_0\text{-}F)/F]=\log Ka + n\log[Q] \quad (2)$$

The intercept and slope in the log (F0 − F) / F with log [Q] plot are the values of Ka and n, respectively (**Figure 4-9**C). In addition, the values of Ka (7.69×105 L/mol) and n (1.29) indicated that the binding strength of resveratrol to MP was stronger than that of some other polyphenols and myosin (Zhang et al., 2020). These results indicate that the MP conformation is significantly deformed when resveratrol interacts with MP.

3.5.3 Molecular modeling

Binding sites were explored by molecular docking to further elucidate the details of the resveratrol's effect on the molecular structure of the protein. Of the 10 calculated results, the best-ranked one by energy is shown in **Figure 4-10**A. The results showed that resveratrol and myosin could effectively bind and change the structure of the protein, and the binding between them was mainly through hydrophobic interaction and hydrogen bonding (**Figure 4-10**B). The specific interaction between the key protein residue active site and resveratrol is shown in **Figure 4-10**C. Resveratrol could interact with MP primarily via hydrophobic forces (Pro569, Ala578, Ala572, Lys575, Glu577, Pro571 and Lys568 residues). This phenomenon indicated that the binding points present in the 556-578 amino acids induced changes in the structure of the protein, and exposed many hydrophobic groups, resulting in higher hydrophobic interaction (Privalov & Gill, 1988). In addition, the Ala567 residue (O) and Tyr556 residue (OH) of myosin formed hydrogen bonds with O3 and O1 atoms of resveratrol, with lengths of 3.06 Å and 2.98 Å, respectively, which affected the stability of the complexes.

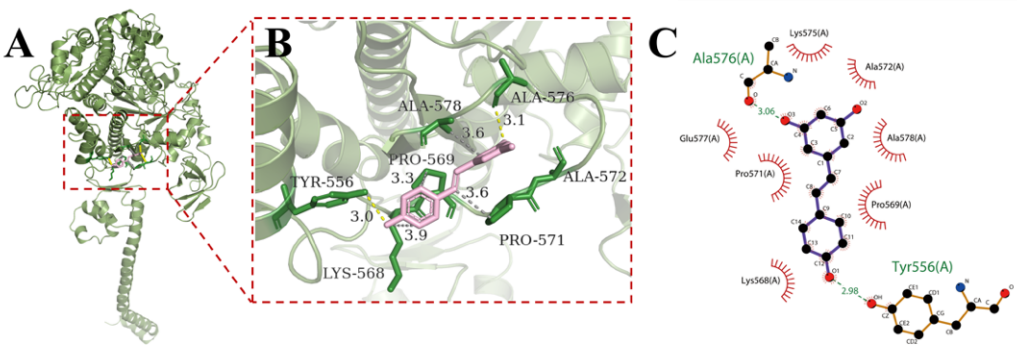


Figure 4-10: Results of molecular docking between resveratrol and myosin. (A) Docking model of resveratrol and myosin with the lowest binding affinity (- 5.18 kcal/mol); (B) The 3D binding mode of the resveratrol-myosin complex (The grey dashed line represents the hydrophobic interaction and the yellow dashed line represents the hydrogen bond); (C) 2D diagrams of resveratrol-myosin complex (The green dashed line represents hydrogen bonding and image of that eyebrows show hydrophobic interaction).

Similar to others' studies results, polyphenols can alter the structure of proteins by binding to them via noncovalent bonds (Quan et al., 2019). These properties are in agreement with the protein solubility results (**Figure 4-8**) and the MP-resveratrol complex formed by this non-covalent binding may cause quenching of the intrinsic fluorescence in the protein. What's more, resveratrol increases exposure to hydrophobic amino acids in myosin, such as Ala, Lys and Pro. It has been shown that some non-precursor amino acids such as Pro and Lys inhibit the formation of HAs (Deng et al., 2022). Thus resveratrol may reduce the production of HAs by altering the structure of the protein to expose large amounts of non-precursor amino acids that inhibit the production of HAs (**Figure 4-11**).

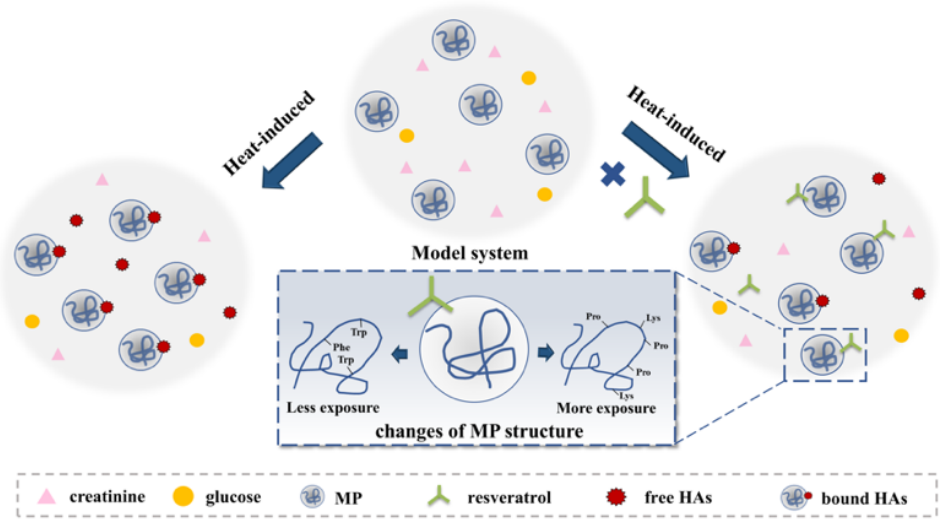


Figure 4-11: Model for the inhibiting effect of resveratrol on MP participation in bound HAs production.

3.6 Mechanism analysis of resveratrol inhibiting SP participation in HAs formation

3.6.1 UV–VIs absorption spectra of resveratrol-SP and HAs-SP systems

Changes in the intensity and position of UV-VIs spectra peaks indicate structural changes in protein as a result of interactions of small molecules with the protein (Ye et al., 2021). **Figure 4-12** illustrates the variations in UV–VIS absorption intensity of SP upon adding different concentrations of resveratrol and free HAs. In the control group, the SP exhibited a maximum absorption peak at 412 nm. The absorbance was gradually increased through the increasing concentrations of HAs and resveratrol, accompanied by a slight blue shift of the peak. Sarcoplasmic proteins are mainly composed of myoglobin and some enzymes involved in glycolysis (Tornberg, 2005). The peak Soret band at 412 nm can reflect the folding of heme protein (Benjakul & Bauer, 2001), and the blue shift observed in the study indicates that myoglobin has been converted to an oxidized state. Masuda et al. (2013) also showed that a variety of phenols promote the oxidation of oxygenated myoglobin. The continued increase in absorption peak intensity near 412 nm with the increase of additive concentration of resveratrol and HAs treatment group indicates enhanced intermolecular interaction and the change of protein structure (Bu et al., 2024). Furthermore, the maximum UV absorption peaks of the combined treatment (resveratrol + HAs) was larger than those of the additions alone, suggesting that resveratrol promoted the interaction between SP and HAs.

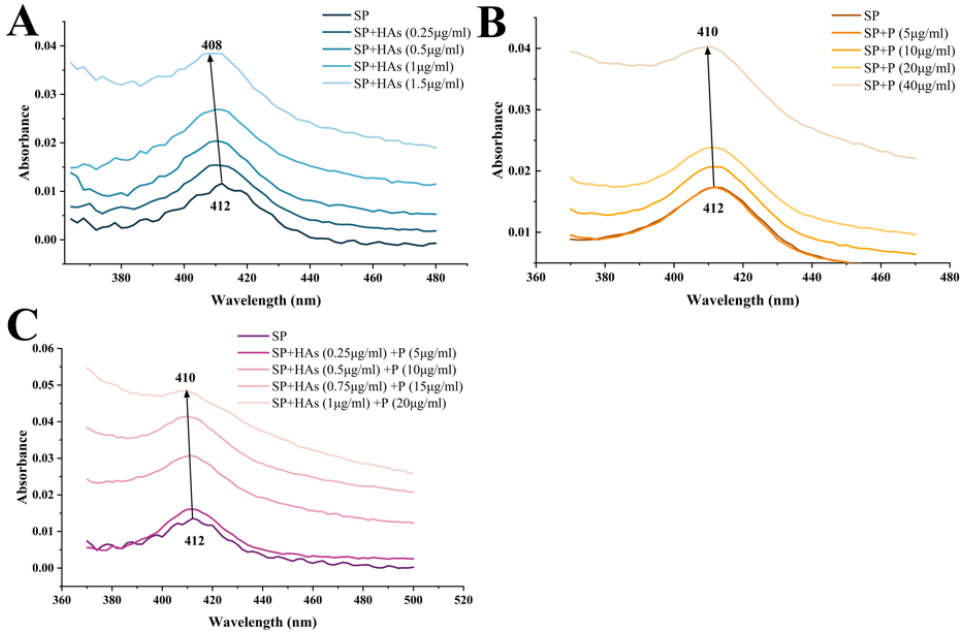


Figure 4-12: Analysis of UV–VIs of SP by resveratrol and HAs. UV–VIs absorbance of SP (0.5 mg/mL) with various concentrations of (A) HAs (0.25, 0.5, 1, 1.5 μg/mL), (B) resveratrol (5, 10, 20, 40 μg/mL) and (C) HAs+P (0.25+5, 0.5+10, 0.75+15, 1+20 μg/mL).

3.6.2 Particle size, ζ- potential and surface hydrophobicity (H0)

Particle size can directly indicate the change of protein aggregation. All samples displayed a single, sharp peak with a narrow particle size distribution, indicating good dispersion, high uniformity, and overall system stability (**Figure 4-13**B). In comparison to the addition of resveratrol alone, the inclusion of HAs significantly increased the average particle size of SP (**Figure 4-13**A). The increase in particle size may result from protein oxidation, which causes the protein structure unfolds in the oxidized state, leading to disulfide bonds formation and protein aggregation (Huang et al., 2022). When resveratrol and HAs were added simultaneously, the protein particle size became larger, indicating that HAs could directly combine with SP to form the bound HAs and the presence of resveratrol promoted the formation. The ζ-potential was used as an indicator to assess the electrostatic interactions between SP and HAs/resveratrol. The results showed that the absolute value of the ζ-potential of the proteins significantly decreased after the addition of HAs ($P < 0.05$) (**Figure 4-13**A). Typically, a higher absolute ζ-potential indicates a more stable system (Li et al., 2020). Therefore, the addition of HAs made the SP solution more unstable and easier to form bound HAs and larger aggregation. Surface hydrophobicity analysis can be used to detect alterations in protein conformation. The addition of resveratrol reduced

H0 compared to control, although not significantly (**Figure 4-13**C). This indicates that the preferential binding of resveratrol prevents ANS from attaching to the hydrophobic sites on the protein surface (Cheng et al., 2020; Ren et al., 2018). In contrast, the addition of HAs significantly increased the H0 of SP, likely due to structural unfolding that exposed more hydrophobic groups. These hydrophobic amino acid groups spontaneously aggregate significantly, thereby increasing the surface hydrophobicity (Wang et al., 2024; Ai et al., 2019). Furthermore, the hydrophobicity of proteins is mainly caused by the mutual repulsion between aggregated nonpolar groups and water molecules (Young, Jernigan, & Covell, 1994). Changes in protein surface hydrophobicity can affect its binding to other molecules, suggesting that the addition of free HAs enhances the interactions between nonpolar groups of proteins and water molecules and promotes the formation and aggregation of bound HAs.

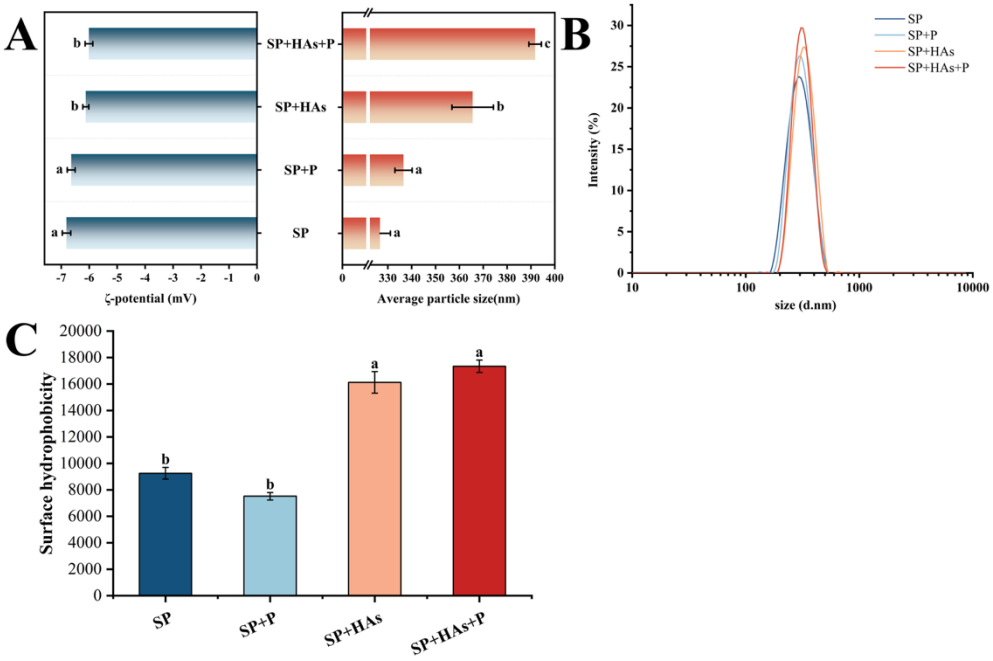


Figure 4-13: Effects of resveratrol and HAs on the particle size, ζ- potential and surface hydrophobicity (H0) of SP. Different letters indicate significant differences among groups ($P < 0.05$).

3.6.3 Effect of resveratrol on intermolecular force of SP

To determine the binding force between polyphenols, SP, and HAs, changes in protein chemical bonds associated with the formation of bound HAs were measured (**Figure 4-14**). Except for disulfide bonds, there was no change or reduction of other

chemical bonds in SP after the addition of HAs, indicating that SP and HAs may mainly rely on disulfide bonds to form bound HAs. In addition, HAs significantly decreased the intermolecular ionic bonding of proteins ($P < 0.05$), whereas resveratrol supplementation significantly increased them ($P < 0.05$), suggesting that polyphenols can influence the binding of SP to free HAs by affecting ionic and disulfide bonds. This weakening of ionic bonds can lead to alterations in protein structure and stability, resulting in protein aggregation behavior (Zhao et al., 2016). In addition to ionic bonds and disulfide bonds, the addition of resveratrol significantly enhanced hydrogen bonds and hydrophobic interactions compared to the blank and HAs-added treatment groups. Enhanced hydrophobic interactions and hydrogen bonds between proteins can also promote the aggregation of protein and increase particle size (Filgueras et al., 2011). These results are consistent with those from SEM and particle size.

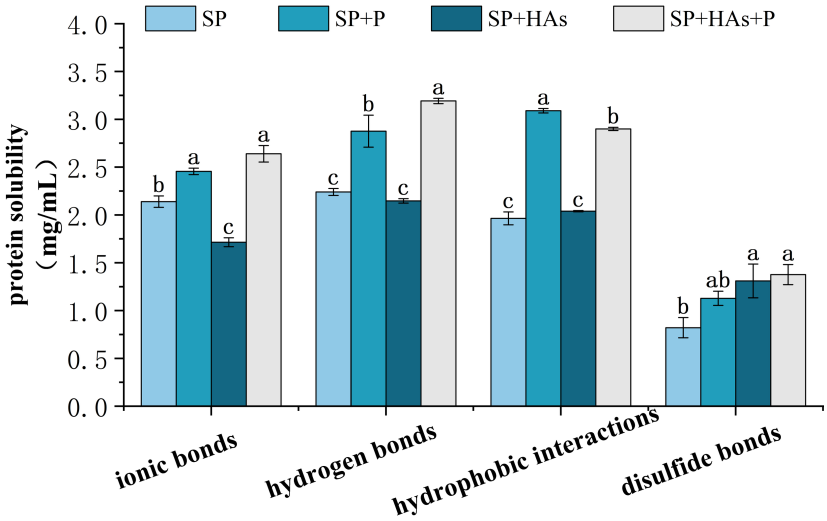


Figure 4-14: Effects of resveratrol on the protein solubility of SP. Different letters indicate significant differences among groups ($P < 0.05$).

