



# Inhibition of the formation of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and 2-amino-3,8-dimethyl-3*H*-imidazo[4,5-*f*]quinoxaline (MeIQx) by phenolics in model systems and beef patties

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## ARTICLE INFO

### Keywords:

Carbonyl-amine reactions  
Carbonyl-phenol reactions  
Food carbonylome  
Heterocyclic aromatic amines  
Maillard reaction  
Reactive carbonyls

### Chemical compounds studied in this article:

Acrolein (PubChem ID: 7847)  
MeIQx (PubChem ID: 62275)  
Phenylacetaldehyde (PubChem ID: 998)  
Phenylalanine (PubChem ID: 6140)  
PhIP (PubChem ID: 1530)  
Threonine (PubChem ID: 6288)

## ABSTRACT

The comparative inhibition of the formation of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) and 2-amino-3,8-dimethyl-3*H*-imidazo[4,5-*f*]quinoxaline (MeIQx) by phenolics in both model systems and beef patties was studied to better understand the protective role of these compounds. Model systems were selected to produce PhIP and MeIQx by carbonyl-amine reactions. Obtained results showed that their formation was inhibited up to 100 % by assayed phenolics. Among simple phenolics, *m*-diphenols were usually more effective than *o*-diphenols, and MeIQx was better inhibited than PhIP because *m*-diphenols both produce the Strecker degradation of amino acids to a lower extent than *o*-diphenols, and trap acrolein (responsible for MeIQx formation) better than phenylacetaldehyde (responsible for PhIP formation). When complex phenolics were assayed, other groups also contributed to the observed protective function. However, relative inhibitions of both compounds in model systems and beef patties were correlated, therefore suggesting that the formation of PhIP and MeIQx also occurs in beef patties by carbonyl-amine reactions.

## 1. Introduction

Food processing has numerous benefits, including a prolonged shelf life and the destruction of harmful bacteria (Chaudhary et al., 2025; Liberal, Fernandes, Ferreira, Vivar-Quintana, & Barros, 2024). On the other hand, when foods are submitted to high processing temperatures, the loss of certain nutrients occurs (Wu et al., 2024) and the formation of a plethora of new compounds, many of them in minute amounts, is produced (He, Wang, Liu, & Sun, 2024). Among them, the appearance of a certain number of substances with potential mutagenic and carcinogenic properties has been described (Lu, Zhang, Zhou, Cai, & Xu, 2024; Mansour, Ibrahim, Zhang, & Farag, 2025). Some of these substances are heterocyclic amines containing several condensed aromatic rings with one or more nitrogen atoms in the ring system, and, usually, one exocyclic amino group. These compounds are known as heterocyclic aromatic amines (HAAs) and have been shown to be mainly produced in

proteinaceous foods upon heating (Oz, 2021). The formation of these compounds has been the objective of many studies because of their potential relationship with the observed association between the appearance of certain types of cancers and the consumption of red and processed meats (Di Maso et al., 2019; Guo et al., 2022).

At present, there is not a common agreement about how these substances are produced, and their formation involving both free radicals (Chen et al., 2024; Pearson, Chen, Gray, & Aust, 1992) and reactive carbonyls (Zamora & Hidalgo, 2020) has been suggested. Nevertheless, recent studies have described the formation pathways for the carcinogenic HAAs with the structure of aminoimidazoazaarene produced to a higher extent in proteinaceous foods: 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ) (Zamora, Lavado-Tena, & Hidalgo, 2020a), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ) (Zamora, Lavado-Tena, & Hidalgo, 2020b), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) (Hidalgo, Lavado-Tena, & Zamora, 2021), and 2-amino-1-

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<https://doi.org/10.1016/j.foodchem.2025.144091>

Received 15 October 2024; Received in revised form 26 January 2025; Accepted 26 March 2025

Available online 28 March 2025

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methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) (Zamora, Alcon, & Hidalgo, 2014). Among them, PhIP and MeIQx are the main carcinogenic HAAs produced in beef patties (Hidalgo & Zamora, 2023).

PhIP (7) and MeIQx (15) have been suggested to be produced by carbonyl-amine reactions as a consequence of the reaction of specific reactive carbonyls (i.e., phenylacetaldehyde (3) for PhIP, and acrolein (13) for MeIQx) with creatinine (4), ammonia (6), formaldehyde (5), and methylglyoxal (14) as shown in Fig. 1. Therefore, reactions that promote the formation of either phenylacetaldehyde or acrolein should increase the formation of PhIP and MeIQx, respectively, and vice versa:

reactions that either inhibit the formation of either phenylacetaldehyde or acrolein, or promote their disappearance, should inhibit the formation of PhIP and MeIQx, respectively.

As shown in Fig. 1, phenylacetaldehyde (3) is mainly produced by the Strecker degradation of phenylalanine (1) initiated by reactive carbonyls, although its production as a consequence of free radical degradation of phenylalanine has also been shown (Hidalgo & Zamora, 2019). Reactive carbonyls responsible for this degradation are usually considered to be carbohydrate-derived (Fontes et al., 2024), but lipid-derived (Hidalgo, León, & Zamora, 2016) or phenolic-derived reactive

