Chapter 5
Heterocyclic Amines in Foods: Analytical Methods, Formation Mechanism, and Mitigation Strategies

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5.1 Introduction

During heating of meat-based food products, a group of structurally close compounds collectively known as heterocyclic amines (HAs) can be formed. Drastic heating conditions such as grilling and roasting could lead to significantly higher HA contents. Precursors for this group of polycyclic aromatic compounds are fundamental food components including sugars, amino acids, and creatine, which undergo the Maillard reaction to form HAs [1]. More than two dozen HAs have been identified in thermally processed foods.

HAs are highly mutagenic in bacteria mutagenicity tests [2], and some of them are classified as potential human carcinogens by the IARC. HAs indeed represent a significant health risk considering the fact that they are widely present in some of our most important dietary components. In this chapter, we will give an overview of key analytical methods for the qualitative and quantitative analysis of HAs, their formation mechanisms, and major strategies for mitigation of HA-associated health risk.

5.2 Analytical Methods for HAs

HAs often occur in the ppb range in foods. Furthermore, they are often present in complex food matrices, which have numerous concurring compounds. Some of these compounds might significantly interfere with the analysis of HAs. Therefore,

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the determination of HAs in food samples requires selective and sensitive methods, which include efficient and robust sample preparation and analytical techniques. One of the most widely used approaches is solid phase extraction (SPE), which in essence incorporates the power of selective types of chromatographic materials packed in one or more solid supports. Chromatography remains the most important analytical technique for HAs. Although some studies also use gas chromatography (GC) for the analysis of HAs, the fact that most HAs are nonvolatile entails a derivatization step prior to quantitative and/or qualitative analysis. Hence, high-performance liquid chromatography (HPLC) coupled to photodiode array detection (LC-PDA) or mass spectrometry (LC-MS) has been the most popular instrument for HA analysis and will be reviewed herein.

5.2.1 Sample Preparation by SPE

SPE is a powerful sample preparation method owing to its capability to accomplish extraction and concentration of target analytes in a single run. This can then be translated into enhanced sensitivity to facilitate subsequent qualitative and quantitative analysis [3, 4]. Depending on the instrumental setup, the SPE process might be completely separated from or coupled to the chromatographic process [5]. Furthermore, the complexity of the sample matrix is also an important determinant of the optimal type of solid adsorbent. For simple matrices, such as those from chemical model reactions using pure precursor compounds of HAs, a combination of a diatomaceous earth cleanup step, followed by a cation exchange and a C-18 reverse phase concentration step, is sufficient to obtain samples amenable to subsequent chromatographic analysis [6, 7]. More recent studies have employed various forms of polymers or composites aiming to enhance the performance of the cleaning and concentration process. For instance, Zhang and co-workers synthesized a derivative of graphene with acrylamide, which exhibited good stability and permeability in both aqueous and organic phases [5]. This modified SPE adsorbent possessed a significantly improved efficiency of extraction of HAs from the sample matrix to allow online coupling to LC analysis.

5.2.2 Determination of HAs by LC-UV and LC-Fluorescence Detection

The presence of a heterocyclic aromatic system in HAs renders them amenable to detection by a UV detector. Although HAs often exhibit very similar chromatographic behavior, especially with most binary mobile phase system, the availability of authentic standards enables unambiguous identification in most cases. Of note, since HAs of the same subclass (e.g., aminoimidazole-azaarenes) only show subtle

difference in their UV absorption spectra, it would be of high importance to have concentrated and clean samples, which faciliate resolution and minimize interference from the sample matrix. Some HAs including the nonpolar ones and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) have an extensive conjugated pi-electron moiety and thus can be detected with a fluorescence detector. The sensitivity was found to be 100–400 times more sensitive than with a UV detector [8, 9]. Some studies have also used a combination of both UV and fluorescence detectors for the analysis of a large number of HAs. For instance, Gibis and Weiss successfully identified and quantified 15 polar and nonpolar HAs in fried beef patties by HPLC coupled to UV and fluorescence detection [9].