3.6.4 Analysis of the correlation between protein structures and HAs in SP

Figure 4-15 presents the Pearson correlation analysis of the association between the inhibition of bound HAs and the interaction between polyphenols and SP. In the figure, blue represents negative correlation, while red represents positive correlation, with darker shades indicating stronger correlations. The analysis revealed that free HAs content was positively correlated with the bound HAs content and surface hydrophobicity, while it was negatively correlated with the ζ-potential, respectively. The addition of polyphenols and HAs resulted in a significant negative correlation between random coil and β-sheet, hydrophobic interaction and hydrogen

bonds among SP. The current study also demonstrates that the β-sheet provides high structural stability through hydrogen bonds, hydrophobic interactions help the protein core to form a tight fold (Dill, 1990), and hydrogen bonds further enhance the stability of the secondary and tertiary structures of protein (Dobson, 2003). The presence of random coil regions may contribute less to or even weakens the overall stability (Wright & Dyson., 1999). These results indicate that free HAs can bind directly to SP to form bound HAs, free HAs promotes the formation of bound HAs by changing the surface hydrophobicity of SP solution and the generation of bound HAs affects the ζ-potential of the SP solution.

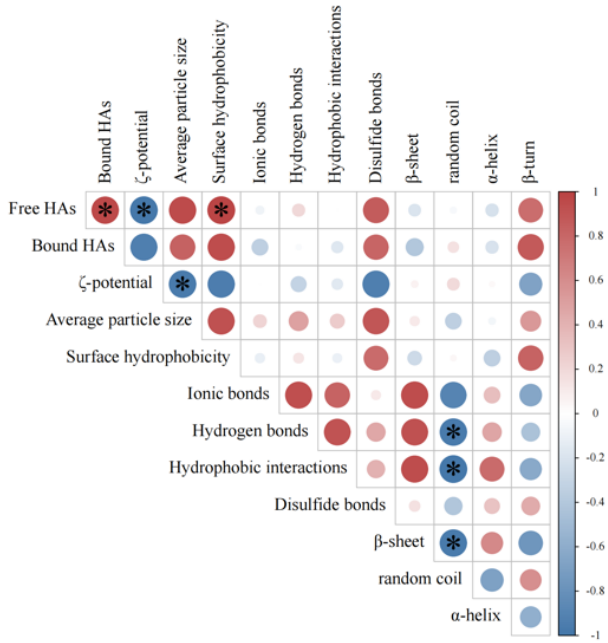


Figure 4-15: Correlation between inhibition of bound HAs and interaction of polyphenols and SP. * indicates a statistically significant correlation.

4.Conclusions

In conclusion, resveratrol not only inhibits the production of heterocyclic amines through the scavenging of free radicals/capture of reactive carbonyl pathway, but also influences the production of bound heterocyclic amines involved in proteins in meat products by altering the protein structure. Resveratrol inhibits the formation of MP-bound HAs mainly by influencing the direct involvement of myofibrillar proteins in the Maillard reaction. Resveratrol changes the structure of MP mainly through hydrophobic interactions and hydrogen bonds. On the one hand, changes in protein structure reduce the exposure of some amino acid residues that are directly involved in the production of HAs, reducing the production of bound HAs. On the other hand,

resveratrol promotes the exposure of certain hydrophobic amino acids in MP. These amino acids are also non-precursor amino acids that inhibit the formation of HAs. Moreover, resveratrol reduces the harm of HAs involved in sarcoplasmic protein by promoting the formation of SP-bound HAs from free HAs. Free HAs form bound HAs by changing the surface hydrophobicity of SP solution. Resveratrol promoted the binding of SP to free HAs by changing protein structure and increasing ionic bond and disulfide bond. Therefore, this study provides a new perspective to understand the mechanism of polyphenol inhibition of HAs formation, not only verifying the way in which MP/SP involved in the formation of bound HAs, but also complementing and perfecting the different inhibition pathways of polyphenols on free/bound HAs in meat. It also lays the foundation for the effective screening of HAs inhibitors and the development of safer heat-processed meat products.

There are also some limitations to this study. The mechanisms were explored using model systems rather than real meat products, which may not fully replicate the complexity of actual food matrices. Additionally, it remains unclear whether the observed inhibitory mechanisms apply universally to other polyphenols with different structural characteristics. Future research should validate these findings in real meat systems, assess the efficacy of resveratrol and structurally diverse polyphenols at food-relevant concentrations within actual food matrices, and assess the in vivo relevance and safety of bound HAs. These efforts will help translate mechanistic findings into practical applications for food safety and public health.

5. References

Alaejos, M.S., & Afonso, A.M. (2011), Factors that affect the content of heterocyclic aromatic amines in foods. *Comprehensive Reviews in Food Science and Food Safety, 10*: 52-108.

Ai, M., Zhou, Q., Guo, S., Ling, Z., Zhou, L., Fan, H., et al. (2019). Effects of tea polyphenol and Ca(OH)2 on the intermolecular forces and mechanical, rheological, and microstructural characteristics of duck egg white gel. *Food Hydrocolloids, 94*, 11–19.

Barzegar, F., Kamankesh, M., & Mohammadi, A. (2019). Heterocyclic aromatic amines in cooked food: A review on formation, health risk-toxicology and their analytical techniques. *Food Chemistry, 280*, 240–254.

Benjakul, S., & Bauer, F. (2001). Biochemical and physicochemical changes in catfish (Silurus glanis Linne) muscle as influenced by different freeze–thaw cycles. *Food Chemistry, 72*(2), 207–217.

Bu, Y., Fan, M., Sun, C., Zhu, W., Li, J., Li, X., & Zhang, Y. (2024). Study on the interaction mechanism between (-)-epigallocatechin-3-gallate and myoglobin: Multi-spectroscopies and molecular simulation. *Food chemistry, 448*, 139208.

Chen, X., Jia, W., Zhu, L., Mao, L., & Zhang, Y. (2020). Recent advances in heterocyclic aromatic amines: An update on food safety and hazardous control

from food processing to dietary intake. *Comprehensive Reviews in Food Science and Food Safety, 19*(1), 124–148.

Chen, Q., Xue, C., He, Z., Wang, Z., Qin, F., Chen, J., & Zeng, M. (2021). Generation of Sarcoplasmic and Myofibrillar Protein-Bound Heterocyclic Amines in Chemical Model Systems under Different Heating Temperatures and Durations. *Journal of Agricultural and Food Chemistry,69*(10), 3232–3246.

Cheng, K. W., Chen, F., & Wang, M. (2007). Inhibitory activities of dietary phenolic compounds on heterocyclic amine formation in both chemical model system and beef patties. *Molecular Nutrition & Food Research, 51*(8), 969–976.

Cheng, K. W., Wong, C. C., Cho, C. K., Chu, I. K., Sze, K. H., Lo, C., Chen, F., & Wang, M. (2008). Trapping of phenylacetaldehyde as a key mechanism responsible for naringenin's inhibitory activity in mutagenic 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine formation. *Chemical Research in Toxicology, 21*(10), 2026–2034.

Cheng, K. W., Wong, C. C., Chao, J., Lo, C., Chen, F., Chu, I. K., Che, C. M., Ho, C. T., & Wang, M. (2009). Inhibition of mutagenic PhIP formation by epigallocatechin gallate via scavenging of phenylacetaldehyde. *Molecular Nutrition & Food Research, 53*(6), 716–725.

Cheng, J., Xu, L., Xiang, R., Liu, X., & Zhu, M. (2020). Effects of mulberry polyphenols on oxidation stability of sarcoplasmic and myofibrillar proteins in dried minced pork slices during processing and storage. *Meat science, 160*, 107973.

Deng, P., Xue, C., He, Z., Wang, Z., Qin, F., Oz, E., Chen, J., El Sheikha, A. F., Proestos, C., Oz, F., & Zeng, M. (2022). Synergistic Inhibitory Effects of Selected Amino Acids on the Formation of 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in both Benzaldehyde- and Phenylacetaldehyde-Creatinine Model Systems. *Journal of Agricultural and Food Chemistry, 70*(35), 10858–10871.

Deng, P., Wei, T., Yu, M., Yang, T., Chen, Q., Wang, Z., He, Z., Chen, J., & Zeng, M. (2024). Investigation on synergistic inhibition of protein-bound heterocyclic amines in sarcoplasmic and myofibrillar model systems by amino acid combinations. *Food chemistry, 460*(Pt 3), 140576.

Dobson C. M. (2003). Protein folding and misfolding. *Nature, 426*(6968), 884–890.

Dong, H., Xian, Y., Li, H., Bai, W., & Zeng, X. (2020). Potential carcinogenic heterocyclic aromatic amines (HAAs) in foodstuffs: Formation, extraction, analytical methods, and mitigation strategies. *Comprehensive Reviews in Food Science and Food Safety, 19*(2), 365–404.

Dubuisson, J. G., Dyess, D. L., & Gaubatz, J. W. (2002). Resveratrol modulates human mammary epithelial cell O-acetyltransferase, sulfotransferase, and kinase activation of the heterocyclic amine carcinogen N-hydroxy-PhIP. *Cancer Letters, 182*(1), 27–32.

Dill K. A. (1990). Dominant forces in protein folding. *Biochemistry, 29*(31), 7133–7155.

Filgueras, R. S., Gatellier, P., Ferreira, C., Rui, C. Z., & Santé-Lhoutellier, V. (2011). Nutritional value and digestion rate of rhea meat proteins in association with storage and cooking processes. *Meat Science, 89*(1), 6–12.

Gibis M. (2016). Heterocyclic aromatic amines in cooked meat products: causes, formation, occurrence, and risk assessment. *Comprehensive Reviews in Food Science and Food Safety, 15*(2), 269–302.

Han, P., An, N., Yang, L., Ren, X., Lu, S., Ji, H., & Dong, J. (2022). Molecular dynamics simulation of the interactions between sesamol and myosin combined with spectroscopy and molecular docking studies. *Food Hydrocolloids, 131*, 107801.

Herrero, A. M. (2008). Raman spectroscopy for monitoring protein structure in muscle food systems. *Critical Reviews in Food Science and Nutrition, 48*(6), 512–523.

Huang, X., Sun, L., Liu, L., Wang, G., Luo, P., Tang, D., & Huang, Q. (2022). Study on the mechanism of mulberry polyphenols inhibiting oxidation of beef myofibrillar protein. *Food chemistry, 372*, 131241.

Joye, I. J., Davidov-Pardo, G., Ludescher, R. D., & McClements, D. J. (2015). Fluorescence quenching study of resveratrol binding to zein and gliadin: Towards a more rational approach to resveratrol encapsulation using water-insoluble proteins. *Food Chemistry, 185*, 261–267.

Kašička V. (2013). Amino acids, peptides and proteins. *Electrophoresis, 34*(18), 2603.

Kataoka, H., Miyake, M., Nishioka, S., Matsumoto, T., Saito, K., & Mitani, K. (2010). Formation of protein adducts of 2-amino-1-methyl-6-phenylimidazo [4, 5-b] pyridine in cooked foods. *Molecular Nutrition & Food Research, 54*(7), 1039–1048.

Klajnert, B., & Bryszewska, M. (2002). Fluorescence studies on PAMAM dendrimers interactions with bovine serum albumin. *Bioelectrochemistry, 55*, 33–35.

Lakowicz, J. R., & Weber, G. (1973). Quenching of protein fluorescence by oxygen. Detection of structural fluctuations in proteins on the nanosecond time scale. *Biochemistry, 12*, 4171–4179.

Laskowski, R. A., & Swindells, M. B. (2011). LigPlot+: multiple ligand-protein interaction diagrams for drug discovery. *Journal of Chemical Information and Modeling, 51*(10), 2778–2786.

Le Marchand, L., Hankin, J. H., Pierce, L. M., Sinha, R., Nerurkar, P. V., Franke, A. A., Wilkens, L. R., Kolonel, L. N., Donlon, T., Seifried, A., Custer, L. J., Lum-Jones, A., & Chang, W. (2002). Well-done red meat, metabolic phenotypes and colorectal cancer in Hawaii. *Mutation Research, 506-507*, 205–214.

Li, Y., Cheng, Y., Zhang, Z., Wang, Y., Mintah, B. K., Dabbour, M., Jiang, H., He, R., & Ma, H. (2020). Modification of rapeseed protein by ultrasound-assisted pH shift treatment: Ultrasonic mode and frequency screening, changes in protein solubility and structural characteristics. *Ultrasonics sonochemistry, 69*, 105240.

Liu, H., Wang, Z., Suleman, R., Shen, Q., & Zhang, D. (2019). Effect of protein thermal stability and protein secondary structure on the roasted mutton texture and colour from different cuts. *Meat Science, 156*, 52–58.

Lu, F., Kuhnle, G. K., & Cheng, Q. (2018). The effect of common spices and meat type on the formation of heterocyclic amines and polycyclic aromatic hydrocarbons in deepfried meatballs. *Food Control, 92*, 399–411.

Masuda, T., Inai, M., Miura, Y., Masuda, A., & Yamauchi, S. (2013). Effect of polyphenols on oxymyoglobin oxidation: prooxidant activity of polyphenols in vitro and inhibition by amino acids. *Journal of Agricultural and Food Chemistry, 61*(5), 1097–1104.

Meurillon, M., Angénieux, M., Mercier, F., Blinet, P., Chaloin, L., Chevolleau, S., Debrauwer, L., & Engel, E. (2020). Mitigation of heterocyclic aromatic amines in cooked meat. Part I: Informed selection of antioxidants based on molecular modeling. *Food Chemistry, 331*, 127264.

Mi, J., Ni, W., Huang, P., Hong, J., Jia, R., Deng, S., Yu, X., Wei, H., & Yang, W. (2022). Effect of acetylated distarch adipate on the physicochemical characteristics and structure of shrimp (Penaeus vannamei) myofibrillar protein. *Food Chemistry, 373*, 131530.

Miriani, M., Keerati-u-rai, M., Corredig, M., Iametti, S., & Bonomi, F. (2011). Denaturation of soy proteins in solution and at the oilewater interface: A fluorescence study. *Food Hydrocolloids, 25*, 620–626.

Morris, G. M., Huey, R., Lindstrom, W., Sanner, M. F., Belew, R. K., Goodsell, D. S., & Olson, A. J. (2009). AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. *Journal of Computational Chemistry, 30*(16), 2785–2791.

Nawaz, A., Irshad, S., Ali Khan, I., Khalifa, I., Walayat, N., Muhammad Aadil, R., Kumar, M., Wang, M., Chen, F., Cheng, K. W., & Lorenzo, J. M. (2022). Protein oxidation in muscle-based products: Effects on physicochemical properties, quality concerns, and challenges to food industry. *Food Research International (Ottawa, Ont.), 157*, 111322.

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

Oguri, A., Suda, M., Totsuka, Y., Sugimura, T., & Wakabayashi, K. (1998). Inhibitory effects of antioxidants on formation of heterocyclic amines. *Mutation Research, 402*(1-2), 237–245.

Oz, E., Aoudeh, E., Murkovic, M., Toldra, F., Gomez-Zavaglia, A., Brennan, C., Proestos, C., Zeng, M., & Oz, F. (2023). Heterocyclic aromatic amines in meat: Formation mechanisms, toxicological implications, occurrence, risk evaluation, and analytical methods. *Meat Science, 205*, 109312.

Privalov, P. L., & Gill, S. J. (1988). Stability of protein structure and hydrophobic interaction. *Advances in Protein Chemistry, 39*, 191–234.

Quan, T. H., Benjakul, S., Sae-leaw, T., Balange, A. K., & Maqsood, S. (2019). Protein–polyphenol conjugates: Antioxidant property, functionalities and their applications. *Trends in Food Science & Technology, 91*, 507–517.

Ren, C., Xiong, W., Peng, D., He, Y., Zhou, P., Li, J., & Li, B. (2018). Effects of thermal sterilization on soy protein isolate/polyphenol complexes: Aspects of structure, in vitro digestibility and antioxidant activity. F*ood Research International (Ottawa, Ont.), 112*, 284–290.

