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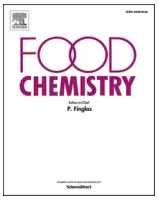

Carbonyl-trapping by phenolics and the inhibition of the formation of carcinogenic heterocyclic aromatic amines with the structure of aminoimidazoazaarene in beef patties

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2-Amino-3,4-dimethyl-3*H*-imidazo[4,5-*f*]quinoline (MeIQ) (PubChem ID: 62274)
2-Amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx) (PubChem ID: 62275)
2-Amino-3-methyl-3*H*-imidazo[4,5-*f*]quinoline (IQ) (PubChem ID: 53462)
2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) (PubChem ID: 1530)
Phenylacetaldehyde (PubChem ID: 998)

ABSTRACT

Carcinogenic heterocyclic aromatic amines (HAAs) with the structure of aminoimidazoazaarene (PhIP, MeIQx, IQ, and MeIQ) are produced by reaction of creatin(in)e, ammonia, and reactive carbonyls (phenylacetaldehyde, acrolein, and crotonaldehyde). In an attempt to provide efficient methodologies for HAA reduction in beef patties, this study: identified phloroglucinol as the most efficient phenolic to reduce HAA formation (76–96% inhibition); isolated and characterized by NMR and MS phloroglucinol/phenylcetaldehyde and phloroglucinol/acrolein adducts; and determined by LC-MS/MS adduct formation in beef patties treated with phloroglucinol. Obtained results suggested that addition of trihydroxyphenols (including phloroglucinol) to beef patties should decrease HAA formation. This was confirmed by both immersing beef patties in apple (or pear) juice before cooking (>90% inhibition) and including wheat bran in patty recipe. All these results confirm the key role of reactive carbonyls in the formation of carcinogenic HAAs and propose carbonyl-trapping as a way for controlling HAA formation in food products.

1. Introduction

Many foods can be consumed in a raw form, but, most frequently, they are submitted to processing. This processing has numerous benefits (van Boekel et al., 2010). On the other hand, it is not free of risks. Thus, in addition to the loss of certain nutrients (Ochieng et al., 2022), the formation of harmful substances with potential mutagenic and carcinogenic properties should be pointed out (Shabbir, Raza, Anjum, Khan, & Suleria, 2015). Among others, the formation in food products of toxic acrylamide (Gazi, Tas, Gorgulu, & Gokmen, 2023), furan (Kim, Park, Moon, Lee, & Kim, 2023), acrolein (Qiu, Lu, Gu, Jia, Wang, Zhang, Lv, 2022), chloropropanols (Eisenreich, Monien, Gotz, Buhrke, Oberemm, Schultrich, Abraham, Braeuning, & Schafer, 2023), and heterocyclic aromatic amines (HAAs) (Nawaz, Shi, Irshad, Suo, Wang, Bi, Wang, Chen, & Cheng, 2023) has received a considerable attention in recent years.

In relation to HAAs, recent studies have described the formation pathways for 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-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) (Zamora, Alcon, & Hidalgo, 2014), which are the carcinogenic HAAs produced to a higher extent in proteinaceous foods upon processing (Meurillon & Engel 2016). These compounds have been suggested to be produced by reaction of specific reactive carbonyls (phenylacetaldehyde for PhIP, acrolein for MeIQx and IQ, and crotonaldehyde for MeIQ and IQ) with ammonia and creatin(in)e (Hidalgo & Zamora, 2022). Therefore, and considering that ammonia and creatin(in)e either are present or are easily produced in proteinaceous foods (Li, Xie, Bai, Wang, Zhou, Gao, Xu, & Zhao, 2022), the inhibition of the formation of these HAAs should be produced if required reactive

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carbonyls are controlled in some way: by inhibiting their formation, by using conditions that limit their reactivity, or by promoting their trapping (Zamora & Hidalgo, 2020).

Among these inhibitory ways, carbonyl-trapping is promising because reactive carbonyls are electrophiles and react easily and rapidly with food nucleophiles, including amines and phenolics. Thus, carbonyl–amine reactions have long been known to play a major role in food safety and quality (Zamora & Hidalgo, 2005). On the other hand, carbonyl-phenol reactions are lesser known (Zamora & Hidalgo, 2016). However, phenolics have been shown to trap the reactive carbonyls responsible for HAA formation (Hidalgo & Zamora, 2014). In addition, they are nowadays usually employed as additives to maintain food quality (Rangaraj, Rambabu, Banat, & Mittal, 2021). Therefore, their use should contribute to reduce HAAs formation. This has been the objective of diverse studies with positive results (see, for example, Vidal, Manful, Pham, Wheeler, Stewart, Keough, & Thomas, 2020). However, not all phenolics have the same carbonyl-trapping abilities, and if phenolics are not well selected, results can be different to those desired (Hidalgo, Navarro, & Zamora, 2018).

The hypothesis of this study is that if a meat is treated with phenolics having a high trapping ability for phenylacetaldehyde, acrolein, and crotonaldehyde, the formation of PhIP, MeIQx, MeIQ, and IQ should be greatly reduced, and the corresponding carbonyl-phenol adducts, responsible for the observed reduction, should be produced and detected. Therefore, the objectives of this study were: firstly, the identification of the phenolic with the highest HAA inhibition ability in beef patties; secondly, the isolation and characterization of carbonyl-phenol adducts produced between the selected phenolic and the reactive carbonyls responsible for HAA formation and their later determination in beef patties treated with the phenolic; and finally, the proposal, and later confirmation, of some food additives, rich in the identified (or analogous) phenolic, which can be appropriate for the reduction of the formation of HAAs in beef patties.

2. Materials and methods

2.1. Materials

Lean beef meat, wheat bran, apples (*Malus domestica*, cv. Granny Smith), and pears (*Pyrus communis*, cv. Conference) were obtained from local markets. For juice preparation, fruits were cut into pieces with a knife. Then, a smooth purée was obtained by using an electric fruit chopper. Finally, juice was expressed from the purée by centrifugation at 10,000 *g* for 30 min and later vacuum-filtering through Whatman no. 1 paper.