Electrochemical detection (ECD) has been a relatively less popular option for the analysis of HAs although it also attains high selectivity and sensitivity, which are key parameters in the method selection for handling samples with complex matrices. An isocratic mobile phase is required for analysis in the high sensitivity range. This criterion, however, made simultaneous analysis of a large number of HAs in one run a daunting task [10]. Improvement in the mobile phase system with the use of ionic liquids such as 1-butyl-3-methylimidazolium tetrafluoroborate, 1-hexyl-3- methylimidazolium tetrafluoroborate, and 1-methyl-3-octylimidazolium tetrafluoroborate (especially the first one) could improve the chromatographic separation of HAs [11].

5.2.3 Determination of HAs by LC-MS

LC-MS is one of the most powerful and versatile hyphenated analytical techniques as it elegantly integrates the separation capacity of column chromatography and the sensitivity and selectivity of MS. This feature makes it a particularly suitable tool for the analysis of HAs. The development of tandem MS has further elevated the resolution power of this already highly efficient analytical tool and enabled the use of much simplified sample preparation procedures [12–14]. As HAs are stable during the ionization process, the protonated molecular ion peaks may thus serve as markers in mass selective detection of HAs [15]. TriMeIQx has been widely used as an internal standard for quantitative analyses [12, 16]. Some studies also used isotopically labeled HAs as internal standards, typically at a hydrogen or carbon atom, and less commonly at nitrogen [12, 15, 17, 18]. The similar chemical properties and mass of these internal standards offer improved accuracy for evaluation of HAs compared to the use of other compounds such as aromatic acids [19].

Various ionization interfaces have been used to couple the liquid chromatograph and mass spectrometer. Major types include thermospray ionization (TSI), atmospheric pressure chemical ionization (APCI), and electrospray ionization (ESI). TSI was used in early studies to detect a single or few HAs [20]. This ionization technique, however, is prone to result in relatively more fragmentation and generate doubly charged ions [21], which would compromise accurate determination of multiple HAs, especially in samples from complex food products. Therefore, ESI and APCI have largely dominated in LC-MS analysis of HAs over the past two decades

due to the soft ionization process. These two interfaces used to be considered complementary to each other since the former is more suitable for analyzing polar HAs while APCI is suitable for less polar ones [22, 23]. Nonetheless, the utilization of ESI and tandem MS has allowed the determination of different categories of HAs from a wider range of food matrices in a single operation. With this configuration, it has become commonplace to simultaneously measure >10 HAs with satisfactory accuracy [14, 15, 24]. More recent studies employing ultra-performance LC tandem MS (UPLC-MS/MS) and principal component analysis have enabled qualitative and quantitative analysis of even larger numbers of different groups of HAs.

Triple quadrupole mass spectrometer has been the most popular mass analyzer for HAs due to its accurate quantitation capability. Operation is almost exclusively in the positive ionization mode given the high proton affinity of HAs, especially with the use of an ammonium salt (e.g., ammonium formate or acetate) in the mobile phase. Data acquisition in multiple reaction monitoring (MRM) mode has accomplished great selectivity and sensitivity (<3.1 ppm) for complex matrices, especially when combined with the use of isotopically labeled reference standards [14]. Further improvement in mass accuracy and sensitivity could be achieved with the use of quadruple time-of-flight (Q-TOF) [25]. The availability of these robust methods makes it feasible to quantitatively monitor total dietary exposure to HAs, and the data generated could be applied to assess long-term health risk.

5.3 Mechanisms of HA Formation

HAs are hazardous by-products of heat treatment in animal products. Their formation is affected by both intrinsic and extrinsic factors. The former mainly include contents and types of amino acids and sugars and characteristic of the food matrix (which could affect interaction among the purported precursors). The latter include heating conditions (e.g., temperature, use of a naked flame, etc.) and sample preparation methods prior to heating (e.g., type of marinade, additives, etc.). Therefore, the same foodstuff might have completely different HA content and profile after different heat treatments and vice versa [26].

According to their chemical structures, HAs can be broadly divided into two categories, namely amino-carbolines and aminoimidazole-azaarenes [27]. Amino-carbolines are generally formed at much higher temperature (>300 °C) via pyrolysis of amino acids or proteins. The formation of aminoimidazole-azaarenes requires lower temperatures. HAs can also be classified based on their polarity. Thus far, important HAs which have demonstrated carcinogenic activity in rodent studies and/or have been associated with increased health risk in epideomiological studies belong to the polar group [28–31]. Structures of polar HAs which have been most extensively studied are shown in Fig. 5.1.