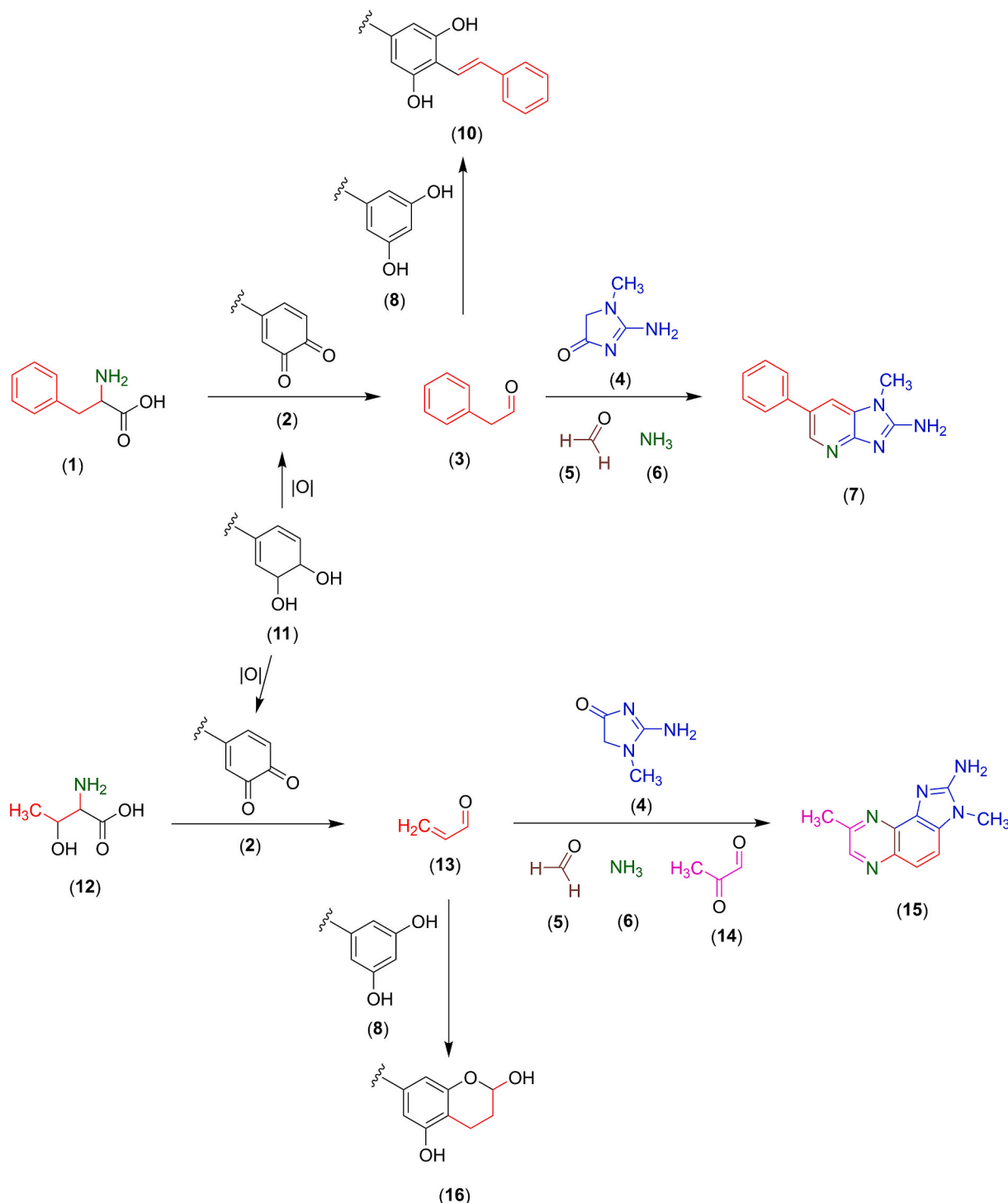


Fig. 1. Reaction pathways for the formation of PhIP (7) from phenylalanine (1), and MeIQx (15) from threonine (12) in the presence of phenolic compounds.

carbonyls (Delgado, Hidalgo, & Zamora, 2016) have also been shown to be very efficient phenylacetaldehyde producers. In Fig. 1, the effect of quinones (2) as phenolic-derived reactive carbonyls is shown. These quinones are produced by oxidation of appropriated phenolics (11).

Differently to phenylacetaldehyde, acrolein (13) is a common dietary and environmental contaminant which is also generated endogenously in foods (Zhou, Jin, Wu, & Zhou, 2024). Chemical reactions responsible for the release of acrolein include heat-induced dehydration of glycerol, retro-aldol cleavage of dehydrated carbohydrates, lipid peroxidation of polyunsaturated fatty acids, and Strecker degradation of methionine and threonine (Stevens & Maier, 2008). If amino acid degradation is considered, the same reactive carbonyls responsible for phenylacetaldehyde formation should also be expected to promote acrolein formation by the Strecker degradation of either threonine (12), as shown in Fig. 1, or methionine.

In addition to their formation as a consequence of food processing, both phenylacetaldehyde (3) and acrolein (13) can also disappear as a consequence of the chemical reactions produced in foods during this processing. Among them, and because of the presence of the carbonyl group, phenylacetaldehyde and acrolein react with nucleophiles, which results in the disappearance of these aldehydes and, therefore, in the reduction of both PhIP and MeIQx. Phenylacetaldehyde- and acrolein-trapping produced by phenolics is one of the defensive barriers described for phenolic compounds in food products (Zamora & Hidalgo, 2016) and it has been shown to occur in beef patties (Hidalgo & Zamora, 2023). As shown in Fig. 1, the reaction of an appropriate phenolic (8) with phenylacetaldehyde (3) produces the corresponding adduct (10) (Hidalgo, Aguilar, & Zamora, 2017), and the reaction of acrolein (13) with the phenolic (8) also produces another adduct (16), which has a different structure to that produced with phenylacetaldehyde (Hidalgo & Zamora, 2014). In addition, aldehydes can be removed by additional reactions, including, for example, the reaction with decarboxylated hydroxycinnamic acids (Bork, Stobernack, Rohn, & Kanzler, 2024). On the other hand, some phenolics can stabilize the produced aldehydes avoiding their natural decomposition, such as their oxidation under oxidative conditions (Wang et al., 2024). Because these different mechanisms take place simultaneously and compete among them, different roles have been found for phenolic compounds and both promotion and inhibition of the formation of heterocyclic aromatic amines have been described (Yang, Blecker, Liu, Zhang, & Wang, 2023; Zeng, Li, He, Qin, & Chen, 2016).

The hypothesis of this study is that the efficiency of phenolic compounds as inhibitors of PhIP and MeIQx formation should be, to a certain extent, a consequence of the balance between their abilities to promote the formation and stabilization of aldehydes (both related to phenolic oxidability) and those that promote aldehyde disappearance (including the above described carbonyl-trapping).

In an attempt to advance in the understanding of the role of phenolics on the inhibition of the formation of HAAs in food products, this investigation describes the effect of the presence of a wide array of phenolics on PhIP and MeIQx formation in both model systems and beef patties, and discusses it as a function of phenolic oxidability and carbonyl-trapping ability of involved phenolics.

## 2. Materials and methods

### 2.1. Materials

Lean beef meat was obtained from a local market. Different simple and complex phenolics were employed in this study. The simple phenolics studied were: catechol (17), 4-methylcatechol (18), 3,4-dihydroxybenzoic acid (19), methyl 3,4-dihydroxybenzoate (20), 1,2,4-trihydroxybenzene (21), 3-(3,4-dihydroxyphenyl)propanoic acid (22), caffeic acid (23), methyl caffeate (24), ferulic acid (25), methyl ferulate (26), resorcinol (27), 2-methylresorcinol (28), orcinol (29), phloroglucinol (30), 2,5-dimethylresorcinol (31), 2,6-dihydroxybenzoic acid

(32), pyrogallol (33), gallic acid (34), methyl gallate (35), propyl gallate (36), and 3-methoxycatechol (37). The structures of all these compounds are given in Fig. 2. The complex phenolics studied involved two flavonols: morin (38) and quercetin (39); one dihydrochalcone: phloretin (40); one flavanone: naringenin (41), one flavone: chrysin (42); two flavan-3-ols: epicatechin (43) and catechin (44); and three stilbenoids: pterostilbene (45); resveratrol (46), and piceatannol (47). The structures of these compounds are given in Fig. 3. The source and purity for all these compounds are given in Table S1 of the Supporting Information.

PhIP and MeIQx were obtained from Toronto Research Chemicals Inc. (North York, Ontario, Canada). Phenylalanine and threonine were purchased from Merck/Sigma-Aldrich (Darmstadt, Germany). All other chemicals used in this research were of the highest available grade and were obtained from reliable commercial sources, including Merck/Sigma-Aldrich (Darmstadt, Germany), Alfa Aesar (Haverhill, Massachusetts), and TCI (Tokyo, Japan), among others.