Deffated wheat bran was obtained from wheat bran as described previously (Zamora & Hidalgo, 2022). Briefly, wheat bran (50 g) was stirred with 400 mL of hexane for 15 min at room temperature, then sonicated for 30 min, and, finally, stirred for another 15 min. The hexane was removed by vacuum-filtering through Whatman no. 1 paper.

Defatted and extracted wheat bran (a defatted wheat bran with a reduced alkylresorcinol content) was obtained by extracting with acetone (900 mL) the defatted wheat bran (obtained from 50 g of wheat bran). This extraction was carried out by successively stirring for 15 min, sonicating for 45 min, and new stirring for 15 min. The defatted and extracted wheat bran residue was vacuum-filtered through Whatman no. 1 paper.

The alkylresorcinol fraction was obtained by taken to dryness with a rotary evaporator the acetone solution obtained after extracting the defatted wheat bran. The obtained residue was dissolved in 50 mL of methanol that was heated at 37 °C, briefly sonicated, and centrifuged at 7500 *g* for 10 min at 20 °C. The obtained supernatant was maintained overnight in the freezer at –23 °C and alkylresorcinols precipitated. The obtained mixture was centrifuged for 10 min at 7500 *g* and –10 °C. Finally, the supernatant was removed and the precipitated was dried by using a slow current of nitrogen.

PhIP, MeIQx, IQ, and MeIQ were obtained from Toronto Research Chemicals Inc. (North York, Ontario, Canada). Phloroglucinol, phenylacetaldehyde, and acrolein 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).

2.2. Preparation and cooking of beef patties

All patties used in this study were prepared similarly, although small differences were included depending on the treatment (see below). Lean beef meat was grinded by using a meat grinder, and grinded meat (containing 5 g of meat and the indicated additive, see below) was formed into patties of 35 mm diameter and 5 mm thickness. Patties were cooked in a Teflon-coated electric griddle at 190 °C for 2.5 min per side. After heating, the patties were minced and 10 mL of 1 mol/L NaOH was added (Shin et al., 2003). Then, a smooth purée was obtained by using an ultra-turrax® disperser and 10 mL of 1 mol/L HCl was added. The mixture was homogenized again and 50 μL of internal standard (6.45 μg of caffeine in 10 mL of water) was added. This mixture was extracted twice with 50 and 30 mL of ethyl acetate, respectively, centrifuged at 7500 *g* for 15 min, and filtered through Whatman no. 1 paper. The joined organic layer (ethyl acetate) was evaporated by using a rotary evaporator and the residue was treated first with 400 μL of acetonitrile and then with 100 μL of 30 mM ammonium formate. The obtained solution was centrifuged at 16,000 *g* for 15 min and studied by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS).

Patties for the study of the effect of phenolic compounds contained 5 g of meat and 50 mg of the phenolic compound.

Patties for the study of the effect of fruit juices were partially immersed in 10 mL of either water (control) or juice at 4 °C for 20 h in a Petri dish of 7 cm diameter. The control water contained citric acid so that the pH of employed water and the pH of the juice were the same (pH ~ 3.5).

Patties for the study of the effect of wheat bran contained 5 g of meat and either 500 mg of wheat bran (or wheat bran fractions) or 50 mg of alkylresorcinols.

2.3. Determination of PhIP, MeIQx, IQ and MeIQ in beef patties

PhIP, MeIQx, IQ, and MeIQ were determined by LC-MS/MS following a procedure similar to that previously described for PhIP determination (Zamora, Alcon, & Hidalgo, 2012). The equipment consisted of an Agilent liquid chromatography system (1200 Series, Agilent Technologies, Santa Clara, CA) connected to a triple-quadrupole API 4500 mass spectrometer (SCIEX, Framingham, MA) using an electrospray ionization interface in positive ionization mode (ESI+). Separations were carried out on a Zorbax Eclipse XDB-C18 (150 mm × 4.6 mm, 5 μm) column from Agilent. The mobile phase was delivered at 0.5 mL/min using 30 mmol/L ammonium formate and acetonitrile as eluents A and B, respectively. These eluents were employed for gradient elution with the following program: 0–15 min, 5–50% B; 15–17 min, 50–100% B; 17–20 min, 100% B; 20–22 min, 100–5% B; and then isocratic reconditioning for 5 min. Acquisition of the mass spectrometric data was performed by using multiple reaction monitoring (MRM). Nitrogen was used for collision gas, curtain gas, and carrier gas. Ion spray voltage was set to 1.5 kV and capillary temperature was set to 700 °C.

Three transitions were acquired for the identification of all determined compounds. To establish the appropriate MRM conditions, mass spectrometric conditions were optimized by 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 S1 of the Supporting Information. The transition in bold for each compound was used for quantification purposes.

Quantification of HAAs was carried out by preparing standard curves of the different compounds in cooked beef patties, and following the whole extraction procedure described above. For each curve, six different concentration levels of the several HAAs were used. HAA contents were directly proportional to the HAA/internal standard area ratios ($r > 0.98$, $p < 0.001$). The coefficients of variation at the different concentrations were always < 15%.

2.4. Preparation of phloroglucinol/phenylacetaldehyde adducts

Forty mixtures of phloroglucinol (50 μmol in 50 μL of methanol), phenylacetaldehyde (50 μmol in 60 μL of ethanol), and 30 μL of 0.3 mol/L sodium phosphate, pH 6, were singly homogenized with 0.063–0.200 mm silica gel (200 mg) (Macherey-Nagel, Düren, Germany). Reaction mixtures were heated at 60 °C for 18 h. Phenylacetaldehyde disappearance during this heating is shown in Figure S1 of the Supporting Information. At the end of the heating time, only 3% of the initial phenylacetaldehyde was present. After cooling, each mixture was treated with 1 mL of ethyl acetate, stirred for 1 min, and the liquid was filtered. All mixtures were pooled, taken to dryness, and the residue dissolved in 30 mL of methanol. The solution was centrifuged at 16,000 *g* for 10 min and produced adduct was isolated by semi-preparative high-performance liquid chromatography.