Schrödinger, L., & DeLano, W. (2020). PyMOL. Retrieved from http://www.pymol.org/pymol.

Sun, X., Yu, Y., Saleh, A. S. M., Yang, X., Ma, J., Li, W., Zhang, D., & Wang, Z. (2023). Understanding interactions among flavor compounds from spices and myofibrillar proteins by multi-spectroscopy and molecular docking simulation. *International Journal of Biological Macromolecules, 229*, 188–198.

Sung, J., Shin, K.J., & Lee, S. (1992). Theory of diffusion-influenced fluorescence quenching. Effects of static quenching on the Stern-Volmer curve. *Chemical Physics, 167*, 17–36.

Szterk A. (2013). Chemical state of heterocyclic aromatic amines in grilled beef: evaluation by in vitro digestion model and comparison of alkaline hydrolysis and organic solvent for extraction. *Food and Chemical Toxicology: an international journal published for the British Industrial Biological Research Association, 62*, 653–660.

Tadpitchayangkoon, P., Park, J. W., Mayer, S. G., & Yongsawatdigul, J. (2010). Structural changes and dynamic rheological properties of sarcoplasmic proteins subjected to pH-shift method. *Journal of Agricultural and Food Chemistry, 58*(7), 4241–4249.

Tornberg E. (2005). Effects of heat on meat proteins - Implications on structure and quality of meat products. *Meat Science, 70*(3), 493–508.

Villaverde, A., & Estévez, M. (2013). Carbonylation of myofibrillar proteins through the maillard pathway: effect of reducing sugars and reaction temperature. *Journal of Agricultural and Food Chemistry, 61*(12), 3140–3147.

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

Wang, M., Kang, J., Chen, L., He, G., Liu, Y., Fan, X., Lv, X., Xu, X., Zhou, G., & Feng, X. (2023). Suppression mechanism of L-lysine on the Epigallocatechin-3-gallate-induced loss of myofibrillar protein gelling potential. *Food Research International, 169*, 112928.

Wang, P., Zou, M., Gu, Z., & Yang, R. (2018). Heat-induced polymerization behavior

variation of frozen-stored gluten. *Food Chemistry, 255*, 242–251.

Wang, S. F., & Smith, D. M. (1994). Dynamic rheological properties and secondary structure of chicken breast myosin as influenced by isothermal heating. *Journal of Agricultural and Food Chemistry, 42*, 1434–1439.

Wang, T., Han, D., Zhao, L., Huang, F., Yang, P., & Zhang, C. (2023). Binding of Selected Aroma Compounds to Myofibrillar Protein, Sarcoplasmic Protein, and Collagen during Thermal Treatment: Role of Conformational Changes and Degradation of Proteins. *Journal of Agricultural and Food Chemistry, 71*(46), 17860–17873.

Wang, D., Li, H., Hou, T. Y., Zhang, Z. J., & Li, H. Z. (2024). Effects of conjugated interactions between Perilla seed meal proteins and different polyphenols on the structural and functional properties of proteins. *Food Chemistry, 433*, 137345.

Wright, P. E., & Dyson, H. J. (1999). Intrinsically unstructured proteins: re-assessing the protein structure-function paradigm. *Journal of Molecular Biology, 293*(2), 321–331.

Wu, D., Wang, H., Guo, X., Zhang, Z., Gao, Z., Gao, S., Liu, Z., Rao, S., & Meng, X. (2023). Insight into the mechanism of enhancing myofibrillar protein gel hardness by ultrasonic treatment combined with insoluble dietary fiber from oat. *LWT-Food Science & Technology, 178*, 114539.

Wu, S. X., Xiong, R. G., Huang, S. Y., Zhou, D. D., Saimaiti, A., Zhao, C. N., Shang, A., Zhang, Y. J., Gan, R. Y., & Li, H. B. (2022). Effects and mechanisms of resveratrol for prevention and management of cancers: An updated review. *Critical Reviews in Food Science and Nutrition, 63*(33), 12422–12440.

Xia, M., Chen, Y., Guo, J., Huang, H., Wang, L., Wu, W., et al. (2019). Water distribution and textual properties of heat-induced pork myofibrillar protein gel as affected by sarcoplasmic protein. *LWT-Food Science & Technology, 103,* 308–315.

Xue, C., He, Z., Qin, F., Chen, J., & Zeng, M. (2020). Effects of amides from pungent spices on the free and protein-bound heterocyclic amine profiles of roast beef patties by UPLC-MS/MS and multivariate statistical analysis. *Food Research International, 135,* 109299.

Xue, C., Chen, Q., He, Z., Qin, F., Wang, Z., Chen, J., & Zeng, M. (2022a). Release mechanism between sarcoplasmic protein-bound and free heterocyclic amines and the effects of dietary additives using an in-vitro digestion model. *Food chemistry, 377*, 131993.

Xue, C., Chen, Q., He, Z., Qin, F., Wang, Z., Chen, J., & Zeng, M. (2022b). Release profiles of beef myofibril protein-bound heterocyclic amines and effects of dietary components on in vitro digestion. *Food Research International, 155*, 111006.

Yan, Y., Fang, J., Zhu, X., Ji, X., Shi, M., & Niu, B. (2024). Effect of extrusion using plasma-activated water on the structural, physicochemical, antioxidant and in vitro digestive properties of yam flour. *Food Chemistry, 460*(Pt 2), 140687.

Yang, X. Y., Blecker, C., Liu, H., Zhang, D. Q., Wang, Z. Y. (2023). Effect of plant polyphenols with different m-hydroxy and o-hydroxy groups on the inhibition of

heterocyclic amines formation in roasted meat. *Food Control, 153*, 109960.

Yang, X., Zhang, D., Blecker, C., Zhang, C., Sun, X., Wang, Z. (2024). Effect of interaction between resveratrol and myofibrillar protein on the production of bound heterocyclic amines. *Food Bioscience, 59*, 104116.

Ye, J., Deng, L., Wang, Y., McClements, D. J., Luo, S., & Liu, C. (2021). Impact of rutin on the foaming properties of soybean protein: Formation and characterization of flavonoid-protein complexes. *Food Chemistry, 362*, 130238.

Yi, X., Pei, Z., Xia, G., Liu, Z., Shi, H., & Shen, X. (2023). Interaction between liposome and myofibrillar protein in surimi: Effect on gel structure and digestive characteristics. *International Journal of Biological Macromolecules, 253*, 126731.

Young, L., Jernigan, R. L., & Covell, D. G. (1994). A role for surface hydrophobicity in protein-protein recognition. *Protein science: a publication of the Protein Society, 3*(5), 717–729.

Yu, C., Shao, Z., Liu, B., Zhang, Y., & Wang, S. (2016). Inhibition of 2-Amino-1-methyl-6-phenylimidazo [4,5-b]pyridine (PhIP) formation by alkoxy radical scavenging of flavonoids and their quantitative structure-activity relationship in a model system. *Journal of Food Science, 81*(8), C1908–C1913.

Zhang, L., Wang, P., Yang, Z., Du, F., Li, Z., Wu, C., Zhou, G. (2020). Molecular dynamics simulation exploration of the interaction between curcumin and myosin combined with the results of spectroscopy techniques. *Food Hydrocolloids, 101*, 105455.

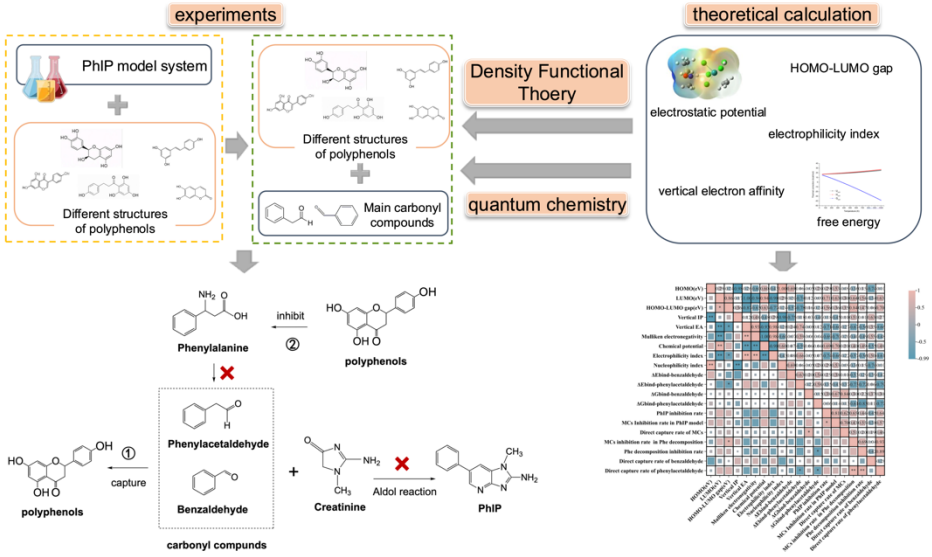
Zhao, Y., Chen, Z., Li, J., Xu, M., Shao, Y., & Tu, Y. (2016). Changes of microstructure characteristics and intermolecular interactions of preserved egg white gel during pickling. *Food Chemistry, 203*, 323–330.

5

Chapter V Mechanistic study on the inhibition of PhIP by polyphenols with different structures in model systems: A combination of experimental and theoretical chemical calculations

Short overview of chapter V

Based on the literature review in chapter III, it is known that polyphenols with m-hydroxyl structures inhibit PhIP generation by trapping active carbonyl compounds, but different polyphenols that all have m-hydroxyl structures inhibit PhIP with different effects, which still need to be explored. Therefore, in this chapter, the relationship between the molecular properties of different polyphenols and their inhibition of PhIP was investigated through a combination of experiments and theoretical calculations.



Graphical abstract: Inhibition mechanism of different structures of polyphenols on PhIP was elucidated through the molecular characteristics of polyphenols.

The work from this chapter was submitted to the journal.

Abstract

In this study, the relationship between the inhibitory effects of seven different types of polyphenols containing m-hydroxyl groups on PhIP formation and their molecular properties and molecular energies were investigated through experimentally and theoretical calculations. The results showed that polyphenols affected the generation of benzaldehyde and phenylacetaldehyde from heated phenylalanine, and the direct trapping effects on benzaldehyde and phenylacetaldehyde were inconsistent with those of both in the PhIP model systems. This suggests that polyphenols inhibit PhIP generation not only by direct trapping of reactive carbonyls, but also by influencing the generation of benzaldehyde and phenylacetaldehyde from the phenylalanine reaction. In addition, the HOMO-LUMO gaps of different polyphenols were significantly and positively correlated with the inhibitory effects of polyphenols on the major carbonyl compounds produced by the thermal degradation of phenylalanine, suggesting that the effectiveness of the polyphenols in inhibiting PhIP can be judged by their HOMO-LUMO gaps.

Keywords: Polyphenols; Heterocyclic amines; Benzaldehyde; Phenylacetaldehyde; Density functional theory (DFT)

1. Introduction

2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), one of the most abundant heterocyclic amines (HAs) formed during high-temperature cooking of meat (Gibis, 2016; Barzegar, Kamankesh, & Mohammadi, 2019; Sugimura, Wakabayashi, Nakagama, & Nagao, 2004; Puangsombat et al., 2012), has been classified as a Group 2B carcinogen (IARC, 1993) and has been reasonably anticipated to be a human carcinogen by the National Toxicology Program of the U.S. (NTP, 2004). Therefore, it is of great significance to further explore the generation and inhibition mechanism of PhIP. The formation mechanism of PhIP has been extensively studied. The carbonyl compounds obtained from the phenylalanine Strecker degradation reaction and creatinine first undergo aldol condensation reaction, after which the hydroxyaldehyde condensation product obtained reacts with formaldehyde and ammonia to form PhIP (Zöchling & Murkovic, 2002). Recent studies have widely demonstrated that reactive carbonyl compounds play a key role in the reaction. Phenylacetaldehyde and benzaldehyde produced by the thermal degradation of phenylalanine are both important intermediates in the production of PhIP (Murkovic et al., 1999; Deng et al., 2022). Accordingly, the addition of exogenous ingredients to bind these intermediates is an effective way to inhibit PhIP and has been widely investigated (Jiang et al., 2022; Zhao et al., 2021).

Among the various additives, polyphenols are the most important and effective inhibitors of PhIP (Alaejos & Afonso, 2011). Quercetin, naringenin, luteolin and chlorogenic acid from spices, and mangiferin from Chinese chive all have significant inhibitory effects on PhIP (Cheng et al., 2008; Zeng et al., 2016; Wang et al., 2021). At present, research on the mechanism by which polyphenols inhibit the formation of PhIP mainly focuses on their ability to form adducts with the intermediate carbonyl compound phenylacetaldehyde, thereby blocking its participation in subsequent reactions (Dong et al., 2024). Moreover, the hydroxyl position of polyphenols has an effect on the inhibitory effect of PhIP, and the m-hydroxyl structure is stronger than the ortho-hydroxyl and para-hydroxyl structures (Salazar et al., 2014). Previous studies have also shown that polyphenols with m-hydroxy structures inhibit the production of PhIP mainly by capturing carbonyl compounds (Yang et al., 2023). However, it was found that different types of polyphenol compounds with the same m-hydroxy structure had significant differences in the inhibitory effects on PhIP, which deserves further exploration. We speculate that the different structures of different polyphenols, except for the m-hydroxyl structure, cause the differences in the ability to capture carbonyl compounds. Whether there is a correlation between the structural characteristics of different polyphenols and the ability to capture carbonyl compounds is still unclear.

In this study, a total of 7 polyphenol compounds (genistein, resveratrol, quercetin, naringenin, epicatechin, luteolin, and phloretin) of three classes (flavonoids, stilbenes, chalcones), which all have the structure of m-hydroxyl group and the effect of

inhibiting PhIP, were selected for investigation. The changes in the contents of PhIP and major carbonyl compounds (benzaldehyde and phenylacetaldehyde) in reaction systems after the addition of polyphenols were determined by HPLC-MS/MS and GC-MS techniques to clarify the mechanism by which polyphenols inhibit PhIP. Then, the adducts structure of polyphenols with phenylalanine and carbonyl compounds were determined using UPLC-Q Exactive-MS. Finally, Density Functional Theory was used to study the correlation between the structure of phenolic compounds and the capture of carbonyl compounds, further clarifying the reasons why the difference of inhibition effect of polyphenols with different structures on PhIP.

2. Materials and methods

2.1 Materials

Standards of PhIP (99.9%), benzaldehyde, phenylacetaldehyde were supplied by TRC (Toronto, ONT, Canada) and Aladdin (Shanghai, China). Resveratrol (98%), quercetin (98%), epicatechin (97%), luteolin (96%), phloretin (98%), genistein (98%), naringenin (98%), glucose and creatinine were sourced from Yuanye (Shanghai, China) and Solarbio (Beijing, China). Phenylalanine was obtained from Sinopharm Chemical Reagent. Diethylene glycol (99%), ammonium acetate (99.9%), ethyl acetate (99.5%), were obtained from Aladdin (Shanghai, China), Merck KGaA (Darmstadt, Hessian, Germany), and Macklin (Shanghai, China), respectively. UPLC-MS grade acetic acid, methanol and acetonitrile were supplied by Thermo Fisher (Waltham, MA, USA). Other analytical-grade reagents utilized in this research were from Sinopharm Chemical Reagent (Shanghai, China).