### 2.2. Formation of PhIP or MeIQx in model reactions of creatinine, phenolics, and phenylalanine or threonine

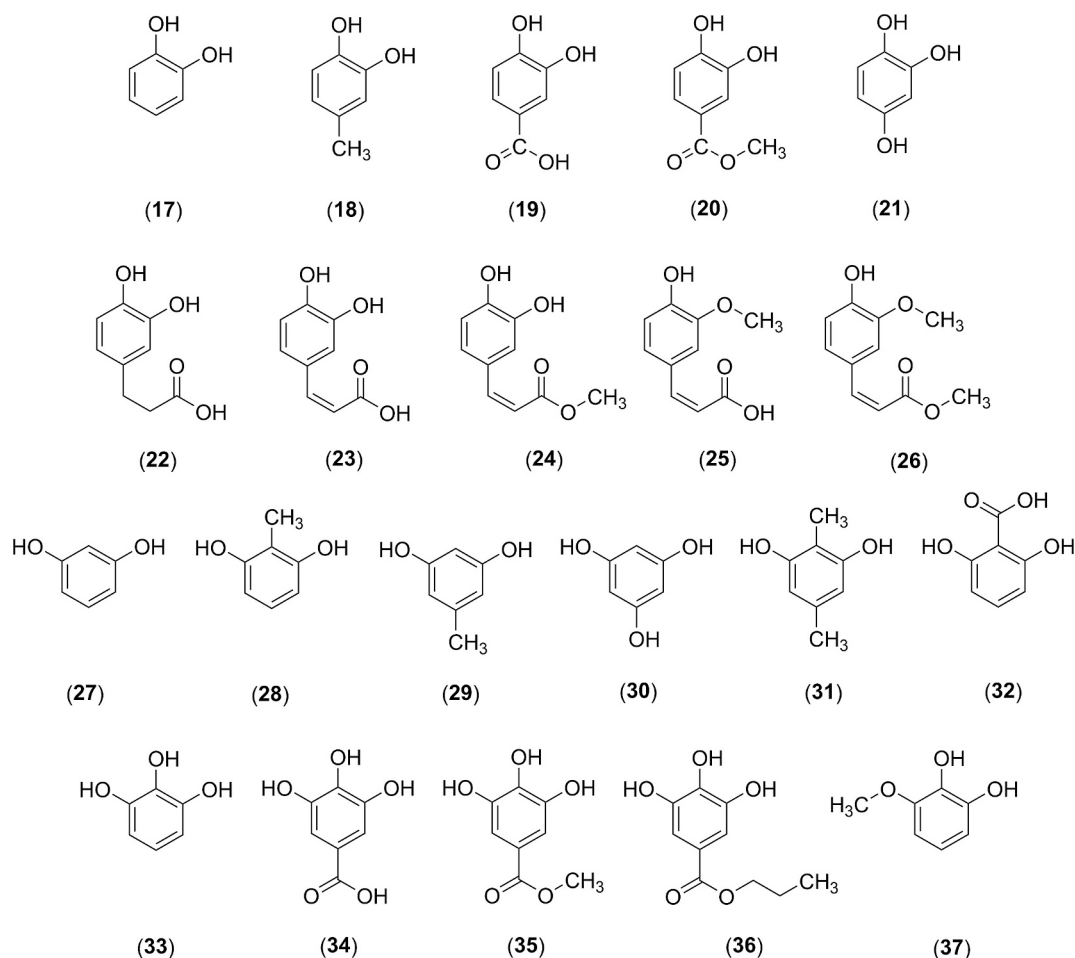
The employed procedure was analogous to that used in the study of the formation of the different HAAs, such as MeIQx (Hidalgo et al., 2021). Briefly, mixtures of creatinine (50  $\mu\text{mol}$ ), phenolic (50  $\mu\text{mol}$ ), and phenylalanine or threonine (50  $\mu\text{mol}$ ) were singly homogenized with 1.5 g of acid-treated and calcined sea sand (Merck, Darmstadt, Germany), used as reaction support, 135  $\mu\text{L}$  of water, and 50  $\mu\text{L}$  of methanol. Reaction mixtures were heated at 180  $^{\circ}\text{C}$  in closed test tubes for 1 h under air. After cooling, 2 mL of ethyl acetate and 30  $\mu\text{L}$  of the internal standard solution (6.45 mg of caffeine in 10 mL of water) were added. Suspensions were stirred for 1 min and centrifuged at 2000g for 10 min. The supernatant was isolated and taken to dryness with nitrogen. The resulting residue was extracted with 250  $\mu\text{L}$  of a 1:1 mixture of 30  $\text{mmol}\cdot\text{L}^{-1}$  ammonium formate and acetonitrile, and the extract was centrifuged at 16000g for 20 min and studied by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) as described in Section 2.4. Controls were prepared similarly, but phenolics were not added.

### 2.3. Preparation and cooking of beef patties

Beef patties were prepared analogously to those described by Hidalgo and Zamora (2023). Briefly, lean beef meat was grinded by using a meat grinder, and grinded meat (containing 5 g of meat and 50 mg of the phenolic) was formed into patties of 35 mm diameter and 5 mm thickness. Patties were cooked in a Teflon-coated electric griddle at 190  $^{\circ}\text{C}$  for 2.5 min per side. After heating, the patties were minced, and 50 mL of ethyl acetate and 50  $\mu\text{L}$  of internal standard (6.45  $\mu\text{g}$  of caffeine in 10 mL of water) were added. Then, a smooth purée was obtained by using an ultra-turrax® disperser for 2 min, which was later sonicated for 3 min and homogenized for 1 min. The organic supernatant was collected and the residue was extracted again with other 50 mL of ethyl acetate and the process was repeated (homogenization for 2 min, sonication for 3 min, and new homogenization for 1 min). The obtained supernatant was mixed with the supernatant obtained in the first extraction, centrifuged at 7500g for 15 min, and then filtered through Whatman no. 1 paper. The obtained organic layer was evaporated by using a rotary evaporator and the residue was treated with 500  $\mu\text{L}$  of a 1:1 mixture of 30  $\text{mmol}\cdot\text{L}^{-1}$  ammonium formate and acetonitrile. The obtained suspension was centrifuged at 16,000g for 20 min and analyzed by LC-MS/MS as described in Section 2.4. Controls were prepared similarly, but phenolics were not added.

### 2.4. Determination of PhIP and MeIQx

PhIP and MeIQx were determined by LC-MS/MS following a



**Fig. 2.** Chemical structures of the simple phenolics used in this study. The simple phenolics studied were: catechol (17), 4-methylcatechol (18), 3,4-dihydroxybenzoic acid (19), methyl 3,4-dihydroxybenzoate (20), 1,2,4-trihydroxybenzene (21), 3-(3,4-dihydroxyphenyl)propanoic acid (22), caffeic acid (23), methyl caffeate (24), ferulic acid (25), methyl ferulate (26), resorcinol (27), 2-methylresorcinol (28), orcinol (29), phloroglucinol (30), 2,5-dimethylresorcinol (31), 2,6-dihydroxybenzoic acid (32), pyrogallol (33), gallic acid (34), methyl gallate (35), propyl gallate (36), and 3-methoxycatechol (37).

previously described procedure (Hidalgo & Zamora, 2023) using an Agilent liquid chromatography system (1200 Series, Agilent Technologies, Santa Clara, CA) connected to a triple-quadrupole API 4500 mass spectrometer (SCIEX, Framingham, MA) equipped with an electrospray ionization interface in the positive ionization mode (ESI+). Separations were carried out on a Zorbax Eclipse XDB-C18 (150 mm × 4.6 mm, 5 μm) column from Agilent using the conditions described in the previous study (Hidalgo & Zamora, 2023).

Three transitions were acquired for the identification of PhIP, MeIQx, and the internal standard. To establish the appropriate multiple reaction monitoring (MRM) conditions, mass spectrometric conditions were optimized using infusion with a syringe pump. Precursor and product ions used for quantification and confirmation purposes, and operating conditions for the different compounds are summarized in Table S2 of the Supporting Information. The transition in bold for each compound was used for quantification purposes.

Quantification of PhIP and MeIQx was carried out by preparing standard curves of both compounds in both the calcinated sea sand and beef patties, and following the whole extraction procedure described above. For each curve, six different concentration levels of PhIP and MeIQx were used. PhIP and MeIQx contents were directly proportional to the HAA/internal standard area ratios ( $r^2 > 0.96$ ,  $p < 0.001$ ). The coefficients of variation at the different concentrations were always < 15%. The limits of detection (LOD) and quantification (LOQ) for PhIP and MeIQx were calculated with signal-to-noise ratios of 3 and 10, respectively. They resulted to be 0.1 and 0.3 ng/g, respectively, for PhIP,

and 0.2 and 0.7 ng/g, respectively, for MeIQx. Recovery rates, determined by the standard addition method, were > 80%.