This reaction mainly produced one adduct. This adduct was isolated on an Agilent Technologies 1260 Infinity II liquid chromatograph (Agilent Technologies, Santa Clara, CA) composed by a G7129A autosampler, a G1311C quaternary pump, a G4212 diode array detector, and a G1346F fraction collector. Samples (400 μL) were fractionated on a Zorbax Eclipse XDB-C18 semi-preparative column (9.4 × 250 mm, 5 μm), from Agilent, held at ambient temperature (22 °C). Phloroglucinol/phenylacetaldehyde adduct was separated at a flow rate of 2.5 mL/min using a gradient of methanol in water. The gradient employed was: 0–3 min, 10% methanol; 3–10 min, 10–50% methanol; 10–25 min, 50–75% methanol; 25–30 min, 75–10% methanol; and, finally, isocratic reconditioning for 5 min. Formed adduct was detected at 280 nm. Fractions containing this adduct were collected and combined. The adduct was characterized by mono- and bi-dimensional nuclear magnetic resonance (NMR) spectroscopy, using the equipment described in section 2.6, and mass spectrometry (MS). In addition, some derivatives were prepared for additional confirmation of its structure. Prepared derivatives were the corresponding trimethylsilyl and acetate derivatives.

Trimethylsilyl derivatives were prepared by dissolving five hundred micrograms of the corresponding adduct in 200 μL of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and heating for 30 min at 60 °C. The produced trimethylsilyl derivative was studied by GC–MS using the equipment and the chromatographic conditions described in section 2.7.

Acetate derivatives were prepared by dissolving five hundred micrograms of the corresponding adduct in 1 mL of dry pyridine and 500 μL of acetic anhydride. The mixture was allowed to react under dark for 20 h at room temperature. After that time, 2 mL of dichlorometane and 2 mL of water were added. The mixture was then stirred for 1 min, and the organic layer was collected and washed 4 times with 2 mL of 5% HCl and once with 2 mL of water. Layers were separated by centrifugation (2000 *g* for 5 min), and the organic layer was studied by gas chromatography coupled to mass spectrometry (GC–MS) using the equipment and the chromatographic conditions described in section 2.7.

2.5. Preparation of phloroglucinol/acrolein adducts

Forty reaction mixtures of phloroglucinol (50 μmol in 50 μL of methanol), acrolein (50 μmol in 50 μL of methanol), and 30 μL of 0.3 mol/L sodium phosphate, pH 6, were singly homogenized with 0.063–0.200 mm silica gel (200 mg) (Macherey-Nagel, Düren, Germany). Reaction mixtures were heated at 100 °C for 1 h. Acrolein disappearance during this heating is shown in Figure S2 of the Supporting Information. At the end of the heating time, only 5% of the initial acrolein was present. After cooling, each mixture was treated with 1 mL of methanol, stirred for 1 min, and the liquid was filtered. All mixtures were pooled and the obtained solution was centrifuged at 16,000 *g* for 10 min and produced adducts were isolated by semi-preparative high-performance liquid chromatography.

This reaction mainly produced three adducts. These adducts were isolated on the same equipment described in section 2.4. This time, 400 μL of samples were fractionated in each injection, the flow rate was 3.0 mL/min, and an isocratic elution was carried out by using 25% acetonitrile in water. Formed adducts were detected at 210 nm. Fractions containing the different adducts were collected and combined. Formed adducts were characterized by mono- and bi-dimensional NMR and MS. In addition, some derivatives were prepared for additional confirmation of its structure. Prepared derivatives were the corresponding trimethylsilyl and acetate derivatives, which were prepared as described in section 2.4.

2.6. Nuclear magnetic resonances (NMR) experiments

NMR spectra were obtained by using a Bruker Advance III spectrometer operating at 500 MHz for protons. Acquisition parameters used for ^{1}H and ^{13}C monodimensional experiments were described previously (Zamora et al., 2016). For helping in structural determinations, bidimensional experiments (mainly COSY, HMQC, and HMBC) were also carried out. All experiments were performed at 22 °C.

2.7. Gas chromatography coupled to mass spectrometry (GC–MS) analyses

GC–MS analyses were carried out in an Agilent 7820A gas chromatograph coupled with an Agilent 5977B mass selective detector (MSD), quadrupole type (Agilent Technologies, Santa Clara, CA). One microliter of sample was injected in the pulsed splitless mode and was fractionated on a fused-silica HP-5MS UI capillary column (30 m length, 0.25 mm inner diameter, 0.25 μm coating thickness). The following conditions were employed: carrier gas, helium (1 mL/min at constant flow); injector, 250 °C; transfer line to mass selective detector, 280 °C; electron ionization (EI), 70 eV; ion source temperature, 230 °C; and mass range, 28–550 amu. The oven was programmed from 50 °C (1 min) to 300 °C at 15 °C/min, and then held at 300 °C for 5 min.

2.8. Determination of phloroglucinol/phenylacetaldehyde and phloroglucinol/acrolein adducts

Adducts were determined in the same analysis that HAAs. Therefore, extraction and chromatographic conditions were described in section 2.3. For MS/MS determination, three transitions were acquired for each adduct. Appropriate MRM conditions were established by using infusion with a syringe pump. Precursor and product ions used for quantification and confirmation purposes, and operating conditions for each compound are summarized in Table S1 of the Supporting Information. The transition in bold for each compound was used for quantification purposes.