2.2 Establishment of reaction systems

The construction of each reaction system was based on previous studies with little modifications (Hui et al., 2021; Yang et al., 2023). (i) PhIP generation model systems: Phenylalanine (0.4 mmol), glucose (0.2 mmol), creatinine (0.4 mmol) and polyphenols (0.2 mmol) were mixed in 4 mL of pH 7.0 diethylene glycol (20% 0.1 M citric acid-phosphate buffer, w/w) without polyphenols as a control, and heated at 150℃ for 60 min. All the reaction solutions were extracted by ethyl acetate, separated and purified by Waters Oasis MCX column, and finally filtered with a 0.22 μm pore size membrane for UHPLC-MS /MS analysis to determine the change of PhIP content. The contents of benzaldehyde and phenylacetaldehyde in the reaction solution were subsequently determined. (ii) Direct capture of carbonyl compound system: 100 μL of the same concentration of benzaldehyde, phenylacetaldehyde in the reaction with different polyphenols (0.2 mmol) in 4 mL of diethylene glycol (150 ℃, 30 min). The contents of benzaldehyde and phenylacetaldehyde were determined after the reaction. (iii) Phenylalanine thermal reaction system: 4 mL phenylalanine solution (0.1mmol/ mL) and different polyphenols (0.2 mmol) were reacted in a closed reaction bottle at 150℃ for 10, 20 and 40 min. The contents of phenylalanine, benzaldehyde and phenylacetaldehyde in the reaction solution were subsequently determined.

2.3 Determination of phenylalanine, benzaldehyde and phenylacetaldehyde

The content of phenylalanine was determined by HPLC. The reaction mixture in Section 2.2 was diluted 10 times with ultra-pure water and filtered by membrane, and then determined by HPLC system (1260, Agilent) equipped with a PDA detector. The determination was performed on Agilent C18 column (5 μm, 250 mm × 4.6 mm) with 5% methanol aqueous solution as mobile phase. The detection wavelength was 260 nm, the flow rate was 1 mL/min, and the sample volume was 10 μL. Standard curves (y=89.597x-7.549, R^2=0.9993) were plotted at six calibration levels (50/100/200/400/800/1650 μg/mL) for phenylalanine.

The contents of benzaldehyde and phenylacetaldehyde were determined by GC-MS. The 2.2 reaction mixture was extracted with 10 mL ethyl acetate and filtered by membrane for further GC-MS analysis. DB wax column (60 m × 250 μm × 0.25 μm) was used with electron ionization (EI) as the ion source, the sample volume was 1 μL, and the column temperature procedures were as follows: 70 °C (1 min), 70-230°C (10°C/min), and 230 °C (2 min), with nitrogen as the carrier gas. The standard curve of benzaldehyde and phenylacetaldehyde was established, and the retention time of samples was compared with that of the standard products to determine benzaldehyde and phenylacetaldehyde contents.

2.4 Structural identification of different polyphenols-intermediates adducts

The reaction mixture of the phenylalanine/polyphenol model systems was centrifuged and filtered through a 0.22 μm membrane before analysis by UPLC-Q Exactive-MS/MS. Separation and identification of adducts were performed using a Q Exactive Orbitrap LC-MS/MS (Thermo Fisher Scientific) system equipped with a Hypersil GOLD C18 column (100 × 2.1 mm; 1.9 μm). Methanol (A) and 0.1% formic acid (B) were used as the mobile phases, with a flow rate of 0.3 mL/min and an injection volume of 2 μL. The elution process was divided into five stages: 0-2 min, 5% A; 2-15 minutes, 90% A; 15-18 minutes, 90% A; 18-18.5 minutes, 5% A; 18.5-20 minutes, 5% A. Total run time is 20 minutes. The operating conditions for MS analysis in positive ion mode were as follows: capillary temperature, 320 °C; sheath gas flow, 45; aux gas flow, 10; resolution of MS, 70k; resolution of MS/MS, 17.5k; spray voltage, 3.50 kV; and scan range: 100–1500 m/z. Thermo Scientific Xcalibur software was used for data acquisition and processing.

2.5 Density functional theory (DFT) calculation

The molecular structures of several polyphenols were optimized using Gaussian09 with the B3LYP/6-31G* functional and basis set (Frisch et al., 1984; Frisch et al., 2009). In addition, by combining Multiwfn with VMD, vertical electron affinity (VEA), electrophilicity index, chemical potential, vertical ionization potential (VIP), nucleophilicity index, HOMO-LUMO gap (eV) were calculated (Lu and Chen, 2012;

Lu, 2024; Lu and Chen, 2021; Lu and Chen, 2022). Further single point energy calculations based on structural optimization were carried out for different polyphenols, benzaldehydes/benzaldehydes and adducts using the M062X-DEF2TZVP functional and basis set. The interaction binding energies (ΔEbind) and binding free energy (ΔGbind) of phenolic compounds with benzaldehyde/phenylacetaldehyde were calculated.

2.6 Statistical analysis

Each experiment was conducted in triplicate, and the results were expressed as mean ± SD. All data were performed by ANOVA followed by Duncan's multiple range tests using SPSS 20 (IBM Corp., New York, USA) to assess significant differences ($P < 0.05$) in results.

3. Results and discussion

3.1 Confirmation of the mechanism of PhIP inhibition by different polyphenols in a model system

The results of inhibition of PhIP and major carbonyl compounds (MCs) by different polyphenols in the model systems (**Figure 5-1**) showed that polyphenols had significant inhibition on PhIP ($P < 0.05$). Luteolin has the weakest inhibitory effect, while epicatechin, resveratrol and phloretin have stronger inhibitory effects, which is similar to the tendency of polyphenols to inhibit the production of HAs in roasted meat (Yang et al., 2023). Benzaldehyde and phenylacetaldehyde are the main intermediate carbonyl compounds produced by PhIP, with phenylacetaldehyde being the most predominant (Deng et al., 2022). It can be seen that the trapping effect of polyphenols with different structures on the main carbonyl compounds in the model systems is basically consistent with the inhibition effect of PhIP except genistein. The trends of benzaldehyde and phenylacetaldehyde content changes were also similar.

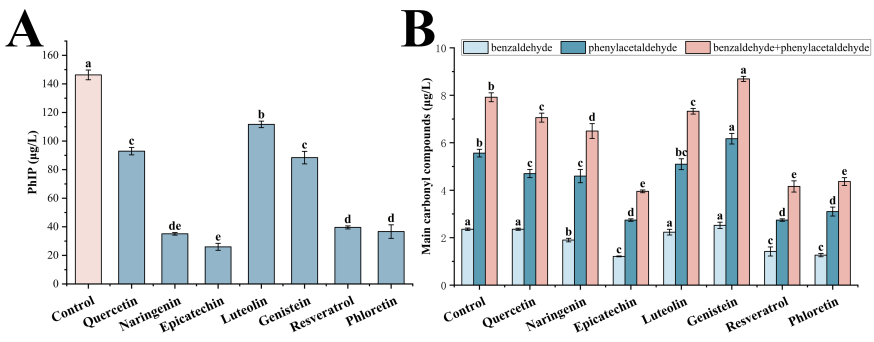


Figure 5-1: Effects of different polyphenols on PhIP (A) and main carbonyl compounds (B) in the model system. Different letters indicate significant differences among groups ($P < 0.05$).

In order to confirm the mechanism that the inhibition of PhIP by different polyphenols is mainly through the capture of reactive carbonyl compounds, the contents of benzaldehyde, phenylacetaldehyde after direct reaction with these polyphenols were determined (**Figure 5-2**). The levels of benzaldehyde and phenylacetaldehyde were reduced with the addition of polyphenols compared to the control group without polyphenols, suggesting that polyphenols do react with these two substances. However, the tendency of different polyphenols to capture benzaldehyde and phenylacetaldehyde directly is significantly different from the change in the content of both measured in the PhIP model systems. These results suggest that polyphenols affect the content of carbonyl compounds through other pathways in addition to their direct trapping of carbonyl compounds remains to be explored. It is known that benzaldehyde and phenylacetaldehyde are produced by the thermal degradation of phenylalanine, so it is hypothesized that polyphenols may contribute to the changes in the level of benzaldehyde and phenylacetaldehyde in the reaction system by affecting the decomposition of phenylalanine into carbonyl compounds.

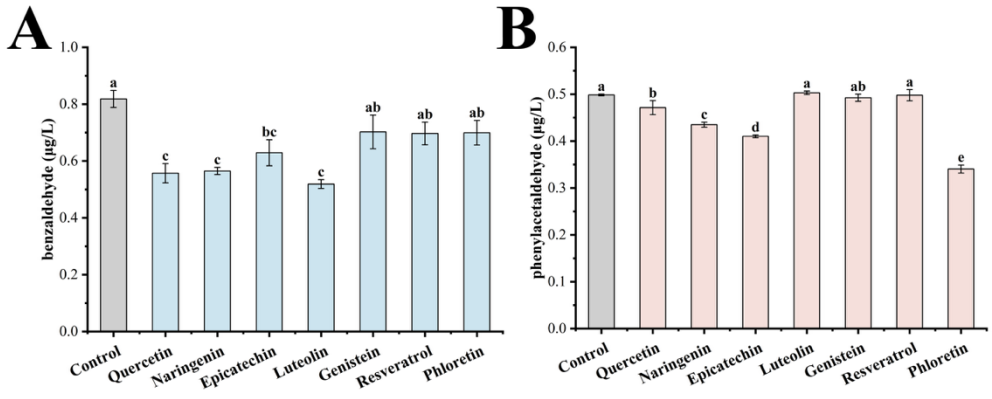


Figure 5-2: Effects of different polyphenols on the direct trapping of benzaldehyde (A), phenylacetaldehyde (B). Different letters indicate significant differences among groups ($P < 0.05$).

3.2 Effect of different polyphenols on the thermal decomposition of phenylalanine

In order to investigate the effect of polyphenols on the pyrolysis process of phenylalanine, changes in the content of phenylalanine and its pyrolysis products, benzaldehyde and phenylacetaldehyde, were systematically determined at different heating times (**Figure 5-3**). With the increase of time, the content of phenylalanine gradually decreased, while the level of benzaldehyde and phenylacetaldehyde

subsequently increased. After 20 min of reaction, the content of phenylalanine was significantly decreased ($P < 0.05$) in all treatment groups with different polyphenols added compared to the control group. In addition, the effects of different polyphenols on the pyrolysis products of phenylalanine varied: the contents of benzaldehyde and phenylacetaldehyde were lower in some treatment groups than in the blank group, whereas the contents of these two carbonyl compounds were higher in some other treatment groups than in the blank group. The reason for this phenomenon may be that on the one hand, the reaction of polyphenols with benzaldehyde and phenylacetaldehyde led to the reduction of the content, and on the other hand, this trapping effect of polyphenols on the carbonyl compounds promoted the positive pyrolysis reaction of phenylalanine. More interestingly, the three polyphenol groups (luteolin, genistein, resveratrol) with the highest levels of benzaldehyde and phenylacetaldehyde after the reaction also had the highest levels of phenylalanine compared to the other polyphenol groups. This may be due to the poor binding of these polyphenols to these two carbonyl compounds especially phenylacetaldehyde. This result was also verified in the direct capture of these two carbonyl compounds by these polyphenols (**Figure 5-2**).

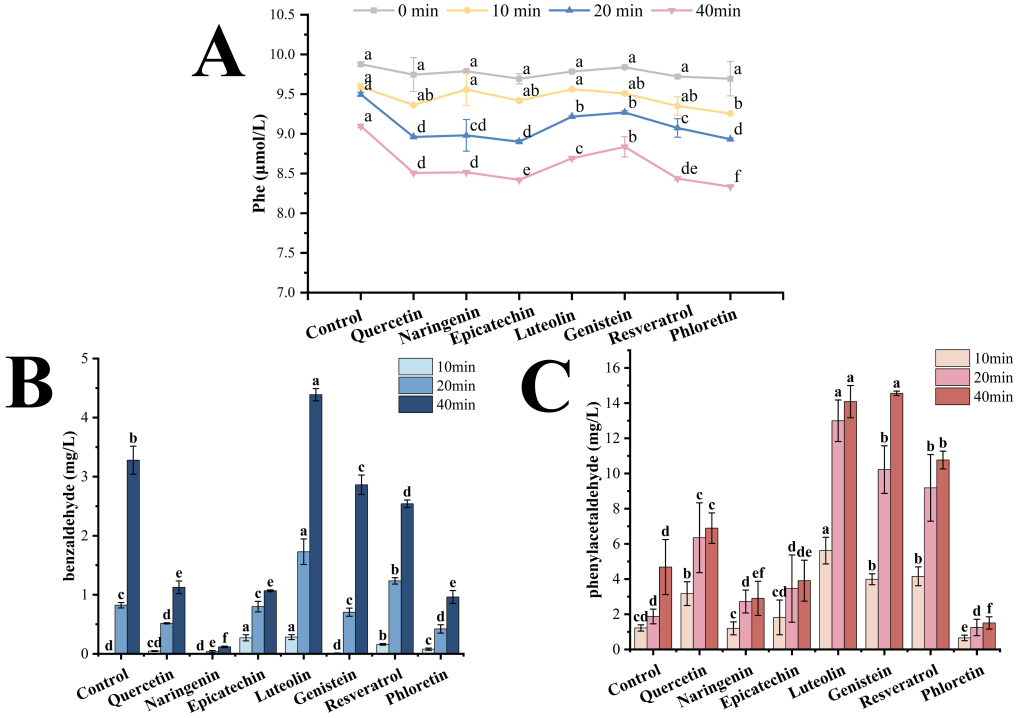


Figure 5-3: Effects of polyphenols on phenylalanine (A), benzaldehyde (B), and phenylacetaldehyde (C) during the thermal degradation of phenylalanine. Different letters indicate significant differences among groups ($P < 0.05$).

3.3 Possible mechanisms of polyphenol inhibition of PhIP production

Based on the above results obtained that the m-hydroxy structure polyphenols not only have a trapping effect on key carbonyl compounds during PhIP formation, but also have an effect on the pyrolysis of the precursor phenylalanine, the mechanism of inhibition of PhIP by polyphenols was further sorted out as shown in **Figure 5-4**. Polyphenol compounds containing m-hydroxyl inhibit the formation of PhIP in two ways: (1) In the PhIP generation reaction, creatinine and carbonyl compounds such as benzaldehyde and phenylacetaldehyde undergo a nucleophilic addition reaction followed by dehydration to form a condensation product, which undergoes a ring-closing reaction in the presence of ammonia and formaldehyde to ultimately form PhIP (Oz et al., 2023). Polyphenols capture the reaction intermediate carbonyl compounds and competitively inhibit their participation in the subsequent reaction to form PhIP; (2) Polyphenols inhibit the thermal degradation process of phenylalanine and reduce the formation of benzaldehyde and phenylacetaldehyde. Previous studies have shown that phenylalanine can generate phenylacetaldehyde by Strecker degradation, thermal decomposition, and oxidative decarboxylation and then hydrolysis (Chu, & Yaylayan, 2008). Moreover, phenylacetaldehyde can generate benzaldehyde through oxidative cleavage induced by free radicals (Hidalgo, & Zamora, 2019). Under high temperature or oxidative conditions, polyphenols (such as epicatechin, quercetin, resveratrol, etc.) are easily oxidized to form quinone compounds (Chedea et al., 2012; Shang et al., 2009). The quinone compounds generated by oxidation can react with phenylethylamine, an intermediate of phenylacetaldehyde, to form a combination product (Monforte, Martins, & Silva Ferreira, 2020; Hidalgo, León, & Zamora, 2016), thereby reducing the production of phenylacetaldehyde and benzaldehyde. In addition, study have shown that flavanols can form non-volatile adducts with amino acids, which can not only change the antioxidant properties of phenolic compounds but also prevent amino acids from participating in Maillard reactions or thermal decomposition reactions (Guerra, & Yaylayan, 2014). Therefore, polyphenols may also combine with phenylalanine to reduce the production of benzaldehyde and phenylacetaldehyde.