### 2.5. Determination of phenolic oxidability

A solution of the phenolic (50 μmol) in dimethylformamide (700 μL) was introduced into a quartz cuvette. Then, 100 μL of a solution of periodic acid in dimethylformamide (50 μmol/mL) was added, and the absorbance at 420 nm was monitored for 10 min in a spectrophotometer Shimadzu UV-2401 PC (Kioto, Japan). The absorbance increased linearly as a function of time. The slope of the line obtained between the minutes 1 and 6 was used as a measure of phenolic oxidability. In the case of strongly colored phenolics, a diluted solution was employed.

### 2.6. Statistical analysis

All data given are mean ± standard deviation (SD) values of, at least, three independent experiments. When needed, obtained means were compared by using analysis of variance. When *F* values were significantly different, statistical differences among groups were evaluated by the Tukey test.

To study the effect of phenolic structure on their ability to inhibit PhIP and MeIQx formation, Multiple Linear Regression (MLR) was used. MLR is a type of linear regression that uses the linear relationship between independent variables and the dependent variable to extract a model equation (Mu et al., 2025; Zhang et al., 2025; Zhang et al., 2025).

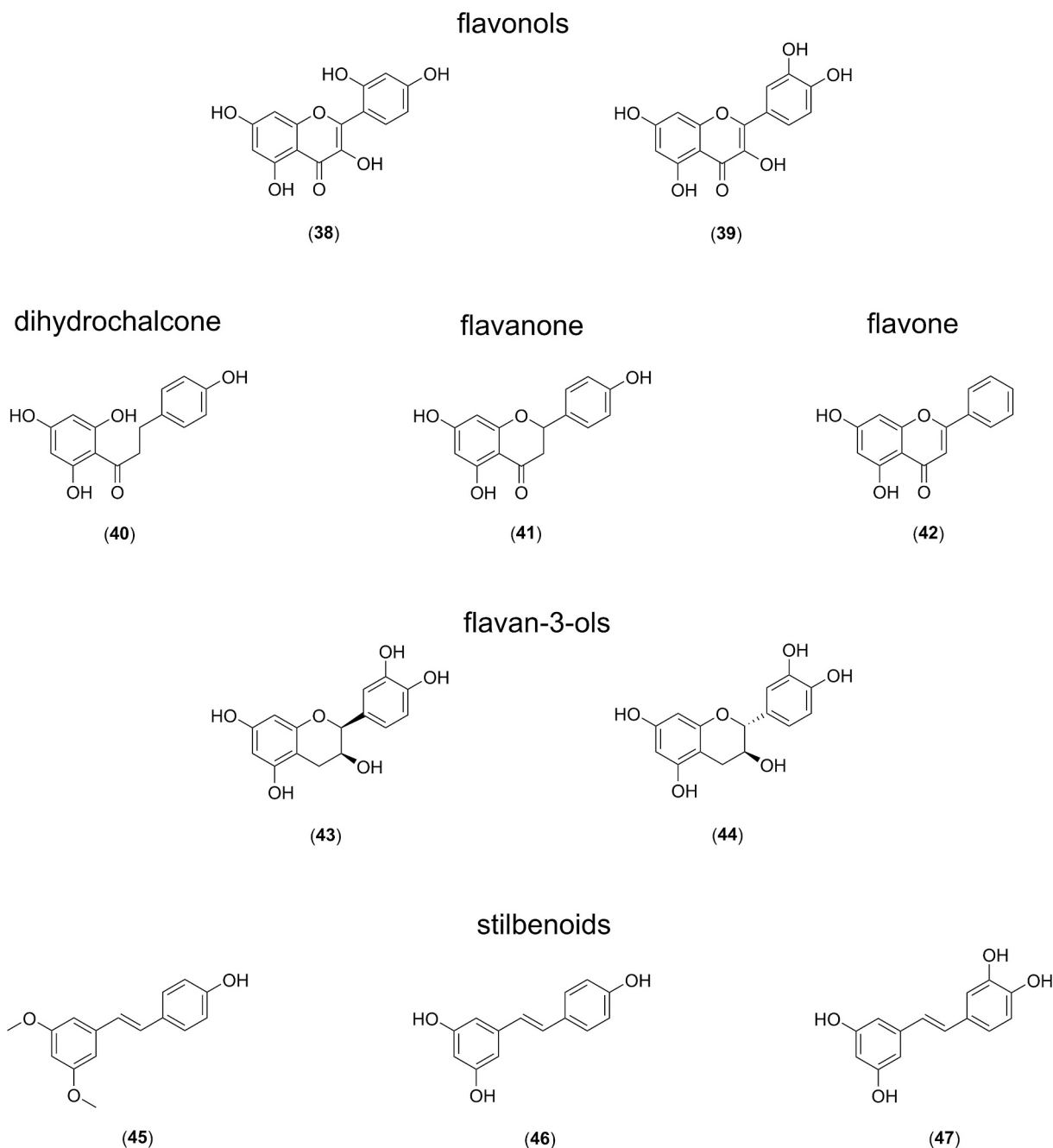


Fig. 3. Chemical structures of the complex phenolics used in this study. The complex phenolics studied were: morin (38), quercetin (39), phloretin (40), naringenin (41), chrysin (42), epicatechin (43), catechin (44), pterostilbene (45), resveratrol (46), and piceatannol (47).

Mean comparisons were carried out with Origin® v. 7.0 (OriginLab Corporation, Northampton, MA). MLR analysis was carried out with R (The R Project for Statistical Computing, The R Foundation, New Zealand). The significance level is  $p < 0.05$  unless otherwise indicated.

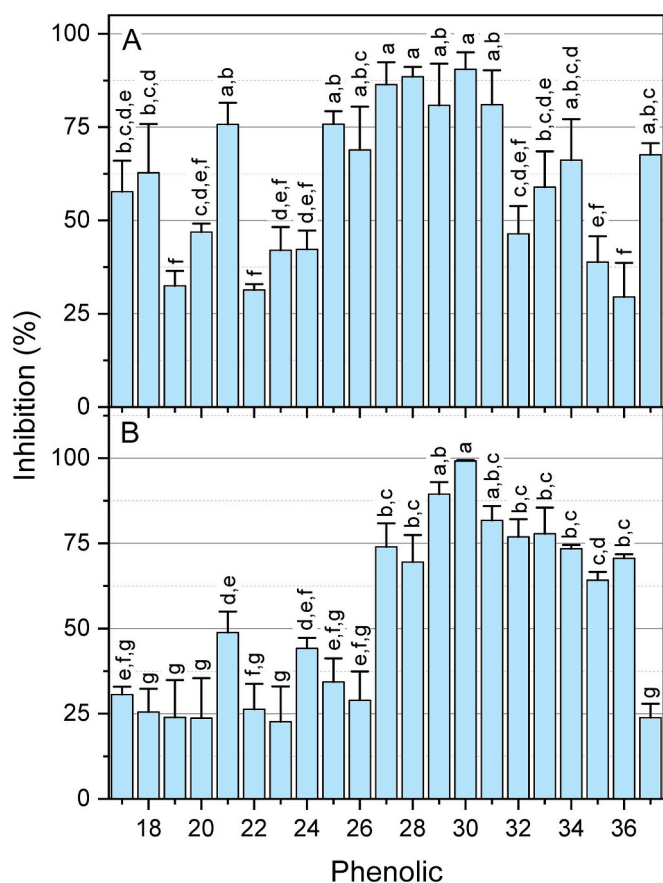
### 3. Results and discussion

#### 3.1. Effect of simple phenolics on the formation of PhIP in mixtures of phenylalanine and creatinine

When phenylalanine and creatinine were heated, PhIP was produced as described previously (Zamora, Alcon, & Hidalgo, 2012). In addition, the amount of produced PhIP decreased when phenolics were also present in the reaction mixtures. However, the protection exhibited by

the different phenolics depended on their structure. Fig. 4A shows the inhibition produced by different simple phenolics. As can be observed, the phenolics that exhibited a higher protection were compounds 21, 25–31, 34, and 37. The compounds with a higher inhibition were those with the letter 'a' in Fig. 4A. These compounds exhibited the highest inhibitions observed in PhIP formation and their inhibitions were not significantly ( $p < 0.05$ ) different among them.