Quantification of the different adducts was carried out by preparing standard curves of them in cooked beef patties and following the whole extraction procedure described in sections 2.2 and 2.3. For each curve, six different concentration levels for each adduct were used. Adducts content was directly proportional to the corresponding adduct/internal standard area ratios ($r > 0.97$, $p < 0.001$). The coefficients of variation at the different concentrations were always < 15%.

2.9. Statistical analysis

All data given are mean ± SD values of, at least, three independent experiments. 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 (Snedecor & Cochran, 1980). These comparisons were carried out by using Origin® v. 7.0 (OriginLab Corporation, Northampton, MA). The significance level is $p < 0.05$ unless otherwise indicated.

3. Results and discussion

3.1. Inhibition of PhIP, MeIQx, MeIQ, and IQ formation in beef patties by phenolics

When beef patties were cooked in an electric frying pan, the formation of HAAs was observed. The main HAA produced, among those carcinogenic HAAs studied, was PhIP (11.5 ± 1.0 ng/g) followed by MeIQx (3.7 ± 0.8 ng/g). In addition, IQ and MeIQ were also produced, but to a much lower extent (0.2 ± 0.2 and 0.5 ± 0.2 ng/g, respectively). These values were similar to those described in previous studies [see, for example, Balogh, Gray, Gomaa, & Booren (2000) or Shi, Park, & Park (2003)].

The amounts of HAAs produced decreased considerably when phenolics were added to beef patties before heating. Different phenolics, having different structures, were assayed: catechol, as an *o*-diphenol; orcinol and olivetol, as *m*-diphenols; phloroglucinol, as a trihydroxy derivative with all hydroxyl groups in *m*-position; hydroxyquinone, as a *p*-diphenol; and *p*-benzoquinone, as a quinone (chemical structures for all these phenolics are given in Figure S3 of the Supporting Information). As observed in Fig. 1, all of them inhibited HAA formation to some extent, but some of them were better inhibitors than others.

The best inhibitor was phloroglucinol. It inhibited PhIP formation by 96%, MeIQx by 88%, IQ by 76% and MeIQ by 83%. This high inhibiting power is likely related to its structure. As observed in previous studies, phenolics having hydroxyl groups in *m*-position exhibit the highest carbonyl-trapping abilities because of the nucleophilicity of their aromatic carbons and hydroxyl groups (Salazar, Arámbula-Villa, Hidalgo, & Zamora, 2014). Because phloroglucinol has three hydroxyl groups at *m*-position, their nucleophilicity is high, and therefore, it is expected to trap reactive carbonyls rapidly and to a high extent.

Other assayed *m*-diphenols (orcinol and olivetol) also inhibited the formation of HAAs, but to a lower extent than phloroglucinol. Thus, orcinol and olivetol inhibited PhIP formation by 38–49% and other HAAs were inhibited to a lower extent (12–27%). There was not any significant difference ($p < 0.05$) in the inhibitory power between both of them. This decreased inhibitory power in relation to that of phloroglucinol is likely related to the existence of only two hydroxyl groups at *m*-position.

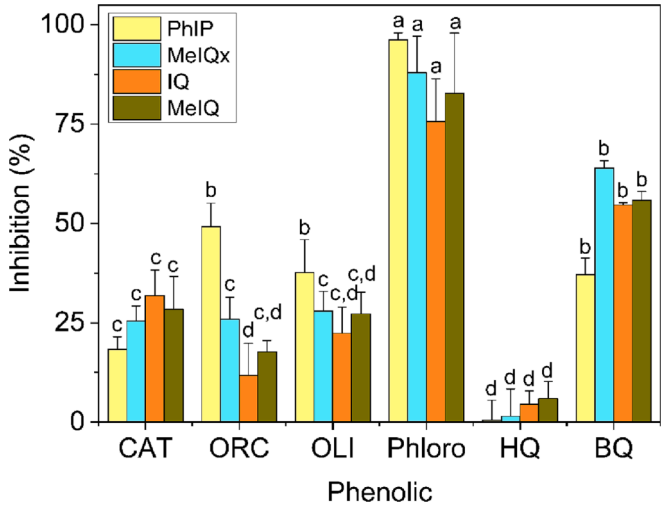

Fig. 1. Inhibiting effect of phenolics on the formation of carcinogenic heterocyclic aromatic amines (HAAs) with the structure of aminoimidazoazaarene in beef patties. Inhibitions with different letters for each determined HAA are significantly different ($p < 0.05$). Abbreviations: CAT, catechol; ORC, orcinol; OLI, olivetol; Phloro, phloroglucinol; HQ, hydroquinone; BQ, benzoquinone.

Benzoquinone also inhibited HAAs formation to a significant extent (37–64%). In fact, it was a better inhibitor than orcinol and olivetol for MeIQx, IQ, and MeIQ, although not for PhIP. However, the mechanism responsible for this inhibition should be different to that above discussed for *m*-diphenols. Differently to *m*-diphenols, quinones are reactive carbonyls (Delgado, Hidalgo, & Zamora, 2016). Therefore, benzoquinone should compete with the reactive carbonyls responsible for HAA formation (phenylacetaldehyde, acrolein, and crotonaldehyde) for the creatinine and ammonia required to produce PhIP, MeIQx, IQ, and MeIQ.

This competition is also likely related to the observed inhibitory effects of catechol (18–32% inhibition) and hydroquinone (1–6% inhibition), although catechol was a better inhibitor than hydroquinone. Both kinds of phenolics can be converted into quinones upon oxidation (Li et al., 2020), and, therefore, they should be able to compete with phenylacetaldehyde, acrolein, and crotonaldehyde for creatinine and ammonia.

3.2. Isolation and characterization of carbonyl-phenol adducts produced in the reaction of phloroglucinol with phenylacetaldehyde and acrolein

Results described in previous section showed that PhIP and MeIQx were the carcinogenic HAAs produced to a higher extent in beef patties. In addition, phloroglucinol was the most powerful phenolic that inhibited almost completely the formation of these HAAs in beef patties. Because PhIP formation requires the participation of phenylacetaldehyde, and MeIQx formation requires the participation of acrolein, the reaction of phloroglucinol with both phenylacetaldehyde and acrolein was studied.