Since amino-carbolines are pyrolytic products and creatine is not required for their formation, they could be present even in foods of plant origins although animal products are the major sources [32]. Thus far, heat-induced generation of free radi-

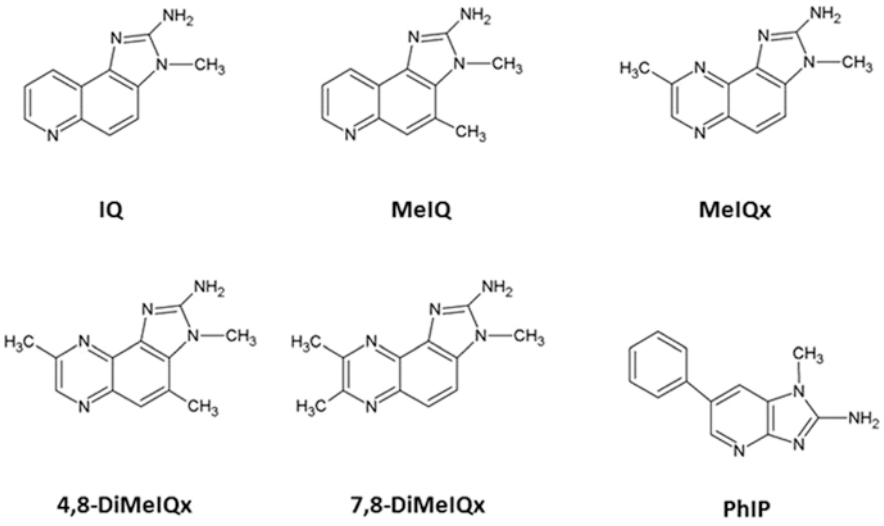

Fig. 5.1 Chemical structures of major polar heterocyclic amines

cals and the subsequent generation of reactive fragments has been proposed to be a major mechanism involved in amino-carboline formation [1]. Condensation of these reactive fragments and rearrangement give rise to various new structures.

In contrast to amino-carbolines, creatin(in)e has been identified to be an important component in the formation pathways of aminoimidazole-azaarenes. In essence, it is creatinine, but not creatine, that contributes to the formation of aminoimidazole-azaarenes including IQ, IQx, and PhIP. The amount of creatinine, a natural breakdown product of creatine phosphate, in muscle is very low. Interestingly, addition of extra creatinine to the surface of meat prior to frying increased the yield of HAs [33]. During heating, creatine can be converted to creatinine via cyclization and water elimination. Meanwhile, intermediary compounds such as pyridines and pyrazines and reactive carbonyls can be formed from the Maillard reaction between amino acids and reducing sugars and Strecker degradation reactions. Creatinine forms the amino-imidazo part which likely joins with the above reactive fragments via aldol condensation, giving rise to different aminoimidazole-azaarenes [32]. Relatively lower heating temperatures (< 300 °C) are sufficient to drive the Maillard reaction and Strecker degradation to generate reactive fragments from the precursors (sugars, amino acids, and their derivatives) [1]. In general, the contents of HAs in foodstuffs and chemical models increase with heating temperature and time [32, 34]. It has also been observed that the content of HAs correlates with the surface color of meat – the darker the color, the higher the HA concentration [35]. Some studies have also reported that radicals, especially pyridine and pyrazine radicals, might also be involved in the formation of aminoimidazole-azaarenes [36]. Nonetheless, findings from more recent studies suggest that free radical-mediated pathway is not likely the principal mechanism of aminoimidazole-azaarene formation [37, 38].

5.4 Mitigation Strategies to Reduce HA-Associated Health Risk

Given the genotoxic potential of HAs and the fact that humans might be exposed to HAs through the consumption of ordinary household dishes on a daily basis, there has been extensive research aiming to develop strategies to reduce HA-associated health risk, which mainly include the following two approaches. The first approach aims to prevent or reduce the content of HAs in our dietary components. This is considered a more desirable approach since it prevents our body tissues and organs from exposing to HAs in the first place. As mentioned above, HA content in foodstuffs is a result of a number of factors, some of which are amenable to manipulation. In other words, it is highly possible to adjust one or more of these parameters to control or interrupt the HA formation process. The second approach targets HAs which have entered the human body through the consumption of foods containing HAs. This approach aims to attenuate the potential pathological consequences of HAs by one or more of the following mechanisms: (1) reducing their bioavailability, (2) suppressing their metabolic activation enzyme systems and/or enhancing their elimination from the body, and (3) modulating metabolic activation at genetic levels [39–41]. The focus of this section will be on the first approach which has been the most widely accepted approach and holds great promise for incorporation into our daily cuisine.