This group mainly included *m*-diphenols (compounds 27–31), which are difficultly oxidized. Table S3 of the Supporting Information shows the oxidability of the different phenolics assayed in this study. As can be observed, all *o*-diphenols exhibited an increase of absorbance at 420 nm  $\geq 0.02$  units/min. On the other hand, the oxidability of *m*-diphenols was much lower ( $< 0.006$  units/min). The absolute values collected in Table S3 of the Supporting Information do not have a direct relationship



**Fig. 4.** Inhibition of the formation of: A, PhIP; and B; MeIQx, by simple phenolics in model systems. The simple phenolics studied were: catechol (17), 4-methylcatechol (18), 3,4-dihydroxybenzoic acid (19), methyl 3,4-dihydroxybenzoate (20), 1,2,4-trihydroxybenzene (21), 3-(3,4-dihydroxyphenyl)propanoic acid (22), caffeic acid (23), methyl caffeate (24), ferulic acid (25), methyl ferulate (26), resorcinol (27), 2-methylresorcinol (28), orcinol (29), phloroglucinol (30), 2,5-dimethylresorcinol (31), 2,6-dihydroxybenzoic acid (32), pyrogallol (33), gallic acid (34), methyl gallate (35), propyl gallate (36), and 3-methoxycatechol (37). Chemical structures of tested phenolics are given in Fig. 2. Means with different letters are significantly ( $p < 0.05$ ) different.

with the ability of the different phenolics to produce quinones because the molar absorptivities of the produced quinones are different for the different phenolics and no attempts were taken to determine these molar absorptivities. However, the assayed phenolics that exhibited a lower inhibition of PhIP formation were easily oxidized. The same occurred with the inhibition of MeIQx formation, as discussed in Section 3.2.

Because of the low oxidability of compounds 27–31, they did not promote the formation of the phenylacetaldehyde needed to produce PhIP (Fig. 1). In addition, and as discussed in a previous study (Hidalgo, Navarro, & Zamora, 2017), *m*-diphenols usually have high carbonyl-trapping abilities. Therefore, they can trap the phenylacetaldehyde required for the formation of PhIP and the formation of this HAA was inhibited. Moreover, compounds 21 and 34 (which also have two hydroxyl groups in *meta* position) also exhibited a high inhibition. On the other hand, compounds 25 and 26 do not have two hydroxyl groups in *meta* but they exhibited a high inhibition. This effect might be related to the trapping ability described for their decarboxylated derivatives (Bork et al., 2024). Differently to all these compounds, compound 37 also exhibited a high inhibition, but it is mainly an *o*-diphenol. The reason for this observed inhibition is unclear at present. In addition, compound 32 exhibited a limited inhibition, but it is a *m*-diphenol. It is the only exception observed among assayed *m*-diphenols. This limited inhibition should be related to the presence of the carboxylic group, which might

cause a steric hindrance for the carbonyl trapping.

Although *o*-diphenols have a more limited ability to trap carbonyls than *m*-diphenols, they still exhibited a significant inhibition of PhIP formation. The reason should be related to the employed model system. PhIP formation requires the participation of phenylacetaldehyde (Fig. 1), and this compound is also produced through free radical degradation of phenylalanine. Therefore, the recognized free radical scavenging function of *o*-diphenols should be related to the observed inhibition, because if phenylacetaldehyde formation is inhibited, PhIP formation is also inhibited. On the other hand, *o*-diphenols are easily oxidized to produce quinones as shown in Table S3 of the Supporting Information, and quinones can also convert phenylalanine into phenylacetaldehyde, as shown in Fig. 1. Therefore, the different oxidability of assayed phenolics is also likely playing a role in observed results.

### 3.2. Effect of simple phenolics on the formation of MeIQx in mixtures of threonine and creatinine

When phenylalanine was changed by threonine, the formation of MeIQx was produced in the place of PhIP. The reason is that threonine degradation produces the acrolein needed to form MeIQx, as shown in Fig. 1, and not the phenylacetaldehyde required for the formation of PhIP. In this case, the difference among the behaviors of the different phenolics was much higher than that observed for PhIP formation in Fig. 4A. As shown in Fig. 4B, the most potent inhibitors were compounds 27–36. All these compounds were the assayed *m*-diphenols. On the other hand, the other assayed phenolics exhibited a lower inhibition. This suggests that the ability of *m*-diphenols to trap the acrolein produced from threonine is more relevant for MeIQx formation than other phenolic functions.

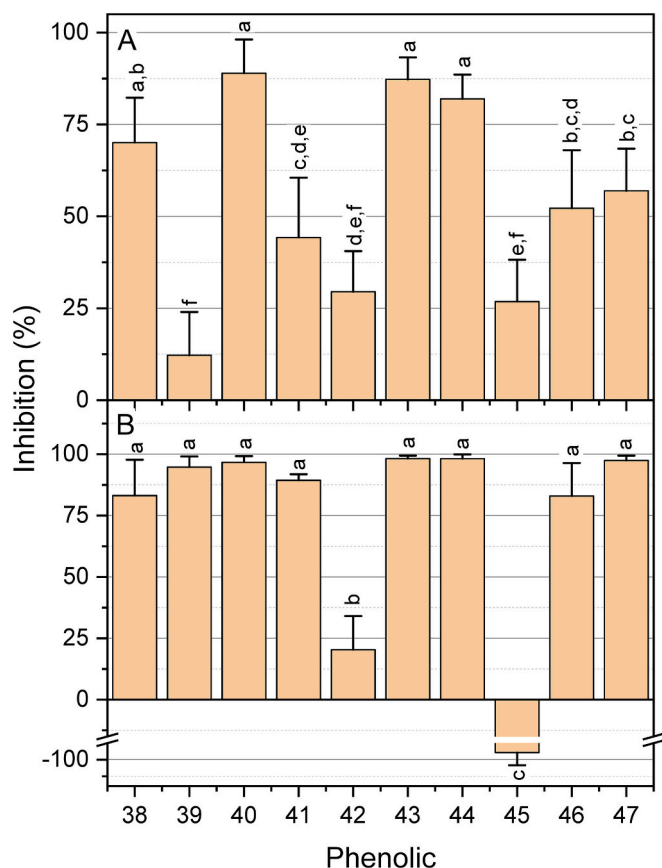
The observed inhibitions for the assayed phenolics shown in Fig. 4A and B are different. In fact, obtained inhibitions were only very slightly correlated ( $r^2 = 0.2$ ,  $p = 0.04$ ). This is likely a consequence of the different ability of phenolics for trapping either saturated aldehydes, like phenylacetaldehyde, or unsaturated aldehydes, like acrolein. As shown in previous studies (Hidalgo, Aguilar, & Zamora, 2017; Hidalgo & Zamora, 2014), the reaction of unsaturated aldehydes with *m*-diphenols occurs at a lower temperature and more rapidly than the reaction of saturated aldehydes with *m*-diphenols. For that reason, when acrolein is produced, it should be rapidly removed by *m*-diphenols and the formation of MeIQx inhibited. In the case of phenylacetaldehyde, the reaction with *m*-diphenols is produced more slowly. Therefore, other reactions may occur.