The reaction between phenylacetaldehyde and phloroglucinol produced one main adduct. This adduct was isolated by semipreparative HPLC and characterized by NMR and MS (key NMR signals are collected in Table 1; complete spectroscopic and spectrometric data are given in the Supporting Information). When studied by either GC–MS or LC-MS/MS, the molecular weight of this adduct was 228 amu. This molecular weight results from the addition of one molecule of phloroglucinol and one molecule of phenylacetaldehyde, and the loss of one molecule of water. In addition, this adduct had three free hydroxyl groups, as could be observed by preparing the corresponding trimethylsilyl and acetate derivatives.

The NMR spectra showed that the aldehyde group of the phenylacetaldehyde had disappeared, a carbon–carbon double bond had been produced, and the reaction involved one of the aromatic carbons of the phloroglucinol. The use of bidimensional NMR experiments allowed to identify the formed adduct as 2-styrylbenzene-1,3,5-triol (**4**). This adduct is suggested to be produced as shown in Fig. 2. Thus, the reaction is initiated by addition of one of the aromatic carbons of the phloroglucinol (**1**) to the carbonyl carbon of phenylacetaldehyde (**2**). The addition product (**3**) is finally dehydrated to produce the most stable adduct (**4**).

Differently to the reaction between phloroglucinol and phenylacetaldehyde, the reaction between phloroglucinol and acrolein produced three main adducts, which could be fractionated by semipreparative LC. The first adduct had a molecular weight of 182 amu. This molecular weight corresponded to the addition of the molecular weights of one molecule of phloroglucinol and one molecule of acrolein. In addition, it had three hydroxyl groups as observed when the trimethylsilyl and the acetate derivatives were prepared. The study of the NMR spectra showed that the carbonyl carbon of the acrolein had disappeared as well as its carbon–carbon double bond, which was converted into a carbon–carbon single bond. These modifications in the acrolein structure were a consequence of its reaction with one of the aromatic carbons and one of the hydroxyl groups of the phloroglucinol.

Table 1
Selected NMR signals of pholoroglucinol/phenylacetaldehyde and phloroglucinol/acrolein adducts.

	Adduct (**4**)		Adduct (**7**)		Adduct (**8**)		Adduct (**10**)	
atom	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1		157.54						
1′	7.41 *d*	120.60						
2		104.71	5.39 dd	92.09	5.46 *m*	92.11	5.45 *m*	91.99/92.07
2′	7.47 *d*	127.42						
3		157.54	1.89 *m*	27.32	1.89 *m*	27.12	1.89 *m*	27.11/27.17
3′		140.23						
4	5.92 *s*	94.35	2.61 *m*	14.97	2.61 *m*	15.06	2.64 *m*	15.01/15.12
4′	7.28 *tt*	128.02		100.93		101.75		101.81/101.83
4″								148.38/148.42
5		157.30		155.54		150.59		
5′	7.44 *m*	125.29				101.75		
6	5.92 *s*	94.35	5.91 *d*	94.79	2.61 *m*	15.06	5.45 *m*	91.99/92.07
6′	7.13 *tt*	125.59						
7				156.18	1.89 *m*	27.12	1.89 *m*	27.11/27.17
7′	7.44 *m*	125.29						
8			5.80 *d*	94.73	5.46 *m*	92.11	2.64 *m*	15.01/15.12
8′	7.28 *tt*	128.02		153.94				101.81/101.83
8″								148.38/148.42
9′						153.35		
10					5.89 *s*	95.01	5.45 *m*	91.99/92.07
10′						153.35		
11							1.89 *m*	27.11/27.17
12							2.64 *m*	15.01/15.12
12′								101.81/101.83
12″								148.38/148.42

Solvent was CD_3OD.

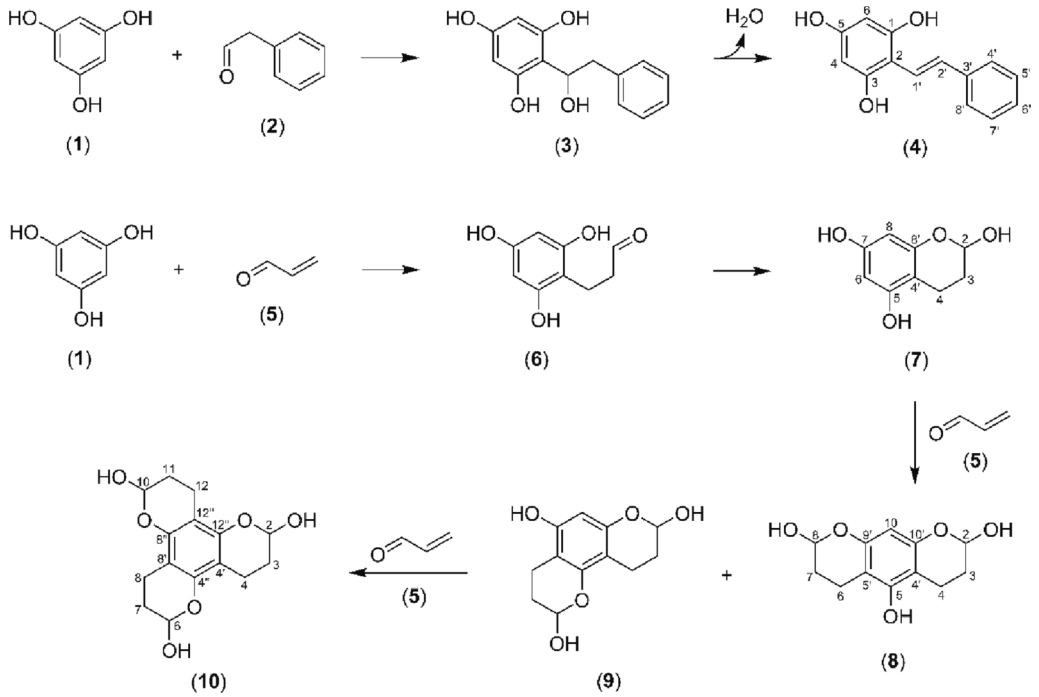

Fig. 2. Proposed pathways for the formation of isolated and characterized phloroglucinol/phenylacetaldehyde adduct (**4**) and phloroglucinol/acrolein adducts (**7**), (**8**), and (**10**).