It has almost become a rule of thumb that lowering heating temperature and/or shortening heating time can effectively reduce the content of HAs in foods. Paradoxically, heat-driven Maillard reaction plays a crucial role in the generation of desirable flavor and aroma compounds. This apparently most straightforward approach might negatively impact sensory quality of food products. Therefore, a general consensus in this area is that complete avoidance of dietary exposure to HAs is infeasible and ideal strategies to mitigate HA-associated health risk should meet the following criteria: (1) significant inhibition of HA formation or reduction of HA content, (2) do not give rise to new genotoxicants or promote the formation of other concurrent hazardous compounds, and (3) do not have negative impact on sensory quality (desirably could enhance it).

This section will give an overview of major inhibitory strategies which hold great promise for application in the food industry or household cooking.

5.4.1 Careful Choice of Sugars in Marinades

It is known that sugars, especially reducing sugars, are principal precursors of HAs. Therefore, change in the content and type of sugars may significantly affect the formation of HAs in foods, and the effect may be completely different at different

heating temperatures. In the lower temperature range (~100 °C), increasing the level of reducing sugars was reported to favor HA formation [42]. In contrast, it was found that addition of reducing sugars beyond a certain concentration range decreased HA formation in ground beef and beef patties heated between 150 and 200 °C [43, 44]. In practice, honey is sometimes used as a substitute for sugar to add sweetness to marinades. A recent study [45] which evaluated the effect of different types of sugar on the formation of HAs in grilled chicken found that marination with honey led to lower HA contents in chicken breast samples than those marinated with brown sugar or table sugar. However, the authors did not further verify whether the observed inhibitory effect was caused by the sugar in honey or was it an integrated effect from sugar and other components.

5.4.2 Addition of Synthetic Antioxidants

Knowledge about the formation mechanism of HAs has greatly facilitated the development of more targeted inhibition methods. Free radicals and other reactive intermediary compounds especially carbonyls have been demonstrated to be among the key participants in HA formation. In accordance with this mechanistic basis, antioxidants have been the most important group of candidate inhibitors. In early proof-of-concept experiments, researchers tested the effect of some common synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG), and *tert*-butylhydroquinone (TBHQ). Interestingly, their effect turned out to be highly dependent on the experimental system employed. In food systems, these antioxidants were able to inhibit HA formation even at low levels of addition [46, 47]. In chemical models containing pure HA precursors, BHT and TBHQ were found to promote HA formation. The effect was particularly strong with TBHQ which increased MeIQx formation by >200% [48–50]. Despite the inconsistent findings on the activity of synthetic antioxidants against HA formation, these proof-of-concept studies at least added to the strength of the evidence that antioxidation is likely to be an important inhibitory mechanism under certain circumstances.

5.4.3 Addition of Natural Antioxidants

Natural agents are generally more preferred to synthetic ones as culinary ingredients or additives. A wide range of natural antioxidants including vitamins, phenolics, and carotenoids have been evaluated for their effect on the formation of HAs. Similar to the aforementioned synthetic antioxidants, vitamins like vitamin C and α-tocopherol failed to demonstrate consistent effects on HA formation [43],

although they possess the capability to interrupt free radical-mediated reaction pathways [51]. As a matter of fact, natural polyphenols are a large group of phytochemicals with a wide range of antioxidant or free radical scavenging capacities. Thus far, reports on the relative effectiveness of natural polyphenols as HA formation inhibitors have been more consistent compared to those on synthetic antioxidants and vitamins. They can be added in the form of pure phytochemicals isolated from plants or in the form of polyphenol-rich extracts. Most of them originated from fruits, vegetables, or spices which could be easily incorporated into daily cuisine.