### 3.3. Effect of complex phenolics on the formation of PhIP in mixtures of phenylalanine and creatinine

When simple phenolics were replaced by complex phenolics, the observed effects were more complex because these phenolics have several *o*- and/or *m*-diphenol groups in their structure, in addition to other groups that can also play a role in the protection of the formation of HAAs.

Fig. 5A shows the effect of the assayed complex phenolics on PhIP inhibition in model systems involving phenylalanine. As can be observed, the phenolics that exhibited the strongest inhibition were morin (38), phloretin (40), epicatechin (43), and catechin (44). On the other hand, the lowest inhibition was observed with quercetin (39), chrysin (42), and pterostilbene (45).

Morin (38) is a flavonol with two *m*-diphenol moieties and no *o*-diphenol group. Therefore, it should have a high carbonyl trapping ability and a low oxidability (as observed in Table S3 of the Supporting Information) that justifies the high inhibition observed. Differently to morin (38), although quercetin (39) is also a flavonol and has a similar structure, it has one *m*- and one *o*-diphenol group. This structural change increased the oxidability of quercetin (see Table S3 of the Supporting Information) and decreased its inhibition ability for PhIP formation



**Fig. 5.** Inhibition of the formation of: A, PhIP; and B, MeIQx, by complex phenolics in model systems. The complex phenolics studied were: morin (38), quercetin (39), phloretin (40), naringenin (41), chrysin (42), epicatechin (43), catechin (44), pterostilbene (45), resveratrol (46), and piceatannol (47). Chemical structures of tested phenolics are given in Fig. 3. Means with different letters are significantly ( $p < 0.05$ ) different.

(Fig. 5A).

Phloretin (40) is a dihydrochalcone with three hydroxyl groups in *meta* position. Its structure is, in this sense, similar to that of phloroglucinol (30), which was found to exhibit a high inhibiting power (Fig. 4A). Therefore, phloretin (40) should be expected to exhibit also a high inhibition, as it was observed experimentally. When the C-ring of phloretin (40) was closed, the dihydrochalcone phloretin (40) was converted into the flavanone naringenin (41). This closure of the C-ring implies the loss of one hydroxyl group. Therefore, a lower inhibitory efficiency of naringenin (41) than that of phloretin (40) should be expected, as it was found. When the conjugation increased from the flavanone naringenin (41) to the flavone chrysin (42), the inhibitory activity decreased. In fact, the inhibitory activity exhibited by chrysin (42) was one of the lowest inhibitory activities determined among the studied phenolics. Differently to naringenin (41), the structure of chrysin (42) is planar and highly conjugated, which is likely contributing to the low inhibitory activity observed.

The flavan-3-ols epicatechin (43) and catechin (44) exhibited a high inhibitory power. These compounds have one *m*-diphenol group, but also one *o*-diphenol group, which make them easily oxidized, analogously to quercetin (39) (see Table S3 of the Supporting Information). However, the absence of an  $\alpha,\beta$ -unsaturated carbonyl group in the C-ring of epicatechin (43) and catechin (44), in comparison with quercetin (39), is likely playing a role in the observed inhibitory effect. Thus, in quercetin (39), the molecule is planar and the produced radicals are conjugated, and it does not occur in either epicatechin (43) or catechin (44).

Stilbenoids did not show a potent inhibitory effect in the assayed systems. The protection exhibited by piceatannol (47) was similar to that of resveratrol (46). Both phenolics have a *m*-diphenol group, which can trap phenylacetaldehyde. On the other hand, piceatannol (47) has also a *o*-diphenol group in the B-ring that is absent in resveratrol (46). This suggests that the presence of the *o*-diphenol group, which increases the oxidability of this phenolic (see Table S3 of the Supporting Information), does not play a major role in the PhIP inhibition observed for piceatannol. On the other hand, the *m*-diphenol group seemed to be very important because the substitution of the hydroxyl groups in resveratrol (46) by methoxy groups in pterostilbene (45) reduced considerably the inhibitory power of this last compound. As described previously, the substitution of a hydroxyl group by a methoxy group decreases the carbonyl trapping ability of the corresponding phenolic (Hidalgo & Zamora, 2014).

#### 3.4. Effect of complex phenolics on the formation of MeIQx in mixtures of threonine and creatinine

When phenylalanine was replaced by threonine, most complex phenolics exhibited an increased inhibitory activity against MeIQx formation (Fig. 5B) in comparison to that observed for the inhibition of PhIP formation (Fig. 5A). In fact, most of them inhibited almost completely the formation of MeIQx under the employed reaction conditions. As discussed in Section 3.2, *m*-diphenol groups seem to be very efficient acrolein scavengers, and this group is present in most of the complex phenolics assayed. Therefore, observed results should have been expected. The only exception was chrysin (42). As discussed above for the inhibition of PhIP, this compound has a planar highly conjugated structure. In addition, the hydroxyl group at position C3 of morin (38) and quercetin (39) is absent. These structural characteristics should be responsible for the low inhibitory activity observed for this compound.

The phenolic that inhibited less the formation of MeIQx was pterostilbene (45). In fact, this compound promoted the formation of this HAA. The absence of inhibition should be related to the absence of the *m*-diphenol group. In addition, the observed increase in the formation of MeIQx might be related to either the increase in the formation of acrolein under the employed reaction conditions or the promotion of MeIQx formation by means of other alternative mechanisms. Additional studies are needed to fully understand this result.

#### 3.5. The inhibitory effect of phenolic compounds in model systems as a balance between their oxidability and their ability to trap carbonyl compounds

The employed model systems were selected so that PhIP and MeIQx were produced by carbonyl-amine reactions, because the heating of amino acids in the presence of phenolics produced the Strecker aldehydes needed to react with creatinine and to produce PhIP and MeIQx (see Fig. 1). To know if the inhibition observed was the result of a balance between the ability of studied phenolics to promote the formation of aldehydes and their ability to remove them from the reaction mixture, a Multiple Linear Regression analysis was carried out. This study correlated the inhibitions observed for PhIP and MeIQx formation with phenolic oxidability and number of *o*-diphenol groups, both related to the promotion of the formation of the aldehydes needed to produce PhIP and MeIQx, and with the number of hydroxyl groups and the number of *m*-diphenol groups, both related with the ability of removing the aldehydes needed to produce PhIP and MeIQx. The number of hydroxyl groups is related to the ability of removing aldehydes because hydroxyl groups can react with the carbonyl group of phenylacetaldehyde and acrolein. In addition, a higher number of hydroxyl groups usually implies a higher number of nucleophilic aromatic carbons that can also react with the aldehyde. In addition to these four characteristics, the number of carbonyl groups present and the planarity of the structure were also considered. The planarity may be important because it extends

the conjugation and can stabilize the formed phenolic radical.

The values used for the different characteristics of the simple and complex phenolics employed in this study are given in Table S4 of the Supporting Information. Some values can be directly obtained from the structures of the different phenolics shown in Figs. 2 and 3 (numbers of hydroxyl groups, *o*-diphenol groups, *m*-diphenol groups, and carbonyl groups). Because values for oxidability in Table S3 of the Supporting Information are not comparable among them (as discussed above, molar absorptivities of the produced quinones are different among them), two values were taken for phenolic oxidability. A value of 1 was given to oxidizable phenolics with values higher than 0.019 absorbance units/min in Table S3 of the Supporting Information. A value of 0 was given to non-oxidizable phenolics with values lower than 0.006 absorbance units/min in Table S3. Finally, a value of 1 was given to complex phenolics having a planar C-ring. If this ring neither exists nor is planar, a value of 0 was given.