The use of bidimensional NMR experiments allowed to identify the formed adduct as chromane-2,5,7-triol (**7**). This adduct is suggested to be produced as shown in Fig. 2. One of the aromatic carbons of phloroglucinol (**1**) is added to the carbon–carbon double bond of acrolein (**5**) to produce adduct (**6**). This last adduct is stabilized by forming the hemiacetal (**7**) with one of the hydroxyl groups of phloroglucinol that is contiguous to the aromatic carbon involved in the initial reaction.

Adduct (**7**) still has reactive aromatic carbons that can react with additional molecules of acrolein. This is the reason for the formation of additional phloroglucinol/acrolein adducts. Thus, the second isolated adduct had a molecular weight of 238 amu, which corresponds to the addition of one molecule of phloroglucinol and two molecules of acrolein. Therefore, it should be expected to be produced by addition of adduct (**7**) to a new molecule of acrolein (**5**). As shown in Fig. 2, two possible isomers can be produced: adduct (**8**) and adduct (**9**). Both adducts can be produced similarly by addition of one of the aromatic

carbons of adduct (**7**) to the carbon–carbon double bond of acrolein in a first step. The only difference between them is the hydroxyl group that participates in the formation of the hemiacetal. If the hydroxyl group at position 7 of adduct (**7**) is implied, then adduct (**8**) is produced. On the other hand, if the hydroxyl group at position 5 of adduct (**7**) is implied, then adduct (**9**) is produced. The involved hydroxyl group is important because it will decide the stability of the adduct. Thus, adduct (**9**) still has one aromatic carbon close to a hydroxyl group. Therefore, it is susceptible to further reactions. On the other hand, the free aromatic carbon and the hydroxyl group are too far away in adduct (**8**) to react with a new molecule of acrolein. For that reason, adduct (**8**) was the isolated adduct. Its structure was confirmed by mono- and bi-dimensional NMR experiments. As observed in Table 1, this compound only showed four resonances in ^{1}H NMR and seven resonances in ^{13}C NMR, which agrees with the symmetry of the molecule. Therefore, this adduct was identified as 3,4,7,8-tetrahydro-2*H*,6*H*-pyrano[3,2-*g*]chromene-2,5,8-triol (**8**).

Finally, the third isolated adduct had a molecular weight of 294 amu. This molecular weight corresponded to the addition of one molecule of phloroglucinol and three molecules of acrolein. As shown in Fig. 2, the addition of the third molecule of acrolein only can occur in adduct (**9**) so that the hemiacetal can be produced. The structure of this compound was also confirmed by mono- and bi-dimensional NMR experiments. As observed in Table 1, this compound only showed three resonances in ^{1}H NMR and five resonances in ^{13}C NMR, which agrees with the symmetry of the molecule. Nevertheless, two signals were observed for each resonance in the ^{13}C NMR spectrum, which suggested the existence of isomers. Because the molecule has three chiral carbons, the existence of two stereoisomers can be suggested. Based on spectroscopic and spectrometric data, this adduct was identified as 3,4,7,8,11,12-hexahydro-2*H*,6*H*,10*H*-dipyrano[2,3-*f*:2′,3′-*h*]chromene-2,6,10-triol (**10**).

3.3. Detection of carbonyl-phenol adducts in beef patties treated with phloroglucinol

Fig. 1 showed that, when beef patties were treated with phloroglucinol, the formation of HAAs was much reduced. According to the hypothesis of this study, it should be a consequence of the ability of phloroglucinol to trap the reactive carbonyls responsible for the formation of these HAAs. Previous section has shown that phloroglucinol effectively catches both phenylacetaldehyde and acrolein, and different adducts are produced. Therefore, the analytical determination of these adducts on beef patties treated with phloroglucinol was carried out. As observed in Fig. 3, control beef patties showed the presence of different HAAs (only PhIP and MeIQx are shown to simplify the figure) and the absence of adducts (phloroglucinol is not present in meat). However, when beef patties were treated with phloroglucinol, the amount of HAAs was greatly reduced and the formation of adducts was observed, therefore confirming both that phenylacetaldehyde and acrolein are produced during beef patties cooking and that phloroglucinol can trap them once that they have been produced, which is likely related to the observed decrease in HAA formation.

Quantitative data of HAAs disappearance and adduct formation are shown in Table 2. As can be observed, two different concentrations of phloroglucinol were assayed. Both concentrations produced the almost complete suppression of HAA formation. However, the amount of produced adducts depended on the amount of added phloroglucinol. The adduct produced to a highest extent was adduct (**7**), which agrees with the high content at which acrolein is produced in food products (Liu et al., 2020). However, because phloroglucinol should be present to a higher extent than that at which acrolein is likely produced, only very small amounts of adduct (**8**) and adduct (**10**) were detected. On the other hand, adduct (**4**) was also produced to a high extent, although it was produced to a much lower extent than adduct (**7**). In spite of that, PhIP –the HAA derived from phenylacetaldehyde– was produced to a higher extent than MeIQx –the HAA derived from acrolein–. This should not be surprising because acrolein is more reactive than phenylacetaldehyde and is expected to be involved in more side reactions than phenylacetaldehyde. In addition, the different HAAs are not equally produced. Therefore, a direct correlation between the amounts at which both reactive carbonyls and HAAs are produced should not be expected. However, proteinaceous foods that can produce phenylacetaldehyde to