Considering the sometimes inconsistent data from chemical model and real food experiments, more recent studies have used both systems or verified the findings from chemical model screening assay in at least one food system. In an attempt to identify potent inhibitors of HA formation, we evaluated 12 food-derived phenolic antioxidants in simple chemical model systems and in fried beef patties [52]. The results did not support a significant positive correlation between antioxidant and HA formation inhibitory activity. Of note, naringenin, a rather weak antioxidant, turned out to have comparable inhibitory capacity to theaflavin-3,3′-digallate and epigallocatechin gallate, which are well-known potent natural antioxidants. At 0.1% (w/w) level of addition, they were able to reduce the content of total HAs by >50% relative to control, and the inhibition was consistent in both chemical model and beef patties. Curcumin, a principal bioactive polyphenol in turmeric, was also found to dose-dependently inhibit mutagenic HA formation in several model systems [53, 54], although it is not a potent free radical scavenger. The discordance between radical scavenging/antioxidant activity and inhibitory activity of HA formation suggests that alternative (if not dominant) mechanism likely contributes to the inhibitory effect of many natural polyphenols.

As mentioned in Sect. 5.3, certain Maillard intermediates, especially reactive carbonyl species (RCS) generated from thermal and/or Strecker degradation reactions, are likely key intermediates in the formation of HAs [32, 49, 55, 56]. Our group were among the first to report the scavenging of reactive carbonyls as a key mechanism responsible for the inhibitory effect of certain natural polyphenols [57]. By doing so, the added polyphenolic inhibitors combine with the reactive HA intermediates to form adducts. PhIP has been the most well-studied HA with regard to this postulated action mechanism, and phenylacetaldehyde is a major reactive carbonyl intermediate involved. A more recent study has further confirmed the importance of this mechanism for the inhibitory activity of polyphenols against HA formation [58]. The authors demonstrated a significant positive correlation between the phenylacetaldehyde-scavenging capacity and PhIP formation inhibitory activity of a group of polyphenols with a diverse range of antioxidant capacities. An example of this recently discovered and yet amply demonstrated inhibitory mechanism is presented in Fig. 5.2. Structures of some potent natural HA formation inhibitors are presented in Fig. 5.3.

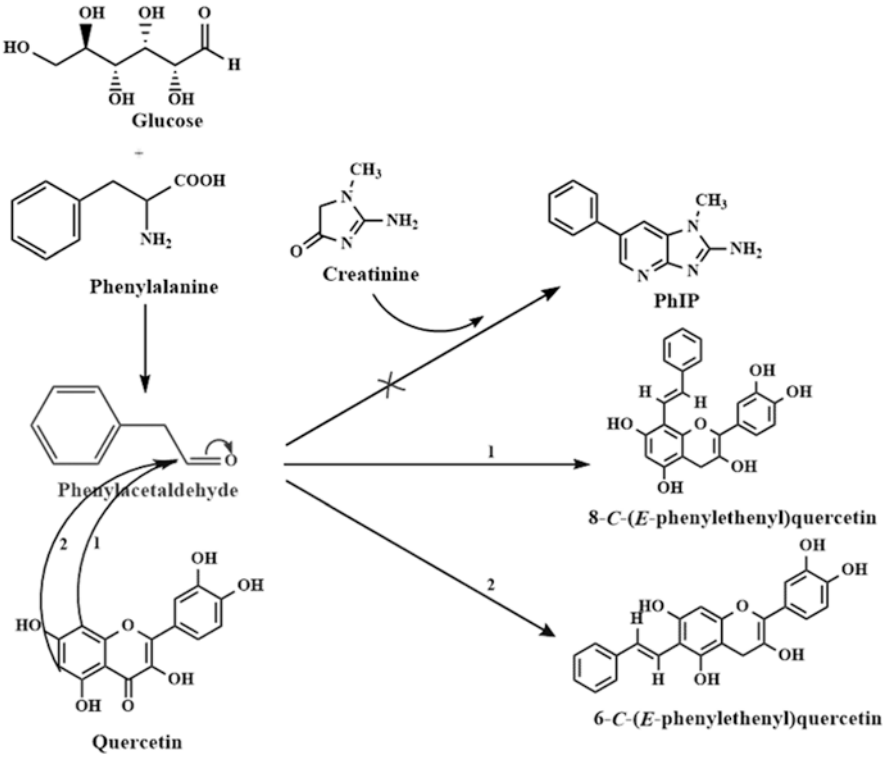

Fig. 5.2 Postulated pathway for the inhibitory activity of quercetin in PhIP formation