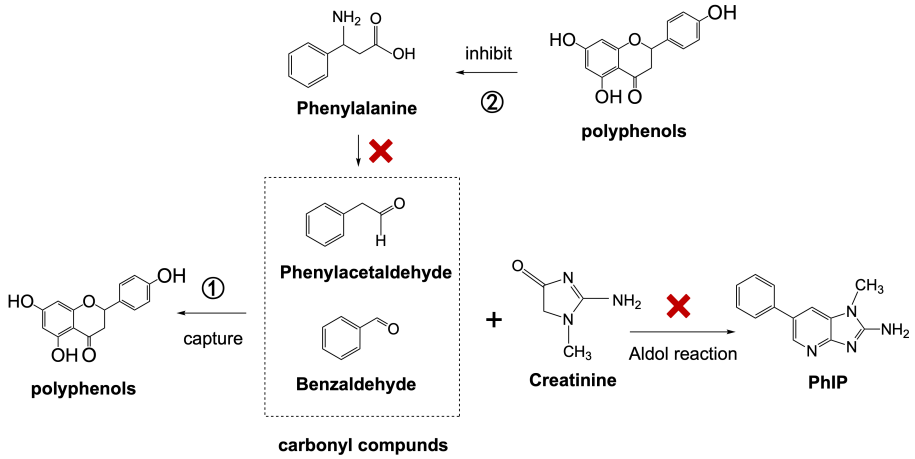


Figure 5-4: Possible mechanism of inhibition of PhIP by polyphenols

3.4 Calculation of common quantities of polyphenols with different structures using conceptual density-functional theory (DFT)

In order to further clarify the relationship between the structure and electronic properties of polyphenols and their capture of carbonyl compounds, DFT geometry optimization and parameter calculation were performed on different polyphenols in the experiment. After optimization, the global energy minimized structures of each polyphenol are shown in **Figure 5-5**, and the electronic properties defined in the conceptual density functional theory were calculated based on the optimal structures (**Table 5-1**). HOMO and LUMO are the energies of the highest occupied electron orbital and the unoccupied electron orbital, respectively. The HOMO-LUMO gap can represent the energy required for electron excitation to a certain extent (Zhou et al., 2024). Among these polyphenols, epicatechin has the largest LUMO energy level (E = - 0.0975 eV) and HOMO-LUMO gap (5.3584 eV), indicating that epicatechin has high stability. Chemical potential and electronegativity represent the tendency of electrons to escape and attract, respectively (Rajan, & Muraleedharan, 2017). Quercetin has the strongest electronegativity and correspondingly the smallest chemical potential. In addition, quercetin has the highest electrophilic index, indicating the strongest electron extraction ability. VIP and VEA represent the energy change of losing an electron and gaining an electron, respectively. It can be seen that the VEA values of the different polyphenols are all negative indicating that they have a hard time getting electrons. And resveratrol has the smallest VIP indicating that it loses electrons most easily.

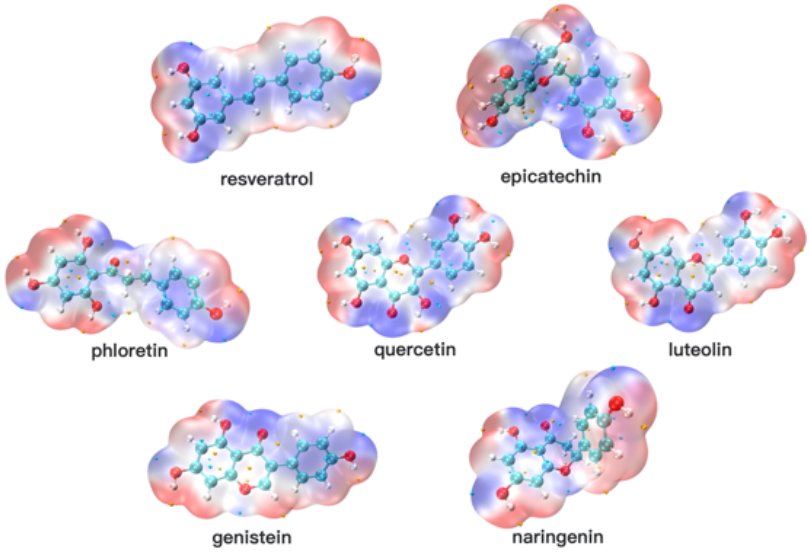


Figure 5-5: The optimal geometry and electrostatic potential diagram of different polyphenols

In addition, the binding energy and binding free energy of different polyphenols and benzaldehyde phenacetaldehyde adducts were calculated (**Table 5-1**)). According to previous studies, the binding between polyphenols and phenylacetaldehyde occurs through a nucleophilic substitution mechanism of phenylacetaldehyde on the hydrogen atom on the carbon atom between the two m-hydroxyl groups on the benzene ring of polyphenols (Cheng et al., 2008) (**Figure 5-6**C). Similarly, benzaldehyde will undergo a nucleophilic addition reaction with the carbon atom between the hydroxyl group of the polyphenol, and the carbonyl carbon of benzaldehyde forms a new C-C bond. Thus, there are two possible structures of the formed polyphenol and benzaldehyde adducts: either the carbonyl group on benzaldehyde is retained (**Figure 5-6**A) or the carbonyl group is reduced to a hydroxyl group (**Figure 5-6**B). And the calculation of the binding energy reveals that the binding energy of the adduct retaining the carbonyl structure on benzaldehyde is positive, indicating that the adduct is not easy to be generated. Therefore, the carbonyl group may be reduced to a hydroxyl group when benzaldehyde binds to the C atom in the polyphenol hydroxyl interstitial position. Similar chemical structure predictions are also reflected in the structures of adducts of benzaldehyde and amino acids (Deng et al., 2022). The binding energy and free energy of binding of polyphenols and benzaldehyde/phenylglyoxal adducts show that resveratrol is least likely to be involved in the formation of the adducts. The binding free energies of the adducts of different polyphenols and phenylacetaldehyde were all significantly higher than those

of polyphenols and benzaldehyde, indicating that polyphenols are more likely to form adducts with phenylacetaldehyde.

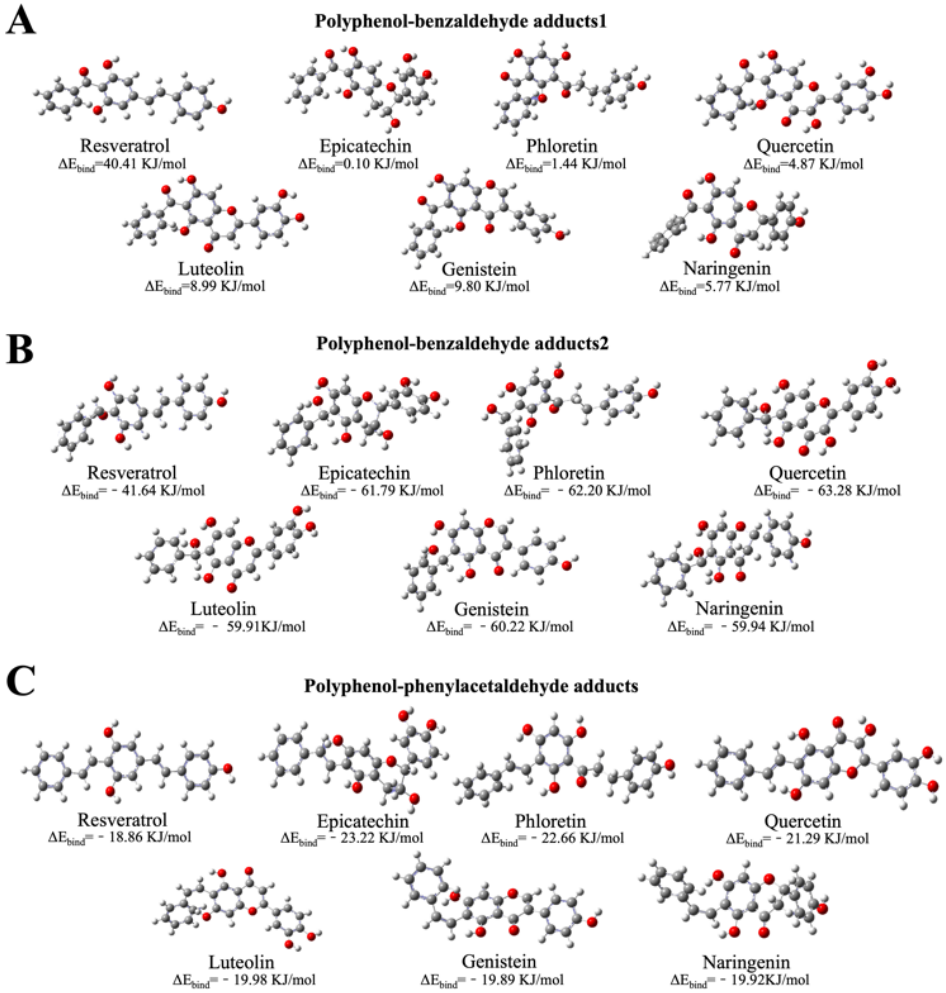


Figure 5-6: Structures of optimized adducts of different polyphenols with benzaldehyde/phenylacetaldehyde

Table 5-1. Calculated values of electronic properties defined in density functional theory of different polyphenols

	resveratrol	**epicatechin**	**phloretin**	**quercetin**	**luteolin**	**genistein**	**naringenin**
Vertical ionization potential	6.7479	7.0390	7.1879	7.2596	7.2081	7.0780	7.4581
Vertical electron affinity	-0.3842	-1.6394	-0.9305	-0.1602	-0.1786	-0.5567	-0.6028
Chemical potential	-3.1818	-2.6998	-3.1287	-3.5497	-3.5147	-3.2607	-3.4277
Electrophilicity index	0.7098	0.4199	0.6029	0.8491	0.8362	0.6963	0.7288
Nucleophilicity index	3.9156	3.6653	3.5581	3.3585	3.4127	3.6108	3.1971
Mulliken electronegativity	3.1818	2.6998	3.1287	3.5497	3.5147	3.2607	3.4277
HOMO(eV)	-5.2056	-5.4559	-5.5631	-5.7627	-5.7085	-5.5104	-5.924
LUMO(eV)	-1.1479	-0.0975	-0.7569	-1.3686	-1.3511	-1.0875	-1.0374
HOMO-LUMO gap(eV)	4.0577	5.3584	4.8062	4.3941	4.3574	4.4229	4.8866
ΔE_{bind} (benzaldehyde)(KJ/mol)	-41.6436	-61.7949	-62.1980	-63.2801	-59.9076	-60.2195	-59.9420
ΔE_{bind} (phenylacetaldehyde)(KJ/mol)	-18.8602	-23.2180	-22.6623	-21.2882	-19.9756	-19.8884	-19.9176
ΔG_{bind} (benzaldehyde)(KJ/mol)	-30.3072	-47.4832	-44.6301	-62.0946	-46.7061	-268.8693	-54.0302
ΔG_{bind}(phenylacetaldehyde)(KJ/mol)	-69.9860	-82.1347	-133.7796	-96.2504	-90.1039	-83.4081	-86.5303

3.5 Structural identification of different polyphenol-intermediate adduct in model system

In the reaction system of phenylalanine and different polyphenols, the adducts formed by the combination of different polyphenols with benzaldehyde and phenylacetaldehyde were further verified. We can see that the adducts of each polyphenol with benzaldehyde and phenylacetaldehyde in positive ion mode were indeed found according to the molecular weight and secondary mass spectrometry data (**Table 5-2**, **Figure 5-7**). Detailed analysis of the adducts listed in **Table 5-2** shows that different polyphenols and benzaldehyde adducts have similar MS/MS fragments, which is related to the similar structure of polyphenols. Almost all polyphenol-benzaldehyde adducts contain a daughter ion at m/z 105, and based on the structural analysis this fragment may be C_7H_5O, which may be a fragment of the benzaldehyde molecule formed during molecular breakage. Similarly, the polyphenol-phenylacetaldehyde adduct daughter ion fragments are all part of the adduct molecule. For example, quercetin, naringenin, and luteolin have similar structures, and the adducts all contain an ionic fragment of m/z 255, which may be $C_5H_{11}O_4$. These results further validate the mechanism of polyphenol inhibition of PhIP generation as the polyphenol traps the carbonyl compounds formed by pyrolysis of phenylalanine to form an adducts, inhibiting its continued participation in the reaction to generate PhIP.

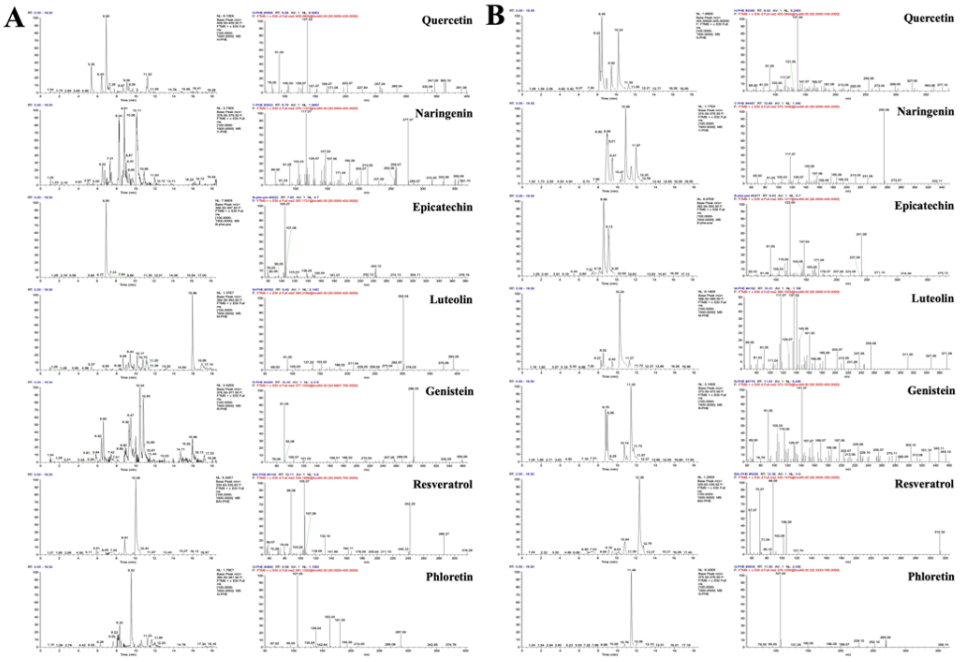


Figure 5-7: Chromatogram and secondary mass spectrometry of different polyphenols with benzaldehyde (A)/ phenylacetaldehyde (B) adducts

Table 5-2. ESI-MS and ESI-MS2 fragment ions of different polyphenols and carbonyl compounds adduction

polyphenols	adducts	RT (min)	MW	MS^1 (*m*/*z*)	MS^2 (*m*/*z*)
Quercetin	Quercetin-benzaldehyde	9.09	408	409$[M+H]^+$	105/137/257
	Quercetin-phenylacetaldehyde	10.28	404	405$[M+H]^+$	103/137/255
Naringenin	Naringenin-benzaldehyde	8.79	378	379$[M+H]^+$	105/117/258
	Naringenin-phenylacetaldehyde	10.88	374	375$[M+H]^+$	145/255
Epicatechin	Epicatechin-benzaldehyde	7.65	396	397$[M+H]^+$	105/138/244
	Epicatechin-phenylacetaldehyde	8.63	392	393$[M+H]^+$	91/123/241
Luteolin	Luteolin-benzaldehyde	9.40	392	393$[M+H]^+$	105/137/153
	Luteolin-phenylacetaldehyde	10.21	388	389$[M+H]^+$	137/161/255
Genistein	Genistein-benzaldehyde	10.45	376	377$[M+H]^+$	105/257/286
	Genistein-phenylacetaldehyde	11.43	372	373$[M+H]^+$	91/115/157
Resveratrol	Resveratrol-benzaldehyde	10.11	334	335$[M+H]^+$	105/132/242
	Resveratrol-phenylacetaldehyde	12.36	330	331$[M+H]^+$	88/106/312
Phloretin	Phloretin-benzaldehyde	9.58	380	381$[M+H]^+$	107/181/287
	Phloretin-phenylacetaldehyde	11.50	376	377$[M+H]^+$	107/269

3.6 Correlation analysis between density functional theory values and inhibition of PhIP for different polyphenols

The correlation between the global molecular parameters of each polyphenol and the experimental results of inhibiting PhIP was analyzed (**Figure 5-8**). It can be clearly seen that EHOMO has a very significant negative correlation with VIP and a very significant positive correlation with nucleophilicity index. ELUMO is extremely significantly positively correlated with chemical potential and HOMO-LUMO gap, extremely significantly negatively correlated with electronegativity and electrophilicity index, and significantly negatively correlated with VEA. In addition, the electrophilicity index is significantly negatively correlated, extremely significantly negatively correlated, significantly positively correlated, and extremely significantly positively correlated with HOMO-LUMO gap, chemical potential, VEA, and electronegativity, respectively. Chemical potential is extremely significantly negatively correlated with VEA and electronegativity. These parameters reflect the ability of molecules to gain or lose electrons and stability and explain the differences in the difficulty of different polyphenols to participate in reactions.