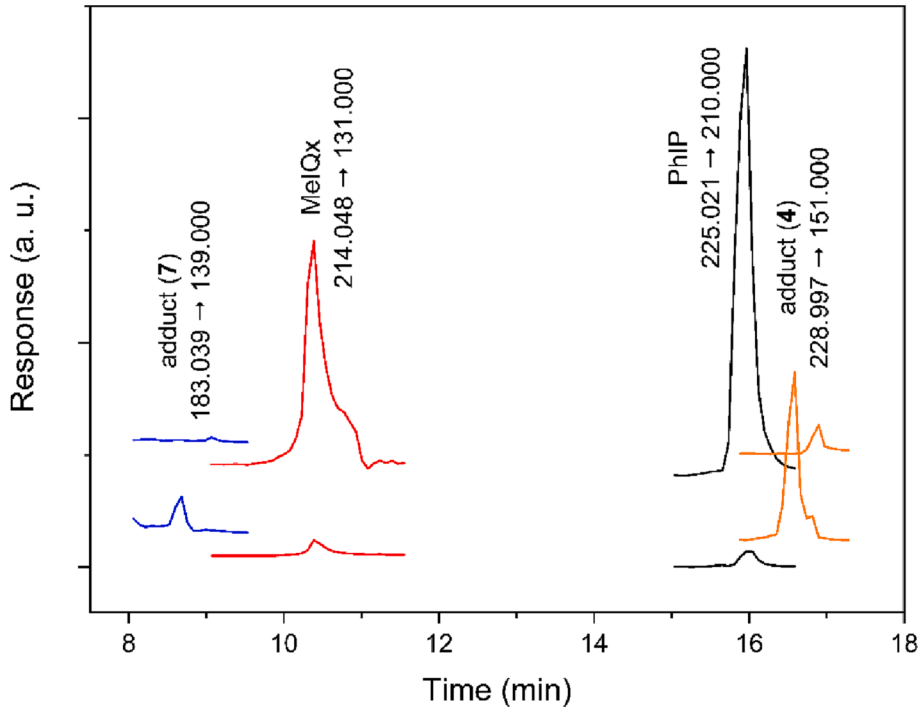

Fig. 3. PhIP (black) and MeIQx (red) disappearance and adduct **4** (orange) and adduct **7** (blue) formation in beef patties treated (bottom) or not (top) with phloroglucinol. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
HAA disappearance and adduct formation in beef patties treated with phloroglucinol.

	Control beef patties	Beef patties treated with phloroglucinol (0.4%)	Beef patties treated with phloroglucinol (1%)
PhIP	11.5 ± 1.0 a	0.6 ± 0.3b	0.4 ± 0.2b
MeIQx	3.7 ± 0.8 a	0.8 ± 0.4b	0.4 ± 0.3b
IQ	0.2 ± 0.2	0.1 ± 0.1	0.1 ± 0.1
MeIQ	0.5 ± 0.2 a	0.1 ± 0.1b	0.1 ± 0.1b
Adduct (**4**)	n. d. a	39 ± 6b	87 ± 10c
Adduct (**7**)	n. d. a	483 ± 21b	854 ± 35c
Adduct (**8**)	n. d. a	0.4 ± 0.2b	0.6 ± 0.4b
Adduct (**10**)	n. d. a	0.2 ± 0.1b	0.4 ± 0.3b

Data are given in ng/g and are mean ± SD of, at least, three independent experiments. Means in the same row with different letters are significantly different ($p < 0.05$). Abbreviation: n. d., not detected.

a significant extent should be expected to produce PhIP to a significant extent, and the same conclusion can also be achieved for acrolein and MeIQx, or for crotonaldehyde and IQ and MeIQ.

3.4. Use of apple and pear juices for inhibiting HAA formation in beef patties

Previous results have shown that phloroglucinol is a very efficient inhibitor of HAA formation because of its high efficiency for trapping the reactive carbonyls responsible for HAA formation. Therefore, any extract rich in this compound should decrease HAA formation to a high extent. Phloroglucinol is not a usual food component, but its glucoside phlorin (3,5-dihydroxyphenyl β-D-glucopyranoside, an orange peel marker) has been detected in the juice of citrus fruits (Louche, Luro, Gaydou, & Lesage, 2000). Furthermore, apple is rich in phloridzin (Suárez-Vallés, Santamaría-Victorero, Mangas- Alonso, & Blanco-Gomis, 1994), a glucoside of the dihydrochalcone phloretin, which has an electronic configuration that favors the nucleophilicity of aromatic carbons. Therefore, it should be very prone to trap reactive carbonyls. For that reason, when beef patties were immersed in apple juice before being cooked, the formation of HAAs was almost completely inhibited (Fig. 4A). An analogous effect was observed when beef patties were partially immersed in commercial apple juices before being cooked (data not shown).

Differently to apples, to the best of our knowledge phloridzin is not a major component of pear phenolics. However, pear juices are rich in the flavan-3-ol epicatechin (Tanrıöven & Ekşi, 2005) which has an A-ring with two hydroxyl groups in *m*-position. Therefore, it should also trap reactive carbonyls, as observed previously (Zhu, Poojary, Andersen, & Lund, 2020), although its trapping ability is likely lower than that of phloridzin (the carbonyl carbon of this last phenolic helps to extend the conjugation and increases the nucleophilicity of aromatic carbons). For that reason, when beef patties were immersed in pear juice, HAAs were also inhibited (Fig. 4A), but some of the studied HAAs were inhibited to a lower extent than when beef patties were immersed in apple juice. In particular, the inhibition of MeIQx was greatly reduced. Additional studies are required to investigate if the difference in the trapping ability of acrolein by both phloridzin and epicatechin is behind the observed differences for inhibiting MeIQx formation between both kinds of juices.