5.4.4 Addition of Phytochemical Extracts and Herbs

Fruits, vegetables, and certain seasoning herbs or spices are known to be valuable natural sources of a wide range of phytochemicals, especially polyphenols. Therefore, it is not surprising that more and more studies have reported the inhibitory effect of plant extracts or herbs on the formation of HAs. As some of the most common seasoning ingredients in Western countries, rosemary, thyme, sage, and garlic were reported to significantly reduce HA content in meat by applying them to the surface prior to heating [59]. Other promising spices include turmeric, torch ginger, pepper, onion, lemongrass, and curry leaves [60, 61]. Fruit and vegetable extracts have also been evaluated for their potential to inhibit HA formation. Our group and others have identified a small number of extracts through systematic comparison using both chemical models and real food systems [61, 62]. One common feature is that they are all rich in polyphenols and could effectively reduce the content of HAs even at low levels of addition. For example, marinating minced beef

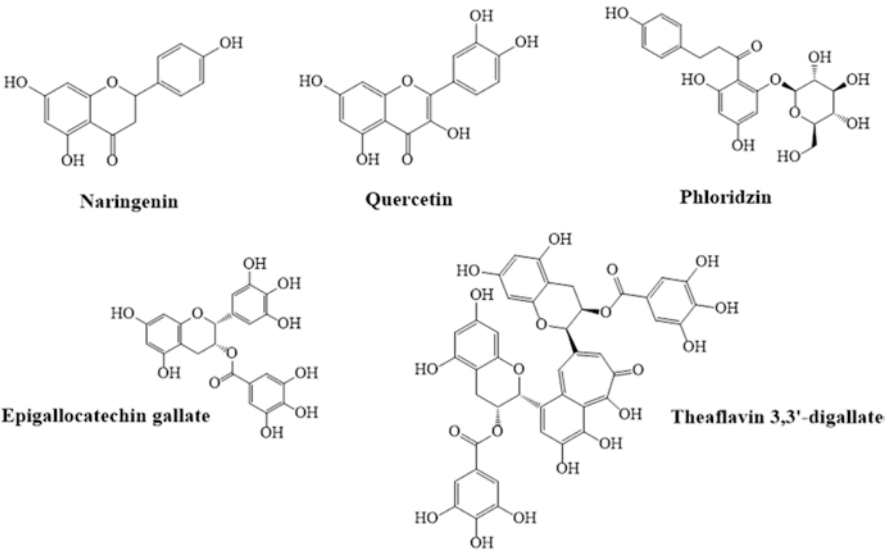

Fig. 5.3 Structures of potent natural HA formation inhibitors

with 0.1% (w/w) apple or grape seed extracts prior to heat treatment decreased individual HAs (MeIQx, 4,8-DiMeIQx and PhIP) and total HA content by >70% in the fried beef patties [62]. Nevertheless, very few studies have performed activity-guided investigation to identify the principal HA formation inhibitors in these extracts, nor has there been sufficient research on the potential synergic effect among some of the purported inhibitors. This warrants further evaluation. It is conceivable that knowledge about the principal inhibitors and/or synergism (if any) among them in these crude extracts would facilitate the preparation of extracts which are selectively concentrated with the inhibitors identified.

5.5 Concluding Remarks

Heating is an important process to ensure microbiological safety, eliminate antinutritional factors, and enable the development of desirable profiles of color and flavor attributes of meat and fish. However, this process also gives rise to HAs and other potentially hazardous by-products which may be a significant health risk for humans in the long term. Robust analytical tools, especially liquid chromatography coupled to diode array detector and mass spectrometer are crucial for the accurate determination of the content of HAs in foods to thus monitor dietary intake and assess the associated health risk. Given the genotoxic potential of HAs and their occurrence in a wide range of major dietary components, development of strategies to inhibit their formation during heat processing or reduce their content in foodstuffs would remain

a key direction in the field of food-borne genotoxicants. Further research should continue to explore how the application of inhibitory strategies could be optimized by integrating with physical conditions such as temperature and duration of cooking, as well as the potential interactions among purported inhibitors of HA formation with various culinary ingredients.

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