The correlation matrix for both PhIP and MeIQx inhibitions is shown in the Figure S1 of the Supporting Information. As observed in the figure, the inhibition of PhIP was positively correlated with the number of *m*-diphenyl groups and also with the number of hydroxyl groups, which agrees with the removal of phenylacetaldehyde promoted by phenolics with these characteristics. On the other hand, the inhibition of PhIP formation was inversely correlated with the oxidability and with the number of *o*-diphenol groups, which agrees with the promotion of phenylacetaldehyde production by phenolics having these characteristics. In addition, the number of carbonyl groups and the planarity of the phenolic were also inversely correlated with the inhibition observed in PhIP formation, therefore suggesting that the stabilization of the phenolic radical contributes to the production of PhIP, most likely by favoring the formation of the phenylacetaldehyde needed to produce this HAA.

Results obtained for MeIQx were similar, although inhibition of MeIQx was strongly inhibited by both the number of hydroxyl groups and the number of *m*-diphenol groups, which is in agreement with the high trapping ability of acrolein by *m*-diphenols. In fact, as discussed in Section 3.2, the trapping ability of acrolein by *m*-diphenols is much higher than the trapping ability of phenylacetaldehyde by the same *m*-diphenols. In this case, the trapping ability of acrolein was so important for MeIQx inhibition that the other phenolic characteristics played a very limited role.

The use of MLR allowed to obtain predicted PhIP and MeIQx inhibition values that could be compared with the experimental values. As shown in Fig. 6A, predicted values were correlated with experimental values for PhIP inhibition ( $r^2 = 0.54$ ,  $p = 0.0027$ ), and a better correlation was observed for MeIQx inhibition ( $r^2 = 0.62$ ,  $p = 0.0003$ ), which was even much better ( $r^2 = 0.80$ ,  $p < 0.0001$ ) if the value of pterostilbene (45) was not considered. The better correlation obtained for MeIQx is consequence of the better trapping of acrolein than phenylacetaldehyde by *m*-diphenols. Furthermore, if number of carbonyl groups and the planarity of the phenolic were not considered, predicted and calculated MeIQx inhibition values were also strongly correlated ( $r^2 = 0.57$ ,  $p = 0.0001$ ) with oxidability and carbonyl-trapping ability, and this correlation improved ( $r^2 = 0.69$ ,  $p = 0.0001$ ) if the value of pterostilbene (45) was not considered. These results suggested that the initial hypothesis was mostly correct and the role of phenolics in the inhibition of the formation of the studied HAAs is related to the balance between their oxidability and their ability to trap carbonyls. In the case of aldehydes that are more difficulty trapped by phenolics, additional phenolic characteristics should also be considered.

The better inhibition of MeIQx than PhIP formation by phenolics was previously observed (Khan et al., 2022; Natale, Gibis, Rodriguez-Estrada, & Weiss, 2014), but, to the best of our knowledge, this observation is satisfactorily explained for the first time in the present study. Furthermore, no previous study pointed out the significant role of phenolic oxidability in the reduction of phenolic inhibitory power, and confusing results were obtained when phenolic inhibitory abilities were

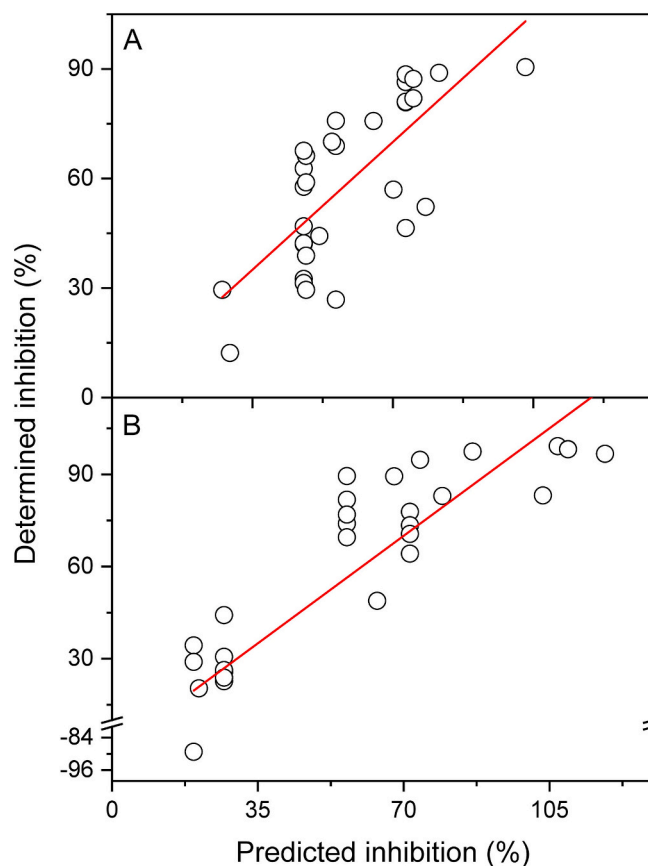
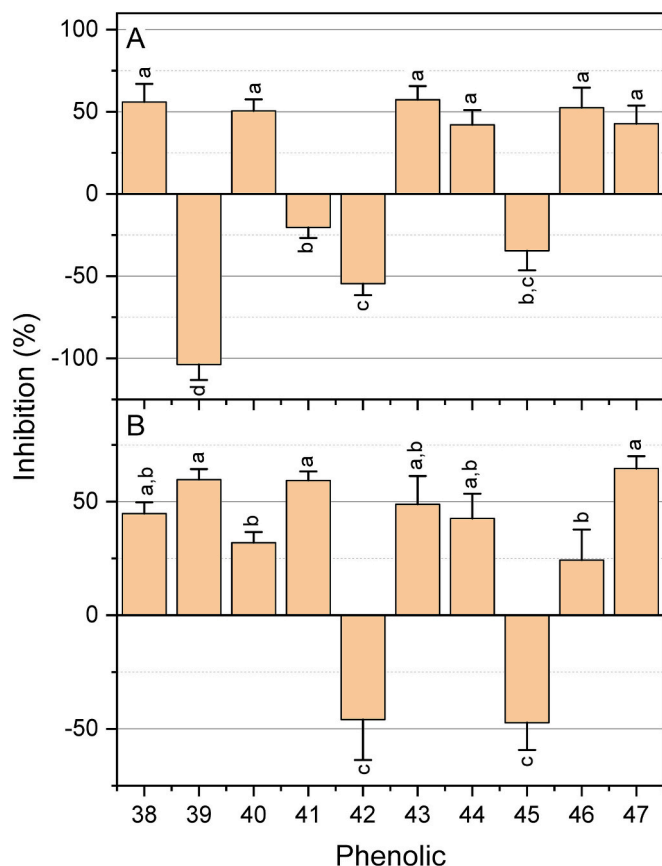


Fig. 6. Correlation between predicted and experimental values of the inhibition produced in model systems by simple and complex phenolics for: A, PhIP; and B, MeIQx.

compared with their antioxidant capabilities (Zhou et al., 2024). On the other hand, a positive correlation between phenylacetaldehyde trapping and PhIP mitigation was previously described (Cheng et al., 2009; Wang, Cheng, Zhang, & Kang, 2021).