3.5. Use of wheat bran for inhibiting HAA formation in beef patties

Epidemiological studies have clearly shown that whole-grain cereals have health benefits and some of them have been partially attributed to wheat's phytochemicals, including phenolic acids, flavonoids,

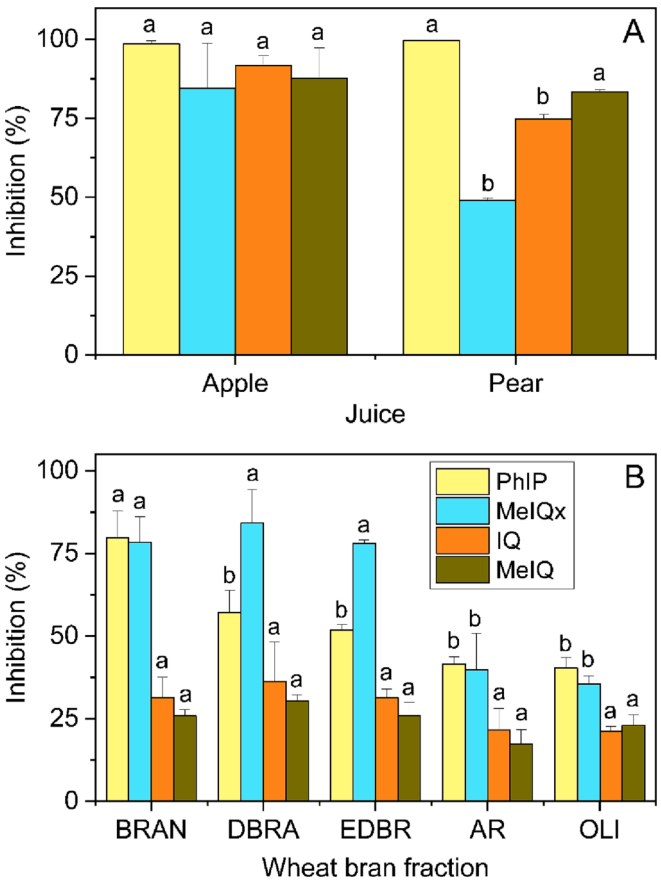

Fig. 4. Inhibiting effect of: A, fruit juices; and B, wheat bran fractions on the formation of carcinogenic heterocyclic aromatic amines (HAAs) with the structure of aminoimidazoazaarene in beef patties. Inhibitions with different letters for each determined HAA are significantly different ($p < 0.05$). Abbreviations: BRAN, wheat bran; DBRA, defatted wheat bran; EDBR, extracted and defatted wheat bran; AR, alkylresolcinols; OLI, olivetol.

alkylresorcinols, carotenoids, phytosterols, tocopherols, and tocotrienols (Tian et al., 2022). Some of these phytochemicals are located in the wheat bran, in particular flavonoids and alkylresorcinols that have exhibited carbonyl-trapping abilities (Zamora & Hidalgo, 2022). Therefore, addition of wheat bran to beef patties should also inhibit the formation of HAAs to some extent. This hypothesis was confirmed when wheat bran was added to beef patties (Fig. 4B). Nevertheless, the effect of wheat bran was lower than that observed for apple juice (Fig. 4A). This is likely due to the complexity of wheat bran composition, which also include components such as metal traces (Onipe, Jideani, & Beswa, 2015) that can also promote the formation of the reactive carbonyls responsible for the formation of HAAs.

When wheat bran was successively extracted with different solvents, its inhibitory power generally decreased and this decrease was not always the same for the different HAAs. Thus, extraction with hexane produced a defatted wheat bran (DBRA in Fig. 4B) that had a reduced inhibition ability for PhIP formation. The extraction with acetone produced an extracted and defatted wheat bran (EDBR in Fig. 4B) that had inhibitory abilities similar to those of DBRA. The alkylresorcinols extracted with acetone (AR in Fig. 4B) also exhibited an inhibitory power for HAA formation, but it was lower than that observed for BRAN, DBRA, and EDBR. The inhibitory power of alkylresorcinols was similar to that of olivetol (OLI in Fig. 4B), which agrees with the *m*-diphenol structure of both of them. As observed in Fig. 1, inhibitory power of *m*-diphenols was lower than that of the trihydroxy derivative phloroglucinol with the three hydroxyl groups placed at *m*-position.

4. Conclusions

The formation of carcinogenic HAAs with the structure of aminoimidazoazaarene is a complex process in which reactive carbonyls, ammonia, and creatin(in)e are involved. Creatin(in)e and ammonia are either present to a high extent in proteinaceous foods or easily produced. Therefore, the easiest way to control HAA formation is trapping the reactive carbonyls required for the formation of these HAAs (phenylacetaldehyde for PhIP, acrolein for MeIQx and IQ, and crotonaldehyde for MeIQ and IQ). This study has shown that these reactive carbonyls can be trapped by phenolics (mainly phenolics with three hydroxyl groups at *m*-position). Thus, when phloroglucinol, as a representative phenolic having three hydroxyl groups at *m*-position, was added to beef patties, HAA formation was reduced and the production of phloroglucinol/acrolein and phloroglucinol/phenylacetaldehyde adducts was observed. Therefore, addition of either analogous phenolics or extracts rich in them to beef patties should decrease the amount of produced HAAs. This was observed when beef patties were immersed in apple (or pear) juice (rich in phenolics with high carbonyl-trapping abilities) for some hours before being cooked, or when wheat bran (also rich in phenolics compounds, including alkylresorcinols) was added to the patty recipe. All these results confirm the key role of reactive carbonyls in the formation of carcinogenic HAAs with the structure of aminoimidazoazaarene and provide a scientific basis for controlling HAA formation in food products.

CRediT authorship contribution statement

Francisco J. Hidalgo: Conceptualization, Software, Validation, Formal analysis, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition. **Rosario Zamora:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.136505.

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