In addition, positive correlations can be seen between the inhibition rate of major carbonyl compounds and PhIP inhibition in the model system. The inhibition of thermal degradation of phenylalanine by polyphenols was highly significantly and negatively correlated with the direct capture of phenylacetaldehyde by polyphenols, and significantly and positively correlated with the free energy of binding of polyphenol-phenylacetaldehyde adducts. These results verify that the mechanism of polyphenol inhibition of PhIP production is closely related to carbonyl compounds: direct capture of carbonyl compounds by polyphenols on the one hand, and influencing the production of benzaldehyde and phenylacetaldehyde from phenylalanine on the other. We can also see a significant negative correlation between the direct trapping effect of phenylacetaldehyde and both the binding energy of polyphenol-phenylacetaldehyde adducts as well as the binding free energy. In addition, the trapping effect showed a highly significant negative correlation with the inhibition rate of phenylalanine thermal degradation by polyphenols, and a positive correlation with the inhibition rate of major carbonyl compounds generated during this process. These results reflect that of the two major carbonyl compounds, benzaldehyde and phenylacetaldehyde, it is phenylacetaldehyde that plays the major role. More importantly, we found a significant positive correlation between the HOMO-LUMO gap of different polyphenols and the rate of inhibition of major carbonyl compounds produced by thermal degradation of phenylalanine by polyphenols, and a highly significant negative correlation with the binding energy of polyphenol-phenylacetaldehyde adducts. This suggests that we may be able to determine the binding effect of polyphenols on phenylacetaldehyde, and thus the magnitude of their inhibitory effect on PhIP, by using the HOMO-LUMO gap of polyphenols.

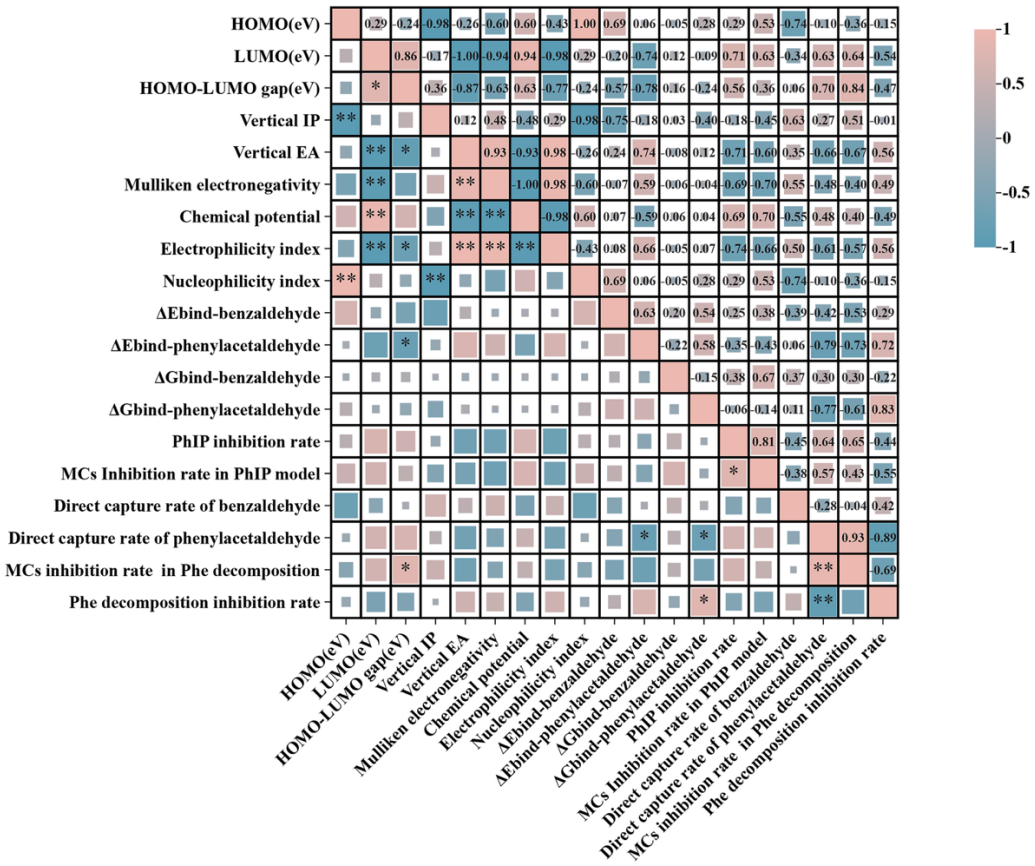


Figure 5-8: Heat map of correlation between inhibition of PhIP by polyphenols and common DFT values. * Indicates a statistically significant correlation; ** indicates a highly significant correlation. MCs represent major carbonyl compounds, and Phe represents phenylalanine.

4.Conclusions

In summary, by studying the effects of different m-hydroxy polyphenols on the contents of precursors and intermediates in the PhIP generation model systems, the carbonyl compound direct capture system and the phenylalanine thermal decomposition reaction system, it was proposed that polyphenols can not only inhibit the formation of PhIP by capturing active carbonyl groups, but also by affecting phenylalanine to produce benzaldehyde and phenylacetaldehyde. The adducts analyzed by UPLC-Q Exactive-MS/MS verified the mechanism. DFT calculations

combined with modeling experiments analyzed that the capture of phenylacetaldehyde by polyphenols is the most critical step to inhibit PhIP generation compared with benzaldehyde. The magnitude of the inhibition of PhIP by polyphenols of known structure can be inferred and compared by calculating their HOMO-LUMO gap. In this study, the molecular structure of polyphenols at the level of theoretical calculations was linked to the inhibitory effects on PhIP in practical experiments, which provided a new theoretical basis for the screening of polyphenols that effectively inhibit PhIP generated during food processing.

5. References

Alaejos, M. S., & Afonso, A. M. (2011). Factors that affect the content of heterocyclic aromatic amines in foods. *Comprehensive Reviews in Food Science and Food Safety, 10* (2), 52–108.

Barzegar, F., Kamankesh, M., & Mohammadi, A. (2019). Heterocyclic aromatic amines in cooked food: A review on formation, health risk-toxicology and their analytical techniques. *Food Chemistry, 280*, 240–254. https://doi.org/10.1016/j.foodchem.2018.12.058

Chedea, V. S., Choueiri, L., Jisaka, M., & Kefalas, P. (2012). o-Quinone involvement in the prooxidant tendency of a mixture of quercetin and caffeic acid. *Food Chemistry, 135*(3), 1999–2004.

Cheng, K. W., Wong, C. C., Cho, C. K., Chu, I. K., Sze, K. H., Lo, C., Chen, F., & Wang, M. (2008). Trapping of phenylacetaldehyde as a key mechanism responsible for naringenin's inhibitory activity in mutagenic 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine formation. *Chemical Research in Toxicology, 21*(10), 2026–2034.

Chu, F. L., & Yaylayan, V. A. (2008). Model studies on the oxygen-induced formation of benzaldehyde from phenylacetaldehyde using pyrolysis GC-MS and FTIR. *Journal of Agricultural and Food Chemistry, 56*(22), 10697–10704.

Deng, P., Xue, C., He, Z., Wang, Z., Qin, F., Oz, E., Chen, J., El Sheikha, A. F., Proestos, C., Oz, F., & Zeng, M. (2022). Synergistic Inhibitory Effects of Selected Amino Acids on the Formation of 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in both Benzaldehyde- and Phenylacetaldehyde-Creatinine Model Systems. *Journal of Agricultural and Food Chemistry, 70*(35), 10858–10871.

Dong, H., Chen, Q., Xu, Y., Li, C., Bai, W., Zeng, X., Wu, Q., Xu, H., & Deng, J. (2024). Effect and mechanism of polyphenols containing m-dihydroxyl structure on 2-amino-1-methyl-6-phenylimidazole [4, 5-b] pyridine (PhIP) formation in chemical models and roast pork patties. *Food Chemistry: X, 23*, 101672.

Frisch M. J., Pople J. A., Binkley J. S. (1984). Self-consistent molecular orbital methods 25. Supplementary functions for Gaussian basis sets, *Journal of Chemical Physics, 80* (7): 3265–3269.

Frisch, M. J., Trucks, G. W., Schlegel, H. B., Scuseria, G. E., Robb, M. A., Cheeseman,

J. R., ..., & Nakatsuji, H. (2009). GAUSSIAN09 (Revision A.01.). Gaussian. Inc.

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

Guerra, P. V., & Yaylayan, V. A. (2014). Interaction of flavanols with amino acids: postoxidative reactivity of the B-ring of catechin with glycine. *Journal of Agricultural and Food Chemistry, 62*(17), 3831–3836.

Hidalgo, F. J., León, M. M., & Zamora, R. (2016). Amino acid decarboxylations produced by lipid-derived reactive carbonyls in amino acid mixtures. *Food Chemistry, 209*, 256–261.

Hidalgo, F. J., & Zamora, R. (2019). Formation of phenylacetic acid and benzaldehyde by degradation of phenylalanine in the presence of lipid hydroperoxides: New routes in the amino acid degradation pathways initiated by lipid oxidation products. *Food Chemistry: X, 2,* 100037.

Hui, T., Li, Y., Chen, R., Yang, X., Liu, H., Wang, Z., & Zhang, D. (2021). Formation and Prediction of PhIP, Harman, and Norharman in Chemical Model Systems Containing Epicatechin under Various Reaction Conditions. *Journal of Agricultural and Food Chemistry, 69*(49), 14975–14984.

International Agency for Research on Cancer (IARC). (1993). Some naturally occurring substance, food items and constituents, heterocyclic aromatic amines and mycotoxins. In IARC Monographs on the evaluation of carcinogenic risks to humans (Vol. 56, pp. 165–244). Lyon, France: WHO Press.

Jiang, L., Li, Y., Xue, C., He, Z., Wang, Z., Qin, F., Chen, J., & Zeng, M. (2022). The inhibitory effects of yellow mustard (Brassica juncea) and its characteristic pungent ingredient allyl isothiocyanate (AITC) on PhIP formation: Focused on the inhibitory pathways of AITC. *Food Chemistry, 373*(Pt A), 131398.

Lu T. (2024). A comprehensive electron wavefunction analysis toolbox for chemists, Multiwfn. *The Journal of Chemical Physics, 161*(8), 082503.

Lu, T., & Chen, F. (2012). Multiwfn: a multifunctional wavefunction analyzer. *Journal of Computational Chemistry, 33*(5), 580–592.

Lu, T., Chen, Q., 2022. Realization of conceptual density functional theory and information-Theoretic approach in multiwfn program, Conceptual Density Functional Theory: Towards a New Chemical Reactivity Theory. pp. 631–647.

Lu, T., & Chen, Q. (2021). Shermo: A general code for calculating molecular thermochemistry properties. *Computational and Theoretical Chemistry, 1200*.

Monforte, A. R., Martins, S. I. F. S., & Silva Ferreira, A. C. (2020). Impact of Phenolic Compounds in Strecker Aldehyde Formation in Wine Model Systems: Target and Untargeted Analysis. *Journal of Agricultural and Food Chemistry, 68*(38), 10281–10286.

Murkovic, M., Weber, H. J., Geiszler, S., Fröhlich, K., & Pfannhauser, W. (1999).

Formation of the food associated carcinogen 2- amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in model systems. *Food Chemistry, 65*, 233–237.

National Toxicology Program (NTP). (2004). NTP 11th report on carcinogens. *Report on Carcinogens: Carcinogen Profiles, 11*, 1–A32.

Oz, E., Aoudeh, E., Murkovic, M., Toldra, F., Gomez-Zavaglia, A., Brennan, C., Proestos, C., Zeng, M., & Oz, F. (2023). Heterocyclic aromatic amines in meat: Formation mechanisms, toxicological implications, occurrence, risk evaluation, and analytical methods. *Meat Science, 205*, 109312.

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

Rajan, V. K., & Muraleedharan, K. (2017). A computational investigation on the structure, global parameters and antioxidant capacity of a polyphenol, Gallic acid. *Food Chemistry, 220*, 93–99.

Salazar, R., Arámbula-Villa, G., Hidalgo, F. J., & Zamora, R. (2014). Structural characteristics that determine the inhibitory role of phenolic compounds on 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) formation. *Food Chemistry, 151*, 480–486.

Shang, Y. J., Qian, Y. P., Liu, X. D., Dai, F., Shang, X. L., Jia, W. Q., Liu, Q., Fang, J. G., & Zhou, B. (2009). Radical-scavenging activity and mechanism of resveratrol-oriented analogues: influence of the solvent, radical, and substitution. *The Journal of Organic Chemistry, 74*(14), 5025–5031.

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

Wang, Q., Cheng, W., Zhang, Y., Kang, Q., Gowd, V., Ren, Y., Chen, F., & Cheng, K. W. (2021). A novel potent inhibitor of 2-amino-1-methyl-6-phenylimidazo[4,5-b] pyridine (PhIP) formation from Chinese chive: Identification, inhibitory effect and action mechanism. *Food Chemistry, 345*, 128753.

Yang, X., Blecker, C., Liu, H., Zhang, D., Wang, Z. (2023). Effect of plant polyphenols with different m-hydroxy and o-hydroxy groups on the inhibition of heterocyclic amines formation in roasted meat. *Food Control, 153*, 109960.

Zhao, Y., Yang, H., Zhang, N., Zhou, Q., Fan, D., & Wang, M. (2021). Effects of the Deacetylation Degree of Chitosan on 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) Formation in Chemical Models and Beef Patties. *Journal of Agricultural and Food Chemistry, 69*(46), 13933–13941.

Zhou, Y., Ma, Y., Ma, Z., Ma, Q., Li, Z., & Wang, S. (2024). Theoretical exploration of the phenolic compounds' inhibition mechanism of heterocyclic aromatic amines in roasted beef patties by density functional theory. *Food Research International (Ottawa, Ont.), 186*, 114394.

Zöchling, S., & Murkovic, M. (2002) Formation of the heterocyclic aromatic amine

PhIP: Identification of precursors and intermediates. *Food Chemistry, 79*, 125–134.

Zeng, M., Li, Y., He, Z., Qin, F., & Chen, J. (2016). Effect of phenolic compounds from spices consumed in China on heterocyclic amine profiles in roast beef patties by UPLC-MS/MS and multivariate analysis. *Meat Science, 116*, 50–57.

6

Chapter VI General discussion, Conclusion and Perspectives

1. General discussion

Roasted meat is loved by people all over the world for its unique flavor. The high temperatures required for roasting, in addition to producing pleasant odors, special flavors and textures, are accompanied by the production of hazardous substances, such as heterocyclic amines (HAs) (Barzegar, Kamankesh, & Mohammadi, 2019). HAs are mainly produced by the precursors (amino acids/proteins, creatinine, and glucose) through a series of reactions under high temperature conditions. At present, more than 30 kinds of HAs have been identified and some of them have been listed as possible carcinogens because of their mutagenic and teratogenic effects on humans (Cheng et al., 2006). Therefore, it is of great significance to explore the inhibition strategy of HAs to ensure the safety and health of roasted meat products. Among the many HAs inhibition strategies, the addition of antioxidants, especially plant polyphenols, is very effective and convenient (Santhakumar, Battino， & Alvarez-Suarez, 2018). The antioxidant activity and inhibition of HAs formation of polyphenols are closely related to the number and location of hydroxyl structures (Hidalgo, Aguilar， & Zamora, 2017). Current studies have focused on the inhibitory effects of flavonoids on HAs in roasted meat (Vitaglione， & Fogliano, 2004), whereas there is a wide variety of polyphenolic compounds, and the patterns and mechanisms by which different types of polyphenols inhibit different types of HAs are still unclear. In addition, protein can not be ignored in meat and in the production of HAs (Chen et al., 2021), the mechanism of HAs production by polyphenols on muscle proteins involved also remains unclear. Therefore, in-depth investigation of the inhibition pattern and mechanism of plant polyphenols on HAs in roasted meat can not only make it more convenient to screen out polyphenol compounds that are more effective in inhibiting HAs, but also provide a theoretical basis for the development of the control technology of HAs in the processing of meat products.