### 3.6. Effect of complex phenolics on the formation of PhIP in beef patties

The effect of the addition of phenolic compounds and phenolic extracts to different types of meats has been the objective of numerous studies in which the inhibition, and also the promotion, of the different heterocyclic aromatic amines has been observed (Khan et al., 2022; Zhang et al., 2023). When the complex phenolics assayed in the model systems described in Sections 3.3 and 3.4 were added to beef patties, the role of phenolics on PhIP formation (Fig. 7A) seemed to be different to that observed in the model systems involving phenylalanine (Fig. 5A). In fact, four of the assayed phenolics [quercetin (39), naringenin (41), chrysin (42), and pterostilbene (45)] promoted the formation of PhIP in beef patties, which was not observed in model systems. The reason for this promotion should be related to the increased complexity of beef patties in relation to assayed model systems. In beef patties, phenylacetaldehyde can be produced by an increased number of routes in addition to those existing in the more simplified model systems employed in this study. In fact, the presence of metal ions in beef is expected to increase the free radical degradation of phenylalanine into phenylacetaldehyde (Xu & Yin, 2024). Furthermore, the contribution of free radicals to PhIP formation might not be discarded. Nevertheless, the compounds that promoted the formation of PhIP in beef patties (Fig. 7A) were also the compounds that exhibited a lower protective effect in model systems (Fig. 5A). In fact, the inhibitions found for the assayed phenolics in model systems were correlated ( $r^2 = 0.80$ ,  $p < 0.0001$ ) with

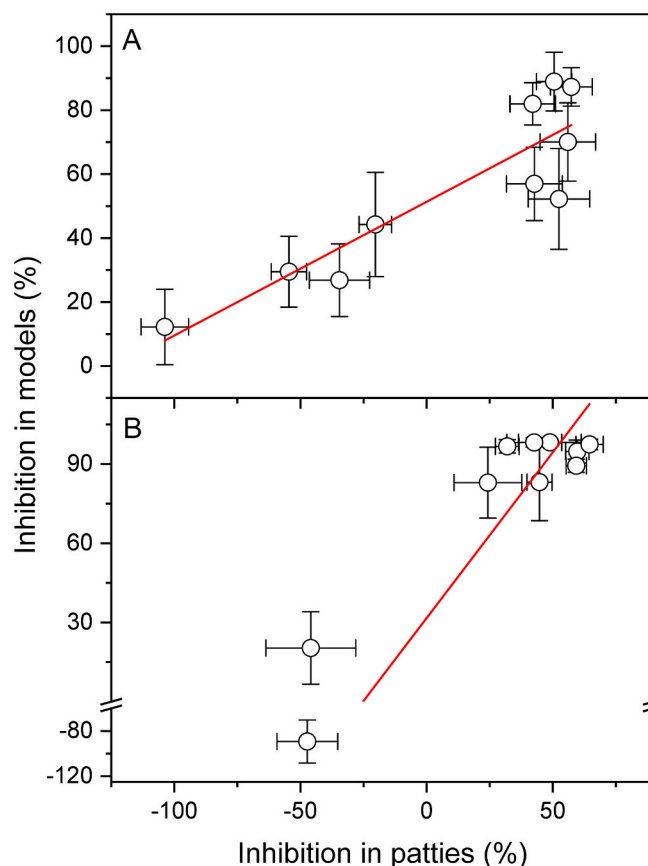


**Fig. 7.** Inhibition of the formation of: A, PhIP; and B, MeIQx, by complex phenolics in beef patties. The complex phenolics studied were: morin (38), quercetin (39), phloretin (40), naringenin (41), chrysin (42), epicatechin (43), catechin (44), pterostilbene (45), resveratrol (46), and piceatannol (47). Chemical structures of tested phenolics are given in Fig. 3. Means with different letters are significantly ( $p < 0.05$ ) different.

the inhibitions found for the same assayed phenolics in beef patties (Fig. 8A). Because the formation of PhIP in model systems occurs by carbonyl-amine reactions according to the pathway shown in Fig. 1, the found correlation between model systems and beef patties suggests that this mechanism is likely the main mechanism responsible for PhIP formation also in beef patties.

### 3.7. Effect of complex phenolics on the formation of MeIQx in beef patties

Differently to PhIP, most previous studies considered a free radical origin for MeIQx (Pearson et al., 1992) and, therefore, its mitigation by phenolics was tried to be explained as a consequence of free radical scavenging (Li et al., 2023; Yang et al., 2023). However, results obtained in this study showed that something similar to that found for the inhibition of PhIP in beef patties (Fig. 7A) was also found for the inhibition of MeIQx (Fig. 7B). Thus, and analogously to that observed in model systems (Fig. 5), more phenolics inhibited the formation of MeIQx than the formation of PhIP. As discussed above, the easier ability of phenolics to trap acrolein than phenylacetaldehyde is likely responsible for obtained results. Only two, among the assayed phenolics, promoted the formation of MeIQx in beef patties. They were chrysin (42) and pterostilbene (45), which also were the same phenolics that exhibited the lowest inhibition in model systems (Fig. 5B). When determined inhibitions in model systems and beef patties were plotted, a correlation was observed ( $r^2 = 0.75$ ,  $p = 0.0003$ ) (Fig. 8B), but this correlation was worse than that observed for PhIP inhibition (Fig. 8A). This is likely due to the different formation mechanisms existing for phenylacetaldehyde



**Fig. 8.** Correlation of the inhibition of the formation of: A, PhIP; and B, MeIQx, by complex phenolics in both model systems and beef patties.

and acrolein. As discussed above, phenylacetaldehyde is mainly produced from phenylalanine. Therefore, the employed model system may be a good way to model PhIP formation in beef patties. On the other hand, there are many alternative pathways for producing acrolein in beef patties in addition to that employed in the assayed model system. Nevertheless, the phenolics that exhibited a lower inhibition in model systems were also the same phenolics that exhibited a lower inhibition in beef patties. This agrees with a major role also in beef patties of the carbonyl-amine reaction pathway for the formation of MeIQx shown in Fig. 1.

## 4. Conclusions

PhIP and MeIQx were formed in model systems by heating creatinine with phenylalanine and threonine, respectively. The formation of these heterocyclic aromatic amines was inhibited by different simple and complex phenolics, but observed inhibition depended on phenolic structure. Among the simple phenolics, *m*-diphenols were usually more effective than *o*-diphenols, and this higher effectivity was especially relevant for MeIQx, most likely because of the better *m*-diphenol-trapping ability for 2-alkenals –such as the acrolein responsible for MeIQx formation–, than for alkanals –such as the phenylacetaldehyde responsible for PhIP formation–.

When complex phenolics were employed, other groups present in the phenolic structure also played a major role in the inhibitory properties observed for these compounds, in addition to those due to the presence of *o*- and *m*-diphenol groups. However, the inhibition produced by the different assayed phenolics could be modelled as a balance between their oxidability and their ability to trap carbonyl compounds, and this modeling was especially satisfactory for MeIQx inhibition. In addition, the relative inhibitions of both PhIP and MeIQx by these complex

phenolics in the assayed model systems were similar to those determined in beef patties. In fact, both inhibitions were correlated. Because the formation of PhIP and MeIQx in the designed model systems occurs by carbonyl-amine reactions, the found correlation between model systems and beef patties suggests that the formation of the studied HAAs in beef patties also occurred by carbonyl-amine reactions.

### CRedit authorship contribution statement

**Angye D. Caro-Cabarcas:** Writing – review & editing, Validation, Resources, Methodology, Investigation, Data curation. **Esmeralda Alcon:** Writing – review & editing, Validation, Resources, Methodology, Investigation, Data curation. **María Salomé Mariotti-Cellis:** Writing – review & editing, Supervision, Funding acquisition. **Franco Pedreschi:** Writing – review & editing, Supervision, Funding acquisition. **Francisco J. Hidalgo:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Project administration, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Rosario Zamora:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

### Acknowledgments

Grant PID2022-137100OB-I00 funded by MICIU/AEI/10.13039/501100011033 and by “ERDF A way of making Europe”. Authors are also indebted to CSIC (Grant 2022AEP022) and to Francisco J. Hidalgo-Zamora, MSc, for the MLR study.

### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2025.144091>.

### Data availability

Data will be made available on request.

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