This research aims to discuss the inhibition mechanism of plant polyphenols with different structures on different HAs, focusing on three aspects of roasted meat, protein and polyphenol molecular characteristics to explore the inhibition pattern and analyze the mechanism. We employed simple models with a single polyphenol in order to clearly dissect the individual inhibitory pathways, establish structure–activity relationships, and avoid confounding interactions that might arise in more complex systems. First, the reactions between classified polyphenols (cover flavonoids, phenolic acids, stilbenes, coumarins, chalcones, and with different hydroxyl groups) and roasted lamb patties were studied. The types and concentrations of HAs in the roasted lamb patties after polyphenol addition were measured. Based on the differences in antioxidant activity among polyphenols, the inhibitory patterns of different polyphenol structures on major HAs were summarized and further validated in an in vitro model system. Next, resveratrol, which exhibited strong inhibitory effects on HAs formation in grilled meat, was selected as an inhibitor. Using a model system of muscle protein participation in HAs production and a direct reaction system between muscle proteins and HAs, the mechanism by which resveratrol inhibits

muscle protein involvement in HAs formation was investigated. Furthermore, protein structural changes were analyzed to determine the underlying factors influencing this inhibition. Finally, based on the differences in PhIP inhibition among various polyphenols, the molecular characteristics of different polyphenols and the energy of their adducts with carbonyl compounds were analyzed to explore the potential mechanisms underlying the variations in their inhibitory effects on PhIP. These data can systematically reveal the inhibition patterns and mechanisms of different structural polyphenols on the generation of HAs in roasted meat, and provide a reference for the screening of potent inhibitors of HAs.

1.1 Patterns and mechanisms of HAs inhibition by polyphenolic structures

Existing studies have shown that polyphenols inhibit the formation of HAs by scavenging and trapping free radicals and active carbonyl groups involved in the formation of HAs (Dong et al., 2024b). Polyphenols with two or three hydroxyl structures on the ortho site of the B-ring (on the 3,4 position) are the most effective radical scavengers (Pannala et al., 2001), as the presence of a catechol group at the B-ring can form a fairly stable ortho-semiquinone radical, which facilitates electron delocalization and thereby enhances free radical scavenging capacity (Zamora & Hidalgo, 2016). In contrast, phenolic compounds with two hydroxyl structures on the meta site of the aromatic nucleus had a high carbonyl scavenging ability (Salazar, Arámbula-Villa, Hidalgo, & Zamora, 2014), since the meta-dihydroxyl configuration generates high electron density carbons, which are essential for efficient nucleophilic addition to reactive carbonyl compounds (Zamora & Hidalgo, 2016). The inhibition of HAs varied widely among different polyphenols, not only because the structures of these compounds affect their inhibitory capacity, but also due to differences in the predominant formation pathways of individual HAs. In roasted lamb patties, the addition of nine types of polyphenols showed different inhibitory effects on the content of HAs (**Table 3-1**), with phenolic acids being the least effective, followed by flavonoids, chalcone, and coumarin, while stilbenes—represented by resveratrol—exhibited the strongest inhibition. The order of inhibition of HAs by several flavonoids was: isoflavones > flavonols > flavanones > flavanols > flavones, which might be related to the structural differences of flavonoids. Flavonoids with substituents at the 3′ position on the C-ring had stronger antioxidant activity and were affected by the double bond between the 2′ and 3′ positions (Cai et al., 2006).

To clarify the relationship between the antioxidant activity of polyphenols and the inhibitory effect of HAs, the inhibitory effects of polyphenols on DPPH and ABTS were determined before and after heating (**Figure 3-2**), and the correlation between the scavenging activity of ABTS or DPPH and the inhibition rate of HAs was analyzed (**Figure 3-3**). The results showed that the free radical scavenging activity of polyphenols with o-hydroxyl structure was significantly stronger than that of polyphenols containing m-hydroxyl structure, which verified that the o-hydroxyl structure was an effective structure for scavenging free radicals (Lucić, Amić, & Trinajstić, 2008). In addition, there was no correlation between the ABTS or DPPH

scavenging rate and the inhibition rate of HAs for polyphenols containing o-hydroxyl structures, in contrast to the positive correlation between the free radical scavenging rate and the inhibition rate of HAs for polyphenols containing only m-hydroxyl structures, suggesting that scavenging free radicals may not be the main pathway for polyphenols to inhibit the generation of HAs. The cluster analysis of the inhibition results of polyphenols on main HAs (Harman, Norharman, PhIP and MeIQx) in roasted meat clarified the inhibition rules of different polyphenols on different kinds of HAs (**Figure 3-4**). Polyphenols containing m-hydroxyl groups showed similar inhibition of Harman, Norharman, and PhIP, suggesting that for these three HAs, capture-active carbonyls are the major inhibitory pathway. The opposite effect was observed for MeIQx inhibition by polyphenols; polyphenol compounds containing o-hydroxyl structures inhibited MeIQx well, suggesting that polyphenols inhibit MeIQx mainly through the scavenging radical pathway. The model systems generating different HAs verified this law by adding the corresponding carbonyl compounds or free radicals as controls.

Among numerous HAs, PhIP has received extensive attention due to its toxicity and richest content (Zhao et al., 2021). Although polyphenols with meta-hydroxyl groups have been shown to inhibit the formation of PhIP by trapping reactive carbonyl compounds, significant differences in their inhibitory effects were still observed among various polyphenols that all possess this structural feature. This suggests that the molecular characteristics of polyphenols may influence their ability to inhibit PhIP formation, warranting further investigation. Therefore, gallic acid and aesculetin, which contain only ortho-hydroxyl groups, were excluded from further analysis. The investigation then focused on the remaining seven polyphenols to examine the relationship between their molecular characteristics and their inhibitory effects on PhIP formation. DFT was employed to calculate the electronic properties and binding energies of each polyphenol. These computational results, combined with experimental data, were used to explore how the molecular characteristics of polyphenols influence their ability to capture key carbonyl intermediates and inhibit PhIP formation.

The effects of different structural polyphenols on PhIP inhibition in the creatinine/phenylalanine/glucose model system were basically the same as in meat, and the inhibitory effects of polyphenols on benzaldehyde and phenylacetaldehyde were also basically the same as those on PhIP inhibition in the model system, which further demonstrated that polyphenols inhibited PhIP generation through trapping of the active carbonyl compounds. However, when polyphenols were used to react directly with benzaldehyde or phenylacetaldehyde, their inhibitory effects were found to be inconsistent with those presented in the model system. Based on the mechanism of PhIP generation, benzaldehyde and phenylacetaldehyde are mainly produced through the thermal degradation reaction of phenylalanine (Chu, & Yaylayan, 2008; Hidalgo, & Zamora, 2019), so it was hypothesized and verified that polyphenols inhibit this process as well. The content of phenylalanine gradually decreased during different time periods of heating, while the content of the reaction products benzaldehyde and phenylacetaldehyde gradually increased. The addition of some

polyphenols significantly inhibited the formation of these two carbonyl compounds, but also the addition of some polyphenols led to an increase in their contents instead. This may be due to the fact that these polyphenols, despite their ability to bind directly to the carbonyl compounds, have a weak reactivity and fail to effectively capture the intermediate products in the reaction system, but instead promote the positive advancement of the reaction, resulting in the generation of more carbonyl compounds. The present study further refined the mechanism of action of polyphenols in inhibiting PhIP generation. On the one hand, polyphenols can exert competitive inhibition by directly forming adducts with carbonyl compounds, thus blocking their participation in subsequent heterocyclic amine generation reactions. On the other hand, polyphenols can also intervene in the pyrolysis process of phenylalanine, affecting the generation of carbonyl compounds during the reaction and reducing the accumulation of the key intermediates of PhIP from the source.

Further, using the quantum chemical parameters of polyphenols and their binding energies with carbonyl compound adducts obtained from density-functional theory calculations, combined with the experimental results, we explored the potential reasons for the differences in inhibition effects of polyphenols, which all contain m-hydroxyl structures, in inhibiting the generation of PhIP. The carbonyl carbon of benzaldehyde/phenylacetaldehyde undergoes nucleophilic substitution reactions with the hydrogen atom between the two hydroxyl groups of the polyphenols to form adducts (Dong et al., 2024a), and these adducts were identified in the reaction between the polyphenols and phenylalanine. More importantly, the correlation between the calculated values of the DFT of the polyphenol electronic properties, the binding energies of the adducts of polyphenols and carbonyl compounds, and the results of the experiments on the inhibition of PhIP by polyphenols and the trapping of reactive carbonyls were analyzed. The relationships between the calculated DFT values of the polyphenol electronic properties all reflect the ability and stability of the polyphenols to gain or lose electrons, which may be the main reason for the differences in PhIP inhibition by different polyphenols. Analysis of the relationship between the adduct binding energy and the ability to inhibit PhIP or trap carbonyl compounds shows that the trapping of phenylacetaldehyde by polyphenols is the most critical step in the inhibition of PhIP compared to benzaldehyde. The HOMO-LUMO gap of polyphenols was significantly and positively correlated with their inhibition of the generation of major carbonyl compounds during phenylalanine pyrolysis, while negatively correlating with the binding energy of polyphenol-phenylacetaldehyde adducts. This result suggests that the HOMO-LUMO gap can be used as a potential predictor of the binding capacity of polyphenols to phenylacetaldehyde, which in turn can be used to assess its inhibitory effect on PhIP generation.

1.2 The multiple inhibitory effect of resveratrol in the formation process of heterocyclic amines

Resveratrol exhibits multiple pathways to inhibit the formation of HAs. In addition to scavenging free radicals and trapping reactive carbonyl compounds to suppress the generation of HAs from free precursors such as amino acids, creatinine, and glucose,

resveratrol also plays a crucial role in regulating protein-involved HAs formation (Xue et al., 2022a). Meat proteins, mainly myofibrillar proteins (55–60%) and sarcoplasmic proteins (30–35%), can either serve as precursors or bind to HAs (Nawaz et al., 2022; Deng et al., 2024; Kataoka, Miyake, Saito, & Mitani, 2012). These HAs bound to proteins will be free after gastrointestinal digestion, and still have great harm to the human body (Xue et al., 2022b).

Our results demonstrate distinct inhibitory behaviors of resveratrol in MP- and SP-associated reactions. In MP systems, resveratrol suppressed the formation of both free and protein-bound HAs, primarily by interfering with MP's role as direct precursors (**Figure 4-1**). Structural analyses revealed that resveratrol increased α-helix and β-turn contents, altered tertiary conformation, and reduced the exposure of aromatic residues such as tryptophan and phenylalanine (Gao et al., 2005). These residues are known HAs precursors (Arvidsson et al., 1997), suggesting that conformational shielding by resveratrol directly reduces precursor availability. In addition, static fluorescence quenching and molecular simulations indicated hydrophobic interactions between resveratrol and MP, which not only modified the protein microenvironment but also promoted the exposure of non-precursor amino acids (e.g., Ala, Lys, Pro) that can inhibit HAs formation (Deng et al., 2022). Thus, the inhibition of MP-related HAs appears to arise from a dual effect: shielding of precursor amino acids and facilitation of inhibitory residue exposure.

In SP systems, resveratrol showed a different inhibitory mode. It mainly reduced free HAs levels while exerting limited effects on SP-bound HAs (**Figure 4-3**). Interestingly, resveratrol promoted the generation of free amino acids such as asparagine, alanine, proline, tryptophan, and glycine, thereby altering the substrate pool available for HAs reactions. Moreover, structural changes induced by resveratrol—such as increased β-sheet and α-helix contents and a higher UV absorption peak—suggested enhanced protein aggregation and HAs-binding capacity. Measurements of particle size, ζ-potential, and surface hydrophobicity further supported this, showing that resveratrol amplified the physicochemical changes caused by HAs binding. Similar effects have been reported for mulberry polyphenols on SP (Cheng et al., 2020), reinforcing the idea that polyphenols can promote HAs sequestration by inducing protein conformational shifts. Therefore, the inhibition of SP-associated HAs formation by resveratrol is mainly achieved through direct pathways such as free radical scavenging and reactive carbonyl trapping to suppress free HAs generation, while simultaneously promoting SP–HAs binding, thereby reducing the potential harm of free HAs.

Taken together, the inhibitory action of resveratrol cannot be attributed to a single mechanism but rather to the synergistic interplay of multiple pathways. Carbonyl trapping limits the initial generation of reactive intermediates, free radical scavenging reduces propagation reactions, and protein structure modulation further decreases the availability of precursors and promotes HAs binding. This integration of chemical reactivity and protein conformational regulation may explain why resveratrol displayed the strongest suppression of HAs among tested polyphenols in roasted meat systems. Such multi-pathway inhibition underscores the importance of considering

both small-molecule interactions and macromolecular environments when elucidating the mechanisms of dietary polyphenols. The combined effects of carbonyl trapping, free radical scavenging, and protein structure modulation work together to effectively suppress HAs formation during the processing and cooking of meat products.

1.3 Industrial application considerations

Beyond mechanistic insights, the practical implementation of polyphenols in the meat industry warrants further attention. Based on existing studies on the inhibitory mechanisms of polyphenols—particularly the role of hydroxyl groups and molecular structure in suppressing HAs formation—future research can further extend to predicting the inhibition potential of unknown polyphenols through structural analysis or comparative evaluation. In this way, a polyphenol screening and evaluation system can be developed to rapidly identify compounds with higher inhibition efficiencies, providing a scientific foundation for targeted and efficient industrial utilization.

In terms of application strategies, technologies such as loading, embedding, and slow-release can be employed to prepare marinades, surface sprays, or coating agents containing polyphenols, thereby enabling their incorporation into meat products and other protein-rich foods. Such approaches not only ensure better distribution and controlled release during processing, but also provide a feasible path to realize the industrial application of polyphenols for improving the safety of thermally processed meat products. From an industrial perspective, cost and availability are crucial; instead of relying solely on purified polyphenols, which may be expensive and difficult to scale up, plant extracts naturally rich in polyphenols—such as grape seed extract, tea polyphenols, or rosemary extract—represent more practical and cost-effective options. These extracts can provide synergistic effects due to their diverse phenolic compositions and may be more suitable for large-scale food processing applications.

At present, no specific regulatory limits for HAs in industrial meat products have been established. This is mainly because HAs are process-induced contaminants whose levels depend strongly on meat type, cooking temperature, and duration, making it difficult to define universal thresholds. A further obstacle is the lack of consistent epidemiological evidence, as dietary HAs exposure is difficult to assess accurately (Iwasaki, & Tsugane, 2021). Although food frequency questionnaire (FFQ) is commonly applied, they only provide approximate estimates. Biomarkers such as PhIP levels in human hair, or DNA, hemoglobin, and albumin adducts, show promise but are still rarely used in large-scale studies (Tang et al., 2013). The absence of standardized exposure assessment hampers risk evaluation and prevents regulatory authorities from establishing clear guidelines. For the industry, practical mitigation strategies—such as optimizing processing parameters or incorporating natural inhibitors like polyphenols—are currently more feasible than enforceable limits, but future regulations may evolve toward guideline values once more comprehensive risk assessment data become available.

2. Conclusions

This study conducts a systematic investigation on the molecular mechanism by which plant polyphenols inhibit the formation of HAs in thermally processed meat products, aiming to reveal the action patterns of polyphenols with different structures in inhibiting different HAs and their influence mechanisms at the molecular level. By adding structurally diverse polyphenols to roasted lamb, the differences in the inhibition of HAs by different types of polyphenols were clarified, and the inhibition effects were in the following order: isoflavones > flavonols > flavanones > flavanols > flavones. Combining the antioxidant capacity and polyphenol structural information, the inhibition of different HAs generation by different structural polyphenols was summarized. The polyphenols containing meso-hydroxyl groups mainly inhibited the generation of PhIP, Harman and Norharman by capturing the reaction intermediate carbonyl compounds. The polyphenols with o-hydroxyl groups mainly inhibited the generation of MeIQx by scavenging free radicals.

Furthermore, by constructing a model system for protein participation in HAs formation, the influence mechanism of polyphenols represented by resveratrol on the participation of MP and SP in the HAs formation process was explored. It was found that it could effectively inhibit the formation of bound and free HAs by changing the protein conformation and binding ability. Polyphenols can interact with myofibrillar proteins to inhibit the direct involvement of alternative amino acid residues in HAs formation by reducing the exposure of key precursor amino acids while promoting the exposure of non-precursor amino acids that exert inhibitory effects. In addition, although polyphenols increased the content of free amino acids in SP, they effectively inhibited the reaction process of HAs generation between free amino acids, creatinine and glucose by scavenging free radicals and trapping reactive carbonyl groups. At the same time, polyphenols also promoted the binding of the generated HAs to SP, thus further reducing the actual content of HAs in the system. Correlation and intermolecular force results indicated that free HAs formed bound HAs by changing the surface hydrophobicity of SP solution, and polyphenols could affect the formation of SP-bound HAs by affecting ions and disulfide bonds.

The experimental results of polyphenol inhibition of PhIP and capture of major carbonyl compounds in the model reaction combined with DFT analysis further explain the variability of the inhibitory effect of polyphenols containing meso-hydroxylated structures on PhIP from a quantum chemical point of view. By constructing a phenylalanine/creatinine/glucose model system, a direct reaction system of polyphenols with benzaldehyde/phenylacetaldehyde, and a thermal degradation system of phenylalanine, the present study systematically evaluated the inhibitory effect of polyphenols on PhIP generation. The results showed that the inhibitory effect of polyphenols on PhIP not only relies on their ability to directly capture reactive carbonyl compounds such as benzaldehyde and phenylacetaldehyde, but also is closely related to their potential role in modulating the generation of carbonyl compounds during the thermal degradation of phenylalanine. In addition, DFT calculations combined with modeling experiments analyzed that the capture of

phenylacetaldehyde by polyphenols is the most critical step in the inhibition of PhIP generation. The degree of inhibition of PhIP by polyphenols with known structures can be inferred and compared by calculating their HOMO-LUMO gaps.

This study integrates chemical modeling, spectroscopic analysis, computational simulations, multivariate statistical analysis, and chemical structure identification to systematically investigate the molecular mechanisms by which polyphenols inhibit the formation of HAs. Each method offers complementary strengths: computational simulations help predict reaction behaviors, spectroscopy provides detailed molecular-level insights, chemical modeling validates theoretical assumptions, multivariate analysis uncovers hidden data rules, and structural identification confirms the chemical structures of key intermediates and target compounds. The combined use of these methodologies enhances the comprehensiveness and reliability of the findings and offers valuable support for developing future quality control and health risk prevention strategies in the meat industry. The present study revealed the structure-dependent inhibitory mechanisms of polyphenols with different structures on both free and protein-bound HAs, providing a theoretical basis for their application as natural inhibitors during meat processing and cooking. The findings may guide the development of natural extracts rich in specific polyphenols for use in functional marinades, coatings, or dietary supplements, as well as for predicting the inhibitory potential of unknown polyphenolic compounds. The research results are of great significance to enhance the food safety of processed meat products and provide a reference for the functionalization of polyphenols in the food industry.

3.Perspectives

The food safety of thermally processed meat products, as an important source of protein in the human diet, is of increasing concern. HAs are widely present in such foods and have strong mutagenicity and potential carcinogenic risk. In view of the potential threat to public health, it is of great scientific value and practical significance to investigate the production pathways of HAs from the molecular mechanism level and develop effective inhibition strategies to control their production at the source during processing to enhance the safety of thermally processed meat products. This study clarified the inhibition rules and mechanisms of polyphenols on some HAs, providing a theoretical basis for the research and development of safer meat products. Future research should address the following critical aspects to further advance this field.

Firstly, this study systematically summarized the main inhibition laws of different types of polyphenols on a variety of HAs, focusing on the relationship between the structural properties of polyphenol molecules and their inhibition of PhIP generation. However, there are many types of HAs, such as Harman, Norharman, and MeIQx, which are also the main representatives of thermally processed foods. The inhibition mechanisms, kinetic characteristics and structure-activity relationships of these HAs by polyphenols with different structures need to be further investigated to reveal the role of polyphenols in the regulation of HAs generation. Future studies can further

combine the molecular level information of polyphenols, such as electronic properties, spatial configuration and reactivity, with the combination of theoretical simulation and experimental validation, to further explore the reaction pathways between polyphenols and various key intermediates or precursors involved in the generation of other HAs.

Secondly, although bound HAs have been shown to exist in large quantities in thermally processed foods and the mechanism of their bound state formation has been initially elucidated, systematic studies on the toxicity of bound HAs are lacking. It has been shown that bound HAs can be released to the free state during gastrointestinal digestion or in the presence of polyphenols. Therefore, the effects of polyphenols on the toxicity of bound HAs and their potential regulatory mechanisms, as well as the toxicity of adducts of polyphenols and proteins or HAs intermediates remain to be further investigated in depth and experimentally confirmed. Future studies should combine in vitro digestion simulation system and animal experiments to systematically evaluate the toxicity characteristics of bound HAs. At the same time, the mechanisms of polyphenols regulating the release and toxicity of bound HAs in vivo should be further explored, with a view to providing theoretical support and practical basis for polyphenolic dietary intervention and safety control of processed meat products.

Thirdly, although polyphenols are widely recognized as effective natural additives to inhibit the generation of HAs, they have certain limitations, especially the poor water solubility of some polyphenols, which to a certain extent restricts their scope of application and actual inhibition effect in food processing. To address this problem, studies have attempted to improve the physicochemical properties of polyphenols through methods such as structural modification or carrier introduction. Therefore, future studies could further explore the composite strategy of polyphenols with other active materials (e.g. polysaccharides, biomolecules, etc.) to enhance their solubility, stability and functional performance in food systems, so as to more effectively fulfill their roles in HAs inhibition.

Finally, future research should move beyond the study of individual polyphenols and explore the combined application of multiple compounds. Different classes of polyphenols may inhibit HAs through distinct pathways, and their mixtures could provide more effective and comprehensive inhibition. It is also important to clarify whether such effects are additive, synergistic, or competitive, particularly under real meat processing conditions. In addition, the impact of polyphenols—alone or in combination—on meat quality attributes such as color, texture, flavor, and nutrition should be systematically assessed, so as to balance safety improvement with consumer acceptance and industrial feasibility. Building on this, research could follow a stepwise approach: from investigating single compounds, to studying mixtures of structurally diverse polyphenols, and ultimately to applying natural extracts that contain a broad spectrum of active components.

Overall, polyphenols, as natural, safe and versatile ingredients, have a broad potential for application as concerns about the safety of processed meat products

increase. Based on the existing research results on the molecular mechanism of plant polyphenols in inhibiting the formation of HAs in roasted meat, future studies can focus on the in-depth analysis of the inhibition mechanism, the systematic evaluation of the toxicity of bound HAs, and the application of polyphenols in the food processing process, to provide a more solid theoretical foundation and technological support for the enhancement of the safety and nutritional quality of high-protein foods.

4. References

Arvidsson, P., Boekel, M. A. J. S., Skog, K., & Jagerstad, M. (1997). Kinetics of formation of polar heterocyclic amines in a meat model system. *Journal of Food Science, 62*(5), 911–916.

Barzegar, F., Kamankesh, M., & Mohammadi, A. (2019). Heterocyclic aromatic amines in cooked food: A review on formation, health risk-toxicology and their analytical techniques. *Food Chemistry, 280*, 240–254.

Cai, Y. Z., Mei Sun, Jie Xing, Luo, Q., & Corke, H. (2006). Structure-radical scavenging activity relationships of phenolic compounds from traditional Chinese medicinal plants. *Life Sciences, 78*(25), 2872–2888.

Chen, Q., Xue, C., He, Z., Wang, Z., Qin, F., Chen, J., & Zeng, M. (2021). Generation of Sarcoplasmic and Myofibrillar Protein-Bound Heterocyclic Amines in Chemical Model Systems under Different Heating Temperatures and Durations. *Journal of Agricultural and Food Chemistry, 69*(10), 3232–3246.

Cheng, J., Xu, L., Xiang, R., Liu, X., & Zhu, M. (2020). Effects of mulberry polyphenols on oxidation stability of sarcoplasmic and myofibrillar proteins in dried minced pork slices during processing and storage. *Meat science, 160*, 107973.

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

Chu, F. L., & Yaylayan, V. A. (2008). Model studies on the oxygen-induced formation of benzaldehyde from phenylacetaldehyde using pyrolysis GC-MS and FTIR. *Journal of Agricultural and Food Chemistry, 56*(22), 10697–10704.

Ding, X., Zhang, D., Liu, H., Wang, Z., & Hui, T. (2022). Chlorogenic acid and Epicatechin: An efficient inhibitor of heterocyclic amines in charcoal roasted lamb meats. *Food Chemistry, 368*, 130865.

Deng, P., Xue, C., He, Z., Wang, Z., Qin, F., Oz, E., Chen, J., El Sheikha, A. F., Proestos, C., Oz, F., & Zeng, M. (2022). Synergistic Inhibitory Effects of Selected Amino Acids on the Formation of 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in both Benzaldehyde- and Phenylacetaldehyde-Creatinine Model Systems. *Journal of Agricultural and Food Chemistry, 70*(35), 10858–10871.

Deng, P., Wei, T., Yu, M., Yang, T., Chen, Q., Wang, Z., He, Z., Chen, J., & Zeng, M. (2024). Investigation on synergistic inhibition of protein-bound heterocyclic amines in sarcoplasmic and myofibrillar model systems by amino acid combinations. *Food Chemistry, 460*(Pt 3), 140576.

Dong, H., Chen, Q., Xu, Y., Li, C., Bai, W., Zeng, X., Wu, Q., Xu, H., & Deng, J. (2024a). Effect and mechanism of polyphenols containing m-dihydroxyl structure on 2-amino-1-methyl-6-phenylimidazole [4, 5-b] pyridine (PhIP) formation in chemical models and roast pork patties. *Food Chemistry: X, 23*, 101672.

Dong, H., Ye, H., Bai, W., Zeng, X., & Wu, Q. (2024b). A comprehensive review of structure-activity relationships and effect mechanisms of polyphenols on heterocyclic aromatic amines formation in thermal-processed food. *Comprehensive Reviews in Food Science and Food Safety, 23*(6), e70032.

Gao, D., Tian, Y., Bi, S., Chen, Y., Yu, A., & Zhang, H. (2005). Studies on the interaction of colloidal gold and serum albumins by spectral methods. Spectrochimica acta. *Part A, Molecular and biomolecular spectroscopy, 62*(4-5), 1203–1208.

Hidalgo, F. J., Aguilar, I., & Zamora, R. (2017). Model studies on the effect of aldehyde structure on their selective trapping by phenolic compounds. *Journal of Agricultural and Food Chemistry, 65*(23), 4736–4743.

Hidalgo, F. J., & Zamora, R. (2019). Formation of phenylacetic acid and benzaldehyde by degradation of phenylalanine in the presence of lipid hydroperoxides: New routes in the amino acid degradation pathways initiated by lipid oxidation products. *Food Chemistry: X, 2*, 100037.

Iwasaki, M., & Tsugane, S. (2021). Dietary heterocyclic aromatic amine intake and cancer risk: epidemiological evidence from Japanese studies. *Genes and Environment: the official journal of the Japanese Environmental Mutagen Society*, 43(1), 33.

Kataoka, H., Miyake, M., Nishioka, S., Matsumoto, T., Saito, K., & Mitani, K. (2010). Formation of protein adducts of 2-amino-1-methyl-6-phenylimidazo 4,5-b pyridine in cooked foods. *Molecular Nutrition & Food Research, 54*(7), 1039–1048.

Lucić, B., Amić, D., & Trinajstić, N. (2008). Antioxidant QSAR modeling as exemplified on polyphenols. *Methods in Molecular Biology (Clifton, N.J.), 477*, 207–218.

Nawaz, A., Irshad, S., Ali Khan, I., Khalifa, I., Walayat, N., Muhammad Aadil, R., Kumar, M., Wang, M., Chen, F., Cheng, K. W., & Lorenzo, J. M. (2022). Protein oxidation in muscle-based products: Effects on physicochemical properties, quality concerns, and challenges to food industry. *Food Research International (Ottawa, Ont.), 157*, 111322.

Pannala, A. S., Chan, T. S., O'Brien, P. J., & Rice-Evans, C. A. (2001). Flavonoid B-ring chemistry and antioxidant activity: Fast reaction kinetics. *Biochemical and Biophysical Research Communications, 282*, 1161-1168.

Salazar, R., Arámbula-Villa, G., Hidalgo, F. J., & Zamora, R. (2014). Structural characteristics that determine the inhibitory role of phenolic compounds on 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) formation. *Food Chemistry*, 151, 480–486.

Santhakumar, A. B., Battino, M., & Alvarez-Suarez, J. M. (2018). Dietary polyphenols: Structures, bioavailability and protective effects against atherosclerosis. *Food and Chemical Toxicology: An International Journal Published for the British Industrial Biological Research Association*, *113*, 49–65.

Tang, D., Kryvenko, O. N., Wang, Y., Trudeau, S., Rundle, A., Takahashi, S., Shirai, T., & Rybicki, B. A. (2013). 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)-DNA adducts in benign prostate and subsequent risk for prostate cancer. *International Journal of Cancer, 133*(4), 961–971.

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

Wang, H., Chu, X., Du, P., He, H., He, F., Liu, Y., Wang, W., Ma, Y., Wen, L., Wang, Y., Oz, F., & Abd El-Aty, A. M. (2023). Unveiling heterocyclic aromatic amines (HAAs) in thermally processed meat products: Formation, toxicity, and strategies for reduction - A comprehensive review. *Food Chemistry: X, 19*, 100833.

Xue, C., Chen, Q., He, Z., Qin, F., Wang, Z., Chen, J., & Zeng, M. (2022a). Release mechanism between sarcoplasmic protein-bound and free heterocyclic amines and the effects of dietary additives using an in-vitro digestion model. *Food Chemistry, 377*, 131993.

Xue, C., Chen, Q., He, Z., Qin, F., Wang, Z., Chen, J., & Zeng, M. (2022b). Release profiles of beef myofibril protein-bound heterocyclic amines and effects of dietary components on in vitro digestion. *Food Research International (Ottawa, Ont.), 155,* 111006.

Zamora, R., & Hidalgo, F. J. (2016). The triple defensive barrier of phenolic compounds against the lipid oxidation-induced damage in food products. *Trends in Food Science & Technology, 54*, 165-174.

Zhao, X., Shao, Z., Zhou, X., Lin, Y., Guo, J., Guo, J., Zhang, Y., & Wang, S. (2021). Sub-chronic exposure to PhIP induces oxidative damage and DNA damage, and disrupts the amino acid metabolism in the colons of Wistar rats. *Food and Chemical Toxicology: an international journal published for the British Industrial Biological Research Association, 153*, 112249.

Appendices

Scientific publications

Appendix-Publications

Yang, X., Blecker, C., Liu, H., Zhang, D., Wang, Z. (2023). Effect of plant polyphenols with different m-hydroxy and o-hydroxy groups on the inhibition of heterocyclic amines formation in roasted meat. ***Food Control****, 153*, 109960. https://doi.org/10.1016/j.foodcont.2023.109960. (Chapter III)

Yang, X., Zhang, D., Blecker, C., Zhang, C., Sun, X., Wang, Z. (2024). Effect of interaction between resveratrol and myofibrillar protein on the production of bound heterocyclic amines. ***Food Bioscience****, 59*, 104116. https://doi.org/10.1016/j.fbio.2024.104166. (Chapter IV)

Yang, X., Blecker, C., Zhang, D., Wang, Z. (2025). Mechanistic insights into the inhibitory effect of resveratrol on heterocyclic amine formation involving sarcoplasmic proteins. ***LWT****, 230*, 118281. https://doi.org/10.1016/j.lwt.2025.118281. (Chapter IV)

Yang, X., Blecker, C., Shi, H., Gao, Z., He, J., Zhang, D., Wang, Z. Mechanistic study on the inhibition of 2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) by polyphenols with different structures in model systems: A combination of experimental and theoretical chemical calculations. (under review) (Chapter V)